Pattern Recognition Receptor and Vaccination Studies in the Murine Model of Lymphatic Filariasis

Dissertation

zur

Erlangung eines Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

Sebastian Ziewer

aus

Bonn

Bonn, September 2012

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der
Rheinischen Friedrich-Wilhelms-Universität Bonn

1. Gutachter: Prof. Dr. med. Achim Hörauf
2. Gutachter: Prof. Dr. rer. nat. Norbert Koch
Tag der Promotion (mündliche Prüfung): 10.01.2013
Erscheinungsjahr: 2013

O saeculum! O litterae!

U.H.

Zusammenfassung

Die lymphatische Filariose ist eine parasitäre Wurmerkrankung, die schätzungsweise 120 Millionen Menschen weltweit betrifft. Etwa ein Drittel der Infizierten entwickelt sichtbare Symptome, z.B. Lymphödeme. Infektionserreger sind Fadenwürmer, die während der Blutmahlzeit von Mücken übertragen werden. In diesen Fadenwürmern befinden sich zudem endosymbiontische Bakterien der Gattung *Wolbachia*, die das Wirtsimmunsystem stimulieren können und deshalb als eine der Ursachen für die Entstehung der Inflammations-abhängigen Pathologie diskutiert werden. Die Behandlungsstrategie beruht derzeit vor allem auf der chemotherapeutischen Massentherapie, die die zirkulierenden Larvenstadien reduziert, aber nur einen geringen Effekt auf die adulten Würmer hat. Trotz der weltweiten Bemühungen die Filariosen auszulöschen, konnte bisher keine Elimination erreicht werden. Da nun auch Resistenzen gegen eingesetzte Mittel beobachtet wurden und eine weitere langjährige Massentherapie hohe Kosten und logistischen Aufwand bedeuten, wäre ein Impfstoff ein weiteres, hilfreiches Mittel bei der Bekämpfung der Erkrankung.

Mustererkennungsrezeptoren (engl. „pattern recognition receptors“, PRRs) sind Bestandteile des Immunsystems, die pathogene Strukturen, z.B. von Viren oder Bakterien, erkennen und dadurch mit Hilfe von Signal weiterleitenden Adapterproteinen (z.B. MyD88) Immunantworten einleiten. Die PRRs TLR2, TLR4 und NOD2 erkennen bakterielle Strukturen und sind deshalb bezüglich ihres Potentials zur Erkennung der Wolbachien diskutiert worden.

Im ersten Teil dieser Arbeit konnte gezeigt werden, dass die proinflammatorischen Antworten (Ausschüttung von TNF und IL-6) bei *in vitro* Stimulation von murinen Antigen präsentierenden Zellen mit einer *Litomosoides sigmodontis* Wurmpräparation nur induziert wurden, wenn diese auch Wolbachien enthielt. Deren Erkennung erfolgte dabei durch das Heterodimer TLR2/6 (nicht aber TLR1/2 oder TLR4) und die Integration des Adaptermoleküls MyD88 war notwendig. Der für seine Interaktion mit TLR2 bekannte PRR NOD2 war allerdings für die TLR2/6 abhängige Antwort nicht notwendig. Um zu untersuchen, ob diese PRRs auch den Verlauf einer Filarieninfektion *in vivo* beeinflussen, wurden Mäuse mit der Nagetierfilarie *L. sigmodontis* infiziert. Während die Wurmlast zwischen TLR bzw. MyD88 kompetenten und den entsprechenden defizienten Tieren ähnlich war, hatten NOD2 defiziente Mäuse eine erhöhte Wurmlast. Zusätzlich hatten NOD2 defiziente Mäuse kürzere Würmer, die sich zudem auch seltener in das adulte Wurmstadium weiterentwickelten. Zusammengefasst konnte also gezeigt werden, dass trotz der TLR-2 und MyD88 abhängigen Fähigkeit zur Erkennung der Wolbachien der Verlauf der Infektion nicht beeinflusst wird. Im Gegensatz dazu scheint NOD2 an der Wurmabwehr beteiligt zu sein, obwohl es für die Erkennung der Endosymbionten *in vitro* nicht notwendig ist. Weitere Untersuchungen sind nötig, um die molekularen Mechanismen hinter diesen Beobachtungen zu identifizieren.

Im zweiten Teil der Arbeit konnte ein erfolgreiches Impfmodell etabliert werden. Die Immunisierung erfolgte subkutan mit Mikrofilarien und dem Adjuvant Alum, und führte zu einer stark reduzierten Larvenlast sowohl im Blut als auch am Infektionsherd (Thoraxhöhle). Diese Reduktion ging einher mit einer bereits im Uterus der weiblichen adulten Würmer gestörten embryonalen Entwicklung der Mikrofilarien. Die Immunisierung verursachte einen Wechsel vom typischerweise mit Wurminfektionen assoziierten Th2 Milieu hin zu einem Th1 Milieu, gekennzeichnet durch erhöhtes IFN-γ und IgG2. Die Ergebnisse dieser Versuche lassen hoffen, dass eine Impfung gegen das für die Transmission verantwortliche Larvenstadium auch in der humanen Infektion eine die medikamentöse Behandlung ergänzende Therapie werden könnte.

Summary

Lymphatic filariasis is a parasitic helminth infection that affects approximately 120 million people. About one third of the infected individuals develop pathological manifestations, e.g. lymphedema. Causative agents of lymphatic filariasis are filarial nematodes, which are transmitted during the blood meal of mosquitoes. Filarial worms contain endosymbiotic bacteria of the genus *Wolbachia*, which are an additional stimulus for the host's immune system and have been intensively discussed to promote pathology due to an exacerbated proinflammatory response of the host. Currently, the available treatment used for mass treatment against filariasis is based on chemotherapeutic intervention that reduces the burden of the blood circulating larval stages but has only limited effects on adult worms. Despite major attempts to eradicate filarial diseases, elimination has not been achieved. Also, with resistance against the chemotherapy being observed and high cost and logistics efforts of mass drug administration, a vaccine would be a desirable tool towards the elimination of the disease. However, despite intensive research there are no vaccines against any human filarial infection.

Pattern recognition receptors (PRRs) are able to sense structures of pathogens, such as viruses or bacteria. In order to investigate, how *Wolbachia* may induce such proinflammatory immune responses, the PRRs TLR2, TLR4 and NOD2 were investigated, since they are known to sense bacterial structures. In the first part of this thesis *in vitro* experiments revealed that proinflammatory responses, measured by the secretion of TNF and IL-6, were induced after *in vitro* stimulation of antigen-presenting cells with filarial *Litomosoides sigmodontis* extract. In contrast, *L. sigmodontis* extract devoid of *Wolbachia* did not induce the secretion of TNF and IL-6. The recognition of *Wolbachia* was transduced by the heterodimer TLR2/6 (but not TLR1/2 or TLR4) and the integration of the intracellular adapter molecule MyD88 was mandatory. In contrast, the intracellular receptor NOD2, which is known for his interaction with TLR2, was not necessary for this proinflammatory response. In addition to the *in vitro* experiments, it was of interest, whether mice deficient for these receptors show an altered course of infection to demonstrate the *in vivo* importance of these PRRs in filarial infections. While the parasite burden after *L. sigmodontis* infection was similar between TLR-, MyD88-deficient and the corresponding receptor-competent mice, NOD2-deficient mice showed a higher worm load. In addition, the worms recovered from NOD2-deficient mice were shorter and showed an impaired development. Taken together, these data show that despite the induction of proinflammatory responses, TLR-2 and MyD88 do not influence the infection *in vivo*. In contrast, the higher worm burden observed in NOD2-deficient mice indicates a role for this PRR in the defense against filarial parasites. Further investigations are needed to identify the molecular mechanism behind these observations.

In the second part of this thesis a successful vaccination against the *L. sigmodontis* microfilarial stage was established. The vaccination presented in the present thesis was performed by subcutaneous injection of microfilariae together with adjuvant alum and led to a strongly reduced microfilarial burden in the blood and at the site of infection. Analysis of filarial embryogenesis revealed that the development of microfilariae was already impaired in the uteri of female worms. The vaccination caused a switch from the Th2 arm of immunity, which is well-known for filarial infections, towards a Th1 milieu, indicated by increased IFN-γ and IgG2 in immunized mice. The results of these experiments not only contribute to the understanding of the immune mechanisms needed to develop a vaccine against filarial parasites, but moreover raise hope for the development of a human vaccination against the transmission stage of lymphatic filariasis.

- Index -

List of abbreviations

APC	antigen-presenting cell also used for the commercially available fluorochrome "allophycozyanine"
AAMO	alternatively activated macrophage
BCECF	2′,7′-Bis(2-carboxyethyl)-5(6)-carboxy-fluorescein-acetoxymethyl ester
BMdAPC	bone marrow-derived antigen-presenting cell
BSA	bovine serum albumin
CCL	chemokine C:C motif ligand
CD	cluster of differentiation
CpG	cytosinphosphatidylguanine
DBP	dibutyl phthalate
DC	dendritic cell
dDC	dermal dendritic cell
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPO	eosin peroxidase
FCS	fetal calf serum
FITC	fluorescine-thioisocyanate
foxp3	forkhead box protein P3
FSC	forward scatter
GM-CSF	granulocyte / monocyte-colony stimulating factor
Gy	Gray
i.p.	intraperitoneally
i.v.	intravenously
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IVM	ivermectin
L3	third larval stage
L4	fourth larval stage
LC	Langerhans cell
ldLN	local draining lymph node
LE	lymphedema
LF	lymphatic filariasis
LN	lymph node
LPS	lipopolysaccharide
LRR	leucine-rich repeats
Ls	*L.sigmodontis* extract
Ls-tet	*Wolbachia*-depleted *L. sigmodontis* extract
MDA	mass drug administration
MDP	muramyl dipeptide
Mf	microfilariae = first larval stage
MHC	major histocompatibility complex

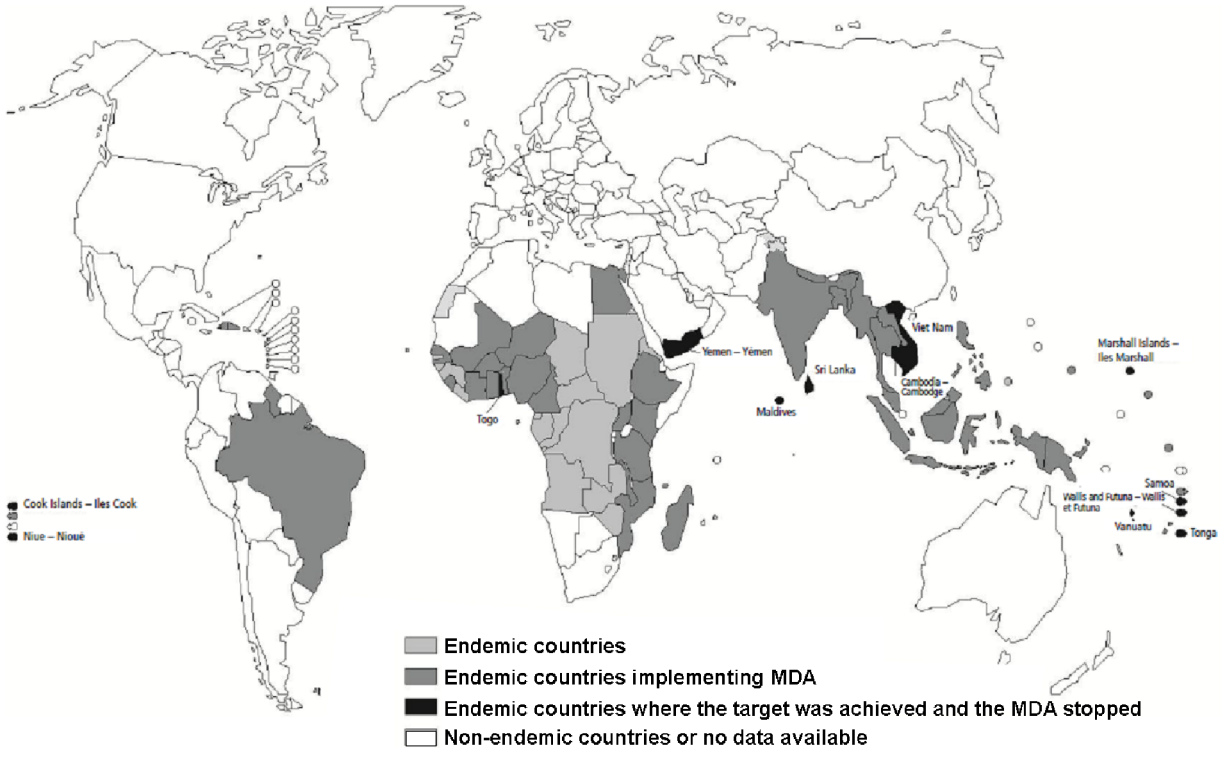
MIP-2	macrophage inflammatory protein 2
MO	macrophage
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor κ B
NK	natural killer cell
NLR	nucleotide-binding domain and leucine rich repeat-containing receptor
NO	nitric oxide
NOD	nucleotide oligomerization domain
NTD	neglected tropical disease
OD	optical density
P3C	tripalmitoyl-S-glycerylcysteine
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PGN	peptidoglycan
PLEC	pleural exudate cell
PRR	pattern recognition receptor
RELM	resistin-like molecule
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
s.c.	subcutaneously
SSC	side scatter
TGF	tumor growth factor
Th1, Th2	T helper cell response of type 1 or 2
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TRIF	Toll-interleukin-1 receptor domain-containing adaptor protein inducing interferon-β
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WT	wild type

1. INTRODUCTION

1.1 Lymphatic Filariasis

1.1.1 Lymphatic filariasis – a major public health problem

Infections with filarial nematodes are classified as neglected tropical diseases (NTDs, (Hotez, Brindley et al. 2008)) and cause serious public health problems in the tropics and subtropics (sub-Saharan Africa, India, Southeast Asia, parts of South America, the Caribbean and the South Pacific; see also map 1 (WHO 2011)) with more than 150 million people infected and many more at risk. Lymphatic filariasis (LF) is caused by the filarial nematodes *Wuchereria bancrofti* and *Brugia* spp. and affects about 120 million people with approximately one third of them suffering from clinical presentations of the infection, namely lymphedema (LE) of the extremities and hydrocele, making LF the second-largest cause of long-term disability (Taylor, Hoerauf et al. 2010) in the world with nearly six million disability-adjusted life years in 2004 (WHO 2004).



Map 1. Global prevalence of lymphatic filariasis and mass drug administration. Map shows endemic countries of lymphatic filariasis and the status of mass drug administration in these countries during 2010. Adapted from "Global Programme to Eliminate Lymphatic Filariasis: progress report on mass drug administration." Wkly Epidemiol Rec 86(35): 377-387.

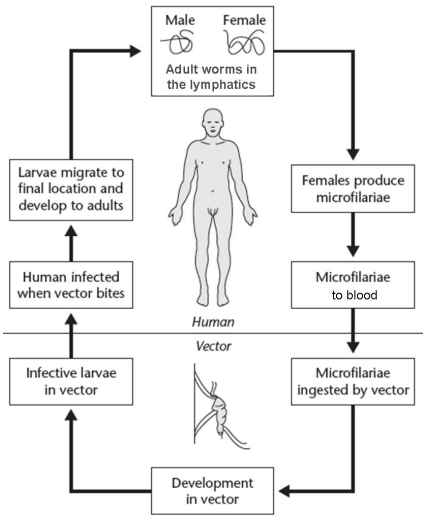


Figure 1. The infectious cycle that leads to human lymphatic filariasis. Shown are the main events in the development of the filariae within the arthropod vector and the human host. Adapted from: Gordon C. Cook & Alimuddin Zumla, Manson's Tropical Diseases, 22 edition, chapter 84: Filariasis (Paul E. Simonsen).

1.1.2 Life cycle

Both filarial species that cause LF are transmitted by blood feeding vectors (genera: *Aedes, Anopheles, Culex* and *Mansonia*) that ingest first stage larvae (microfilariae, Mf) from infected patients (Fig. 1). Within the vector the Mf undergo two obligatory molts to become infective third stage larvae (L3). After their transmission into a new host, infectious L3 migrate to and reside within the lymphatic vessels, most exclusively in the extremities and the male genitalia or the female breasts. The larvae molt further two times into diecious adults, which are 3-4 cm (male) and 8-10 cm (females) long and can reside in the host for several years. After mating, the females release thousands of Mf that eventually enter the peripheral blood stream (microfilaraemia), where they can be taken up again by the transmitting vector (Hoerauf, Pfarr et al. 2011). Microfilariae were first described by Jean-Nicolas Demarquay and Otto Henry Wucherer in the 1860s, but their relevance for the filarial disease was not understood until the life cycle was mapped by Patrick Manson and George Carmichael Low several years later. The adult worms were described by Joseph Bancroft in 1876 (Cox 2002).

1.1.3 Pathology

Due to the parasite's ability to evade host immunity and the permanent risk of reinfection LF is a chronic infection. However, not all infected individuals develop disease manifestations; the majority is even free of any clinical signs. Furthermore, some people with lifelong exposure to the transmitting vector do not acquire the infection at all (a group referred to as endemic normals) (Pfarr, Debrah et al. 2009).

Infected individuals can be categorized into the following groups: (1) asymptomatic infection is characterized by the presence of blood-circulating Mf, in conjunction with several immune regulatory processes that promote worm survival and prevent onset of pathology. Of note, this group is the greatest reservoir of transmission, due to the high microfilarial load in the blood. (2) Latent infected (in older publications also referred to as cryptic infected (Lawrence 2001)) patients, who are characterized by the presence of adult worms but absence of Mf are free of any obvious clinical signs.

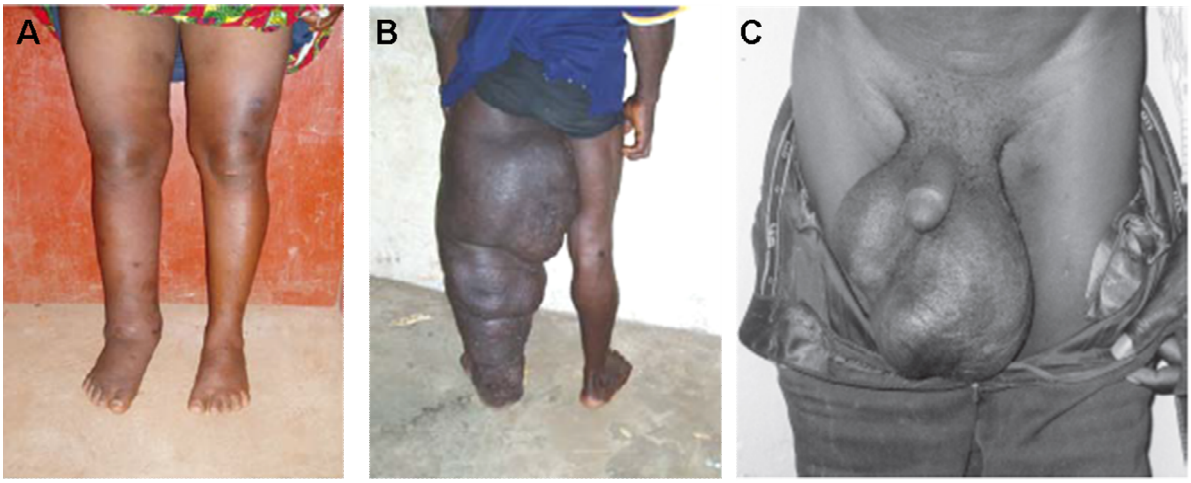


Figure 2. The pathological manifestations of lymphatic filariasis. Examples of disease manifestations are shown. (A) lymphedema, (B) elephantiasis and (C) hydrocoele. (A, B) from Debrah et al (2006), (C) from Gordon C. Cook & Alimuddin Zumla: Manson's Tropical Diseases, 22 edition, chapter 84: Filariasis (Paul E. Simonsen).

Finally, (3) symptomatic individuals are characterized by presence of low numbers of parasites and even absence of parasites at later stages of infection, but vigorous host immune reactions that promote pathology due to dying parasites (Pfarr, Debrah et al. 2009; Taylor, Hoerauf et al. 2010).

With regard to pathology, in the early phase episodes of lymphangitis and lymphadenitis are present with localized pain and swelling and recurrent debilitating fever. In this acute, but retrograde phase that lasts for several days, individuals are often extremely weak and immobilized. Pathology develops progressively but not all individuals will suffer from the most severe forms. In turn, the subclinical changes - lymph vessel dilation and thickening of the lymph vessel wall - are almost abundantly found in all infected individuals (Pfarr, Debrah et al. 2009). Due to the infection-associated enlargement, the lymph vessels become less efficient in transporting lymph fluid, which leads to extravasation and eventually results in severe forms of pathology, the LE (Fig. 2A) and the hydrocele of the scrotum (Fig. 2C; only in *W. bancrofti* infected individuals). The LE mainly occur in the legs, arms and breasts and in some cases will further progress towards the most severe form with irreversible LE, called elephantiasis (Fig. 2B) (Pfarr, Debrah et al. 2009).

There is impressive evidence that susceptibility to infection *per se* as well as the degree of infection and pathology have genetic traits. For example, the analysis of vascular endothelial growth factor (VEGF)-A (VEGF-A is a rapid and potent inducer of vascular permeability and has also functions in embryo- and angiogenesis, (Takahashi and Shibuya 2005)) single-nucleotide-polymorphisms revealed a higher frequency of cases of hydrocele than in those without the nucleotide exchange (Debrah, Mand et al. 2007).

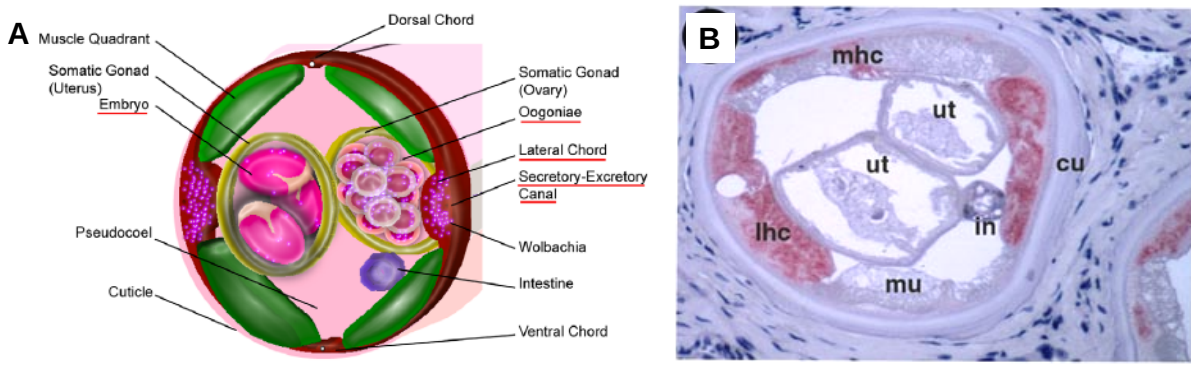


Figure 3. The endosymbiotic *Wolbachia* bacteria of filarial nematodes. (A) Schematic midbody cross section giving an overview of *Wolbachia* distribution in *B. malayi* tissue; *Wolbachia*-containing tissue underlined in red. Adapted from Landmann et al (2010). (B) Histological midbody cross section of a host tissue embedded *Onchocerca volvulus* filariae. The *Wolbachia* are stained red, host cell nuclei blue. 150 x magnification (adapted from http://www15.bni-hamburg.de/bni/bni2/english/annualrep/ 1998/ jb4_b1.jpg). Abbr.: ut uteri, cu cuticle, in intestine, mhc / lhc median / lateral hypodermal cords, mu musculature.

1.1.4 The endosymbiont *Wolbachia*

Decades ago "unusual bodies" were observed in the hypodermis of filarial nematodes, which could be identified as endosymbiotic bacteria of the genus *Wolbachia* (order *Rickettsiales*) (McLaren, Worms et al. 1975). These bacteria are found in the hypodermis of adult worms as well as embryos and are transmitted transovarially (Kozek 1977). The relationship between filariae and bacteria is an obligate mutual symbiosis, since the endosymbionts are essential for worm fertility, reproduction, larval molting and survival (Hoerauf, Nissen-Pahle et al. 1999; Smith and Rajan 2000; Hoerauf, Mand et al. 2001; Hoerauf, Mand et al. 2003; Debrah, Mand et al. 2007). At least for *Brugia malayi* it has been shown that this is caused by the induction of apoptosis in reproductive cells, developing embryos, Mf and developing larvae after depletion of *Wolbachia* from the filariae (Landmann, Voronin et al. 2011).

Wolbachia are present in most filarial species (exception for example are *Loa loa and Acanthocheilonema viteae*) and can be a provocative driver of inflammation in the filariae-infected host (Brattig, Buttner et al. 2001). Especially upon death of parasites, the immune system gets access to proinflammatory stimuli, including large numbers of bacteria. For human filarial infections medicamentous depletion of the bacteria has proven that exposure to *Wolbachia* is associated with lympgangiogenesis, lymphatic endothelial proliferation, and dilation of lymphatic vessels, shown by the improvement of the situation after their depletion (Pfarr, Debrah et al. 2009). Figure 3 gives an overview of *Wolbachia* in the filarial tissue.

1.1.5 Diagnosis and Treatment

Diagnosis of LF traditionally relies on the detection of peripheral Mf (Taylor, Hoerauf et al. 2010). However, taking into account that (1) symptomatic individuals have only few or no Mf, (2)

latent infected individuals are amicrofilaraemic, (3) acceptability of blood taking is low and (4) Mf burden may be decreased after microfilaricidal drug intake, this method is limited. Therefore, other tests have been developed, as detection of *W. bancrofti* filarial antigen (Taylor, Hoerauf et al. 2010), serological assays for *Brugia*-specific immunoglobulin (Ig) or visualization of active *W. bancrofti* nematodes via ultrasonography (Amaral, Dreyer et al. 1994).

Current elimination strategies of the WHO such as the Global Programme to Eliminate LF are based on the mass drug administration (MDA) of albendazole together with either ivermectin (IVM) or diethylcarbamazine (see also Map 1). This strategy has been impressively successful in reducing Mf-burden (WHO 2011). However, in most cases only IVM and albendazole are used in MDA programs against LF in Africa, because diethylcarbamazine causes fast death of Mf, thereby increasing chances of adverse reactions, such as ocular damages in individuals co-infected with another filariae, namely *O. volvulus* (in some *O. volvulus* non-endemic areas diethylcarbamazine sometimes is used, e.g. in Tanzania; (Hoerauf, Pfarr et al. 2011)). In addition, doxycycline has been introduced for individual drug administration (Hoerauf 2008) directed against the *Wolbachia* bacteria of the filariae (Hoerauf, Volkmann et al. 2000; Hoerauf, Mand et al. 2001). The antibiotic inhibits filarial embryogenesis and has been proven to be macrofilaricidal and to stop or reduce pathology (Hoerauf, Specht et al. 2008; Mand, Pfarr et al. 2009; Mand, Debrah et al. 2012). However, doxycycline is contraindicated in children ≤ 9 years and pregnant woman. Therefore, a major focus of ongoing research is improvement of anti-wolbachial chemotherapy to be used in public health control programs (Hoerauf, Pfarr et al. 2011).

Despite the success of anti-helmintic drugs used in MDA, certain drawbacks have to be considered. Ivermectin has only limited macrofilaricidal efficacy and repeated treatments for the life of the adult worm are needed to stop transmission (Hoerauf, Pfarr et al. 2011). Together with the limited logistics, especially in areas with civil unrest, the incidence of adverse events after treatment such as scrotal pain or systemic inflammation can considerably corrupt the level of compliance to therapy (Taylor, Hoerauf et al. 2010). Finally, emerging resistance to IVM (Osei-Atweneboana, Awadzi et al. 2011) reinforces the urgent need for alternative ways of disease control.

1.1.6 Human immune reactions

Infected individuals are exposed to filarial material, due to incoming larvae, the persisting presence of filarial larvae and adults, as well as secreted or released filarial products and *Wolbachia*. This permanent parasite / host interface has features of immunogenicity, regulation and pathogenesis.

1.1.6.1 The innate host defense

After entering the host, the infective larvae are confronted with entities of the innate host defense system. This system includes (1) physical, mechanical and (bio)chemical barriers, (2) soluble immune factors, and (3) pattern recognition receptor (PRR)-bearing sentinel cells (de Veer, Kemp et

al. 2007). For human infections it is not known how the interaction of the incoming larvae with the innate host defense system takes place *in vivo*, but *in vitro* experiments suggest that there is interaction already at this early phase of infection. For example *B. pahangi* L3 polarize natural killer (NK) T cells towards the T helper cell (Th) 2 arm of immunity (Balmer and Devaney 2002). Also, human Toll-like receptors (TLRs) of antigen-presenting cells (APCs) have the potential to sense the *Wolbachia* of the filariae *ex vivo*, and their expression and signaling is known to be altered by the filariae *in vivo* (Venugopal, Nutman et al. 2008).

1.1.6.2 Adaptive responses

Two major adaptive responses are documented: (1) asymptomatic individuals are characterized by down regulated Th1 responses and a dominant Th2 immunity and this regulation involves T cell hyporesponsiveness with decreased production of interferon (IFN)-γ and interleukin (IL)-2 but increased levels of IL-4 an IL-5. Furthermore, increased regulatory T cell (Treg) responses with high levels of immunosuppressive IL-10 and tumor growth factor (TGF)-ß are observed (summarized in (Pfarr, Debrah et al. 2009)). With regard to antibody responses, microfilaraemic patients are characterized by higher IgG4 but limited IgE production (Hoerauf, Satoguina et al. 2005). It is thought that this is induced by the filariae itself in order to evade host defenses and ensure the own survival (Maizels, Pearce et al. 2009; Allen and Maizels 2011). (2) In contrast, symptomatic individuals with pathology mount a dominant Th1 response with IFN-γ (and in part Th17 responses) and are characterized by higher IgE:IgG4 ratios (Hoerauf, Satoguina et al. 2005; Pfarr, Debrah et al. 2009; Taylor, Hoerauf et al. 2010).

1.2 The Murine Model of Lymphatic Filariasis

In 1992 Petit and colleagues could show that the rodent filariae *L. sigmodontis* (formerly known as *L. carinii*), which parasitizes the cotton rat as natural host, undergoes it's complete developmental cycle including patency in the common laboratory BALB/c mouse (Petit, Diagne et al. 1992). The filariae *L. sigmodontis* belongs to the same family as the human-pathogenic filariae *Wuchereria* and *Brugia* (*Onchocercidae*). The finding that immune responses elicited in BALB/c mice often mirror the known responses in human individuals (Lawrence and Devaney 2001) makes this model a valuable tool for filarial research. The fact that not all BALB/c mice become patent (about 30 to 50 % remain amicrofilaraemic, despite the presence of adults) can further be seen as a close match to latently infected individuals (characterized by adults, but neither peripheral Mf nor obvious pathology). Indeed, understanding the immunological basis of amicrofilaraemia in the murine model of LF helps to understand the mechanisms behind latent infections.

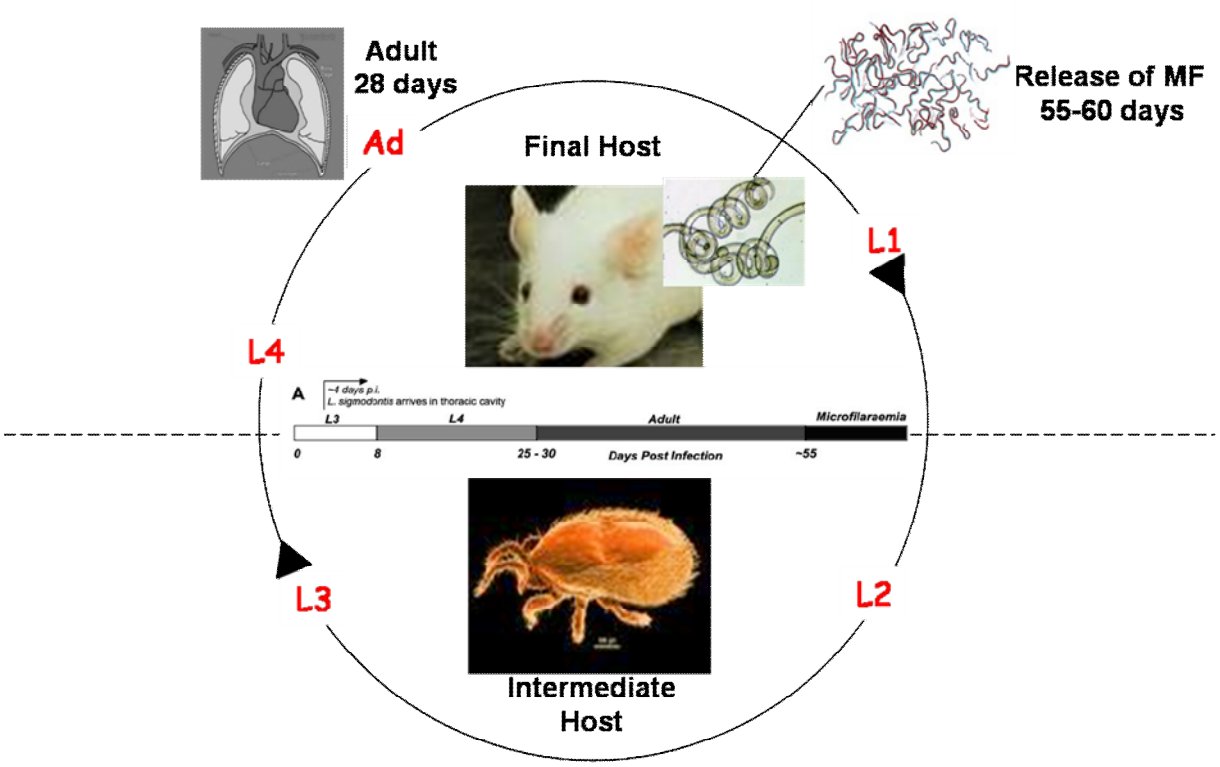


Figure 4. The laboratory life cycle of the rodent filariae *L. sigmodontis*. The life cycle for the permissive BALB/c mouse strain is illustrated. Inset shows time frame of *L. sigmodontis* larval development within the mouse. L1 to L4 indicates larval stages one to four, Ad indicates adults.

1.2.1 *L. sigmodontis* infection cycle

The laboratory cycle of the murine model of LF is illustrated in figure 4. The mite *Ornithonyssus bacoti* is used as vector. During the blood meal of the mite (hereafter termed "natural" infection) the infective L3 enters the host through the skin, actively enters a lymphatic vessel (unpublished observation of Coralie Martin, Muséum National d'Histoire Naturelle, Paris), migrates through the lymphatics and reaches the pleural space of the thoracic cavity after approximately four days. Studies with a defined number of inoculated L3 revealed that a great proportion of L3 (approx. 60 %) does not reach the pleural space (Hoffmann, Petit et al. 2000). Once in the pleural space L3 develop into the L4 (around days 8 to 9) and eventually into adults (from the third week after infection onwards). Male (1 to 2 cm in length) and female (approx. 10 cm) adult filariae mate and afterwards filarial embryos develop in the female filariae. Around days 50 to 55 post infection the first Mf (approx. 80 µm) appear in the peripheral blood (Hoffmann, Petit et al. 2000).

1.2.2 Immune mechanisms in the murine model of lymphatic filariasis

Immune reactions to the infection with *L. sigmodontis* depend on the susceptibility of the genetic mouse strain (Petit, Diagne et al. 1992), may be directed towards the filariae and / or the endosymbiotic *Wolbachia* bacteria and many reactions of the host immune system can also be understood as an attempt to avoid parasite-induced pathology and *control* rather than *reject* the

infection. This kind of "stand off" has been explained as a result of the long lasting co-evolution of parasite and host (Allen and Maizels 2011). Although adaptive immune responses influence the filariae also in the later course of infection, several authors claim that events during the early phase define the overall outcome of the infection (Babayan, Ungeheuer et al. 2003; Taylor, van der Werf et al. 2008). This idea is indeed supported by the fact that the grade of parasite burden is established within days and recovery rate does not dramatically change afterwards in most mice strains (in some mice with genetic deficiencies the worm burden can change at later time points in the infection). After this initial drop there is a second phase of adult worm death (Hoffmann, Petit et al. 2000); depending on the mouse strain this drop starts around the time of L4 / adult molting (C57BL/6) or the beginning of patency (BALB/c), suggesting successful adaptive host responses.

1.2.2.1 Immune responses to incoming *L. sigmodontis* larvae

The key ambition for the infective *L. sigmodontis* L3 is to enter the host during the blood meal of the vector and to migrate to the main site of infection (Bain and Babayan 2003). During this passage the infective larvae are confronted with members of the innate immune system in the skin and the lymphatics. Since the murine model of LF is known for decades, it may come as a surprise that only a few studies have dealt with these early events. After infection, the inflammatory responses, which are exacerbated by the incoming L3 and / or the bite of the vector lead to destruction of many larvae (Bain and Babayan 2003). That may be due to neutrophils that infiltrate the penetration site within six hours after infection in BALB/c mice (Martin, Saeftel et al. 2001). Another study showed that the *Wolbachia* of the filariae are recognized by skin mast cells in a TLR2-dependent manner and that this recognition leads to mast cell degranulation and alteration of vascular permeability under the control of the chemokine C-C motif ligand (CCL)17, a chemokine that is produced by dendritic cells (DCs) upon microbial challenge (Specht, Frank et al. 2011).

Since the larval burden in the pleural space is stable for several days after arrival at the site of infection (Hoffmann, Petit et al. 2000), indirect conclusions about immune reactions against incoming and migrating larvae can be drawn from studies, that assess the parasite burden very early; differences in this early phase worm numbers very likely reason in processes along the passage from the skin through lymphatics into the thoracic cavity. For example seven days post infection C57BL/6 mice deficient for granzyme A have elevated L4 numbers compared to wild type (WT) mice (Hartmann, Marsland et al. 2011), suggesting a pivotal role of granzyme A at the early parasite / host interface. However, taking into account that deficiencies in the Th hallmark cytokines IFN-γ (Saeftel, Volkmann et al. 2001) and IL-5 (Al Qaoud, Pearlman et al. 2000) in BALB/c mice, as well as transgenic over-expression of IL-5 (Martin, Le Goff et al. 2000) in semi resistant[1] CBA/Ca mice do not influence the

[1] Unfortunately, literature knows various (and confusing) terms to determine the susceptibility to *L. sigmodontis* infection. In this work mice are termed as "resistant", when no infection is established at all, "semi resistant" when infection takes place but not all developmental stages occur (e.g. C57BL/6), and "permissive" when complete development including peripheral Mf is present (e.g. BALB/c).

early parasite burden and that duration of filarial migration and recovery rate even between permissive BALB/c and semi resistant C57BL/6 mice are only moderately different (Babayan, Ungeheuer et al. 2003) it seems to be more the genetic background than the weaponry of innate immunological responses that defines susceptibility.

1.2.2.2 Immune responses at the site of infection

Immune responses in the later course of *L. sigmodontis* infection occur and during the first month of infection a variety of immune cells starts to be present at the site of infection. Most recently it has been shown that one of the first of these cellular responses after parasite arrival is the local proliferation of alternatively activated macrophages (AAMOs) (Jenkins, Ruckerl et al. 2011), which has revised the assumption that MOs mainly get recruited from outside the pleural space (Taylor, Harris et al. 2006). After appearing within a few days, the AAMOs stay present in the pleural space until day 60 post infection (Nair, Gallagher et al. 2005). Although AAMOs are classically attributed to Th2 immunity (Martinez 2011), they have been shown to have a suppressive capacity in filarial infections by inducing cluster of differentiation (CD)4^+ T cell hyporesponsiveness at the site of *L. sigmodontis* infection (see section below; (Taylor, Harris et al. 2006)). Furthermore, expression of resistin-like molecule (RELM)-α, which has been attributed to immune regulation, gets elevated in MOs after infection of BALB/c mice with *L. sigmodontis* (Nair, Gallagher et al. 2005). The findings that C57BL/6 mice deficient for the induction of the nitric oxide (NO) synthase, which is a major MO effector function against various extra cellular parasites, as well as mice treated with NO synthesis inhibitors do not have decreased Mf clearance rates further indicate that rather the regulatory than the effector functions of MOs are involved in responses at least to the Mf of *L. sigmodontis* (Pfaff, Schulz-Key et al. 2000).

Granuloma formation is a hallmark response after *L. sigmodontis* infection. In an extensive study Attout and colleagues elucidated the manner of granuloma formation within the first few days after parasite arrival (Attout, Martin et al. 2008). These early granulomas are found in permissive BALB/c and semi resistant C57BL/6 mice, form only around shed third and fourth stage cuticles but never around healthy larvae, and mainly consist of eosinophils but only less neutrophils. In turn, later granulomas around adult filariae consist mainly of neutrophils in BALB/c mice, but of eosinophils in semi resistant C57BL/6 mice. Of note, the composition of granulomas by no means reflects the composition of the pleural exudate cell (PLEC) population. This suggests that mechanisms of pleural space recruitment / local proliferation and granuloma formation differ. Interestingly, the late granuloma formation in BALB/c mice is under control of IFN-γ as well as IL-5, because IFN$\gamma^{-/-}$ mice have less or no granuloma due to the lack of neutrophil activating cytokines tumor necrosis factor (TNF, former TNF-α) and granulocyte / monocyte colony-stimulating factor (GM-CSF) and reduced neutrophil chemotactic and phagocytic activity (Saeftel, Volkmann et al. 2001); IL-5 in turn is

essential for the induction of murine neutrophils chemo attractants as TNF, GM-CSF and the murine homologue of human IL-8 (keratinocyte chemo attractant, KC (Bozic, Gerard et al. 1994)) and depression of neutrophil-deactivating IL-10, as shown by depletion of IL-5 in BALB/c WT mice (Al Qaoud, Pearlman et al. 2000). With respect to granuloma formation, as well as chemotactic and phagocytic activity this overall picture was also confirmed for IFN-$\gamma^{-/-}$IL-$5^{-/-}$ double deficient BALB/c mice (Saeftel, Arndt et al. 2003). In all three studies the worm burden stayed high in the deficient mice compared to the BALB/c WT littermates eighty days post infection (in contrast to C57BL/6 IFN-$\gamma^{-/-}$ mice with similar parasite load (Specht, Volkmann et al. 2004)). In line with these elevated worm burdens, IL-5 has been shown to be critical for the control of adult parasite burden in BALB/c mice, most prominent from day 60 to 200 post infection (Volkmann, Bain et al. 2003). However, beside these beneficial roles for the host, IL-5 has also been discussed as a promoter of parasite development. Ten days post infection mice with transgenic over expression of IL-5 have longer male and female larvae and have developed into the fourth larval stage to a greater extend than in the WT littermates (Martin, Le Goff et al. 2000). Recently this effect has been pinned down to very early IL-5-driven eosinophilia (Babayan, Read et al. 2010). Contrary to this, the absence of the another important Th2 cytokine, IL-4, led to longer adult filariae at day 77 post infection (Volkmann, Saeftel et al. 2001). Interestingly, the role of IL-4 with respect to parasite burden at all depends on the genetic background of infected mice: whereas it does not reduce worm burden in permissive BALB/c mice, it does in semi resistant C57BL/6 (Le Goff, Lamb et al. 2002). This parasite controlling mode of IL-4 in semi resistant mice is due to overriding possible counter regulation by IL-10, since IL-4 and IL-10 double deficient C57BL/6 mice display the phenotype of the WT again (Specht, Volkmann et al. 2004).

The role of T and B effector cells in the murine *L. sigmodontis* model of LF was already elucidated in the 1990's. Depletion of $CD4^+$ T cells from infected BALB/c mice led to increased adult parasite burden already at day 28 post infection. In this study, depletion went along with diminished Th2 cytokines (IL-4 and IL-5), eosinophilia, and filarial-specific IgE (Al Qaoud, Taubert et al. 1997). In contrast, the depletion of $CD8^+$ cells did not influence the adult parasite burden (Korten, Volkmann et al. 2002). The role of B cells remains controversial: BALB.Xid mice, which lack B1 cells have elevated parasite burden at day 28 post infection, associated with diminished IgG and IgM in the blood and reduced IL-4 and IL-5 secretion of restimulated pleural space cells (Al Qaoud, Fleischer et al. 1998). In turn, µMT mice that have no mature B cells at all have similar worm burden until day 60 post infection in BALB/c mice (Martin, Saeftel et al. 2001) but no parasites at all in C57BL/6 mice (Le Goff, Lamb et al. 2002), suggesting a complex role of the different B cell subpopulations.

The role of granulocytes has been addressed as well. In semi resistant 129/SvJ mice absence of the eosinophil granule proteins eosin peroxidase (EPO) and major basic protein lead to higher worm burdens than in the WT littermates 28 days post infection. In addition to elevated IL-10 levels, these $EPO^{-/-}$ mice display increased amounts of the Th2 hallmark cytokine IL-5 secreted by $CD4^+$ T cells as well as a enhanced eosinophilia (Specht, Saeftel et al. 2006). Basophils in turn get activated only in a

narrow time frame of infection around eight weeks post infection and amplify the pre-present type 2 immune response, but do not serve a protective role in terms of reducing parasite burden (Torrero, Hubner et al. 2010). Finally, NK cells present a small subset of pleural space lymphocytes in naïve BALB/c mice but start to raise three weeks post infection. The depletion of these cells after onset of patency leads to elevated worm burden 63 and 84 days post infection without affecting granuloma formation (Korten, Volkmann et al. 2002).

1.2.2.3 Immune responses against microfilariae

The ability of naïve recipient mice to clear intravenously (i.v.)-injected Mf depends on the genetic background, as extensively studied by Hoffmann and colleagues (Hoffmann, Pfaff et al. 2001). The same study revealed the significance of the major histocompatibility complex (MHC)-II locus, since C57BL/6 MHC-II$^{-/-}$ mice loose their ability to clear the Mf. Moreover, the semi resistance of C57BL/6 mice is broken in those MHC-II$^{-/-}$ mice with mature parasites and onset of patency. Of note, in this study the presence of only one single implanted adult *L. sigmodontis* female (but not male) worm rendered all mice permissive, irrespective of their genetic background. This finding is of fundamental importance: it clearly proves that susceptibility to patency always has to be seen in context with the mechanisms induced by the filariae itself, which are obviously of a suppressing nature, thus facilitating survival and persistence of Mf in the host (Hoffmann, Pfaff et al. 2001). *Id est* mice strains that are commonly defined as susceptible to infection *could* reject Mf, if these suppressive mechanisms would be *absent*. However, several studies have revealed interesting findings of host immune responses against Mf. First, the onset of patency leads to fundamental changes with respect to these responses. Ribonucleic acid (RNA) levels of IFN-γ of restimulated splenocytes obtained from *L. sigmodontis*-infected BALB/c mice are strongly elevated within only a few days after the onset of patency (Taubert and Zahner 2001). The significance of IFN-γ in mounting immune responses against Mf in infected BALB/c mice is further underlined by the finding that IFN-γ$^{-/-}$ mice have increased numbers of circulating *L. sigmodontis* Mf compared to the WT littermates (Saeftel, Volkmann et al. 2001). Importantly this enhanced microfilaraemia was not due to the elevated adult parasite burden, since the increase of Mf load exceeded the increase of adult filariae about three times and furthermore it is known that only few inseminated female filariae can establish full microfilaraemia. Beside the Th1 hallmark cytokine IFN-γ the agents of Th2 immunity play a role in controlling microfilaraemia as well. In BALB/c mice absence of both IL-4 and IL-5 leads to dramatically increased Mf load in the peripheral blood (Volkmann, Bain et al. 2003). At least for IL-4 this role has been clearly confirmed in another study (Volkmann, Saeftel et al. 2001): in the typically semi resistant C57BL/6 moue strain IL-4$^{-/-}$ mice do have Mf. Due to missing patency in the WT littermates this result can not be interpreted with respect to responses against Mf, since the WT has no patent adults at all (Specht, Volkmann et al. 2004). In contrast to IL-4, the picture for IL-5 is not as clear, because one study found increased peripheral Mf load after treatment of BALB/c mice with recombinant IL-5 (Babayan, Read

et al. 2010), what is contradictory to the aforementioned study from Volkmann and colleagues. Since T cells are important producers of IL-5, their role in the response to Mf has been investigated: BALB/c mice depleted from $CD4^+$ T cells have a increased microfilarial burden. As in the mentioned experiments with $IFN\gamma^{-/-}$ mice (Saeftel, Volkmann et al. 2001), that importantly was not due to higher adult worm burden in this mice; increase in Mf load clearly outnumbered the increase of adult worms (Al Qaoud, Taubert et al. 1997). Finally, same correlation could be found in B1 cell lacking BALB/c.Xid mice (Al Qaoud, Fleischer et al. 1998).

1.2.2.4 Immune regulation

Filarial parasitic nematodes facilitate their survival by down regulation of host immune responses and this regulation includes inducing Treg cells and AAMOs, which suppress both Th1 and Th2 responses (Hoerauf, Satoguina et al. 2005; Maizels, Pearce et al. 2009). In the *L. sigmodontis* model both regulatory cell populations appear very fast within a few days after infection: as mentioned above, MOs of the alternatively activated phenotype proliferate rapidly at the site of infection (Jenkins, Ruckerl et al. 2011) and are able to render $CD4^+$ T cells hyporesponsive to the filariae (Taylor, Harris et al. 2006). Since MOs are important producers of IL-10, several studies focused on the role of IL-10 in suppressing the responses to *L. sigmodontis*. For example semi resistant C57BL/6 mice that had been rendered "permissive" by implanting adult *L. sigmodontis* filariae (see section above) clear i.v.-injected Mf faster when implantation was performed in $IL\text{-}10^{-/-}$ mice (Hoffmann, Pfaff et al. 2001). Very similar to this result is the observed reconversion to resistance of permissive C57BL/6 $IL\text{-}4^{-/-}$ mice upon additional genetic IL-10 deficiency (Specht, Volkmann et al. 2004). Finally, in the semi resistant FVB mouse strain (adults, but no embryogenesis) over expression of IL-10 in MOs increases parasite burden and converts the mice into a permissive state with peripheral Mf (Specht, Taylor et al. 2011).

As fast as MOs, $CD4^+$ $CD25^+$ forkhead box protein P3 $(foxp3)^+$ Treg cells appear; intensive studies of Taylor and colleagues have shed light how Treg cells act: at the site of infection, the local $CD4^+$ T-cell response to *L. sigmodontis* infection becomes rapidly biased towards a regulatory phenotype independently of the susceptibility of the monitored strain (BALB/c and C57BL/6) until seven days post infection (Taylor, van der Werf et al. 2008). This time frame is of interest, because it means that between arrival of larvae and Treg expansion only few days elapse. If Treg were depleted prior to infection, larval establishment was unaltered but parasite burden reduced after the onset of patency. Depletion was further associated with impaired embryogenesis and subsequent lower microfilaraemia.

The suppressive potential of *L. sigmodontis* is also impressively underlined by various studies that show suppression of host responses in the setting of co-infections or out-of-control immunity. For example in the setting of an malaria co-infection *L. sigmodontis*-derived IL-10 protects mice from developing malaria-associated pathology (Fernandez, Dubben et al. 2009). In the murine model of

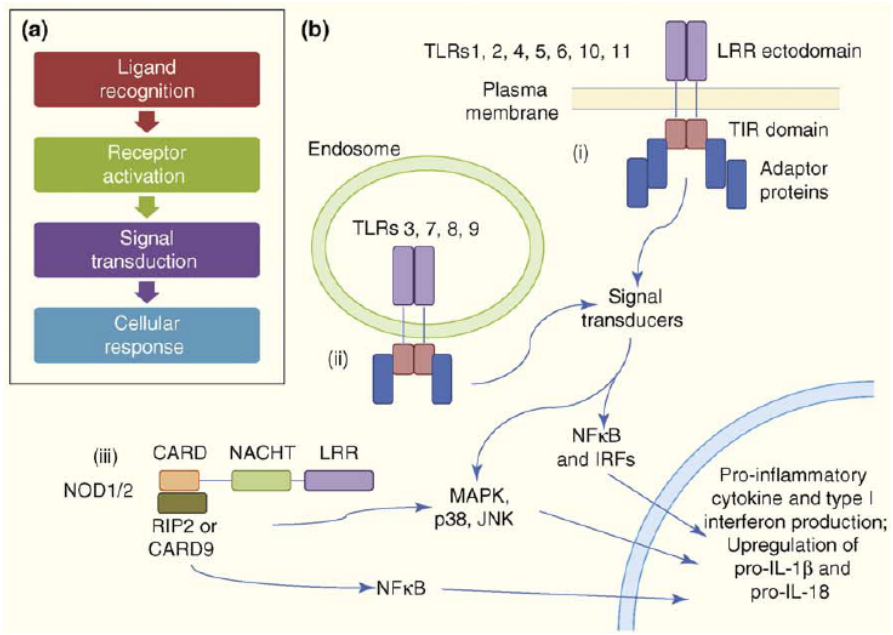


Figure 5. Signal transduction of TLRs and NLRs. (a) Scheme of receptor activation. (b) Simplified view of TLR and NLR signaling. The TLRs sense ligands via their LRR ectodomains (purple). Different adaptor proteins (blue) are recruited to the TIR domain (red) scaffold, creating a multiprotein complex located at either the plasma (i) or endosomal (ii) membrane. Signal transduction is propagated through the action of signal transducers, what finally leads to an increase in the NFκB level. NOD1/2 (iii) stimulates receptor oligomerization, followed by interactions between NOD1/2 and adaptor proteins, what also leads to higher NFκB or other pathways. The TLR, NOD1 and NOD2 signaling pathways lead to a cellular response that includes the production of pro-inflammatory cytokines and the upregulation of pro-IL-1b and pro-IL-18. Adapted from: Monie, T. P., C. E. Bryant, et al. (2009). "Activating immunity: lessons from the TLRs and NLRs." Trends Biochem.Sci. 34(11): 553-561

asthma, infection with *L. sigmodontis* suppresses all aspects of the asthmatic phenotype as Ag-specific Ig and pulmonary eosinophilia (Dittrich, Erbacher et al. 2008). Most recently it could be shown that natural infection with *L. sigmodontis* suppresses the humoral response to bystander antigen in the C57BL/6 host due to IL-10-mediated foxp3 induction in T cells (Hartmann, Haben et al. 2011).

1.3 Pathogen Recognition Through Pattern Recognition Receptors

1.3.1 Toll-like Receptors

Toll-like receptors are one class of the germ-line encoded PRRs of the innate immune system that sense conserved non-self structures called pathogen-associated molecular patterns (PAMPs) and eventually can direct adaptive immunity. They recognize pathogens by their ability to sense a variety of PAMPs as lipids, proteins, nucleic acids, lipopolysaccharides, and lipopeptides. The TLRs 3, 7, 8 and 9 are located in intracellular compartments (endoplasmatic reticulum, endosomes, lysosomes, and endolysosomes), all other TLRs are inserted in the plasma membrane (Fig. 5). Toll-like receptors are expressed not only in professional immune cells (APCs and immune effector cells), but also in other

cells, e.g. in epithelial cells. They are transmembrane proteins with an ectodomain that contains leucine-rich repeats (LRR) and an endodomain with TLR-unique Toll-IL-1 receptors (TIRs). Upon ligand recognition through these LRR domains, the receptor gets activated and transduces the signal via the TIRs to adapter proteins (e.g. to the myeloid differentiation factor 88 (MyD88)). After integration of a highly complex network of several further adapter proteins (see below), nuclear factors (e.g. nuclear factor κB, NFκB) act on gene encoding, thus inducing proinflammatory cellular responses (Kawai and Akira 2010).

The founding member of the TLR family is TLR4. It recognizes the lipopolysaccharide (LPS) of the bacterial outer cell membrane. The receptor only functions together with the co-receptor MD2. The TLR4/MD2 heterodimer alone is not able to sense LPS; a complex of a soluble LPS-binding protein and the membrane-standing CD14 has to deliver the LPS to the receptor (Akashi-Takamura and Miyake 2008). Another member of the family, TLR2, acts as a heterodimer together with TLR1 or TLR6. The heterodimer TLR1/2 recognizes triacetylated (Jin, Kim et al. 2007), TLR2/6 diacetylated lipopeptides (Kang, Nan et al. 2009). This specificity allows the sensing of a variety of bacterial, viral, fungal and parasite-related molecules, e.g. peptidoglycan (PGN) or lipoteichoic acid. Toll-like receptor 2 can cooperate with co receptors as CD36 (Hoebe, Georgel et al. 2005) or dectin-1 (Goodridge and Underhill 2008) that assist PAMP recognition. Both heterodimers integrate MyD88 in their downstream signaling and induce NFκB-dependent induction of proinflammatory cytokines (Kawai and Akira 2010). The MyD88 protein is a key adapter in down streaming of TLR signaling since it is part of all TLR pathways except TLR3. After ligand binding, a conformal change in the TLR receptors allows the recruitment of MyD88. Afterwards MyD88 itself assembles with a variety of proteins of several families (IL-1R-assiociated kinases, TNF receptor-associated factors and others) and rapidly activates NFκB. This activation leads then to expression of proinflammatory cytokines like TNF and IFN (O'Neill and Bowie 2007). At least TLR4 can be also activated MyD88-independently via TRIF (Toll-IL-1 receptor domain-containing adaptor protein inducing IFN-β), another adapter protein (O'Neill and Bowie 2007), and most recently, MyD88-independent activation has also been suggested for TLR2 (Gao, Qi et al. 2012). Besides the NFκB activation, MyD88 has also been shown to be involved in activation of other transcription factors of the IFN regulatory factor family and in IFN-γ signaling. Furthermore, the impaired response of $MyD88^{-/-}$ mice to IL-1 and IL-18 implicates an accordant role of the adapter (O'Neill and Bowie 2007).

Despite their ligand specificity TLRs can also be activated by nonspecific endogenous agonist; most of these agonists are products of cell injury or cell death or mediators of danger and stress (Kawai and Akira 2010). For example TLR4 gets activated by the antimicrobial peptide ß-defensin 2 (Biragyn, Ruffini et al. 2002). The molecular mechanisms behind this nonspecific (in terms of the main TLR specificity) co-activation are not well understood.

Finally, besides their important role in mounting an inflammatory response against pathogens, it has become apparent that TLR signaling has to be regulated to avoid pathogenesis or even recognition of self structures.

1.3.2 Nucleotide oligomerization domain receptors

The nucleotide-binding oligomerization domain (NOD) receptors belong to another class of PRRs, the cytoplasmatic nucleotide-binding domain and leucine rich repeat-containing receptors (NLRs, sometimes also referred as NOD-like receptors). The intracellular membrane standing NLRs have emerged to play central roles in innate and adaptive immunity, with the induction of the inflammasome referred as one of the main actions (Shaw, Lamkanfi et al. 2010; Kufer and Sansonetti 2011). Due to their localization NLRs are sensors of intracellular events. Importantly, NLRs respond not only to pathogens, but moreover to a general status of stress and danger (e.g. irradiation) and xenogenous compounds (e.g. environmental pollutants) (Benko, Philpott et al. 2008). Like the TLRs nearly all NLRs contain a C-terminal LRR domain that most likely also mediates the ligand binding, although doubtless evidence for direct binding is missing. In turn, heterogeneity is a feature of the N-terminal effector domains of the NLRs, which specify the function of the receptor (Kufer and Sansonetti 2011). Recent studies have shown, that the interaction between TLRs and NLRs can be crucial for mounting an effective immune response, e.g. in terms of acting together in an additive fashion (synergism) or even that way, that the signal of one is mandatory for the other one's action (licensing) (Kawai and Akira 2010).

The NOD2 receptor is as a cytosolic, membrane standing mediator of activation after recognition of the muramyl dipeptide (MDP) motif in the bacterial cell wall component PGN (Benko, Philpott et al. 2008), thereby complementing the task of soluble PGN receptors (Dziarski and Gupta 2010). Of note, it is not known if MDP is the direct ligand for NOD2 or if NOD2 enters the signaling cascade downstream to a not known MDP receptor (Sorbara and Philpott 2011). However, after its activation NOD2 is mainly consigned to induce a signal cascade that mounts in NFκB-induced proinflammatory responses (Fig. 5). These proinflammatory responses include cytokines but also other mediators of inflammation (e.g. antimicrobial ß-defensin or reactive oxygen species (ROS) (Sorbara and Philpott 2011)). Additionally, recent studies have added diverse functions as autophagy, antiviral responses and T cell activation to the activities of NOD2 (Shaw, Kamada et al. 2011).

1.3.3 Pattern recognition receptors and lymphatic filariasis

Since TLR2 and TLR4 recognize microbial membrane components, and *W. bancrofti* and *B. malayi* harbor obligate bacterial endosymbionts of the rickettsial *Wolbachia* genus (Taylor and Hoerauf 1999), attention has drawn to possible TLR-dependent immunity in filarial infections (Venugopal, Nutman et al. 2008). Indeed, human individuals carrying a definite TLR2 haplotype were more likely to present bancroftian filariasis (Junpee, Tencomnao et al. 2010). Furthermore, the

development of human pathology was shown to be associated with TLR2-dependent proinflammatory cytokine production (Babu, Anuradha et al. 2011). In mice, *in vitro* responses against *Wolbachia* bacteria are elicited predominantly through TLR2 with integration of MyD88 (Hise, Daehnel et al. 2007; Turner, Langley et al. 2009). Infection with *Rickettsia conorii* (an intracellular bacterium closely related to *Wolbachia*) is controlled *in vivo* by TLR4 (Jordan, Woods et al. 2008), suggesting also a possible role for this TLR in sensing rickettsial bacteria.

The role of TLR signaling in the murine model of LF was investigated only in two studies: the first showed that naturally LPS-nonresponsive C3H/HeJ mice have a similar parasite burden (Pfarr, Fischer et al. 2003), the second, that the control of vascular permeability by CCL17 after *L. sigmodontis* infection is dependent on TLR2 (Specht, Frank et al. 2011). The role of NOD2 and its impact on the disease has only been addressed in one single study: Babu and colleagues could show that peripheral blood mononuclear cells of asymptomatic infected LF individuals presented reduced NOD2 expression after restimulation with filarial extract compared to not stimulated cells, whereas patients with chronic pathology did not (Babu, Bhat et al. 2009).

1.4 Vaccination

The concept of vaccination is known for more than a millennium (when dried material of smallpox was inhaled in China). The Western world defines the famous small pox experiments of Edward Jenner in 1796 as the beginning of vaccination research. It is remarkable that despite this long experience the mechanisms (especially in terms of antigen-specific effector cell responses) that form the basis for a successful vaccination are still not fully understood (Boog 2009).

1.4.1 Principles and mechanisms of vaccination

The main scope of a vaccination is to introduce long time immunity against the disease-inducing pathogen. The responses induced by the vaccination should be similar to those produced in the natural infection but not expose the recipient to the disease and its possible complications. A good efficacy of a vaccine is based on stimulation of antigen-specific T and B cells and on establishment of immunological memory (Boog 2009; CDC 2011).

Two main forms of vaccines can be distinguished: (1) the vaccine contains the whole live but attenuated pathogen or (2) only an inactivated form, either complete or fractionated (e.g. peptides, nucleic acids). A general rule for the success of vaccination reads: “the more similar a vaccine is to the disease-causing form of the organism, the better the immune response to the vaccine”. That implies that attenuated vaccines are preferable to inactivated ones. However, severe reactions to attenuated vaccines may occur and handling and storage of those is challenging. In turn, vaccination with inactivated vaccines requires several doses, responses are in most cases only humoral and antibody

titer declines with time (CDC 2011). Nevertheless, comparing all pros and cons, vaccination is thought to be the best way of disease prevention and has consequently also been discussed for helminth infections (Bergquist and Lustigman 2010; Harris 2011; Hotez 2011).

Adjuvants are substances that are able to amplify the immunogenicity of vaccines. Although in animal models several adjuvants are used, for humans only the adjuvant alum has been approved. However, recent advances in adjuvant research have proposed new candidates (Tagliabue and Rappuoli 2008). As for the vaccination *per se* the mechanisms underlying the mode of action of adjuvants still remain to be settled in detail (Kwissa, Kasturi et al. 2007).

1.4.2 Vaccination against lymphatic filariasis

Based on the early success of vaccines against viral and bacterial infections, it was assumed that vaccines against parasites would be likewise. However, there is no vaccine against any filarial infection available so far (Bergquist and Lustigman 2010; Bethony, Cole et al. 2011). Addressing this issue, several animal immunization studies using different approaches have been performed. For example the group of Odile Bain used *L. sigmodontis* L3 to immunize mice and achieved up to 58% protection (Babayan, Attout et al. 2006). The protection was established within a few days after challenge infection and was characterized by L3-specific Ig, eosinophilia and high levels of IL-5. In the experiments of Dixit and colleagues (Dixit, Gaur et al. 2006), immunization of multimammate mice (*Mastomys coucha*) with a fraction of adult *B. malayi* extract reduced the recovery rate of adult worms by 85.7%. Other groups used recombinant peptides instead of complete extracts (Dabir, Dabir et al. 2008; Rathaur, Yadav et al. 2008; Vedi, Dangi et al. 2008; Makepeace, Jensen et al. 2009). The immunization with *B. malayi* heavy chain myosin for example generated a high level of protection against challenge infection in jirds and *M. coucha* (Vedi, Dangi et al. 2008). Different to all these setups, Anand and colleagues used a cocktail of *B. malayi* DNA to immunize mice and found high cytotoxcicity against *B. malayi* Mf in immunized mice, associated with specific Ig and increased IFN-γ responses (Anand, Murugan et al. 2008). However, none of these studies reported complete clearance of adults or peripheral Mf.

1.5 Aims and Objectives of this Work

Lymphatic filariasis is a major public health concern in many parts of the world. About 120 million people are already infected with the disease and hundreds of million remain at risk. The clinical manifestations of the infection can severely impair the quality of life of the infected individual. Although the administration of drugs has already improved the situation in terms of patient's pathology and spread of the disease, further efforts are needed to reach the WHO's objective to eliminate the disease.

In this thesis two major concerns are addressed:

(1) To further understand the fundamental interaction between the filarial worm and the host's immune system, experiments in the murine model of LF compared the response to infection between mice that are able to sense foreign pathogenic structures via a special class of immune receptors and adapter molecules (TLR2, TLR4, NOD2, and MyD88) to those with deficiencies in that signaling pathways. The *in vitro* capacity of APCs, the immunological events *in situ* in the skin and the local lymph nodes after infection, and the *in vivo* outcome after a natural infection were investigated.

(2) Spread of the disease depends on the transmission of the blood-circulating filarial larval stages. To reduce this larval burden in the blood of infected mice, a possible vaccination against this stage was investigated. Different immunization schemes were examined and the reaction to vaccination in terms of larval and adult parasite burden, as well as immunological changes and responses in the immunized host monitored during different time point of the infection.

2. MATERIAL AND METHODS

2.1 Mice

All mice *(Mus musculus)* were maintained, breaded and crossed in the animal facility of the Institute of Medical Microbiology, Immunology and Parasitology of the University Hospital Bonn, Germany. C3H/HeN WT mice (Janvier, La Genest St. Isle, France), C3H/HeJ mice deficient for LPS signaling via TLR4 (no genetic knock out, but a receptor modification - therefore hereafter termed $TLR4^{def}$), $C3H/TLR2^{-/-}$ mice (both from Andre Gessner, Institute of Medical Microbiology and Hygiene, University Regensburg, Germany), $C3H/TLR4^{def}TLR2^{-/-}$ mice (crossed from $TLR4^{def}$ and $TLR2^{-/-}$ mice), $C3H/MyD88^{-/-}$ mice (Andre Gessner), $C3H/MyD88^{+/-}$ mice (crossed from C3H/WT and $C3H/MyD88^{-/-}$ mice), C57BL/6 WT mice (Janvier), C57BL/6 $NOD2^{-/-}$ mice (Thomas Kufer, Institute of Medical Microbiology, Immunology and Hygiene, University Hospital Cologne, Germany), and BALB/c WT (Janvier) were maintained under specific pathogen-free conditions, according to animal welfare guidelines.

2.2 Parasites and infection

The *L. sigmodontis* strain was maintained by passage in cotton rats (*Sigmodon hispidus*) as follows: *O. bacoti* mites (Habedank 2002) serving as vectors were kept in animal bedding at 28°C and 80 % humidity in the dark and fed with murine neonates at least twice a week. Passage of Mf was performed by an over night blood meal on microfilaraemic (≥ 1,000 Mf / µl peripheral blood) cotton rats as final host. Bedding was collected and stored at above mentioned conditions for twelve days allowing development of Mf into infective L3. Prior to infection of experiment mice mites were checked for L3 content under the binocular.

Mice were infected either via the natural route, by injection of L3 or locally restricted as follows and only females were infected since male and female mice differ in their susceptibility to infection (Petit, Diagne et al. 1992):

For the natural infection infective L3 were transmitted during the blood meal of *O. bacoti* twelve days after the Mf transmitting blood meal on cotton rats. To ensure comparability of infection load, mice were always exposed to the same population of mites, e.g. in the same cage with mite-containing bedding. If mice were immunized, the natural infection was performed one week after last immunization injection.

For injection of a defined number of L3, mites containing the infective stages were opened under the binocular twelve days after the Mf transmitting blood meal on cotton rats, motile larvae collected, washed several times and adjusted to L3 / ml PBS as required by experimental design. Finally, L3 were injected subcutaneously (s.c.) into the footpad using a syringe with an 27¾ G injection needle. The sham-treated contra lateral footpad received PBS.

For the locally restricted natural infection (hereafter termed "locally restricted infection", illustrated in figure 13) twelve days after the Mf transmitting blood meal on cotton rats thirty mites for each mouse were collected in a 2 ml plastic tube, which was transferred into a 15 ml plastic tube, the latter one fixed at the foot of an anaesthetized (Ketanest®, Medistar, Germany / Rompun®, Bayer, Leverkusen, Germany) mouse. To avoid escape of mites the open end was sealed with wool. The locally restricted infection was performed for thirty minutes in the dark (what increased the bite frequency of mites). Control mites of the batch used for infection and mites used for locally restricted infection were controlled for L3 content under the binocular.

2.3 Ethics Statement

All animal experiments were approved by and conducted in accordance with guidelines of the appropriate committee (Landesamt für Natur, Umwelt und Verbraucherschutz, Köln, Germany).

2.4 Immunization

The Mf were purified from the peripheral blood of infected cotton rats on a Percoll gradient as described earlier (Chandrashekar, Rao et al. 1986). In brief, isoosmotic Percoll (Sigma-Aldrich, Munich, Germany) was prepared by mixing nine parts of Percoll (1.130 g/ml) with one part of 2.5 M D-sucrose (Sigma-Aldrich). Dilutions of the isoosmotic Percoll in 0.25 M sucrose were made to obtain 25% and 30% solutions. Each 3 ml of both gradient dilutions were layered in 15 ml tubes. The peripheral blood was diluted 1:2 in phosphate-buffered saline (PBS) (PAA, Cölbe, Germany) and layered on top. Then the tubes were centrifuged at 400 x g for 30 min at room temperature (RT) without brakes. Recovered Mf (between the 25% and 30% layers) were washed twice with PBS and 1 x 10^5 viable Mf each mouse were injected to immunize. Injection was performed via different administration routes as indicated in the corresponding text. For Mf attenuation, 1 x 10^6 Mf / ml were irradiated 40 min at 140 kV and 25 mA (corresponding to an absorbed dose of 400 Gray (Gy)) in the RS2000 biological research irradiator (Rad Source Technologies Inc., Suwanee, GA, USA). Irradiation was performed in the Facility of Experimental Therapy of the University Hospital Bonn. Microscopic analysis of irradiated Mf confirmed their paralysis by monitoring their motility (see Fig. 24, page 45).

For IVM (Merck, Darmstadt, Germany) treatment after immunization, mice received 800 µg per kg mouse body weight. For immunization with alum (Thermo Scientific, Bremen, Germany), Mf were mixed with adjuvant drop-wise to a final adjuvant concentration of 25% and then mixed on an automatic shaker at 1,000 rpm for 30 min. After this procedure Mf were morphologically intact, but amotile and motility was not reconstituted after 96 hours at 37°C and 5% CO_2, suggesting that Mf were not viable anymore (data not shown). Directly before injection, suspension was intensively vortexed again. Control mice received alum alone. Directly before injection, suspension was intensively vortexed. For sham injection control mice received alum or PBS. In all experiments,

second and third immunization injections were performed two and three weeks after initial immunization.

2.5 Bone marrow-derived antigen-presenting cells, human embryonic kidney cells and *in vitro* stimulation

Mice were sacrificed with Isofluran (Abbott, Wiesbaden, Germany). Femur and tibia were separated from tissue, opened at both ends and bone marrow cells flushed out with 5 ml 1 x cold PBS using a 5 ml syringe with a $27^3/_4$ G needle. Cells were washed twice with PBS, filtered through sterile 41 µm gaze (Bueckmann, Moenchengladbach, Germany), and incubated in 10 cm culture dishes (VWR, Darmstadt, Germany) with IMDM medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% penicillin / streptomycin, 1% non-essential amino acids, 1% sodium bicarbonate, and 1% sodium pyruvate (all PAA) at 37°C and 5% CO_2. Differentiation was induced with 2 µg / ml GM-CSF (PeproTech, Hamburg, Germany). After four days confluent cells were collected, washed, transferred back into dishes and all cells provided with new medium and GM-CSF. After another four days of incubation the supernatant was discarded and adherent cells were detached by incubating with 2 mM disodium ethylenediaminetetraacetic acid (EDTA, Carl Roth, Karlsruhe, Germany) for 10 min at 37°C and 5% CO_2. All procedures were conducted under sterile conditions. Flow cytometry using antibodies against CD11c and F4/80 (eBioscience, Frankfurt, Germany) revealed a mixture of MOs and DCs (data not shown).

Human embryonic kidney (HEK) cells transfected with either TLR1/2 (HEK293/hTLR1/hTLR2), TLR2/6 (HEK293/hTLR2/hTLR6, both kindly provided by Dr. Clifford Harding (Case Western Reserve University, Cleveland, OH, USA) or TLR3 (HEK293/hTLR3 flag, kindly provided by Dr. Amy Hise (Case Western Reserve University) were permanently cultured in L-glutamine-containing high glucose DMEM Medium (PAA) supplemented with 10 % FCS, 10 µg / ml blasticidin (Invitrogen) and 10 µg / ml ciprofloxacin (VWR). Passage and stimulation as described for BMdAPCs.

For preparation of complete (Ls) and *Wolbachia*-free (Ls-tet, (Arumugam, Pfarr et al. 2008)), *L. sigmodontis* antigen, freshly isolated adult worms were rinsed in sterile PBS before being mechanically minced. Insoluble material was removed by centrifugation of 300 x g for 10 min at 4°C. Protein concentrations of crude extracts were determined using the Advanced Protein Assay (Cytoskeleton, Denver, USA). All procedures were conducted under sterile conditions.

For *in vitro* stimulation 100 µl with 2.5 x 10^5 cells were incubated in a 96 well plate (Greiner BioOne, Frickenhausen, Germany) together with 100 µl medium alone or (depending on experiment) with 100 ng / ml tripalmitoyl-*S*-glycerylcysteine (P3C), 100 ng / ml polyinosinic-polycytidylic acid (pIC,), 100 ng / ml ultra pure LPS (all from InvivoGen, Toulouse, France), 10 µg / ml cytosinphosphatidylguanine (CpG-ODN1668, TIB Molbiol, Berlin, Germany), 25 µM MDP (InvivoGen), 50 - 100 µg / ml Ls or Ls-tet (depending on results of control titration with BMdAPCs,

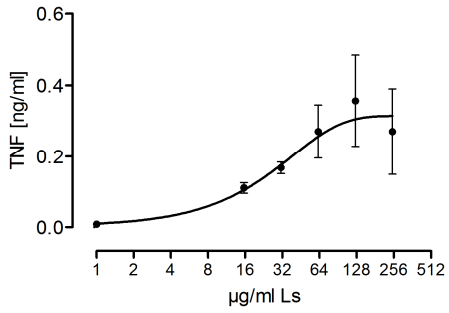


Figure 6. Exemplary titration of the filarial *L. sigmodontis* extract used for stimulations. The TNF secretion 24 hours after stimulation of $2.5*10^5$ BMdAPCs with *L. sigmodontis* crude extract (Ls) of given concentration. Data presented as mean ± SEM. The black curve shows the Boltzman regression.

see figure 6). As MyD88-deficient BMdAPCs poorly responded to LPS they where additionally stimulated with human saliva containing a mixed bacterial suspension. After 24 hours supernatants were taken and TNF and IL-6 quantified per enzyme-linked immunosorbent assay (ELISA) according to manufacturers instructions (DuoSet, R&D, Wiesbaden, Germany).

2.6 Mf Monitor

Peripheral blood was taken from the tail vein and directly transferred into 500 µl Hinkelmann solution (0.5% [wt/vol] eosin Y, 0.5% [wt/vol] phenol (both Merck) and 0.185% [vol/vol] formaldehyde (Sigma-Aldrich) in distilled water). After centrifugation (5 min, 250 x g) supernatant was discarded and pellet suspended in 20 µl PBS before counting under the microscope (40 x). For monitoring Mf in the pleural space, 50 µl from 1 ml pleural cavity lavage (see below) were added to 450 µl Hinkelmann solution and treated as described for peripheral Mf.

2.7 Autopsy and pleural space lavage

Mice were euthanized with Isofluran (Abbott, Wiesbaden, Germany). Thoracic cavity was prepared free of skin and the pleural space was opened by inserting two holes at the upper area of both sides of the diaphragm. Lavage was performed with 10 ml of cold PBS and lavage containing cells and filariae put on ice until further usage.

2.8 Assessment of parasite burden, developmental stage, size and gender

Fourth stage larvae were separated from cells by 15 min sedimentation of the pleural space lavage, adults by using tweezers for direct isolation by eyes. Developmental stage and gender was defined microscopically by analysis of the anterior buccal capsule and designation of male spiculae and female vulva (Fig. 7). Length of L4 was determined with the microscope analysis software Diskus 4.6 (Technisches Buero Hilgers, Koenigswinter, Germany), adult length by manual measurement, respectively.

2.9 Embryogram

Each single female worm was transferred into 80 µl PBS, cut into several pieces and embryonic stages squeezed out of the uterus using a 1.5 ml plastic tube and plastic pestle. The

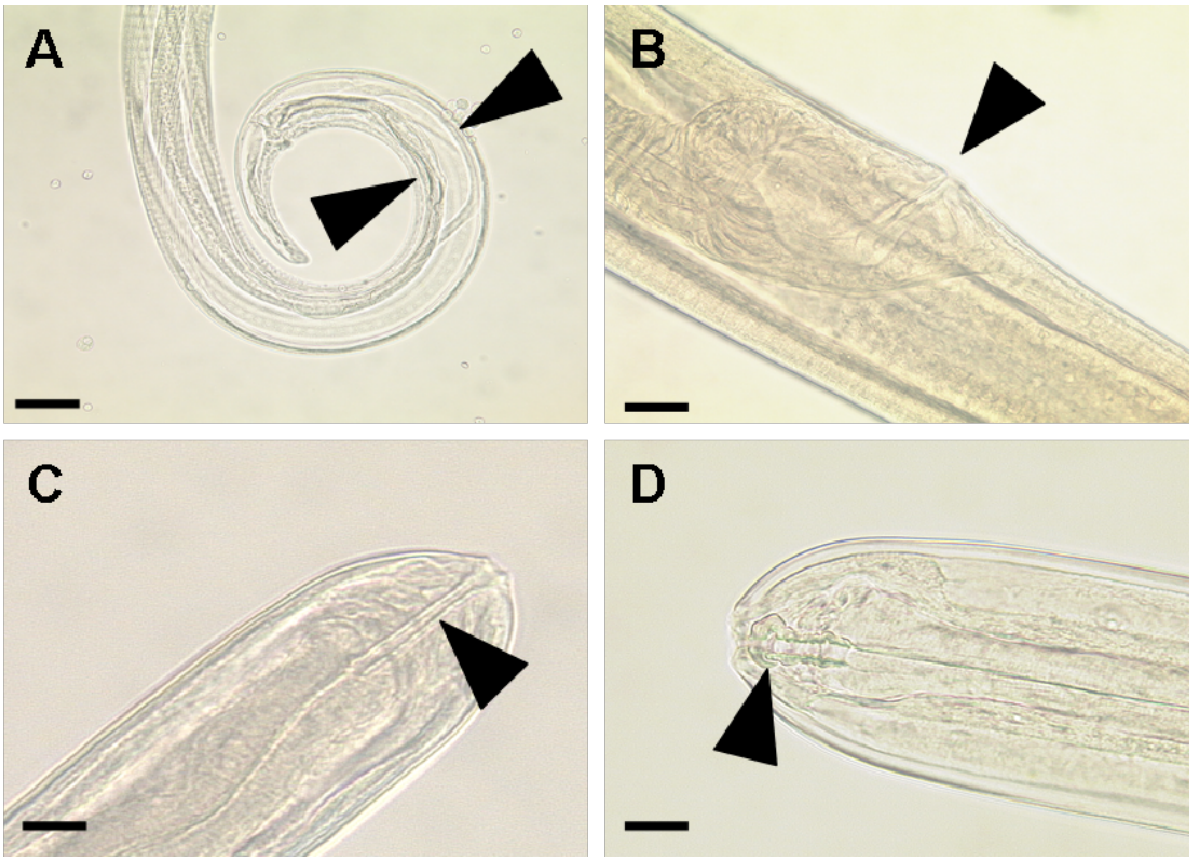


Figure 7. Anatomical features to determine *L. sigmodontis* larval stage and gender. (A) Male genital structure with spiculae (arrows). (B) Female genital structure with vulva (arrow). (C) L4 stage: anterior end with buccal capsule (arrow). (D) Adult stage: anterior end with buccal capsule (arrow). Light microscope pictures, 40-fold magnification, micron bars 50 µm.

embryonic stages were stained by adding 20 µl Hinkelmann solution and 10 µl of this preparation (thus 10 % of total uterine content of each analyzed female filariae) were analyzed under the microscope (the rather small error bars indicate data generated this way are representative). If possible, three female worms of each mouse were investigated. However, as worm burden is already reduced in immunized mice and not all female worms are inseminated or start embryogenesis, total numbers of analyzed filariae varied between experiments, as indicated in the corresponding figure captions. Damaged females, females without any embryonic stages or with only oocytes were excluded from analysis (percentage of those did not differ between the groups, data not shown).

2.10 Polymerase chain reaction

The TLR and MyD88 genetic knockdowns were checked by PCR as follows: tissue for genetic determination was received by tail capping; the end of the tail (approx. 0.5 cm) was transferred into a 0.5 ml reaction tube (Eppendorf, Hamburg, Germany) and incubated over night at 55°C in 180 µl DirectPCR-Tail solution (PeqLab, Erlangen, Germany) together with 20 µl proteinkinase K (Qiagen, Hilden, Germany). After the enzymatic reaction was stopped by 45 min incubation at 85°C, hairs and residing tissue were pelleted by a short time centrifugation at low g, and the DNA-containing sample

either stored at -20°C or directly used for genotyping PCR. Each reaction (15 µl) for PCR as follows: 6.1 µl A. dest, 3 µl 5 x Q-Solution, 1.5 µl 15 nM $MgCl_2$ 10 x PCR Puffer, 1.5 µl Yellowsub©, 0.1 ml deoxyribonucleosidtriphosphate (all Qiagen), 1.2 µl primer (Invitrogen, Karlsuhe, Germany), 0.1 µl Taq-Polymerase (Qiagen). Wild type and knockdown PCR for MyD88 and TLR2 could be done in the same reaction, PCR for TLR4 WT and knockdown had to be performed in separate reactions, due to differing reaction temperatures. 5'→ 3' primer sequences and PCR reaction conditions as follows:

TLR2 forward primer sequence: CTT CCT GAA TTT GTC CAG TAC AGG G
TLR2 reverse primer sequence: TCG ACC TCG ATC AAC AGG AGA AGG G
TLR2 knock down primer sequence: GGG CCA GCT CAT TCC TCC CAC TCA T
With 35 cycles at [10sec 94°C – 30sec 60°C – 180sec 72°C]

TLR4 WT forward primer sequence: GGT GGC TGT GGA GAC AAA AT
TLR4 WT reverse primer sequence: CAG CCA GCA ATA AGT ATC AGG
With 45 cycles at [30sec 95°C – 30sec 55°C – 180sec 72°C]
TLR4 mutation forward primer sequence: GTT TAG AGA ATC TGG TGG CTG TGG AGA C
TLR4 mutation reverse primer sequence: TGT TGG GTC GTT TGT TCG GAT CCG TCG
With 35 cycles at [15sec 95°C – 30sec 60°C – 180sec 72°C]

MyD88 forward primer sequence: TGG CAT GCC TCC ATC ATA GTT AAC C
MyD88 reverse primer sequence: GTC AGA AAC AAC CAC CAC CAT GC
MyD88 neo primer sequence: ATC GCC TTC TAT CGC CTT GAC G
With 40 cycles at [10sec 94°C – 30sec 60°C – 180sec 72°C]

Products were separated by size using a corresponding DNA ladder (New England BioLabs, Frankfurt, Germany) in a 1% agarose gel (MBI Fermentas, St. Leon-Rot, Germany) together with 2.5 µl RedSafe (iNtRON Biotechnology, Freiburg, Germany) at 125 V. Pictures were made in a UV chamber (Biometra, Goettingen, Germany).

2.11 CD4 depletion

In vivo clearance of $CD4^+$ T cells was achieved by intraperitoneally (i.p.) injection of 100 µg of a depleting antibody against CD4 (BioLegend, Fell, Germany) at days 0, 6 and 12 after infection. Depletion was controlled by flow cytometry (see also Fig.20).

2.12 Cromolyn treatment

Mast cell stabilizer disodium cromoglycate (hereafter termed "cromolyn") dissolved in 0.9% saline (both Sigma-Aldrich) was injected into mice i.p. at 8 mg / kg body weight 48 and 24 hours before and 24 hours after infection.

2.13 Flow cytometry

Prior to staining unspecific protein binding sites were saturated by taking up 250,000 – 500,000 (depending on experimental setup) cells in 100 µl PBS containing 1% bovine serum albumin (BSA). The Fc receptor was additionally blocked with rat IgG or incubation with anti-CD16/32 (clone 2.4G2, BD Pharmingen) at 1 µg / ml for 15 minutes. Staining with antibodies against surface marker was performed on ice in the dark for 30 minutes with titrated antibody concentrations. Afterwards cells were washed twice with 2 ml 1% BSA-PBS and finally taken up in 200 µl for analysis at the cytometer. For intracellular staining of foxp3 500,000 cells were initially fixed and permeabilized with 2 ml fixation / permeabilization buffer for 30 minutes on ice. Afterwards cells were washed in 1 ml permeabilization buffer and intracellular Fc receptors blocked as described above. Incubation with anti-foxp3 was performed in permeabilization buffer for 30 minutes in the dark on ice. After intracellular staining the cells were washed twice with permeabilization buffer and finally resuspended in PBS for measurement at the cytometer. All measurements were performed at a BD Canto 1 (BD Biosciences, Heidelberg, Germany) as follows. The flow speed was adjusted to ≤ 5,000 events per second. The data were measured at logarithmic scale, integrating the forward and sideward scatter (FSC, SSC) width and height and plotted bi-exponential. Duplets were excluded by their scatter properties. Fluorescence sensitivity and signal-to-noise ratio were optimized by positioning the highest fluorescence events in the last decade of the respective dot plot. An initial live / dead staining using probidium iodide and 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxy-fluorescein-acetoxymethyl ester (BCECF) revealed that less than 10% of PLECs are dead after the thoracic cavity lavage procedure, therefore exclusion of dead cells was skipped in most stainings (data not shown).

2.14 Analysis of lymph node-immigrating skin dendritic cells

Skin DC emigration from footpad skin was induced by skin painting with 100 µl of 1:1 aceton / dibutyl phthalate (DBP) (both Merck, Darmstadt, Germany). The contra-lateral foot served as control and was treated with acetone alone. Skin DC labeling was achieved by skin painting with 0.5% fluorescine-thioisocyanate (FITC, Sigma-Aldrich, Munich, Germany) in acetone 30 minutes after applying the contact allergen. Forty-eight and 72 hours later mice were sacrificed, and the popliteal lymph node (LN) surgically removed, mechanically opened and digested within 500 µl DNAse [180 U / ml] / collagenase [1 mg / ml] (both Sigma-Aldrich, Munich, Germany) for 20 minutes at 37°C. Afterwards cells were washed with medium, filtered through gaze, counted and analyzed per flow cytometry. Lymph node T- and B-cells were quantified by staining with antibodies against

CD3ε-FITC (clone 145-2C11, BD Pharmingen, Heidelberg, Germany) and CD19-PE (clone eBio1D3, eBioscience). Immigration of skin DCs into the local draining LNs (ldLN) was analyzed according to Stutte et al (Stutte, Jux et al. 2008) using MHC-II-PE (clone M5/114.15.2, eBioscience) and CD24a-PerCP-Cy5.5 (clone M1/69, eBioscience).

2.15 *Ex vivo* restimulation

Ex vivo stimulations were performed at days 22, 70 or 90 post infection. Red blood cell lysis of pleural space exudate cells was done by 5 min incubation with trisammoniumchloride (Sigma-Aldrich). Cells were washed twice with PBS, filtered through sterile 41 µm gaze (Bueckmann, Moenchengladbach, Germany), and 2.5 x 10^5 cells per well in RPMI medium (supplemented with 10% FCS, 1% L-glutamine, 1% penicillin / streptomycin, 1% non-essential amino acids, 1% sodium bicarbonate, and 1% sodium pyruvate (all PAA)) were stimulated with 5 µg / ml concanavalin A (Sigma-Aldrich) in a 96 well plate (Greiner Bio-One, Frickenhausen, Germany) or the respective crude worm extracts for 72 hours at 37°C and 5% CO_2.

2.16 Mf-specific Ig and cytokine ELISA

Systemic Mf-specific IgG was measured from plasma of mice directly before immunization injections (28, 14 and 7 days before infection) and in weekly intervals after infection (days 0, 7 and 14). Blood was taken submandibular from anesthetized (Ketanest®, Medistar, Ascheberg, Germany / Rompun®, Bayer, Leverkusen, Germany) mice. After centrifugation (5 min at 6,500 x g), supernatant was taken and stored at -20°C until further usage. Microfilarial-specific Ig of the pleural space was measured from the lavage at days 22 and 70 post infection. Polysorb enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 µg / ml of Mf crude extract in PBS at pH 9. After blocking 1 hour with 1% BSA-PBS (all from PAA), plates were washed with PBS containing 0.05% Tween20® (Sigma-Aldrich) and incubated for 2 hours at RT with either 50 µl of pleural space lavage or a 1:1 to 1:100,000 plasma dilution and only values from the linear binding phase were used for analysis. After another washing step, biotinylated detection Ig (BD Pharmingen, Heidelberg, Germany) was added as recommended by the manufacturer. After a final wash, alkaline phosphatase-conjugated streptavidin (Roche, Grenzach, Germany) was added; tetramethylene benzidine (Carl Roth, Karlsruhe, Germany) was used as substrate. The reaction w as stopped by adding 1 M H_2SO_4 (Merck) and the absorbance (optical density, OD) was measured using a SpectraMax 340pc ELISA plate reader at 450 nm and the SOFTmax Pro software (both Molecular Devices, Ismaning, Germany).

Interferon-γ (eBioscience, Frankfurt, Germany), IL-5 (BD Pharmingen), IL-13, MO inflammatory protein 2 (MIP)-2α, CCL5, granzyme B, eotaxin-1 and eotaxin-2 (R&D Systems, Wiesbaden, Germany) ELISA of the pleural space lavage and the supernatants of restimulated cells were performed according to manufacturer's instructions.

2.17 Statistics

Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA), using the Student's unpaired t-test for parametric, the Mann Whitney t-test (u-test) for non parametric data, and Welch's correction for data sets with different variances. Variances were tested with the D'Agostino & Pearson omnibus normality test. If three or more groups were analyzed one-way ANOVA for parametric and Kruskal-Wallis test for nonparametric data were performed (with Bonferroni's multiple comparison post hoc test). Although parasite burden in some experiments are not Gaussian distributed, interexperimental comparison shows that parasite burden is a normally distributed element; therefore all parasite burdens are analyzed with the t-test (< three groups) and one-way ANOVA (≥ three groups). Microfilaraemia and Ig kinetics were analyzed with regular two-way ANOVA and Bonferroni's multiple comparison post hoc test. Integer values for parasite burden (adult and all larval stages) are shown as median ± range and were mentioned whole number rounded in the text. Al other data are shown as means ± standard error of mean (SEM). *P*-values ≤ 0.05 were considered as significant.

2.18 Referencing methods

References were managed with the EndNote X4 bibliography software (Thomson Reuters, New York, USA), licensed for the Institute of Medical Microbiology, Immunology and Parasitology of the University Hospital Bonn. Citations in this work are apostrophized; all other adopted contents are referenced.

2.19 Text processing

Word and tabular processing was done with Microsoft Office 2003 (Microsoft, Unterschleißheim, Germany), licensed for the Institute of Medical Microbiology, Immunology and Parasitology of the University Hospital Bonn. This work is written, grammar and spell-checked in American English.

2.20 Funding

This work was funded by the European Commission: Enhanced Protective Immunity Against Filariasis (EPIAF), agreement number 242131. This work was further supported by the bilateral programme ''PROCOPE'', which is financed through the German Academic Exchange Service (DAAD, with funds from the BMBF), and the Ministere des Affaires Etrangeres. This work was also supported by funds from the German Research Foundation (Sonderforschungsbereich 704, Teilprojekt A1, A3).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

3. RESULTS

3.1 The Role of the Pattern Recognition Receptors TLR2, TLR4, NOD2 and the Adapter Molecule MyD88 in the Murine Model of Lymphatic Filariasis

Due to the presence of *Wolbachia*, TLRs have been under investigation concerning their role during human helminth infection. In order to investigate aspects of this function, we carried out *in vitro*, *in situ* and *in vivo* experiments with C3H WT mice and those that have deficiencies in the signaling of either TLR2 or TLR4, both of these TLRs, or the downstream adapter molecule MyD88. The role of the NOD2 receptor was investigated as well.

3.1.1 The proinflammatory response of BMdAPCs against *L.sigmodontis* depends on the endosymbiotic *Wolbachia* bacteria

To investigate whether professional APCs from C3H mice are able to respond to *L. sigmodontis* (in terms of antigen recognition and proinflammatory cytokine response), and whether this response depends on filarial components or on the *Wolbachia*, C3H WT BMdAPCs were stimulated *in vitro* with medium alone, with *Wolbachia*-containing or *Wolbachia*-depleted filarial crude *L. sigmodontis* extract. Control stimulations for TLRs were used as well (P3C for TLR2; LPS for TLR4). The proinflammatory cytokine response was analyzed 24 hours later in the culture supernatants (Fig. 8A, F). Interestingly, the proinflammatory TNF and IL-6 secretion that was seen for Ls-stimulated WT BMdAPCs (both $P<0.001$ compared to medium control) was greatly diminished when stimulation was conducted with *Wolbachia*-depleted Ls-tet (Ls vs. Ls-tet $P<0.001$). The control stimulations with P3C, LPS and CpG strongly induced TNF and IL-6 secretion (all $P<0.001$).

3.1.2 Recognition of endosymbiotic *Wolbachia* depends on MyD88 and the TLR2/6 heterodimer, but not on TLR3, TLR4 and NOD2

To examine whether TLR signaling is required for the observed recognition of *Wolbachia*, BMdAPCs of MyD88$^{-/-}$ mice were stimulated, since this adapter molecule is integrated in all TLR signaling pathways except TLR3; in most of these pathways MyD88 is even indispensable for downstream transducing of TLR signals (Kawai and Akira 2010). As figures 8B and G illustrate, the MyD88$^{-/-}$ BMdAPCs did not respond to Ls with proinflammatory TNF and IL-6. But, since the MyD88$^{-/-}$ BMdAPCs responded only weakly to LPS via the TRIF path of activation, which functions independently of MyD88 (O'Neill and Bowie 2007), this absent cytokine secretion might be due to dead cells, rather than lacking MyD88 signaling. However, the TNF and IL-6 response to human saliva (Sal vs. Med; $P<0.001$) clearly indicated that the cells were alive and had the potential to respond (Fig. 8B, G).

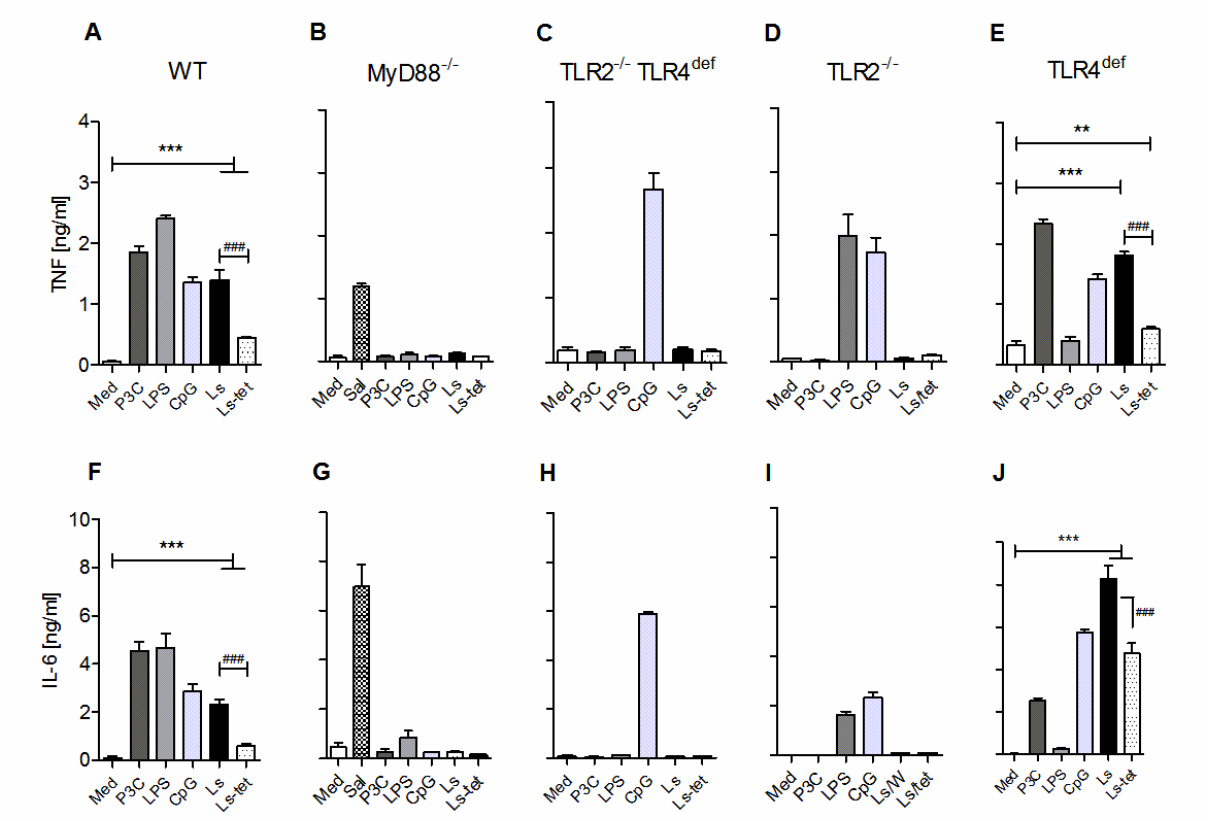


Figure 8. Proinflammatory cytokine responses of C3H BMdAPCs to *L. sigmodontis* crude extract are mainly dependent on the endosymbiotic *Wolbachia* bacteria and require MyD88 and TLR2, but not TLR4 signaling. $2.5*10^5$ BMdAPCs of C3H WT mice (A, F) or mice deficient for signaling via MyD88 (B, G) or TLR (C – E, H - J) were incubated with either medium alone or with 100 ng / ml of the TLR2 ligand P3C, 100 ng / ml of the TLR4 ligand LPS, 10 µg / ml of the TLR9 ligand CpG, 100 µg / ml complete filarial (Ls) or 100 µg / ml *Wolbachia*-depleted filarial extract (Ls-tet). The MyD88$^{-/-}$ BMdAPCs were additionally stimulated with human multipathogenic saliva (Sal). After 24 hours supernatants were taken and analyzed by ELISA for TNF (A – E) and IL-6 (F – J) secretion. Representatives of at least two independent experiments for each genotype. Statistical analysis with ANOVA and Bonferroni post hoc test, the mean ± SEM is shown, **, *** indicate $P<0.01$, $P<0.001$, respectively. Statistical significances of stimulation with TLR control ligands compared to the medium control (all $P < 0.05$) are not depicted in the graph for a better view.

Having shown that MyD88 is necessary for response to Ls, we wanted to decipher which of the upstream TLR receptors might be integrated in this sensing, because literature shows conflicting data concerning whether TLR2 or TLR4 recognizes *Wolbachia* (Brattig, Bazzocchi et al. 2004; Hise, Daehnel et al. 2007). Therefore, BMdAPCs of TLR2$^{-/-}$TLR4^{def} mice that lack a functional response to LPS and all TLR2 ligands were stimulated (Fig. 8C, H). Strikingly, for TLR2$^{-/-}$TLR4^{def} mice the proinflammatory response to Ls was completely abolished whereas the cells responded well to the control ligand CpG (via TLR9) with TNF ($P<0.001$) and IL-6 ($P<0.01$) production. Next, to elucidate which of the both investigated TLRs is involved in sensing *Wolbachia*, BMdAPCs defective for signaling either via TLR2 (TLR2$^{-/-}$) or via TLR4 (TLR4^{def}) were stimulated. Cells from TLR2$^{-/-}$ mice responded apparently weaker (all $p<0.001$) to Ls and Ls-tet (Fig. 8D, I). In turn, TLR4 was

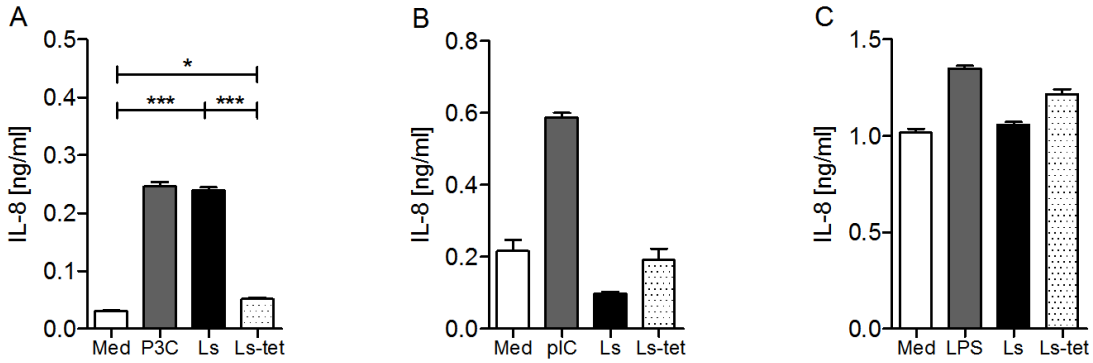


Figure 9. *Wolbachia* components of *L. sigmodontis* crude extracts are sensed by HEK cells through the TLR2/6 heterodimer, but not through TLR3 and TLR4. $2.5*10^5$ HEK cells transfected with either TLR2/6 (A), TLR3 (B) or TLR4 (C) were incubated with 100 ng / ml P3C, LPS or pIC, 100 µg / ml of complete filarial (Ls) or 100 µg / ml of *Wolbachia*-depleted (Ls-tet) extract or left unstimulated (Med). After 24 hours supernatants were taken and analyzed by ELISA for reporter cytokine IL-8. Single experiments. Statistical analysis with ANOVA and Bonferroni post hoc test, the mean ± SEM is shown. *, *** indicate $P<0.05$, $P<0.001$, respectively. Statistical significance of stimulation with TLR control ligands compared to the medium control (all $P < 0.05$) are not depicted in the graph for a better view.

dispensable for responding to complete filarial antigen and *Wolbachia*-depleted extract (Fig. 8E, J, compared to medium all $P<0.001$).

To confirm these results we stimulated HEK cells transfected with different TLRs, which secrete IL-8 after receptor triggering. As figure 9 shows, only TLR2 but not TLR3 and TLR4 competent HEK cells responded with IL-8 after stimulation with *Wolbachia*-containing filarial extract (Med vs. Ls $P<0.001$). As seen for BMdAPCs this response depended mainly on the endosymbiotic *Wolbachia* as it was greatly decreased for the *Wolbachia*-free extract (Fig. 9A; Ls vs. Ls-tet $P<0.001$).

Toll-like receptor 2 signaling occurs via the two different heterodimers TLR1/2 and TLR2/6 (Kawai and Akira 2010). To elucidate which of both heterodimers senses the *Wolbachia* we stimulated TLR1/2 or TLR2/6 transfected HEK cells and found the TLR2/6 but not the TLR1/2 heterodimer to be sensitive for stimulation with complete filarial extract (Fig. 10; Med vs. Ls $P<0.001$). Again, this response was due to the endosymbiotic bacteria, since no IL-8 was secreted after stimulation with *Wolbachia*-depleted extract.

A growing field of studies revealed a complex cross talk between TLRs and NODs, either enhancing (Netea, Ferwerda et al. 2005; Magalhaes, Fritz et al. 2008; Petterson, Jendholm et al. 2011) or dampening (Watanabe, Kitani et al. 2004; Dahiya, Pandey et al. 2011; Hedl and Abraham 2011; Tsai, Huang et al. 2011) each other. To elucidate a possible integration of NOD2 signaling in response to filarial *L. sigmodontis* crude extract, murine $NOD2^{-/-}$ BMdAPCs were stimulated. As figure 11 shows, both, the WT and the $NOD2^{-/-}$ BMdAPCs responded with proinflammatory TNF and IL-6 after exposure to the complete filarial extract (all $P<0.001$). Proinflammatory responses again depended on the endosymbiotic *Wolbachia* bacteria, since they were abolished for *Wolbachia*-depleted extract (all

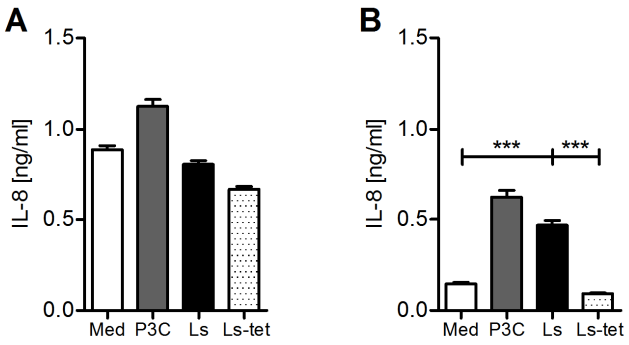


Figure 10. *Wolbachia* components of *L. sigmodontis* crude extract are sensed by HEK cells through the TLR2/6 but not the TLR1/2 heterodimer. 2.5*10^5 HEK cells transfected with either TLR1/2 (A) or TLR2/6 were incubated with 100 ng / ml P3C, 100 µg / ml complete filarial (Ls) or 100 µg / ml *Wolbachia*-depleted (Ls-tet) filarial extract or left unstimulated (Med). After 24 hours supernatants were taken and analyzed by ELISA for the reporter cytokine IL-8. Single experiment. Statistical analysis with ANOVA and Bonferroni post hoc test, the mean ± SEM is shown. *** indicates $P<0.001$. Statistical significance of stimulation with TLR control ligand P3C compared to the medium control (both P in (A) and (B) < 0.001) is not depicted in the graph for better view.

$P<0.001$). A further increase of cytokine secretion after additional treatment with the NOD2 activator MDP was only observed for WT BMdAPCs both for TNF (Ls vs. Ls + MDP; $P<0.001$) and IL-6 ($P<0.001$) but not for NOD2$^{-/-}$ BMdAPCs, confirming the phenotype of these cells.

Taken together, the data of the *in vitro* stimulation of BMdAPCs and HEK cells presented in this section show, that the proinflammatory response to *L. sigmodontis* crude extract depends on signaling via the TLR2/6, but not the TLR1/2 heterodimer, and, that the endosymbiotic *Wolbachia* are the main provocative driver of this response. Furthermore, signaling depends on the downstream adapter molecule MyD88, but does not need cooperative help from TLR3, TLR4 and NOD2.

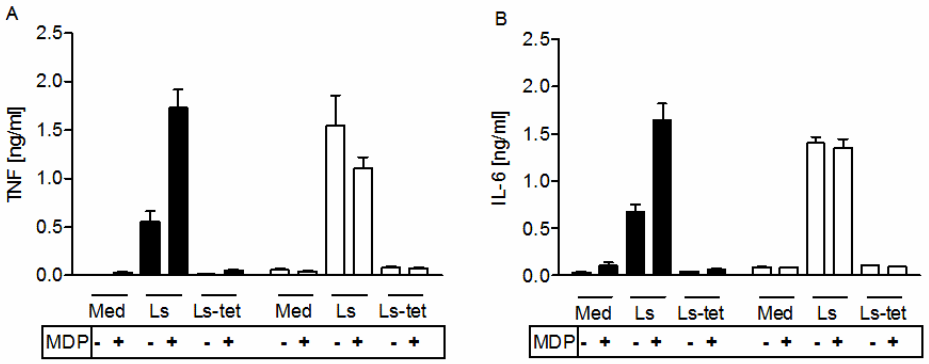


Figure 11. The NOD2 receptor is dispensable for the proinflammatory responses of C57Bl/6 BMdAPCs to *L.sigmodontis* antigenic preparations. 2.5*10^5 BMdAPCs of C57Bl/6 WT (black bars) or NOD2$^{-/-}$ mice (white bars) were incubated with either medium alone (Med), with 100 µg / ml complete filarial (Ls) or 100 µg / ml *Wolbachia*-depleted filarial (Ls-tet) extract or with 25 µM MDP as indicated. After 24 hours supernatants were taken and analyzed by ELISA for TNF (A) and IL-6 (B) secretion. Representatives of three independent stimulations are shown. Statistical analysis with ANOVA and Bonferroni post hoc test, the mean ± SEM is shown, for *P*-values see text in result section 3.1.2, page 30f.

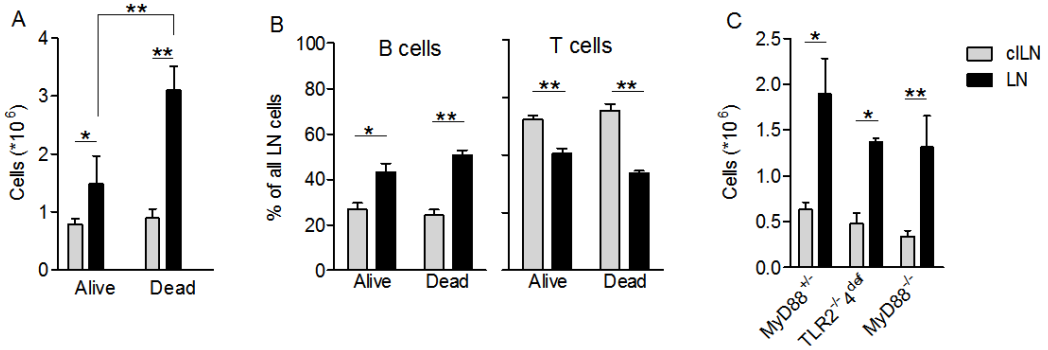


Figure 12. Cellular responses in the local draining lymph nodes are elicited independently of MyD88, TLR2 and TLR4 after skin challenge with *L. sigmodontis*. Absolute cell number of the local draining (popliteal) lymph node (LN, black bars) eight days after injection of either 25 living or dead third stage larvae ((A, B), L3) or complete *L. sigmodontis* crude extract (C) into the footpad of anaesthetized C3H mice of indicated genotype; the contra lateral lymph node (clLN, grey bars) received PBS. (B) Percentage of B and T cells in the lymph node, defined by flow cytometry. Representatives of at least two independent experiments with at least three mice each group. Statistical analysis with ANOVA and Bonferroni post hoc test, the mean ± SEM is shown. *, ** indicate *P*-values < 0.05, 0.01, respectively.

3.1.3 L3-induced proliferation in the local draining lymph node is independent of TLR2, TLR4 and MyD88

The results of the BMdAPC experiments showed that APCs are able to recognize and process *Wolbachia* components of *L. sigmodontis* extract under *in vitro* conditions. Therefore we addressed whether this capacity influences the immune responses at the site of larval entry into the host, since the skin is populated with sentinel DCs, namely dermal DCs (dDCs) and Langerhans cells (LCs), which both express TLRs (Kupper and Fuhlbrigge 2004). When dDCs and LCs encounter antigen they emigrate from the skin and home to the ldLN, where they eventually induce germinal centre formation (Kupper and Fuhlbrigge 2004). Since filarial L3 enter the host's body through the skin, we hypothesized that incoming L3 may be recognized by skin-resident DCs, which than could eventually induce lymphocyte proliferation in the ldLN. We therefore injected 25 L3 intradermaly into one footpad of a C3H WT mouse and compared the responses to the contra lateral LN (clLN; PBS was injected as control) eight days after this injection, a time point when antigen-experienced cell proliferation in the ldLN has already started (Kupper and Fuhlbrigge 2004). Figure 12A shows that injection of both living ($P<0.05$) and dead ($P<0.01$) L3 led to increased number of cells in the ldLN compared to the contra lateral one that drains the sham-treated footpad. The cell number within the clLN was similar to that of naïve LNs (data not shown). The cellular increase was lower when living L3 were injected ($P<0.01$). In order to identify which cell populations contribute to the observed increase in cell numbers, we next analyzed B and T cells in the corresponding LN and found that after injection of live L3 increase of B cells was more prominent than that of T cells (Fig. 12B).

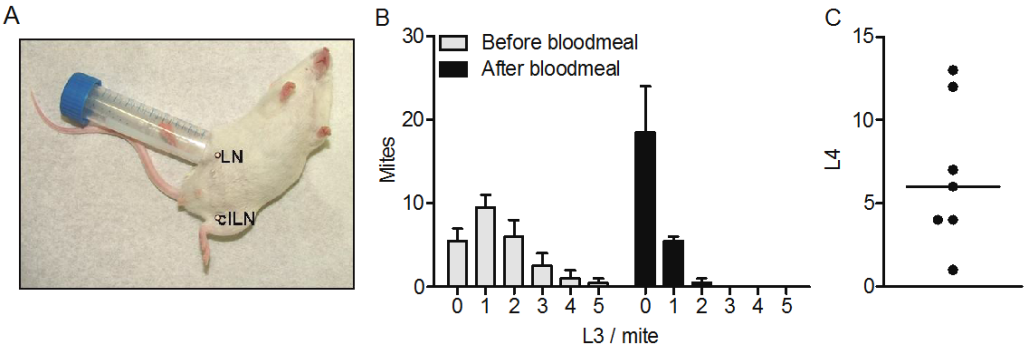


Figure 13. The model of a locally restricted natural infection establishes a full infection with larvae in the pleural space of the thoracic cavity. (A) Mites with infective third stage larvae (L3) were forced to bite in a restricted area on the footpad of an anaesthetized mouse, as described in detail in materials and methods, section 2.2, page 20. The position of the local draining (LN) and the contra lateral lymph node (clLN) are indicated. (B) Number of L3 in mites before (control mites of the same batch, grey bars,) and after (black bars) locally restricted infection. Representative of three independent experiments with at least 20 mites per group. Bar graphs with the median ± range. (C) Number of fourth larval stage (L4) burden of C3H mice 15 days after locally restricted natural infection with 30 mites, single experiment with seven mice, the line represents the median.

Next, to elucidate the role of TLR signaling in the observed induction of cellular increase, mice deficient in TLR2 and TLR4 or in MyD88 signaling were challenged with filarial extract. However, compared to the MyD88-competent control mice (in this experiment $MyD88^{+/-}$ mice) the cellular increase was not reduced in the $TLR2^{-/-}TLR4^{def}$ or in the $MyD88^{-/-}$ mice (Fig. 12C).

3.1.4 The locally restricted natural *L.sigmodontis* infection does not induce enhanced skin DC immigration into the local draining lymph node

Injection of L3 does not exactly mirror the *in situ* situation of L3 transfer through the skin during the natural blood meal of the arthropod vector. Therefore, a new model of infection overcoming this disadvantage was established. Figure 13A illustrates how *L. sigmodontis*-infected *O. bacoti* were restricted to have their blood meal at one hind leg, the contra lateral hind leg thereby serving as control in the same mouse. Larval transfer into the host was confirmed by counting remaining L3 in mites after their blood-meal (Fig. 13B). Establishment of infection was confirmed by isolating larvae from the pleural space 15 days after the locally restricted natural infection (Fig. 13C). With this model the *in situ* fate of skin DCs after local *L. sigmodontis* challenge was elucidated: after a locally restricted infection the skin was painted with the fluorescent dye FITC and the immigration of that way labeled MHC-II^{hi} skin DCs (according to (Stutte, Jux et al. 2008)) into the ldLN was assessed by flow cytometry 48 and 72 hours after infection; time points when skin DC enter the ldLN after antigen uptake (Kissenpfennig, Henri et al. 2005). As positive control for skin DC emigration the skin was treated with the contact allergen DBP. The dot plot of figure 14A shows how incoming skin DCs were identified by their expression of surface MHC-II expression and their FITC signal. As figure 14B shows, 48 hours after infection analysis of skin DCs in WT control mice revealed similar numbers

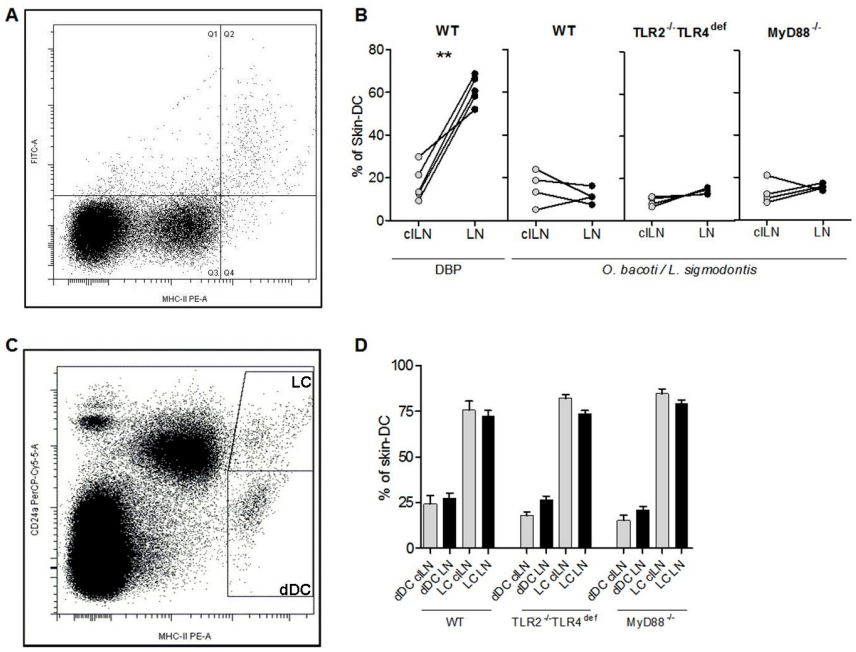


Figure 14. Skin DC immigration into the local draining lymph node is not altered after locally restricted natural infection. (A) Dot plot showing the staining strategy for identifying skin-emigrated DCs in the local draining (popliteal) lymph node using FITC skin painting and a PE-conjugated antibody against MHC-II. (B) Before-after graphs showing percentage of FITC positive MHC-II^{hi} skin DCs in the local draining lymph node of mice of indicated genotype 48 hours after locally restricted natural infection, clLN indicates lymph node of the sham-treated contra lateral footpad. As control, emigration of skin DCs was provoked with DBP / acetone treatment (DBP). Four mice each group, data were analyzed with Student's t-test. ** indicates $P<0.01$. (C) Dot plot showing how skin-emigrated dermal DCs (dDC) and Langerhans cells (LC) were defined via flow cytometry using MHC-II-PE and CD24a-PerCP-Cy5.5. (D) Percentage of lymph node immigrated dDCs and LCs 48 hours after locally restricted natural infection (LN, black bars) or sham treatment (clLN, grey bars). Statistical analysis with ANOVA and Bonferroni post hoc test, bar graphs with mean ± SEM are shown. (A – D) Data of a single experiment with four mice each group.

compared to steady state condition, although mites had transferred almost all L3 into the skin, as confirmed by counting remaining L3 in the mites after the blood meal (data not shown). Similar to WT mice, the TLR2$^{-/-}$TLR4^{def} and MyD88$^{-/-}$ mice did not present significantly increased numbers of skin DCs. Importantly, increased immigration of FITC^{+} skin DCs 48 hours after control DBP treatment into the ldLN compared to the clLN without DBP was observed ($P<0.01$). In addition to the absolute cell number, composition of incoming DCs 48 hours after local challenge infection was similar in ldLN and the contra lateral one with a dDC:LC ratio of about 1:3 in all groups (Fig. 14D). Results for immigration 72 hours after local infection were similar (data not shown).

The data shown here reveal that the L3 transmitted by the vector during its blood meal do not elicit substantial skin DC immigration into the ldLN. However, the positive control substance DBP is a very strong inductor of skin-DC emigration; more experiments should be carried out, which titrate the number the amount of mites and larvae and analyze more cell types in order to monitor even subtle changes in cell migration patterns. Absolute number and composition of B and T cells were not altered

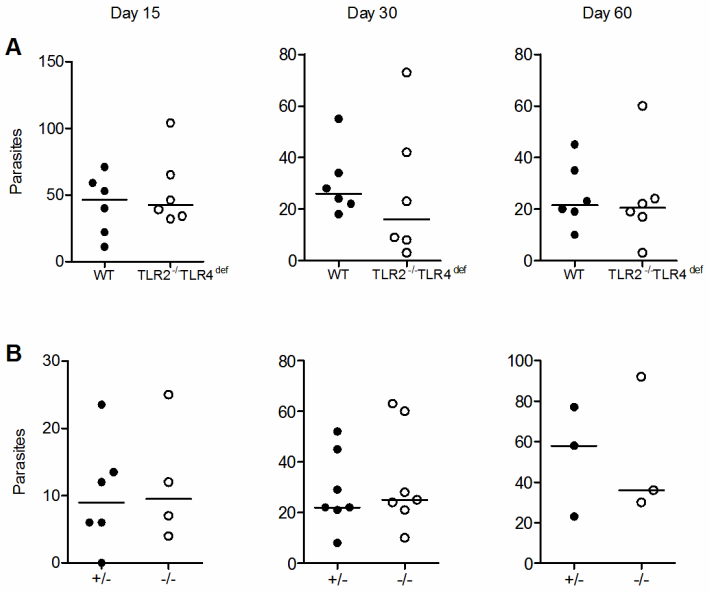


Figure 15. C3H TLR2$^{-/-}$TLR4^{def} and C3H MyD88$^{-/-}$ mice combat natural *L. sigmodontis* infection as successful as the control littermates (A) C3H/WT and C3H/TLR2$^{-/-}$TLR4^{def} mice or (B) C3H/MyD88$^{+/-}$ and MyD88$^{-/-}$ mice were naturally infected and worm burden in the pleural space was determined at indicated time points after infection. One representative of at least two independent experiments is shown, each circle represents worm burden of one mouse. Statistical analysis with the Student's t-test, the line represents the median.

as well. This is in contrast to the cellular increase after intradermal injection of L3 or filarial crude extract and emphasizes the differences between both models. It could also been shown that the observed cellular increases after the injection of L3 is independent of TLR2, TLR4 and MyD88, signaling components which have been shown to be important for the sensing of Ls under *in vitro* conditions, as shown in section 3.1.2.

3.1.5 Natural *L. sigmodontis* infection of C3H WT and CH TLR2$^{-/-}$TLR4^{def} mice

Next, we wanted to investigate whether TLR play a role during later time points of *L. sigmodontis* infection, i.e. when filariae have reached the pleural space. TLR signaling of immune cells may influence the course of infection independently of the observed absent response towards the incoming L3 in the skin and the ldLN. We therefore assessed the worm burden of naturally infected WT and signaling-deficient mice. As figure 15A shows, number of parasites in mice deficient in TLR2 and TLR4 signaling were similar to those in WT mice on day 15 post infection, and this comparable worm burden was still found on days 30 and 60 post infection. Also, MyD88$^{-/-}$ mice that lack all TLR pathways except TLR3 showed no significant differences (Fig. 15B).

Despite the similar parasite burden in WT, MyD88$^{-/-}$ and TLR2$^{-/-}$TLR4^{def} C3H mice we investigated the cellular responses at the site of infection in order to detect possible differences that could play a role in the later course of infection. Therefore, we extensively analyzed the cellular content of the pleural space lavage (tables 1 and 2), but found no consistent cellular differences in

A. Percentages (% of all cells)

		d15 (1st Exp.)		d15 (2nd Exp.)		d15 (3rd Exp.)		d30 (1st Exp.)	
		Value	*P* =Value	Value	*P* =Value	Value	*P* -Value	Value	*P* -Value
DC	WT	0.12 ± 0.02 N=6	0.0983	nd		4.17 ± 0.54 N=8	0.0033	nd	
	TLR2-/-TLR4def	0.17 ± 0.01 N=3		nd		2.24 ± 0.21 N=9		nd	
MO	WT	48.76 ± 2.70 N=6	0.0085	26.53 ± 1.94 N=6	0.1466	31.65 ± 1.74 N=8	0.3204	23.80 ± 2.42 N=3	0.7817
	TLR2-/-TLR4def	34.35 ± 0.72 N=3		21.06 ± 2.98 N=5		29.43 ± 1.33 N=9		25.27 ± 4.32 N=3	
BC	WT	14.29 ± 2.12 N=6	0.0028	20.34 ± 1.54 N=6	0.0106	34.62 ± 2.97 N=8	0.0033	9.63 ± 0.92 N=3	0.0229
	TLR2-/-TLR4def	29.14 ± 1.75 N=3		31.55 ± 3.38 N=5		20.32 ± 2.82 N=9		13.73 ± 0.67 N=3	
TC	WT	5.67 ± 0.96 N=6	0.5222	2.86 ± 0.45 N=6	0.0085	8.99 ± 0.87 N=8	< 0.0001	6.10 ± 0.61 N=3	0.6352
	TLR2-/-TLR4def	6.70 ± 0.89 N=3		5.89 ± 0.84 N=5		3.51 ± 0.53 N=9		5.67 ± 0.58 N=3	
EO	WT	28.22 ± 4.52 N=6	0.3682	29.21 ± 2.34 N=6	0.0123	11.71 ± 2.24 N=8	< 0.0001	54.73 ± 1.64 N=3	0.1495
	TLR2-/-TLR4def	21.73 ± 2.05 N=3		19.93 ± 1.62 N=5		35.40 ± 2.69 N=9		48.53 ± 3.07 N=3	

B. Absolute (*10^6)

		d15 (1st Exp.)		d15 (2nd Exp.)		d15 (3rd Exp.)		d30 (1st Exp.)	
		Value	*P* =Value	Value	*P* =Value	Value	*P* -Value	Value	*P* -Value
Cells	WT	4.483 ± 0.8384 N=6	0.3931	5.217 ± 0.5809 N=6	0.0036	4.875 ± 0.7042 N=8	0.001	36.83 ± 6.521 N=3	0.4797
	TLR2-/-TLR4def	5.667 ± 0.6566 N=3		8.593 ± 0.6809 N=6		10.05 ± 1.021 N=9		30.83 ± 4.106 N=3	
DC	WT	0.004988 ± 0.001069 N=6	0.0388	nd		0.2028 ± 0.03334 N=8	0.7481	nd	
	TLR2-/-TLR4def	0.00974 ± 0.001571 N=3		nd		0.2144 ± 0.01553 N=9		nd	
MO	WT	2.116 ± 0.3662 N=6	0.7587	1.395 ± 0.2097 N=6	0.3841	1.579 ± 0.3042 N=8	0.0144	8.939 ± 2.312 N=3	0.6438
	TLR2-/-TLR4def	1.939 ± 0.2016 N=3		1.723 ± 0.3037 N=5		3.005 ± 0.4032 N=9		7.649 ± 1.155 N=3	
BC	WT	0.5865 ± 0.1036 N=6	0.0008	1.074 ± 0.1542 N=6	0.0019	1.626 ± 0.2164 N=8	0.3574	3.429 ± 0.3161 N=3	0.1789
	TLR2-/-TLR4def	1.639 ± 0.1676 N=3		2.532 ± 0.3215 N=5		1.960 ± 0.2695 N=9		4.181 ± 0.3369 N=3	
TC	WT	0.2330 ± 0.04276 N=6	0.0792	0.1557 ± 0.03241 N=6	0.0013	0.3999 ± 0.03042 N=8	0.3894	2.239 ± 0.4136 N=3	0.2648
	TLR2-/-TLR4def	0.3711 ± 0.03775 N=3		0.4643 ± 0.06340 N=5		0.3435 ± 0.05334 N=9		1.700 ± 0.04439 N=3	
EO	WT	1.403 ± 0.3741 N=6	0.7874	1.491 ± 0.1388 N=6	0.616	0.6199 ± 0.1928 N=8	0.0002	19.96 ± 3.071 N=3	0.2936
	TLR2-/-TLR4def	1.243 ± 0.2306 N=3		1.598 ± 0.1514 N=5		3.643 ± 0.5554 N=9		15.08 ± 2.626 N=3	

A. Percentages (% of all cells)

		d30 (2nd Exp.)		d30 (3rd Exp.)		d30 (4th Exp.)		d60	
		Value	*P* -Value	Value	*P* -Value	Value	*P* -Value	Value	*P* -Value
DC	WT	0.36 ± 0.04 N=6	0.0299	1.04 ± 0.026 N=6	0.6649	4.59 ± 0.53 N=7	0.6156	nd	
	TLR2-/-TLR4def	0.58 ± 0.07 N=6		1.118 ± 0.1698 N=6		4.30 ± 0.21 N=7		nd	
MO	WT	16.47 ± 0.77 N=6	0.0923	29.68 ± 2.87 N=6	0.9923	35.10 ± 1.07 N=7	0.0598	83.78 ± 0.82 N=5	0.1203
	TLR2-/-TLR4def	13.42 ± 1.45 N=6		29.63 ± 4.18 N=6		32.23 ± 0.88 N=7		76.78 ± 3.63 N=6	
BC	WT	11.95 ± 0.62 N=6	0.0323	5.83 ± 0.43 N=6	0.0021	13.17 ± 1.61 N=7	0.0910	nd	
	TLR2-/-TLR4def	17.53 ± 2.16 N=6		11.53 ± 1.32 N=6		16.57 ± 0.91 N=7		nd	
TC	WT	2.53 ± 0.36 N=6	0.9899	2.08 ± 0.34 N=6	0.0976	2.80 ± 0.27 N=7	0.1436	nd	
	TLR2-/-TLR4def	2.52 ± 0.37 N=6		3.03 ± 0.40 N=6		3.60 ± 0.43 N=7		nd	
EO	WT	44.13 ± 2.34 N=6	0.5193	36.48 ± 3.82 N=6	0.3105	46.72 ± 1.92 N=7	0.4812	16.50 ± 1.04 N=5	0.1943
	TLR2-/-TLR4def	46.52 ± 2.70 N=6		42.07 ± 3.58 N=6		44.75 ± 1.92 N=7		21.60 ± 3.18 N=6	

B. Absolute (*10^6)

		d30 (2nd Exp.)		d30 (3rd Exp.)		d30 (4th Exp.)		d60	
		Value	*P* -Value	Value	*P* -Value	Value	*P* -Value	Value	*P* -Value
Cells	WT	23.67 ± 2.235 N=6	0.0213	52.00 ± 3.621 N=6	0.0218	43.96 ± 6.812 N=7	0.1615		
	TLR2-/-TLR4def	38.17 ± 4.823 N=6		37.12 ± 4.118 N=6		33.10 ± 2.558 N=7			
DC	WT	0.08765 ± 0.01459 N=6	0.0128	0.3092 ± 0.03134 N=6	0.8147	2.045 ± 0.3604 N=7	0.1229	nd	
	TLR2-/-TLR4def	0.2219 ± 0.04189 N=6		0.3284 ± 0.07330 N=6		1.417 ± 0.1161 N=7		nd	
MO	WT	3.875 ± 0.3755 N=6	0.2163	15.66 ± 1.999 N=6	0.0309	15.77 ± 2.828 N=7	0.0989	15.41 ± 1.911 N=5	0.8633
	TLR2-/-TLR4def	5.169 ± 0.9061 N=6		10.30 ± 0.7539 N=6		10.57 ± 0.6671 N=7		15.91 ± 2.043 N=6	
BC	WT	2.874 ± 0.3658 N=6	0.0008	1.707 ± 0.1916 N=6	0.0099	5.348 ± 0.5857 N=7	0.9459	nd	
	TLR2-/-TLR4def	6.274 ± 0.6176 N=6		3.378 ± 0.4906 N=6		5.394 ± 0.3181 N=7		nd	
TC	WT	0.6063 ± 0.1122 N=6	0.0732	0.5878 ± 0.09044 N=6	0.1287	1.167 ± 0.1283 N=7	0.9290	nd	
	TLR2-/-TLR4def	0.8942 ± 0.08999 N=6		0.9320 ± 0.1872 N=6		1.187 ± 0.1710 N=7		nd	
EO	WT	10.54 ± 1.207 N=6	0.0406	10.63 ± 1.411 N=6	0.4719	21.03 ± 4.062 N=7	0.1995	3.010 ± 0.3712 N=5	0.1574
	TLR2-/-TLR4def	18.32 ± 3.082 N=6		13.09 ± 2.977 N=6		15.06 ± 1.687 N=7		4.483 ± 0.8095 N=6	

Table 1. Flowcytometric analysis of pleural exudate space cells after natural infection of WT and TLR2-/-TLR4def C3H mice. Mice were infected with *L. sigmodontis* and pleural exudate cells were analyzed 15, 30 and 60 days post infection for percentages (A) and absolute numbers (B) of dendritic cells (DC), macrophages (MO), B-cells (BC), T-cells (TC) and eosinophils (EO). Staining was performed according to standard protocols with fluorochrome-conjugated antibodies to the surface markers F4/80, SiglecF, CD3ε, CD11c, and CD19. "nd" indicates that no data are available for the given time point. Data for each single experiment are shown. Statistical analysis for each population was performed with the Student's t-test.

A. Percentages (% of all cells)							
		d15		d30		d30	
		Value	*P*-Value	Value	*P*-Value	Value	*P*-Value
DC	Ctrl	0.78 ± 0.10 N=6	0.2131	nd		0.45 ± 0.07 N=4	0.7908
	MyD88$^{-/-}$	0.63 ± 0.05 N=6		nd		0.43 ± 0.06 N=4	
MO	Ctrl	58.88 ± 2.15 N=6	0.1760	23.80 ± 2.42 N=3	0.7646	56.75 ± 11.64 N=4	0.6889
	MyD88$^{-/-}$	62.30 ± 0.95 N=6		25.77 ± 5.64 N=3		50.73 ± 8.37 N=4	
BC	Ctrl	52.70 ± 5.01 N=6	0.0323	9.63 ± 0.92 N=3	0.1567	4.40 ± 0.96 N=4	0.6752
	MyD88$^{-/-}$	37.57 ± 3.46 N=6		6.73 ± 1.39 N=3		3.93 ± 0.49 N=4	
TC	Ctrl	nd		6.10 ± 0.61 N=3	0.5260	1.95 ± 0.64 N=4	0.1834
	MyD88$^{-/-}$	nd		5.33 ± 0.92 N=3		2.98 ± 0.25 N=4	
EO	Ctrl	14.23 ± 3.88 N=6	0.3705	54.73 ± 1.64 N=3	0.6699	36.78 ± 11.23 N=4	0.7044
	MyD88$^{-/-}$	18.93 ± 3.18 N=6		57.53 ± 5.87 N=3		42.30 ± 8.16 N=4	

B. Absolute (*10^6)							
		d15		d30		d30	
		Value	*P*-Value	Value	*P*-Value	Value	*P*-Value
Cells	Ctrl	14.08 ± 2.557 N=6	0.7060	36.83 ± 6.521 N=3	0.4797	31.59 ± 10.59 N=4	0.3608
	MyD88$^{-/-}$	12.92 ± 1.578 N=6		30.83 ± 4.106 N=3		19.93 ± 5.185 N=4	
DC	Ctrl	0.1149 ± 0.03190 N=6	0.3814	nd		0.1467 ± 0.05587 N=4	0.2956
	MyD88$^{-/-}$	0.08308 ± 0.01382 N=6		nd		0.0790 ± 0.01922 N=4	
MO	Ctrl	8.140 ± 1.264 N=6	0.9276	8.939 ± 2.312 N=3	0.3920	14.82 ± 2.162 N=4	0.0685
	MyD88$^{-/-}$	7.996 ± 0.8939 N=6		6.470 ± 1.135 N=3		8.987 ± 1.497 N=4	
BC	Ctrl	6.935 ± 0.8301 N=6	0.0692	3.429 ± 0.3161 N=3	0.0069	1.267 ± 0.3785 N=4	0.2236
	MyD88$^{-/-}$	4.804 ± 0.6379 N=6		1.641 ± 0.1487 N=3		0.7236 ± 0.1308 N=4	
TC	Ctrl	nd		2.239 ± 0.4136 N=3	0.0877	0.6172 ± 0.2991 N=4	0.9967
	MyD88$^{-/-}$	nd		1.304 ± 0.04000 N=3		0.6156 ± 0.2056 N=4	
EO	Ctrl	2.460 ± 1.029 N=6	0.9578	19.96 ± 3.071 N=3	0.5021	14.62 ± 7.673 N=4	0.5763
	MyD88$^{-/-}$	2.523 ± 0.5570 N=6		15.74 ± 4.840 N=3		9.543 ± 3.872 N=4	

Table 2. Flowcytometric analysis of pleural exudate space cells after natural infection of wild type (WT) and MyD88-deficient (MyD88$^{-/-}$) C3H mice. Mice were infected with *L. sigmodontis* and pleural exudate cells were analyzed 15 and 30 days post infection for percentages (A) and absolute numbers (B) of dendritic cells (DC), macrophages (MO), B-cells (BC), T-cells (TC) and eosinophils (EO). Staining was performed according to standard protocols with fluorochrome-conjugated antibodies to the surface markers F4/80, SiglecF, CD3ε, CD11c, and CD19. "nd" indicates that no data are available for the given time point. Statistical analysis for each population was performed with the Student's t-test.

terms of absolute cell number and percentages of DCs, MOs, eosinophils, and TCs. The higher percentage of B cells in TLR2$^{-/-}$TLR4^{def} mice thirty days post infection was not consistently mirrored by a higher absolute numbers of B cells at the site of infection.

The experiments presented in this section show that establishment of *L. sigmodontis* in the pleural space of the thoracic cavity is not enhanced when TLR2 and TLR4 or MyD88 signaling is hampered.

3.1.6 NOD2$^{-/-}$ mice are more susceptible to *L. sigmodontis* infection than the wild type littermates

In contrast to TLR2, the *in vitro* experiments with BMdAPCs did not reveal a essential role for NOD2 in the response to filarial extracts. However, since *in vitro* systems have fundamental limitations and can never mimic the complex situation *in vivo* in the infected host (O'Neill and Wilson 2004) we infected C57BL/6 WT and C57BL/6 NOD2$^{-/-}$ mice naturally and assessed the parasite burden. As figure 16A shows, the NOD2$^{-/-}$ mice contained more L4 than the WT mice already 15 days

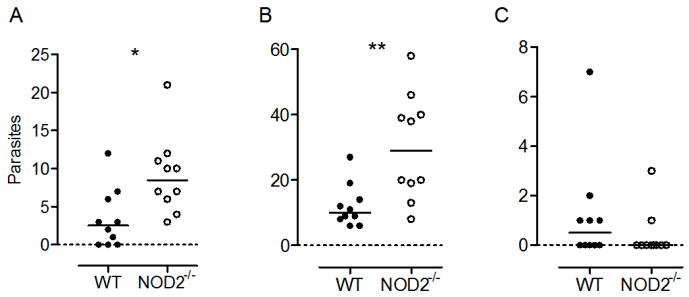


Figure 16. C57BL/6 NOD2$^{-/-}$ mice have higher worm burden. Mice were infected via the natural route and worm burden in the pleural space was assessed at days 15 (A), 30 (B) and 60 (C) post infection. Representatives of three (A), four (B) or two (C) independent experiments, each symbol represents the worm burden of one mouse. Statistical analysis with Student's t-test, the line represents the median. *, ** indicate P<0.05, 0.01, respectively.

post infection. In two of three independent experiments this increased parasite load reached statistical significance (figure 16A shows one representative; P<0.05), and a trend could be observed in a third one (P=0.1812). A combined analysis taking into account the experiment to experiment variations was carried out using ANOVA and revealed that the increase in parasite loads was indeed due to the lack of NOD2 (P<0.05). Thirty days post infection differences in parasite burden were even higher with a significant increase of parasite burden in four out of five independent experiments; figure 16B shows one exemplary experiment (P<0.01). Sixty days post infection both groups had cleared the infection in a similar manner, with hardly any adult worms left (Fig. 16C). Neither WT nor NOD2$^{-/-}$ mice developed a patent infection, as no Mf could be found in the peripheral blood.

3.1.7 Worms of *L. sigmodontis* infected NOD2$^{-/-}$ mice are retarded in development and growth

In addition to the establishment of infection *per se*, the development of *L. sigmodontis* might be influenced in NOD2$^{-/-}$ mice. We therefore analyzed length, stage and gender of the filariae. The L4 in NOD2$^{-/-}$ mice were significantly shorter than those of WT mice at day 15 post infection and this significance was reached both when analyzing the length of each single L4 (Fig. 17A; P<0.0001) or comparing the mean L4 lengths of each mouse (P<0.05; data not shown). In C57Bl/6 WT mice, most remaining parasites have developed into adults by day 30 post infection (Hoffmann, Petit et al. 2000). Strikingly, this development into adults was repressed in NOD2$^{-/-}$ mice (Fig. 17D; P<0.0001). Furthermore (as observed for day 15), remaining L4 were shorter also on day 30 post infection (Fig. 17B; P<0.05) and the few parasites that had molted into the adult stage in the NOD2$^{-/-}$ group were significantly shorter as well (Fig. 17C; P<0.001). The gender balance was similar in both groups (data not shown).

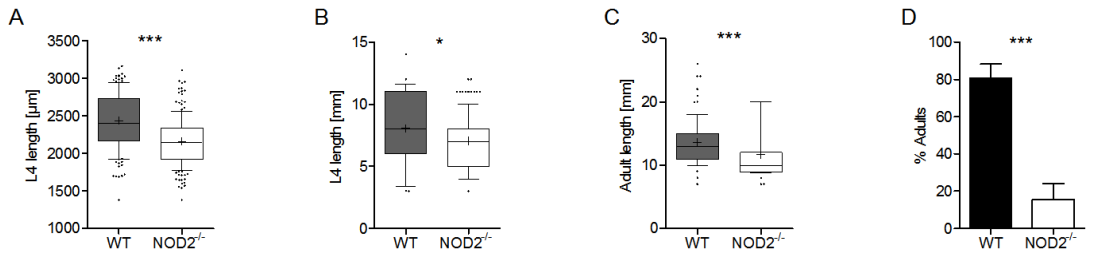


Figure 17. Worms in NOD2$^{-/-}$ mice are retarded in development. (A) L4 length at day 15 post infection. Representative one of two independent experiments with at least six mice each group. (B) – (D) Parasitological data of worms 30 days post infection. Representative one of three independent experiments with at least six mice per group. Length of L4 (B) and adults (C) and percentage of worms that have undergone moltage into adult stage (D) until day 30 are shown. All quartile box plots (median, 25 and 75%) show the 10-90 percentile and outliers are indicated. Bar graph in (D) shows mean ± SEM. Significances were calculated with students t-test, *, *** indicate $P<0.05$, $P<0.001$, respectively.

3.1.8 Flowcytometric analysis of the pleural space lavage cells of *L. sigmodontis* infected NOD2$^{-/-}$ mice.

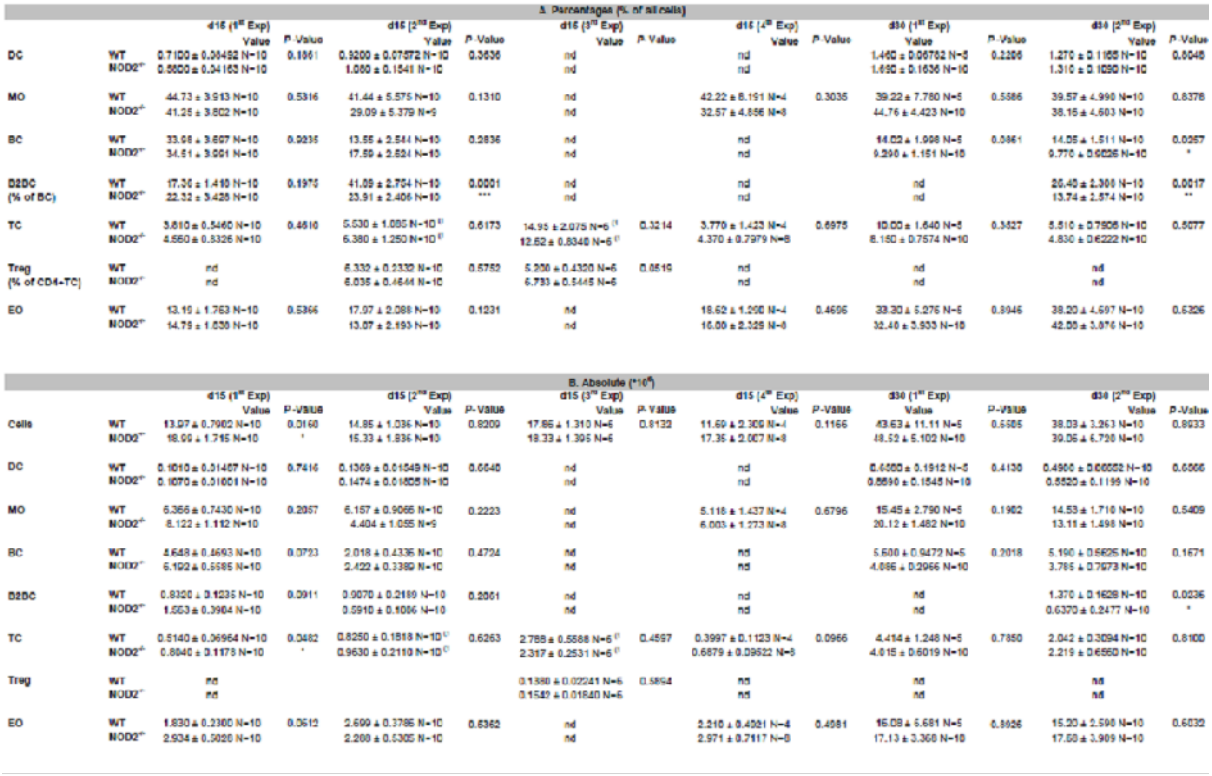
To investigate whether the observed changes in worm development in NOD2$^{-/-}$ mice might be associated with altered cellular responses against *L. sigmodontis*, we analyzed the PLECs of the thoracic cavity lavage by flow cytometry (table 3).

We did not find any consistent differences in number or percentages of DCs, MOs, T cells, or eosinophils, but significant lower amounts of conventional B cells (B2 cells) in NOD2$^{-/-}$ mice compared to the WT control (Fig. 18, $P<0.001$). However, this difference was not reflected by differing absolute numbers of B2 cells at the site of infection. Moreover, analysis of B2 cells in naïve mice revealed that the lower percentage is already present without any infection (data not shown), indicating that this is rather an intrinsic effect of the genetic NOD2 knock out in C57BL/6 mice, than a differing response to the *L. sigmodontis* infection.

The results show that NOD2$^{-/-}$ mice are more susceptible to *L. sigmodontis* infection, despite this receptor does not seem to be involved in innate proinflammatory responses upon stimulation with filarial antigen (see 3.1.2). Moreover, in the NOD2$^{-/-}$ mice L4 and adult worms are inhibited in growth, and molting of the L4 into the adult stage is impaired, indicating that very early mechanisms must be responsible for this phenotype.

3.1.9 NOD2$^{-/-}$ pleural exudate cells respond with increased IL-5 secretion after *ex vivo* restimulation

To further characterize the immune responses at time points when parasite numbers are increased we analyzed the pleural space lavage for the amount of various cytokines and Ig on days 15 and 30 post infection, but did not find any differences for the cytokines IL-1ß, IL-4, IL-5, IL-6, IL-10, TNF and IFN-γ, or the filarial-specific IgA, IgE, IgG1, IgG2a/b and IgM (data not shown).



A. Percentages (% of all cells)

		d15 (1st Exp) Value	P-Value	d15 (2nd Exp) Value	P-Value	d15 (3rd Exp) Value	P-Value	d15 (4th Exp) Value	P-Value	d30 (1st Exp) Value	P-Value	d30 (2nd Exp) Value	P-Value
DC	WT	0.7100 ± 0.04452 N=10	0.1561	0.9200 ± 0.07572 N=10	0.3636	nd		nd		1.460 ± 0.06752 N=5	0.2286	1.270 ± 0.1165 N=10	0.6046
	NOD2-/-	0.5600 ± 0.04163 N=10		1.060 ± 0.1541 N=10		nd		nd		1.650 ± 0.1636 N=10		1.310 ± 0.1690 N=10	
MO	WT	44.73 ± 3.913 N=10	0.5316	41.44 ± 5.575 N=10	0.1310	nd		42.22 ± 6.151 N=4	0.3035	39.22 ± 7.760 N=5	0.5586	39.57 ± 4.990 N=10	0.8378
	NOD2-/-	41.25 ± 3.802 N=10		29.09 ± 5.379 N=5		nd		32.57 ± 4.856 N=8		44.76 ± 4.423 N=10		38.15 ± 4.603 N=10	
BC	WT	33.98 ± 3.627 N=10	0.9235	13.55 ± 2.544 N=10	0.2836	nd		nd		14.62 ± 1.998 N=5	0.0961	14.05 ± 1.511 N=10	0.0357
	NOD2-/-	34.51 ± 3.001 N=10		17.59 ± 2.824 N=10		nd		nd		9.290 ± 1.161 N=10		9.770 ± 0.6626 N=10	*
B2BC (% of BC)	WT	17.36 ± 1.410 N=10	0.1978	41.09 ± 2.704 N=10	0.0001	nd		nd		nd		26.40 ± 2.398 N=10	0.0017
	NOD2-/-	22.32 ± 3.428 N=10		23.91 ± 2.406 N=10	***	nd		nd		nd		13.74 ± 2.574 N=10	**
TC	WT	3.610 ± 0.5460 N=10	0.4616	5.530 ± 1.095 N=10 [1]	0.6173	14.95 ± 2.075 N=6 [1]	0.3214	3.770 ± 1.423 N=4	0.6975	10.00 ± 1.640 N=5	0.3627	5.510 ± 0.7606 N=10	0.5077
	NOD2-/-	4.560 ± 0.5326 N=10		6.380 ± 1.250 N=10 [1]		12.62 ± 0.8348 N=6 [1]		4.370 ± 0.7979 N=8		8.150 ± 0.7574 N=10		4.830 ± 0.6222 N=10	
Treg (% of CD4+TC)	WT	nd		6.332 ± 0.5330 N=10	0.6783	5.256 ± 0.4356 N=6	0.8619	nd		nd		nd	
	NOD2-/-	nd		6.035 ± 0.4844 N=10		5.793 ± 0.5445 N=6		nd		nd		nd	
EO	WT	13.15 ± 1.763 N=10	0.5366	17.07 ± 2.088 N=10	0.1231	nd		18.62 ± 1.200 N=4	0.4696	39.30 ± 5.276 N=5	0.9946	39.20 ± 4.597 N=10	0.6326
	NOD2-/-	14.75 ± 1.638 N=10		13.07 ± 2.193 N=10		nd		16.00 ± 2.328 N=8		39.40 ± 3.933 N=10		42.05 ± 3.976 N=10	

B. Absolute (*10^6)

		d15 (1st Exp) Value	P-Value	d15 (2nd Exp) Value	P-Value	d15 (3rd Exp) Value	P-Value	d15 (4th Exp) Value	P-Value	d30 (1st Exp) Value	P-Value	d30 (2nd Exp) Value	P-Value
Cells	WT	13.97 ± 0.7902 N=10	0.0166	14.85 ± 1.036 N=10	0.8209	17.65 ± 1.310 N=6	0.8133	11.69 ± 2.309 N=4	0.1166	43.63 ± 11.11 N=5	0.5685	39.03 ± 3.263 N=10	0.9633
	NOD2-/-	18.99 ± 1.715 N=10	*	15.33 ± 1.836 N=10		18.33 ± 1.395 N=6		17.35 ± 2.067 N=8		48.52 ± 5.102 N=10		39.05 ± 6.730 N=10	
DC	WT	0.1010 ± 0.01457 N=10	0.7416	0.1369 ± 0.01549 N=10	0.6646	nd		nd		0.6660 ± 0.1612 N=5	0.4136	0.4960 ± 0.06552 N=10	0.6666
	NOD2-/-	0.1070 ± 0.01601 N=10		0.1474 ± 0.01825 N=10		nd		nd		0.5690 ± 0.1545 N=10		0.5520 ± 0.1199 N=10	
MO	WT	6.366 ± 0.7430 N=10	0.2957	6.157 ± 0.9065 N=10	0.2223	nd		5.118 ± 1.437 N=4	0.6796	15.45 ± 2.790 N=5	0.1902	14.53 ± 1.716 N=10	0.5409
	NOD2-/-	6.122 ± 1.112 N=10		4.404 ± 1.055 N=5		nd		6.003 ± 1.273 N=8		20.12 ± 1.482 N=10		13.11 ± 1.498 N=10	
BC	WT	5.648 ± 0.8693 N=10	0.0723	2.018 ± 0.4336 N=10	0.4724	nd		nd		5.600 ± 0.9472 N=5	0.2018	5.190 ± 0.6625 N=10	0.1671
	NOD2-/-	6.192 ± 0.9585 N=10		2.422 ± 0.3382 N=10		nd		nd		4.086 ± 0.2966 N=10		3.786 ± 0.7673 N=10	
B2BC	WT	0.9300 ± 0.1235 N=10	0.0911	0.9070 ± 0.2189 N=10	0.2661	nd		nd		nd		1.370 ± 0.1628 N=10	0.0236
	NOD2-/-	1.553 ± 0.3964 N=10		0.5910 ± 0.1086 N=10		nd		nd		nd		0.6370 ± 0.2477 N=10	*
TC	WT	0.5140 ± 0.06964 N=10	0.0482	0.8250 ± 0.1518 N=10 [1]	0.6263	2.788 ± 0.5588 N=6 [1]	0.4567	0.3997 ± 0.1123 N=4	0.0966	4.414 ± 1.248 N=5	0.7850	2.042 ± 0.3094 N=10	0.6100
	NOD2-/-	0.8940 ± 0.1173 N=10	*	0.9630 ± 0.2119 N=10 [1]		2.317 ± 0.2531 N=6 [1]		0.6879 ± 0.09522 N=8		4.015 ± 0.6019 N=10		2.219 ± 0.6560 N=10	
Treg	WT	nd				0.1380 ± 0.02241 N=6	0.5894	nd		nd		nd	
	NOD2-/-	nd				0.1542 ± 0.01840 N=6		nd		nd		nd	
EO	WT	1.830 ± 0.2360 N=10	0.0612	2.699 ± 0.3786 N=10	0.6362	nd		2.216 ± 0.4581 N=4	0.4681	16.08 ± 5.681 N=5	0.9926	15.20 ± 2.590 N=10	0.6032
	NOD2-/-	2.934 ± 0.5020 N=10		2.200 ± 0.5305 N=10		nd		2.971 ± 0.7617 N=8		17.13 ± 3.368 N=10		17.60 ± 3.909 N=10	

A. Percentages (% of all cells)

		d30 (3rd Exp) Value	P-Value	d30 (4th Exp) Value	P-Value	d30 (5th Exp) Value	P-Value	d60 (1st Exp) Value	P-Value	d60 (2nd Exp) Value	P-Value
DC	WT	0.4400 ± 0.03399 N=10	0.0454	0.1150 ± 0.06217 N=6	0.9069	nd		1.690 ± 0.2991 N=10	0.0295	2.855 ± 0.3517 N=8	0.6992
	NOD2-/-	0.7300 ± 0.1334 N=10	*	0.1050 ± 0.05560 N=6		nd		2.950 ± 0.3341 N=10	*	2.725 ± 0.2144 N=8	
MO	WT	36.60 ± 4.264 N=10	0.1429	37.65 ± 5.336 N=6	0.6230	nd		60.16 ± 3.016 N=10	0.0874	72.68 ± 3.903 N=8	0.7461
	NOD2-/-	45.13 ± 5.409 N=10		34.72 ± 2.231 N=6		nd		69.67 ± 4.297 N=10		74.13 ± 2.671 N=8	
BC	WT	10.33 ± 1.734 N=10	0.0064	27.15 ± 2.029 N=6	0.5799	nd		16.66 ± 2.644 N=10	0.9541	7.238 ± 0.7933 N=8	0.3409
	NOD2-/-	19.44 ± 2.121 N=10	**	29.97 ± 2.436 N=6		nd		17.09 ± 3.964 N=10		9.350 ± 1.990 N=8	
B2BC (% of BC)	WT	15.64 ± 2.211 N=10	0.1523	24.05 ± 2.973 N=6	0.1150	nd		16.99 ± 3.961 N=10	0.7606	15.53 ± 2.348 N=8	0.1677
	NOD2-/-	15.02 ± 0.9575 N=10		17.93 ± 1.962 N=6		nd		21.61 ± 4.586 N=10		21.63 ± 3.473 N=8	
TC	WT	3.420 ± 0.3915 N=10	0.1070	2.967 ± 0.6989 N=6	0.2391	4.383 ± 0.3646 N=6 [1]	0.0321	1.790 ± 0.1750 N=10	<0.0001	nd	
	NOD2-/-	5.790 ± 1.341 N=10		4.333 ± 0.8386 N=6		5.883 ± 0.4799 N=6 [1]	*	3.390 ± 0.2597 N=10	****	nd	
Treg (% of CD4+TC)	WT	nd				7.083 ± 1.002 N=5	0.0852	nd		nd	
	NOD2-/-	nd				10.22 ± 1.307 N=5		nd		nd	
EO	WT	45.40 ± 4.750 N=10	0.0079	25.82 ± 3.706 N=6	0.4593	nd		8.020 ± 1.313 N=10	0.1232	12.50 ± 2.547 N=8	0.3705
	NOD2-/-	25.27 ± 4.785 N=10	**	32.55 ± 3.302 N=6		nd		13.46 ± 3.097 N=10		9.513 ± 1.396 N=8	

B. Absolute (*10^6)

		d30 (3rd Exp) Value	P-Value	d30 (4th Exp) Value	P-Value	d30 (5th Exp) Value	P-Value	d60 (1st Exp) Value	P-Value	d60 (2nd Exp) Value	P-Value
Cells	WT	31.72 ± 2.448 N=10	0.2964	50.18 ± 4.534 N=6	0.6748	36.43 ± 6.669 N=5	0.1377	17.39 ± 3.030 N=10	0.7478	18.79 ± 3.768 N=8	0.3015
	NOD2-/-	27.88 ± 2.599 N=10		52.52 ± 2.771 N=6		52.83 ± 5.949 N=5		15.76 ± 3.968 N=10		24.60 ± 3.876 N=8	
DC	WT	0.1400 ± 0.01619 N=10	0.2113	0.05333 ± 0.02661 N=6	0.9662	nd		0.3322 ± 0.05192 N=10	0.4471	0.5563 ± 0.1378 N=8	0.4655
	NOD2-/-	0.2110 ± 0.05233 N=10		0.05167 ± 0.02613 N=6		nd		0.4507 ± 0.1257 N=10		0.7019 ± 0.1368 N=8	
MO	WT	11.29 ± 1.526 N=10	0.5476	18.19 ± 1.964 N=6	0.9305	nd		11.89 ± 2.017 N=10	0.2500	13.14 ± 2.458 N=8	0.2057
	NOD2-/-	12.76 ± 1.758 N=10		18.00 ± 0.7027 N=6		nd		8.566 ± 1.948 N=10		18.11 ± 2.841 N=8	
BC	WT	3.112 ± 0.3513 N=10	0.0063	13.93 ± 1.997 N=6	0.6134	nd		2.745 ± 0.5553 N=10	0.9029	1.400 ± 0.3583 N=8	0.1670
	NOD2-/-	5.102 ± 0.8064 N=10	*	15.36 ± 1.883 N=6		nd		2.667 ± 0.7994 N=10		2.199 ± 0.4145 N=8	
B2BC	WT	0.5790 ± 0.07965 N=10	0.2334	3.362 ± 0.6170 N=6	0.4914	nd		0.5248 ± 0.1444 N=10	0.9954	0.2471 ± 0.08426 N=8	0.1499
	NOD2-/-	0.7140 ± 0.07510 N=10		2.513 ± 0.5025 N=6		nd		0.5253 ± 0.1786 N=10		0.5260 ± 0.1625 N=8	
TC	WT	1.049 ± 0.1221 N=10	0.1758	1.497 ± 0.3470 N=6	0.1877	1.765 ± 0.4203 N=6 [1]	0.0326	0.3120 ± 0.05573 N=10	0.1726	nd	
	NOD2-/-	1.468 ± 0.2711 N=10		2.247 ± 0.4012 N=6		3.083 ± 0.2700 N=6 [1]	*	0.5956 ± 0.1882 N=10		nd	
Treg	WT	nd		nd		0.1338 ± 0.04398 N=6	0.0226	nd		nd	
	NOD2-/-	nd		nd		0.3096 ± 0.04817 N=6	*	nd		nd	
EO	WT	14.04 ± 1.805 N=10	0.0080	14.80 ± 2.219 N=6	0.4594	nd		1.602 ± 0.4020 N=10	0.3926	2.683 ± 0.9400 N=8	0.9099
	NOD2-/-	7.573 ± 1.589 N=10	**	17.17 ± 2.127 N=6		nd		2.770 ± 1.239 N=10		2.553 ± 0.6225 N=8	

[1] only CD4+ TCs

Table 3. Flowcytometric analysis of pleural space cells after natural infection of C57BL/6 WT and NOD2$^{-/-}$ mice. Mice were infected with *L. sigmodontis* and pleural exudate cells analyzed 15, 30 and 60 days post infection for percentages (A) and absolute numbers (B) of dendritic cells (DC), macrophages (MO), B-cells (BC), B2 B cells (B2BC), T-cells (TC), regulatory T cells (Treg) and eosinophils (EO). Staining was performed according to standard protocols with fluorochrome-conjugated antibodies to the surface markers F4/80, SiglecF, CD3ε, CD11c, CD23 and CD19, as well as to the intracellular foxp3 domain. "nd" indicates that no data are available. Statistical analysis for each population with the Student's t-test.

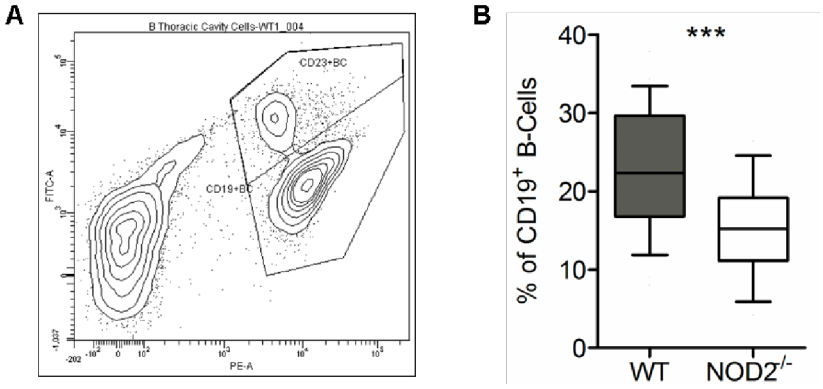


Figure 18. NOD2$^{-/-}$ mice have an altered B cell composition at the site of infection. Mice were infected via the natural route and composition of the B cell compartment was analyzed via flow cytometry using staining against CD19 and CD23. (A) Representative dot plot showing staining and gating strategy. (B) Percentage of conventional B2 cells within the B cell compartment of the pleural space lavage 30 days post infection. Data are presented as quartile box plots (median, 25 and 75%) and show the 10-90 percentiles. Statistical analysis of pooled data of three independent experiments with the Student's t-test, *** indicates P<0.001.

However, as the cytokine responses at the site of infection might be influenced by the presence of parasites itself and cytokine patterns may be difficult to assess due to a strong dilution during the process of the lavage, we restimulated PLECs antigen-specifically *ex vivo* and analyzed the secretion of cytokines by the ELISA method. Interestingly, we found the NOD2$^{-/-}$ PLECs to secrete more IL-5 compared to the cells from the WT littermates. These differences were significant in two out of three independent experiments (Fig. 19; all P<0.001). In contrast, we found no differences for TNF and IFN-γ secretion after restimulation. Furthermore we observed no differences for these cytokines on day 15 post infection (data not shown).

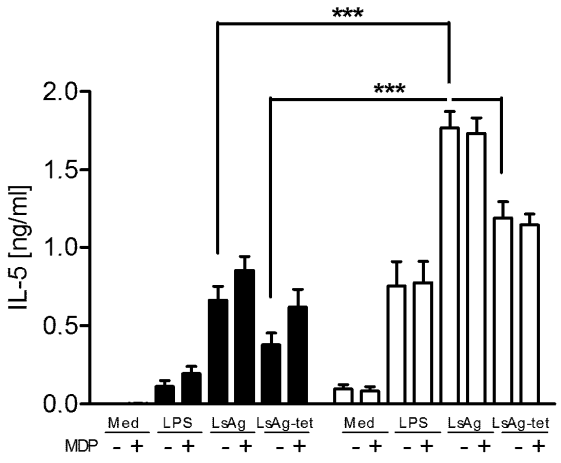


Figure 19. PLECs of NOD2$^{-/-}$ mice secrete more IL-5 after *ex vivo* restimulation. (A) At day 15 post infection pleural space exudate cells of C57BL/6 WT (black bars) or NOD2$^{-/-}$ mice (white bars) were isolated by lavage of the thoracic cavity, filtered, washed and restimulated at 2.5*10^5 cells in 200 µl medium per well in a 96 well plate with positive TLR4 ligand LPS, complete filarial extract (Ls), *Wolbachia*-depleted extract (Ls-tet) or left unstimulated (Med) for 72 hours. The NOD2 deficiency was confirmed by stimulation with 25 µM MDP as indicated. Supernatants were analyzed for IL-5 using ELISA. Representative of two experiments with a significant difference with six mice each group. Analysis was done with ANOVA and the mean ± SEM is depicted. *** indicates P<0.001; the significant differences of the LPS and stimulus + MDP responses are not depicted for a better view (compared to Med all P<0.001).

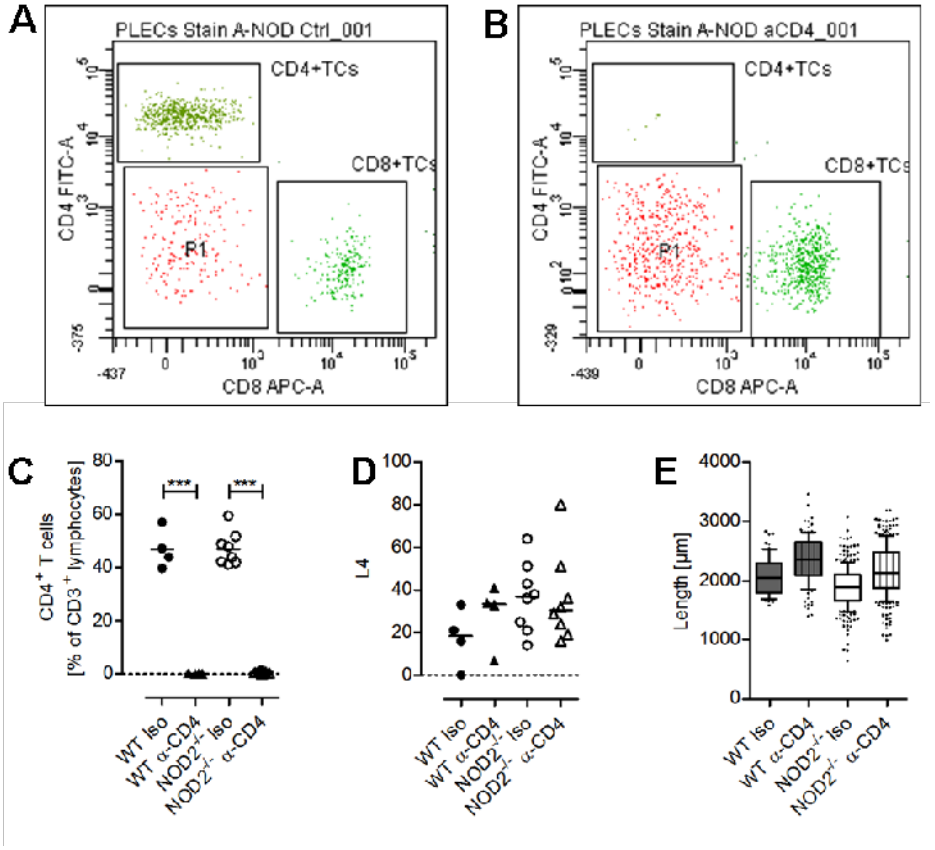


Figure 20. Depletion of CD4 T cells does not revert the phenotype of NOD2$^{-/-}$ mice. Mice received a CD4-depleting antibody (α-CD4) or control Isotype Ig (Iso) at days 0, 6 and 12 after natural infection. At day 15 post infection the pleural space lavage was analyzed for the presence of T cells by flow cytometry and the L4 burden was quantified. Single experiment with four (WT) and eight (NOD2$^{-/-}$) mice each group. (A, B) Representative dot plots for a control (A) and a CD4-depleted (B) mouse. (C) Percentage of CD4^{+} T cells within the CD3^{+} T cell compartment. Statistical analysis with ANOVA, the line represents the mean. *** indicate P<0.001. (D, E) Parasitological outcome with L4 burden (D) and larvae length (E). Statistical analysis with ANOVA and Bonferroni post hoc test, the line in (D) represents the median. In (E) data are presented as quartile box plots (median, 25 and 75%) and show the 10-90 percentile. All groups differed statistically from each other (P at least <0.01), except WT Iso vs. NOD2$^{-/-}$ α-CD4; significances are not depicted for a better view.

3.1.10 Depletion of CD4^{+} T cells in NOD2$^{-/-}$ mice does not increase the parasite burden as it does in the WT littermates

CD4^{+} T cells are involved in the murine immune response against *L. sigmodontis* infection and are main producers of IL-5 in this helminth infection model (Al Qaoud, Taubert et al. 1997). Interestingly, most recently it has been shown that T cell activation can be mediated by the NOD2 receptor (Shaw, Kamada et al. 2011). Therefore, we wanted to elucidate a possible role of the NOD2 receptor on CD4^{+} T cells in the responses to *L. sigmodontis* infection and depleted CD4^{+} cells in mice by injection of a depleting Ab to the CD4 surface receptor (Fig. 20). As figure 20D shows, the L4 burden in WT mice was slightly increased after depletion of CD4 T cells (P=0.3070). Most interestingly, the elevated parasite burden observed in NOD2$^{-/-}$ mice (see 3.1.6) was not increased or reversed when CD4^{+} T cells were depleted from those NOD2$^{-/-}$ mice.

We again found differences in the length of the L4 between WT and NOD2$^{-/-}$ mice (Fig. 20E; P<0.01; similar to experiments above, see figure 17A). In addition, the length of the larvae was

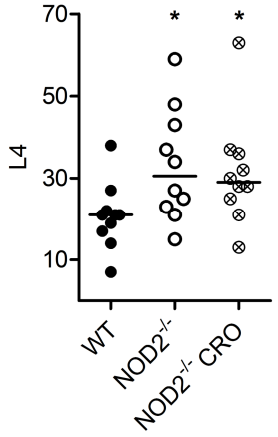


Figure 21. Mast cell stabilization does not revert the phenotype of NOD2$^{-/-}$ mice. C57Bl/6 NOD2$^{-/-}$ mice received 8 mg / kg body weigth of the mast cell stabilizer cromolyn i.p. (NOD2$^{-/-}$ CRO) or PBS as control (WT and NOD2$^{-/-}$) 48 and 24 hours before and 24 hours after natural infection. At day 15 post infection the L4 burden was assesed. Single experiment with ten mice each group. Statistical analysis with the Students t-test, the line represents the median. * indicates $P<0.05$.

increased in the CD4-depleted NOD2$^{-/-}$ mice ($P<0.001$), as was the length of the L4 recovered from CD4-depleted WT mice compared to WT controls ($P<0.001$).

3.1.11 Inhibition of mast cell degranulation does not revert the NOD2$^{-/-}$ phenotype

Mast cells with a mutation in a MDP receptor have been associated with increased vascular permeability in the skin of mice (Nakamura, Kambe et al. 2009). In the murine model of LF enhanced vascular permeability depends on degranulation of mast cells and is further associated with increased larval establishment within the first few days after infection (Specht, Frank et al. 2011). We therefore wanted to know whether enhanced worm burden in NOD2$^{-/-}$ mice may be attributed to mast cell controlled enhanced vascular permeability.

We blocked the degranulation of mast cells *in vivo* by injection of the mast cell stabilizer cromolyn and assessed the larval establishment 15 days post infection. However, as figure 21 shows, the enhanced L4 burden of NOD2$^{-/-}$ mice ($P<0.05$) on day 15 post infection was not reverted to the lower WT level after this treatment ($P<0.05$ compared to WT control; $P=0.7600$ compared to the untreated NOD2$^{-/-}$ mice).

The results show that *ex vivo* restimulated NOD2$^{-/-}$ PLECs secrete higher amounts of IL-5 than WT PLECs, indicating an interaction of NOD2 with Th2 pathways. However, depletion of CD4^{+} T cells (the major source of IL-5) *in vivo* did not revert the phenotype of NOD2$^{-/-}$ mice. Furthermore, inhibition of mast cell degranulation did not revert the phenotype.

3.2 Immunization of BALB/c Mice Against the First Larval Stage of *L. sigmodontis*

A successful immunization is the best way of preventing a disease, since it does not only treat the symptoms and combats the pathogen (as drug therapy does) but in fact enables the host's immune system to cope with the threat of infection before pathogens start to spread in the body. Despite the severity of infection and the vast number of infected people and individuals at risk, there are no available vaccines against any filarial infection, even though various attempts have been undertaken (Bergquist and Lustigman 2010; Bethony, Cole et al. 2011).

Already in the 1960s, Wenk and colleagues found that cotton rats immunized with *L. sigmodontis* Mf had fewer blood-circulating Mf, although adult worms were present (Haas and Wenk 1981; Kimmig and Wenk 1982; Wenk and Wegerhof 1982). In this study we have taken this approach another step forward using the fully permissive BALB/c mouse model to study filarial infections.

3.2.1 Subcutaneous immunization with Mf in alum prevents peripheral microfilaraemia

Initially, we immunized BALB/c mice three times i.v. with 100,000 viable Mf (four, two and one week before natural *L. sigmodontis* infection). This injection resulted in a transient presence of Mf in the peripheral blood lasting about two weeks (Fig. 23A, page 45). After challenge infection, natural Mf levels in the peripheral blood were monitored from the onset of peripheral microfilaraemia at day 50 post infection until the end of patency around day 90 post infection. This immunization neither delayed the onset of natural microfilaraemia nor significantly changed the Mf levels in the peripheral blood after challenge infection compared to control animals at any time point during patency (Fig. 22A).

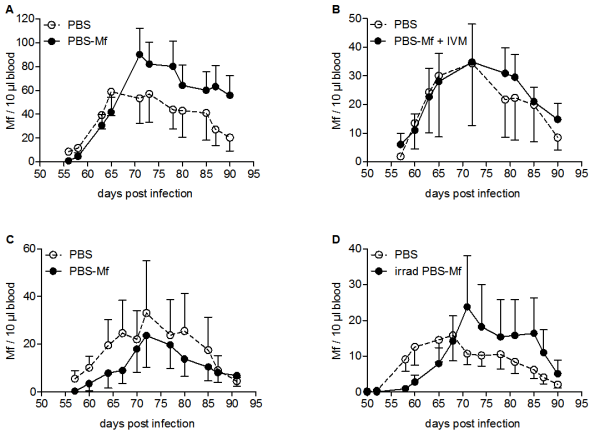


Figure 22. Immunization strategies that failed to protect mice from peripheral microfilaraemia. Mice were immunized with 100,000 Mf either three times i.v. (A, B) or first s.c. followed by an i.p. and i.v. immunization (C, D). All control mice received PBS. *L. sigmodontis* challenge infection was performed one week after the last immunization. (B) After immunization mice were treated i.v. with Ivermectin (IVM). (D) Mice were immunized with irradiated (400 Gy) Mf. Microfilaraemia was monitored throughout patency. Data obtained from single experiments with at least six mice per group are shown. Two-way ANOVA was used for statistical analysis, mean ± SEM is shown, integrating both Mf^- and Mf^+ mice.

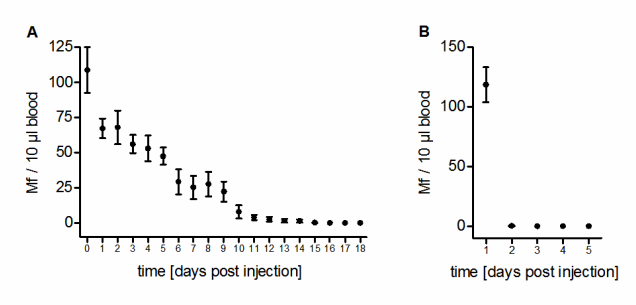


Figure 23. Kinetic of i.v.-injected Mf in the peripheral blood. 100,000 Mf were injected i.v. into the tail vein and circulation in the peripheral blood was monitored daily. (A) Representative data of two independent injections with seven mice per group, data presented as mean ± SEM. (B) One hour after injection mice were treated with Ivermectin at 800 µg per kg body weight. Representative of two independent injections with eight mice per group, data presented as mean ± SEM.

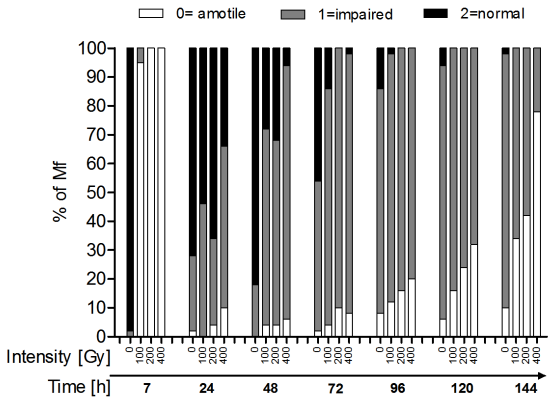


Figure 24. Irradiation of *L. sigmodontis* Mf paralyzes and immobilizes the first stage larvae. Mf were isolated from the blood of cotton rats, irradiated with doses as indicated or left non-irradiated (0 Gy) and cultured for 148 hours at 37°C and 5% CO_2. Motility was defined as normal, impaired or absent (amotile). Motility was checked under the microscope and examiner was blind to samples. Representative of two independent experiments.

Next, since healthy Mf may modulate immune responses in the immunized host, and in order to enrich immunogenic material, mice were treated with the microfilaricide IVM after immunization. Ivermectin suppresses the ability of Mf to secrete immunomodulatory proteins (Moreno, Nabhan et al. 2010) and inhibits their neuromuscular control (Wolstenholme and Rogers 2005). Accordingly, after IVM injection, Mf disappeared from the peripheral blood within one day after injection (Fig. 23B). However, microfilaraemia after challenge infection was not reduced in mice (Figure 22B).

According to the successful scheme originally used in cotton rats (Haas and Wenk 1981), we then immunized mice first s.c., followed by an i.p. immunization two weeks later and an i.v. immunization three weeks after primary immunization. As with the two former schemes, this route of immunization failed to protect mice and this was independent of the usage of either healthy (Fig. 22C) or irradiation-attenuated Mf (Fig. 22D), although the latter treatment led to paralysis of Mf until seven hours after irradiation (Fig. 24).

Finally, to investigate whether a standard adjuvant is able to establish protective immunity, mice were then immunized three times (time schedule as above) s.c. with 100,000 Mf together with the adjuvant alum. Mice immunized with Mf in alum had significantly reduced numbers of circulating Mf after challenge infection when compared to control animals throughout patency (Fig. 25A; $P<0.005$). Furthermore, the frequency of mice that became microfilaraemic until the end of observation was significantly reduced in the immunized group (Fig. 25B; $P<0.05$).

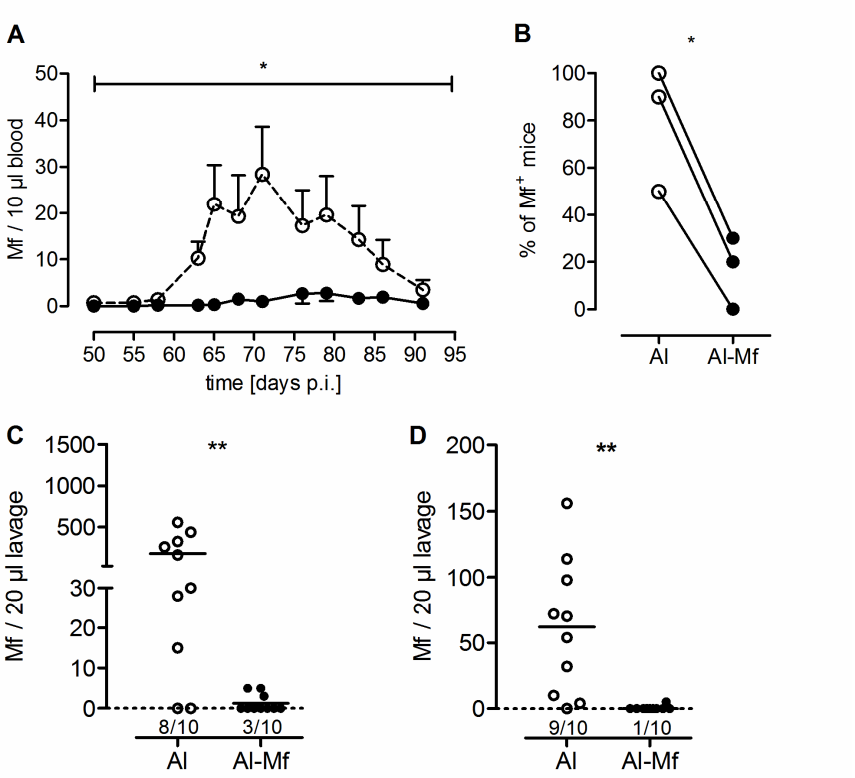


Figure 25. Mice immunized with Mf in alum have reduced numbers of Mf. Mice were immunized three times s.c. with 100,000 Mf in alum 28, 14, and 7 days before *L.sigmodontis* challenge infection. Control mice received alum alone. Microfilaraemia was monitored twice a week throughout patency. (A) Kinetics of Mf load of sham-treated (dashed line) and immunized (black line) mice in the peripheral blood. One representative of three independent experiments with ten mice per group. Two-way ANOVA was used for statistical analysis, mean ± SEM is shown, integrating both Mf^- and Mf^+ mice. (B) Percentage of Mf^+ mice at the end of observation (day 90 post infection) in the three independent experiments, analyzed with Student's t-test. Each mouse with peripheral Mf at any given time point was defined as Mf^+. (C, D) Mf burden in the pleural space at days 70 (C) 90 (D) post infection. Graphs show one representative of three (C) and two (D) independent experiments (at least seven mice each group) and were analyzed with Welch-corrected t-test. Numbers below symbols indicate the number of Mf^+. Line represents the median. *, ** indicate significances <0.05, <0.005, respectively.

Taken together, effective vaccination of 70 - 100% was observed in mice after s.c. immunization with Mf in alum, but not after immunization with Mf alone, irrespective of the administration route, irradiation of Mf or IVM treatment of mice after immunization. Consequently, all further experiments were performed with 100,000 s.c.-administrated Mf in alum.

3.2.2 Immunization blocks embryogenesis of female worms

To investigate whether immunization inhibits the ability of filarial females to generate and release Mf *per se* or just hinders the Mf migration into the blood, the pleural space lavage was analyzed for the presence of Mf on days 70 and 90 post infection. Figures 25C and D show, that the number of Mf in the pleural space was significantly reduced after immunization compared to the alum-treated control group, the latter showing the wide range of microfilaraemia that is well described

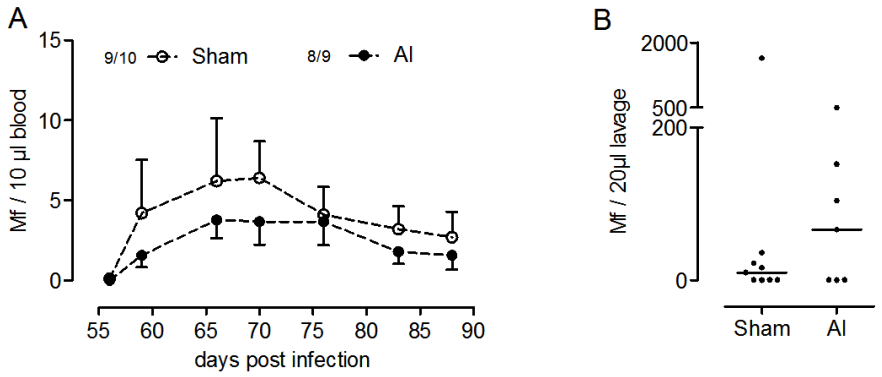


Figure 26. The adjuvants alum itself does not alter the course of microfilaraemia. Mice were treated times s.c. with 100 µl PBS (Sham) or 100 µl 25 % adjuvants (Alum) in PBS (= the concentration that was used for immunization with Mf). *L. sigmodontis* infection was performed one week after last injection. Single experiment. (A) Microfilaraemia was monitored throughout patency; numbers next to legend indicate number of Mf^+ mice in the group. Two-way ANOVA was used for statistical analysis, mean ± SEM is shown, integrating both Mf^- and Mf^+ mice. (B) Number of Mf in the pleural space at the end of observation, analyzed with Student's t-test, the line represents the median.

for this model (Al Qaoud, Taubert et al. 1997). The few immunized mice that were Mf^+ at day 70 post infection had only low Mf levels with a mean of only four Mf compared to 226 Mf / 20 µl lavage in the alum treated control group (Fig. 25 C; $P<0.005$). Furthermore, at day 90 post infection 90% of immunized mice were free of Mf with only one mouse having five Mf / 20 µl lavage, but 90% of control mice still harbored Mf with a mean of 68 Mf / 20 µl lavage (Fig. 25D; $P<0.005$,). To rule out that alum itself influences the course of infection, we compared mice injected s.c. with either alum alone or PBS and did not find significant differences in the course of microfilaraemia neither in the blood (Fig. 26A; $P=0.4898$), nor in at the site of infection (Fig. 26B; $P=0.7377$).

The reduction of Mf levels in the pleural space suggested that either Mf were cleared immediately after being released or the output of Mf by fecund female worms was reduced. Consequently, the embryogenesis in the uteri of female worms was analyzed. During the filarial embryogenesis four main developmental stages can be distinguished in the uteri of female worms (Fig. 27A-D): oocyte, divided egg (fulfilled first cell division), pretzel and stretched Mf (Hoerauf, Nissen-Pahle et al. 1999; Goldstein 2001). Female worms of each mouse were investigated and females with an empty uterus or females with oocytes only were excluded from analysis (in three independent experiments percentage of those excluded females was similar between immunized (33, 20 and 27%) and non-immunized mice (16, 18 and 28%), indicating that immunization did not interfere with insemination). As figure 27 illustrates, we mainly found the first two developmental stages (oocytes and divided eggs) but rarely pretzel stages ($P<0.001$) and fully developed Mf ($P<0.01$) in the uteri of female worms from immunized mice at day 70 post infection, whereas females of control mice contained all stages. To confirm this result, any remaining worms were checked for the presence or absence of later stages such as fully stretched Mf and pretzel stages. Only two out of 23 (second experiment: 0/6) female filariae were positive for later stages in the Mf-vaccinated group,

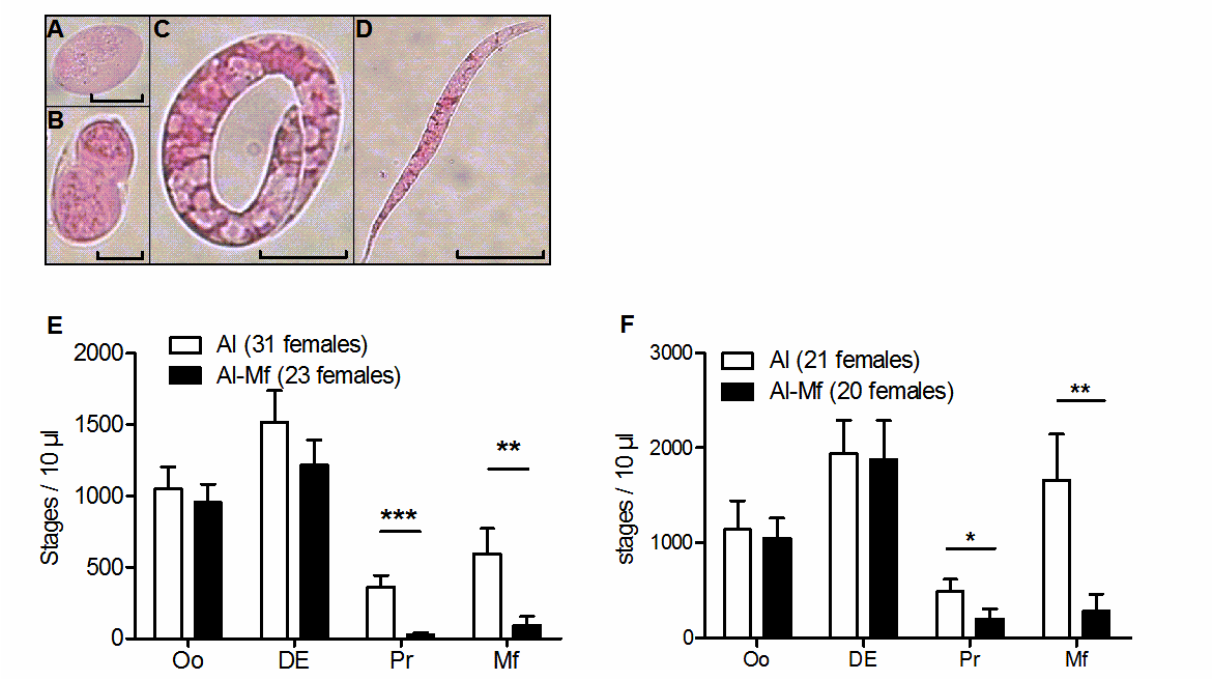


Figure 27. Immunization inhibits embryogenesis in female worms. Mice were immunized three times s.c. with 100,000 Mf in alum 28, 14, and 7 days before *L.sigmodontis* challenge infection. Control mice received alum alone. Seventy (E) and 56 (F) days after infection female worms were analyzed for their embryonic stages. Representative pictures of oocyte (A; micron bar 10 µm), divided egg (B; 10 µm), pretzel stage (C; 15 µm) and stretched Mf (D; 30 µm) are shown. (E, F) Embryograms illustrating the composition of embryonic stages in female worms. For analysis each single female worm was transferred into 80 µl PBS, cut into several pieces and embryonic stages squeezed out of the uterus. Stages were stained with Hinkelmann solution and analyzed under the microscope. The number of analyzed females in each group is indicated. Statistical analysis with Mann-Whitney t-test, mean ± SEM is shown, **, **, *** indicate $P<0.05$, $P<0.01$, $P<0.001$ respectively.

whereas 25 of 31 (second experiment: 14/26) females contained stretched Mf in the control (data not shown). Inhibition of embryogenesis at day 70 post infection was exemplarily documented by live video analysis of the uteri of freshly isolated healthy females (Videos 1 and 2; provided on supplementary CD). Finally, inhibition of embryogenesis could already be observed at the beginning of patency at day 56 post challenge infection (Fig. 27F).

3.2.3 Immunization reduces adult worm burden

Cross reactive protection with respect to other developmental stages is known for immunization with *L. sigmodontis* L3 (Specht and Hoerauf 2009). Thus, we asked whether Mf immunization may also affect other stages than the Mf. Analysis of the parasite burden at days 15 and 56 post challenge infection showed that immunized mice had similar worm numbers as control animals (Fig. 28A, B). However, on days 70 (Fig. 28C; $P<0.005$) and 90 post infection (Fig. 28D; $P<0.0001$) immunized mice contained significantly fewer adult worms and this reduction was associated with decreased numbers of both males and females, as the gender balance was similar in

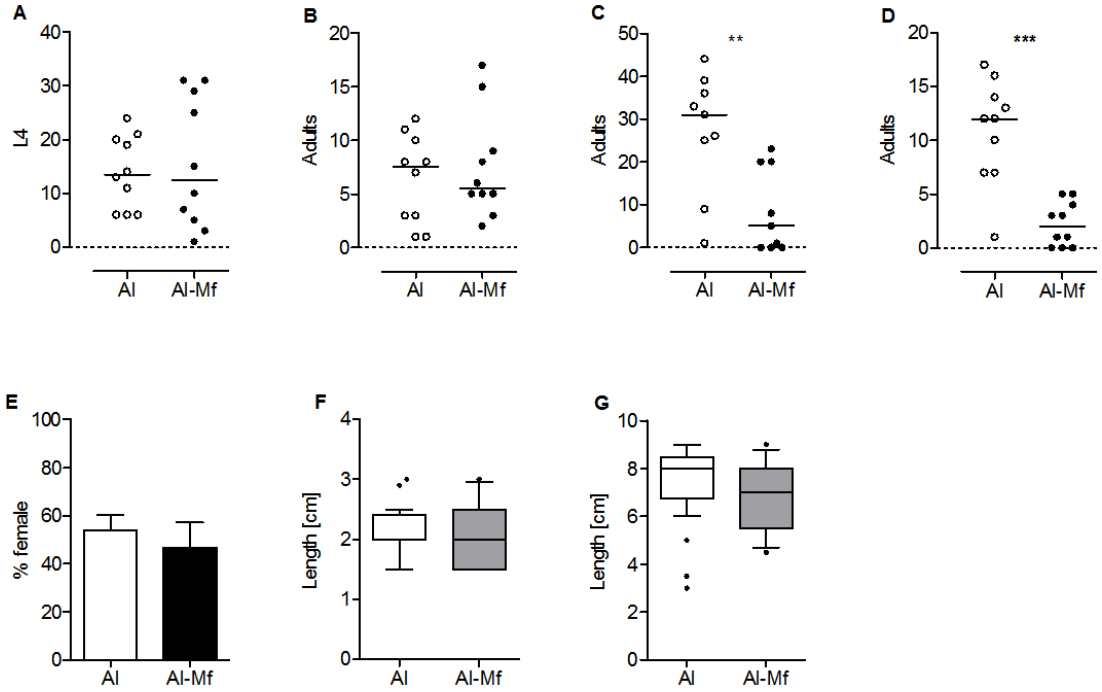


Figure 28. Immunization reduces adult worm burden, but does not affect their development. Mice were immunized three times s.c. with 100,000 Mf in alum. Control mice received alum alone. *L. sigmodontis* challenge infection was performed one week after the last immunization. Numbers of worms at days 15 (A), 56 (B), 70 (C) and 90 (D) post infection were analyzed for statistical significance using the Student's t-test. Single (A, B), representative of two (C) or three (D) independent experiments, line represents the median. (E) Gender balance of worms, pooled data from two independent experiments with ten mice each group, the mean ± SEM is shown. Male (F) and female (G) worm length at day 90 post infection, data presented as quartile box plots (median, 25 and 75%) with the 10-90 percentile, outliers are indicated. Pooled data from two independent experiments with ten mice per group are shown.

immunized and control mice (Fig. 28E). Male and female worms did not differ in length to the corresponding worms obtained from infected control mice (Fig. 28F, G).

Taken together, these data suggest that immunization induces the inhibition of intrauterine larval development. This inhibited embryogenesis is already present at the onset of filarial patency. Furthermore, the data show that immunization also reduced adult worm burden from that time point onwards.

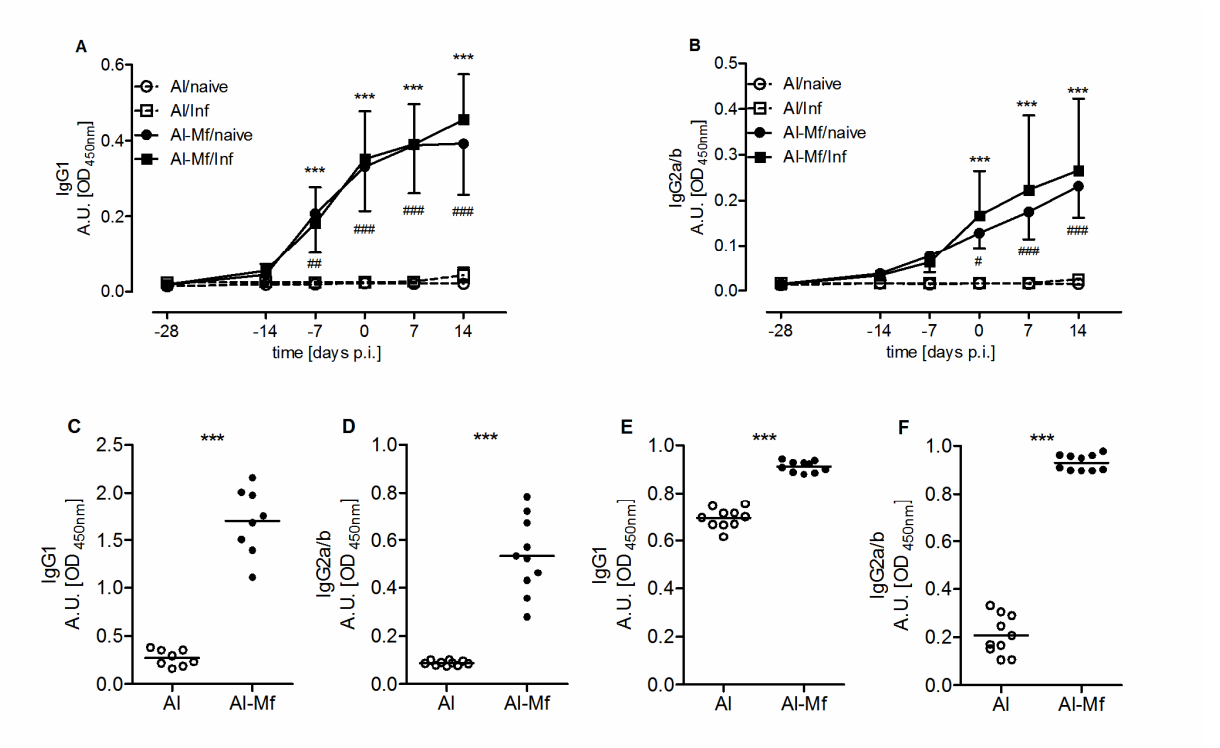


Figure 29. Immunization induces Mf-specific IgG1 and IgG2. Mice were immunized three times s.c. with 100,000 Mf in alum (Al-Mf/naïve, Al-Mf/Inf). Control mice received alum alone (Al/naïve, Al/Inf). *L. sigmodontis* challenge infection was performed one week after the last immunization (Al/Inf, Al-Mf/Inf) or left uninfected (Al/naïve, Al-Mf/naïve). Plasma levels of Mf-specific IgG1 (A) and IgG2a/b (B) were measured. Two-way ANOVA was used for statistical analysis, day 0 indicates day of challenge infection. Asterisks indicate significant differences between the immunized and infected, and the corresponding control group (*** $P<0.001$) and pound signs between the immunized but uninfected, and the corresponding control group ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$). (C - F) Pleural space lavage was analyzed for specific IgG1 and IgG2a/b at d22 (C, D) and d70 p.i. (E, F). Data analyzed with Student's t-test, line represents mean, *** indicates $P<0.001$. Graphs show representatives of three independent experiments with at least five mice each group.

3.2.4 Humoral responses induced by immunization

To investigate whether immunization-induced Mf-specific Ig responses were associated with protection, Mf-specific IgE, IgG1 and IgG2 levels were measured in the plasma and pleural space lavage at different time points throughout immunization and infection. As figure 29A and B illustrate, the immunization induced a Mf-specific humoral response and both IgG1 and IgG2 antibodies were elevated in the blood. The most prominent increase was observed after the boost immunizations, as indicated by the levels seven days before the challenge infection. A comparison of both immunized groups (infected vs. uninfected) revealed that these humoral responses were not further enhanced by the infection itself. The same picture was found at the site of infection with Mf-specific IgG1 and IgG2 levels being significantly elevated in immunized mice compared to controls on day 22 (Fig. 29C, D; $P<0.001$) as well as on day 70 post infection (Fig. 29E, F; $P<0.001$). Albeit the differences in IgG1 levels remained significantly higher in immunized mice at day 70 post infection, the IgG1 levels of infected but non-immunized mice increased on days 28 and 42 compared to day 22 post infection (data

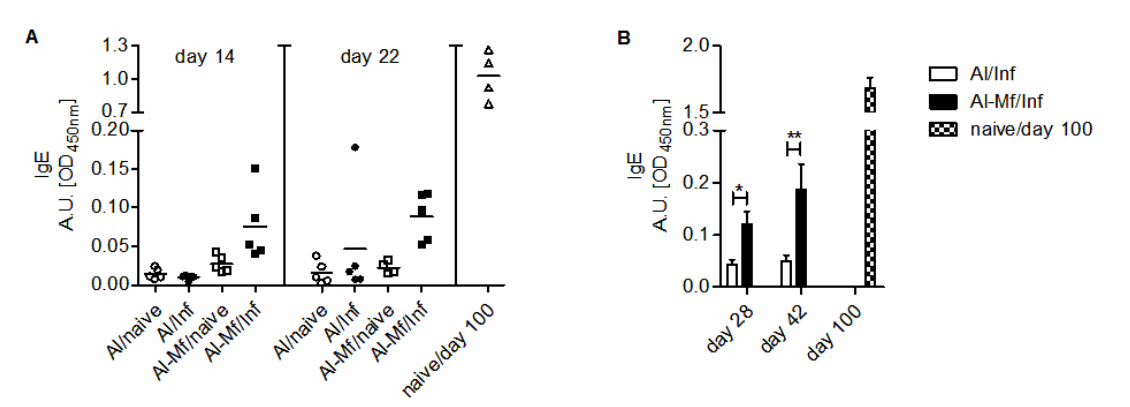


Figure 30. Immunization induces moderate Mf-specific IgE. Pleural space lavage (A) or serum (B) was analyzed for Mf-specific IgE for mice that were either immunized three times s.c. with 100,000 Mf in alum (Al-Mf) or received alum alone as control (Al) and were infected with *L. sigmodontis* one week after the last immunization (Inf) or left uninfected (naive). Samples from chronically infected mice served as control for high Mf-specific IgE. ANOVA was used for statistical analysis. *, ** indicate $P<0.01$, $P<0.001$ respectively.

not shown). This however indicates a Th2 shift induced by the parasite itself and is well-known for primary infected BALB/c mice (Marechal, Le Goff et al. 1997; Babayan, Attout et al. 2005).

The amount of Mf-specific IgE was not strongly increased until day 22 post infection in the pleural space of immunized mice compared to non-immunized mice (Fig. 30A). Later during infection, Mf-specific IgE was elevated in the blood of immunized mice with a mean OD of 0.121 (day 28 post infection) and 0.187 (day 42 post infection) in immunized mice, and a mean OD of 0.043 and 0.050 in control mice, respectively (Fig. 30B). However, these levels of Mf-specific IgE clearly did not reach the IgE levels of chronically infected mice (OD on day 100 post infection 1.683) suggesting that immunization *per se* does not lead to a rough IgE induction.

3.2.5 Cellular responses induced by immunization

To classify cellular responses that have been initiated by the vaccination, we analyzed major cell populations and their cytokine response in the pleural space. Flow cytometric analysis of cell populations revealed no consistent differences (table 4). However, analysis of IFN-γ and IL-5 cytokine amounts showed that immunized mice had significant more IFN-γ in the pleural space ($P<0.001$), whereas the level of IL-5 was low irrespective of the immunization (Fig. 31A). A similar picture was observed when cells recovered from the pleural space were restimulated with worm extracts (Fig. 31B, C). Strikingly, 22 days post infection, a time point when parasites are already present in the pleural space, only cells of immunized mice secreted IFN-γ regardless of whether they were infected or not.

A. Percentages (% of all cells)

		d15		d22		d70 (1st Exp.)		d70 (2nd Exp.)		d90 (1st Exp.)		d90 (2nd Exp.)	
		Value	*P*-Value	Value	*P*-Value	Value	*P*-Value	Value	*P*-Value	Value	*P*-Value	Value	*P*-Value
DC	AI	2.23 ± 0.26		nd		nd		3.49 ± 0.32		2.83 ± 0.25		1.83 ± 0.09	
	AI-Mf	1.55 ± 0.16	0.0856	nd		nd		5.44 ± 0.58	0.0088	4.06 ± 0.46	0.0227	1.83 ± 0.07	0.9689
MO	AI	14.18 ± 0.98		11.30 ± 2.04		23.39 ± 2.44		7.42 ± 0.74		25.44 ± 2.08		9.89 ± 1.61	
	AI-Mf	15.68 ± 1.04	0.3315	14.76 ± 1.18	0.1811	22.36 ± 1.21	0.7090	8.60 ± 1.02	0.3604	21.06 ± 2.05	0.2071	10.62 ± 2.58	0.8086
BC	AI	50.43 ± 4.13		24.50 ± 1.47		1.10 ± 0.35		3.17 ± 0.52		5.38 ± 1.61		1.34 ± 0.27	
	AI-Mf	61.35 ± 2.94	0.0831	23.74 ± 1.01	0.6807	1.42 ± 0.26	0.4743	4.91 ± 0.88	0.1050	4.02 ± 0.81	0.5779	1.83 ± 0.40	0.3197
B2 BC (% of BC)	AI	24.89 ± 2.26		13.76 ± 1.84		46.83 ± 3.65		61.43 ± 4.38		76.12 ± 2.20		81.96 ± 2.30	
	AI-Mf	31.13 ± 2.79	0.1057	15.32 ± 1.86	0.5674	50.52 ± 4.01	0.5045	44.75 ± 4.22	0.0133	71.44 ± 2.57	0.2188	66.62 ± 6.73	0.0421
TC	AI	8.02 ± 0.49		6.92 ± 0.63		4.75 ± 1.49		15.79 ± 2.34		5.31 ± 0.99		4.06 ± 0.58	
	AI-Mf	9.68 ± 1.16	0.1486	6.96 ± 1.40	0.9798	5.31 ± 0.70	0.7375	14.80 ± 1.48	0.7247	7.36 ± 0.80	0.2031	6.75 ± 1.02	0.037
EO	AI	21.12 ± 3.69		41.74 ± 1.47		56.83 ± 5.53		46.08 ± 4.36		34.93 ± 2.10		65.09 ± 3.18	
	AI-Mf	7.88 ± 1.28	0.0177	45.34 ± 3.04	0.3180	41.27 ± 1.61	0.0147	43.80 ± 3.64	0.6926	30.88 ± 2.0	0.2431	55.20 ± 3.34	0.0558

B. Absolute (*10^6)

		d15		d22		d70 (1st Exp.)		d70 (2nd Exp.)		d90 (1st Exp.)		d90 (2nd Exp.)	
		Value	*P*-Value	Value	*P*-Value	Value	*P*-Value	Value	*P*-Value	Value	*P*-Value	Value	*P*-Value
Cells	AI	2.07 ± 0.41		13.61 ± 1.79		17.15 ± 3.24	0.1580	25.12 ± 2.48		58.10 ± 15.68		41.73 ± 7.68	
	AI-Mf	1.57 ± 0.13	0.3705	25.85 ± 1.08	0.0004	11.97 ± 1.37		12.68 ± 2.79	0.0037	30.08 ± 12.21	0.1756	26.20 ± 10.88	0.2580
DC	AI	0.048 ± 0.02		nd		nd		0.86 ± 0.11		1.48 ± 0.35		0.76 ± 0.14	
	AI-Mf	0.024 ± 0.00	0.2465	nd		nd		0.69 ± 0.18	0.4155	1.14 ± 0.44	0.5565	0.45 ± 0.17	0.1937
MO	AI	0.29 ± 0.06		1.62 ± 0.37		4.25 ± 1.29		1.85 ± 0.27		14.85 ± 4.82		4.26 ± 1.16	
	AI-Mf	0.25 ± 0.03	0.6305	3.79 ± 0.30	0.0018	2.75 ± 0.38	0.2768	1.10 ± 0.31	0.0844	6.58 ± 3.04	0.1637	2.18 ± 0.71	0.1696
BC	AI	0.98 ± 0.19		3.35 ± 0.49		0.17 ± 0.05		0.79 ± 0.15		3.15 ± 1.46		0.64 ± 0.21	
	AI-Mf	0.96 ± 0.10	0.9458	6.11 ± 0.22	0.0009	0.15 ± 0.02	0.6559	0.69 ± 0.30	0.6676	0.88 ± 0.32	0.1465	0.56 ± 0.26	0.8224
B2 BC	AI	0.23 ± 0.04		0.44 ± 0.06		0.08 ± 0.02		0.53 ± 0.11		2.49 ± 1.19		0.54 ± 0.19	
	AI-Mf	0.30 ± 0.04	0.3752	0.94 ± 0.13	0.0080	0.08 ± 0.02	0.9585	0.37 ± 0.18	0.4633	0.60 ± 0.11	0.2891	0.42 ± 0.21	0.6611
TC	AI	0.17 ± 0.03		0.98 ± 0.18		0.66 ± 0.13		4.00 ± 0.65		2.85 ± 0.95		1.55 ± 0.26	
	AI-Mf	0.15 ± 0.01	0.6612	1.83 ± 0.41	0.0973	0.64 ± 0.10	0.8357	1.8 ± 0.46	0.0134	2.24 ± 0.89	0.6435	2.03 ± 0.97	0.6149
EO	AI	0.50 ± 0.14		5.76 ± 0.84		9.78 ± 1.66		11.56 ± 1.80		19.88 ± 5.40		27.29 ± 5.39	
	AI-Mf	0.13 ± 0.02	0.0708	11.69 ± 0.82	0.0010	5.03 ± 0.69	0.0167	5.16 ± 1.11	0.0073	8.44 ± 3.15	0.0840	14.34 ± 5.95	0.1342

Table 4. Flow cytometric analysis of pleural space exudate cells 15, 22, 70 and 90 days post infection. Mice were immunized three times s.c. with 100,000 Mf in alum. Control mice received alum alone. *L. sigmodontis* challenge infection was performed one week after last immunization. Percentages (A) and absolute numbers (B) for dendritic cells (DC), macrophages (MO), B-cells (BC) and B2 B-cells (B2 BC), T-cells (TC) and eosinophils (EO) are shown. Staining was performed according to standard protocols with fluorochrome-conjugated antibodies to the surface markers F4/80, SiglecF, CD3ε, CD11c, CD19, and CD23. "nd" indicates that no data are available for that time point.

This effect was seen after specific restimulation with crude extract of adult worms and Mf, as well as with non-specific stimulation by concanavalin A (Fig. 31A, B; $P<0.001$). Although less pronounced, enhanced IFN-γ responses after restimulation were also present throughout patency (Fig. 32A, B). Different to IFN-γ, the IL-5 responses were dependent on the infection itself, as only cells from infected mice secreted IL-5 after restimulation irrespective of immunization (Fig. 31C, Fig. 32C, D).

Finally, since eosinophils are known effector cells in helminth infections (Specht, Saeftel et al. 2006), we measured molecules involved in eosinophils recruitment or activity, i.e. IL-13, macrophage inflammatory protein (MIP)-2α, CCL5, granzyme B, eotaxin-1 and eotaxin-2. Results from three independent experiments did not reveal any significant differences between immunized mice and control animals (data not shown).

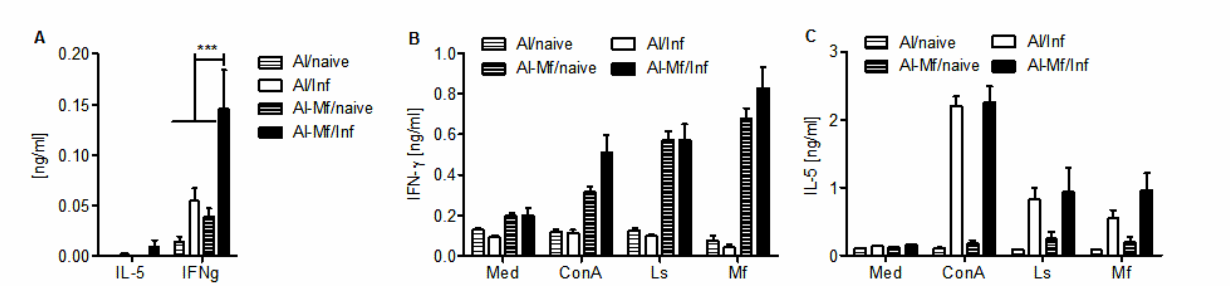


Figure 31. Immunization enhances IFN-γ responses. (A) At day 22 post infection the pleural space lavage was analyzed for absolute amounts of IL-5 and IFN-γ. Pooled data of three independent experiments with five mice each group. Analysis was done with one-way ANOVA; mean ± SEM is depicted, *** indicates $P<0.001$. (B, C) At day 22 post infection 2.5 x 10^5 cells from the site of infection were restimulated 72 hours with 5 µg / ml Concanavalin A (ConA), 100 µg / ml complete adult (Ls) or microfilarial (Mf) crude extract of *L. sigmodontis* (B, C) and IFN-γ (B) and IL-5 (C) secretion were measured. Data presented as mean ± SEM. Representative data of two independent experiments with five mice each group (restimulation with Mf extract in only one experiment). Analysis was done with two-way ANOVA, for significances see text.

Taken together, these results shown here illustrate that the immunization induced high amounts of Mf-specific IgG, but only minor IgE. Since the immunization induced IFN-γ and the level of Th1-associated IgG2a was high, it appears that immunization skews immunity towards the Th1 arm.

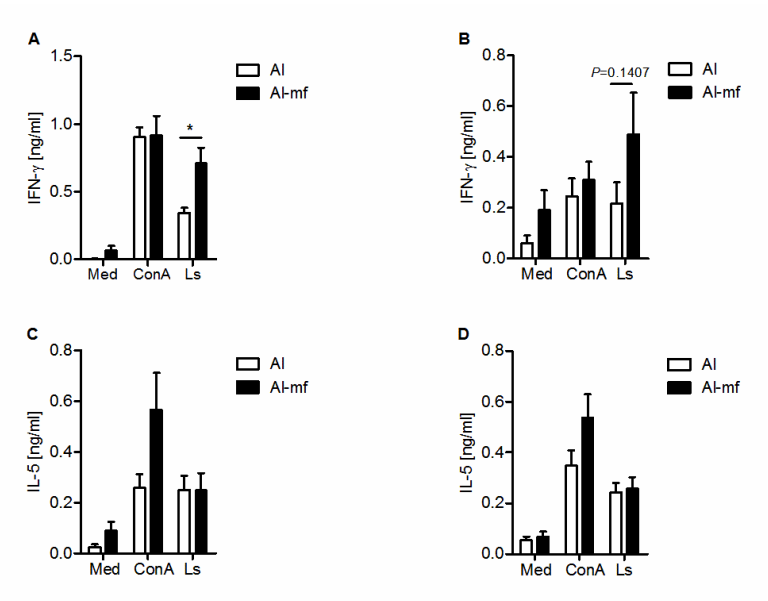


Figure 32. IFN-γ and IL-5 responses of pleural space exudate cells. 2.5*10^5 cells from the site of infection were restimulated 72 hours with 5 µg / ml Concanavalin A (ConA) or 100 µg / ml complete adult crude extract of *L. sigmodontis* (Ls). Restimulation at days 70 (A, C) and 90 (B, D) post infection. Pooled data of two independent experiments with ten mice per group in each experiment. Absolute IFN-γ and IL-5 secretion is shown. Data are presented as mean ± SEM. Analysis were done with two-way ANOVA.

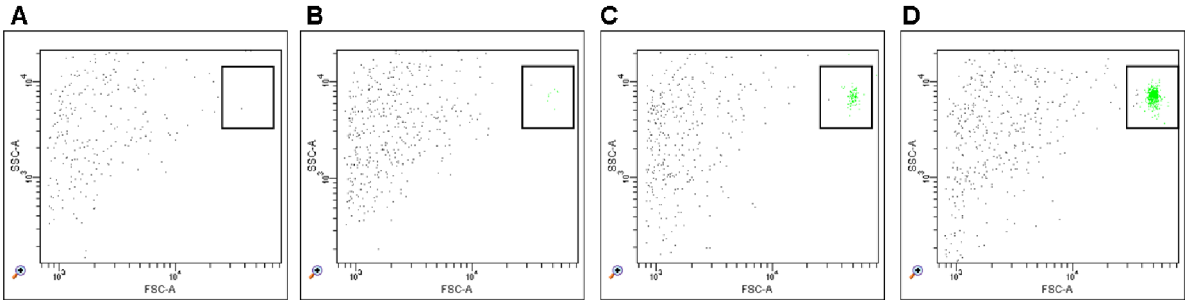


Figure 33. *L. sigmodontis* Mf can be depicted at single larvae level with a flow cytometer at standard configuration. The 80 – 100 µm long and 4 µm wide Mf were isolated from the blood of an infected cotton rat, washed twice, counted and defined numbers of Mf added to 200 µl PBS prior to measurement with a BD Canto 1 flow cytometer. Scatter dot plots are plotted as forward scatter area (FSC-A) against sideward scatter area (SSC-A) showing 200 µl PBS without Mf (A) or with 10 (B), 100 (C) or 1,000 (D) Mf. Gates show area with the Mf.

3.3 Flow cytometric analysis of *L. sigmodontis* Mf

Microfilariae are one main focus of filarial research. For example, it is of interest to analyze the status of the filarial offspring after treatment with microfilaricidal drugs. The data presented in the following section show for the first time that flow cytometry can be used to analyze the filarial Mf stage of *L. sigmodontis*.

Flow cytometer at standard configuration are mainly focused on measurement of events with the size of a cell (~ 1 - 10 µm) or below (the used BD Canto 1 cytometer is limited to 0.5 – 50 µM size at standard configuration) and the pass width of the flow cell, as well as the voltage adjustments defines this range at least at standard instrument configurations (Shapiro 2003). With respect to this restriction a stretched multi cellular Mf (80-100 µm tall) apparently exceeds this size. However, since an Mf is only 4 µm wide and may align in length with the laminar flow, we hypothesized that they could be measured at the flow cytometer. To prove this, we isolated Mf from a microfilaraemic cotton rat, washed them twice, counted them and measured different numbers with the flow cytometer. Figure 33 illustrates, that it was possible to measure the Mf with highest accuracy. The two-dimensional dot plot presentation of events allowed the depiction of a single Mf and was therefore superior to displaying with the one-dimensional histogram of the lab's Casyton cell counter (data not shown). Mf could be measured at maximum flow speed of 10,000 events per second.

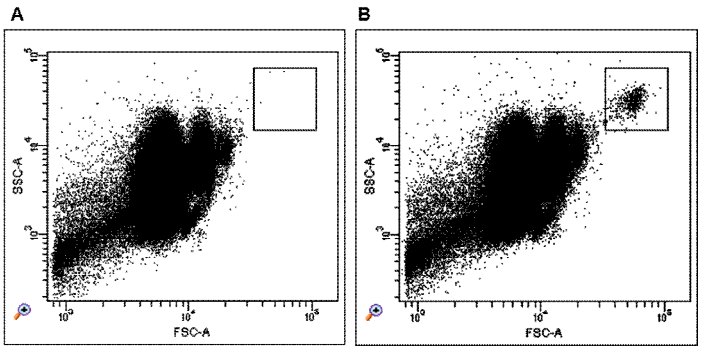


Figure 34. *L. sigmodontis* Mf can be analyzed by flow cytometry directly from peripheral blood. FSC-A vs. SSC-A dot plots of either 10 µl whole blood within 200 µl PBS as control (A) or 1,000 isolated Mf mixed with 10 µl whole blood within 200 µl PBS (B).

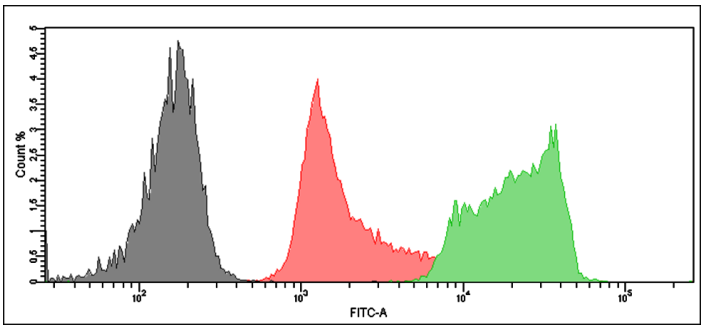


Figure 35. The metabolic activity of *L. sigmodontis* Mf can be analyzed with a commonly used metabolic marker dye. Mf were isolated from the blood of a microfilaraemic cotton rat, washed and counted. Afterwards 50,000 Mf in 100 µl PBS were either left untreated and unstained (grey histogram) or left untreated but stained with 10 ng of the common metabolic activity marker BCECF (green histogram) or were inactivated with 70% ethanol and stained with BCECF (red histogram).

Isolating Mf from the blood is time-consuming; measuring directly from the blood would meet the request of a fast and simple analysis. However, although we could display Mf with high accuracy when analyzed in PBS, the measurement of blood with Mf could be problematic due to high amounts of other events as cells or debris. Therefore, we mixed 200 µl PBS with 10 µl blood of a either a microfilaraemic cotton rat or an uninfected cotton rat without blood-circulating Mf and measured this preparation with the flow cytometer. Figure 34B shows that the Mf grouped as a well-separated population that was larger and more granular than cells of whole blood, as shown by the related scatter signals. However, the blood of the amicrofilaraemic cotton rat (Fig. 34A) displayed also a few false positive events of unknown origin, although the rate was extremely low (< 10 events / 10 µl whole blood).

Having shown that Mf can be depicted by flow cytometry we went one step further and wanted to test a functional flow cytometry assay. The ester BCECF is hydrolyzed by cytosolic esterases into a fluorescent product and is commonly used for analysis of cellular metabolic activity in flow cytometry. We wanted to know whether *L. sigmodontis* Mf can also be analyzed using BCECF. After isolation of Mf from a microfilaraemic cotton rat, we set up two different groups of Mf. In one group the isolated Mf were inactivated by incubation with 70% ethanol, in a second group they were left untreated as control. Afterwards the Mf were stained with BCECF or left unstained. Figure 35 shows that untreated BCECF-stained Mf were clearly distinguishable from inactivated Mf by an increase of fluorescence intensity of more than one decade. Interestingly, the inactivated and BCECF-stained Mf had a higher fluorescence signal compared to the untreated and unstained negative control. This may indicate residual metabolic activity due to incomplete inactivation.

Taken together, we could demonstrate for the first time, that the first larval stage of the rodent filariae *L. sigmodontis* can be analyzed at a flow cytometer at standard configuration without the need to manipulate any constructional feature. Flow cytometry was used not only to count the Mf, but furthermore to analyze a functional attribute of the larvae.

4. DISCUSSION

4.1 The Role of the Pattern Recognition Receptors TLR2, TLR4, NOD2 and of the Adapter Molecule MyD88 in the Murine Model of Lymphatic Filariasis

Filarial infections affect millions of people and many infected individuals present severe disease manifestations. Understanding the host's immune response to infection including the role of the endosymbiotic *Wolbachia* bacteria of the filariae is important to understand the mechanisms of disease pathology. Unravelling these underlying mechanisms is also important to find effective drugs against the infection but also to cure the disease symptoms. The data presented in the first part of this thesis elucidated the role of the PRRs TLR2 and TLR4, and their downstream adapter MyD88 in the murine model of LF. These molecules are thought to play a role in the response after filarial infection. In addition another PRR was investigated: NOD2, a receptor which has emerged as an important part of innate immunity in the last years.

The experiments revealed that the endosymbiotic *Wolbachia* are sensed by the TLR2/6 heterodimer on BMdAPCs *in vitro*, but that this ability is not reflected by enhanced parasite clearance in TLR2-competent WT mice after natural infection *in vivo* (compared to the $TLR2^{-/-}$ littermates). In contrast, mice deficient for the NOD2 receptor have an increased parasite burden and altered filarial development *in vivo*, although this receptor is not necessary for innate responses *in vitro*. The following section integrates these results into the existing body of literature and presents possible explanations for the observed outcomes.

4.1.1 *Wolbachia* are the main provocative driver of inflammatory *in vitro* APC responses

To elucidate the meaning of the endosymbiotic *Wolbachia* in the BMdAPC and HEK cell response to *L. sigmodontis* extracts, we stimulated *in vitro* either with complete filarial extract or with *Wolbachia*-depleted preparation. Doing so, *Wolbachia* components of the *L. sigmodontis* extracts clearly were identified as the main trigger of proinflammatory responses in these experiments with BMdAPCs and HEK cells (Fig. 8A, F; 9A; 10B; 11A and B), because the proinflammatory responses was nearly abolished when the endosymbionts were depleted. However, in some experiments the endosymbionts were not the exclusive stimulus, because response to *Wolbachia*-free extract was still significant higher compared to the medium control. The grade of tetracycline depletion may be a reason for this observation, because very low amounts of *Wolbachia* can remain in the Ls-tet extracts (Arumugam, Pfarr et al. 2008). Another explanation may be the response of other PRRs against non-Wolbachial structures in the preparations (e.g. immunogenic lipids, sugars or others). Nevertheless, the endosymbionts were the main source of proinflammatory TNF and IL-6 response. This is in accordance with findings of other groups, e.g. Taylor et al (Taylor, Cross et al. 2000), who found that

adherent peritoneal exudate cells respond to *Wolbachia*-containing *B. malayi*, but not to *Wolbachia*-free *A. viteae* extract.

4.1.2 The TLR2/6 capacity to sense *Wolbachia*

Having shown that the *Wolbachia* are the main driver of the proinflammatory *in vitro* response, we wanted to know which of the PRRs TLR2, TLR4 and NOD2 may be responsible for this sensing of the bacteria.

First we found the general TLR downstream adapter MyD88 to be essential for the BMdAPC response (Fig. 8B und G), indicating towards TLR-dependent sensing. Following *in vitro* experiments with BMdAPCs and HEK cells revealed that the *Wolbachia*-induced proinflammatory responses after stimulation with filarial extracts indeed depended on TLR2 but not on TLR4 (Fig. 8D, E, I, and J).

Consistent with these findings, BMdDCs generated from C57BL/6 WT and TLR4$^{-/-}$ but not those from TLR2$^{-/-}$ mice respond with elevated expression of CD40 and secretion of proinflammatory IL-6 when stimulated with antigenic preparations from *Wolbachia*-containing *O. volvulus* and *B. malayi*, as shown by a study from Daehnel and colleagues (Daehnel, Gillette-Ferguson et al. 2007). In accordance, *O. volvulus* and *B. malayi* filarial extracts directly stimulate HEK cells that express TLR2 but not those that express TLR3 or TLR4 (Hise, Daehnel et al. 2007). The same study revealed that MOs from C57BL/6 TLR2$^{-/-}$, TLR6$^{-/-}$ or MyD88$^{-/-}$ mice can not be stimulated by these extracts, whereas this is possible for WT and TLR4$^{-/-}$ MOs. These findings are underlined by the fact that the genome of *B. malayi Wolbachia* contains genes for the di-, but not for the triacetylation of proteins (Turner, Langley et al. 2009), because those diacetylated motifs are recognized by the TLR2/6 heterodimer (Kang, Nan et al. 2009). Beside these studies, which promote a pivotal role for TLR2 in the recognition of *Wolbachia* components, importance of TLR4 in this process has also been described: for example, peritoneal MOs isolated from TLR4^{def} mice that were stimulated with recombinant *Wolbachia* surface protein have a reduced TNF secretion (Brattig, Bazzocchi et al. 2004). Interestingly, this reduced TNF secretion after stimulation gets completely abolished when MOs are additionally deficient for TLR2 signaling. In line with this, the NFκB-dependent reporter gene activation, which is observed in TLR4/MD2-transfected HEK cells upon challenge with recombinant *Wolbachia* surface protein, requires higher concentrations for TLR4/MD2-transfected cells than for TLR2/MD2-transfected ones (Turner, Langley et al. 2009). Finally, in a single study the response dependent on TLR4 but not on TLR2: when peritoneal exudate MOs where stimulated with soluble *B. malayi* extract or extracts derived from *Wolbachia*-infected insect cells, the TNF and NO release was completely abolished in the LPS-nonresponsive C3H/HeJ mice, although these mice have a functional TLR2 receptor (Taylor, Cross et al. 2000). Different sources of *Wolbachia* antigen (extracts from various *Wolbachia*-infected filarial worms, extracts from *Wolbachia*-infected insect cell lines, production of recombinant *Wolbachia* protein) and different origins of stimulated cells (bone-marrow

derived cells, peritoneal exudate cells, transfected HEK cells) may account for these conflicting data. Also, additive responses of both receptors may be possible.

4.1.3 The NOD2 capacity to sense *Wolbachia*

In addition to TLR-dependent responses we wanted top elucidate a possible role of the NOD2 receptor, since this receptor has emerged as an important component of innate immunity. Three facts led us to speculate about a possible role of NOD2 signaling: (1) As already mentioned it is documented that TLR2 and NOD2 can act in cooperation, (2) although a PGN layer (and thus MDP) has not been detected so far in *Wolbachia*, the bacteria contain a functional enzymatic machinery to synthesize Lipid II, the final PGN precursor (Henrichfreise, Schiefer et al. 2009) and the PGN component MDP is sensed by NOD2, and (3) the remarkably striking coincidence of the evolutionary distribution of NOD2 and *Wolbachia*: only arthropods and nematodes contain the endosymbiotic bacteria (Fenn and Blaxter 2006) and exactly only these two genera lack the NOD2 receptor (Saleh 2011).

However, stimulation of $NOD2^{-/-}$ BMdAPCs with filarial extract led to secretion of proinflammatory TNF and IL-6 (Fig. 11). This shows that the *in vitro* TLR2 response to *Wolbachia* in $NOD2^{-/-}$ mice is not absent, or in other words: TLR2 does not necessarily need cooperative help from NOD2 to sense the *Wolbachia* and to initiate proinflammatory cytokine secretion. This fits to many studies, which show that NOD2 signaling can synergize with TLR2, but only rarely licenses TLR2 signaling (Magalhaes, Fritz et al. 2008). Importantly, these findings are not in contrast to the observation that NOD2-transfected HEK cells (Zurek, Bielig et al. 2011) respond well with NFκB activation to the lysate of *Wolbachia*-infected insect cells, but only weak to the *Wolbachia*-free lysate (unpublished observation of Thomas Kufer, Institute of Medical Microbiology, Immunology and Hygiene, University Hospital Cologne, Germany), because (1) NFκB activation not necessarily leads to proinflammatory cytokine response and (2) the HEK cells in this assay lack TLR2, thus any interaction between both receptors as they occur *in vivo* are missing.

Taken together, the results of the *in vitro* stimulations of BMdAPCs and HEK cells show that sensing of *L. sigmodontis* crude extract is mainly due to the endosymbiotic *Wolbachia* and that this recognition depends on the TLR2/6, but not the TLR1/2 heterodimer. Furthermore, it does not need the cooperative help of the TLR4 and NOD2 axes, but depends on the downstream adapter MyD88. These results are in line with the understanding, that the TLR2/6 heterodimer senses a diacetylated protein of the endosymbiont and TLR2 signaling in most cases needs downstream MyD88 signaling (Gao, Qi et al. 2012), but rarely NOD2 licensing. Future experiments should address the diacetylated molecular target of recognition, which originates most likely from the outer *Wolbachia* membrane. This is also of importance, because TLR ligands have been discussed as amplifier of vaccine-induced responses to provide not only prophylactic, but furthermore therapeutic protection against infectious

diseases (Duthie, Windish et al. 2011). Furthermore, the interaction of TLR2 and NOD2 should be elucidated by comparative receptor studies of WT, $TLR2^{-/-}$, $NOD2^{-/-}$ and $TLR2^{-/-}NOD2^{-/-}$-deficient cells of the same genetic mouse background.

4.1.4 The responses in the local draining lymph node after *L. sigmodontis* infection

The results from our *in vitro* stimulation experiments led us hypothesize that the absence of TLR2 signaling may impair the responses of skin-DC to incoming *L. sigmodontis* L3. Such skin-DC responsibilities have already been shown for other parasitic infections; e.g. skin-DCs can promote immunity by presenting antigen in the ldLN as shown for leishmaniasis (Moll, Fuchs et al. 1993).

In our experiments, an injection of L3 induced proliferation of both B and T cells in the ldLN and the injection of dead L3 led to greater cell numbers than injection of viable L3 (Fig. 12A, B). This may be due to (1) absent possible inhibitory effects elicited by live L3 (e.g. cystatin (Pfaff, Schulz-Key et al. 2002)) and (2) due to more L3 material left in the case of injection of dead (thus not migrating) larvae. The observed cellular increase in the $MyD88^{-/-}$ and $TLR2^{-/-}$ ldLN (Fig. 12C) revealed, that this process was independent of TLR2 and MyD88, suggesting that signaling through other PRRs (thus of other filarial motifs) or PRR-independent processes (e.g. inflammation, danger signals) may have induced this increase.

In order to mimic a natural infection we established a new model of infection - the locally restricted natural infection - in which L3 are naturally transferred by the bite of *O. bacoti* (Fig. 13) and not injected. This model led to a fully established infection, indicated by the presence of viable larvae in the pleural space 15 days post infection (Fig. 13C). Since the infected *O. bacoti* mites used in our experiments contained 1- 2 infective L3 (mean of 1.4 L3 in all three independent experiments) per mite, and these L3 were nearly all were transferred into mice (Fig. 13B) we could calculate a recovery rate of ~17% for the pleural space on day 15 post infection. This recovery rate is lower than the well documented rates of ~40 % for injected L3 (Hoffmann, Petit et al. 2000), suggesting that the route of infection plays an important role for the establishment of the parasite. To our knowledge this is the first time that a recovery rate has been calculated for a natural *L. sigmodontis* infection in the murine model of filariasis.

Strikingly, we did not find elevated numbers of skin-DC in the ldLN of WT mice using this model (Fig. 14B), indicating that incoming L3 may not elicit (or prevent) substantial emigration of skin-DCs. This is in contrast to Semnani et al., who showed elevated numbers of emigrating LCs in *B. malayi* L3-exposed human epithelial tissue explants ("skin blisters") (Semnani, Law et al. 2004). However, in this study even non-exposed emigrating LCs showed an activated phenotype, i.e. surface expression of activation markers CD80, CD86 and CD40, and elevated expression of MHC class I and II molecules. This indicates that blistering itself might induce emigration, as it has already been shown for other skin-irritating experimental methods, e.g. the tape stripping (Krueger and Emam 1984); therefore slight differences in emigration patterns may be difficult to observe under such non-steady

state conditions. Furthermore *in vitro* exposure of LCs (or other cells) to isolated worms excludes possible additional immunomodulatory mechanisms induced by the arthropod vector itself, e.g. mediators in transferred saliva during the blood meal (Demeure, Brahimi et al. 2005; Depinay, Hacini et al. 2006). However, as we have analyzed immigration to the ldLN rather than the exit of DCs out of the skin, more investigations (especially a more detailed analysis of migration markers) are needed to understand the complete migratory pattern of skin-derived cells during filarial larval entry. With regard to this it is interesting that it has been shown that the blood-circulating *B. malayi* first larval stages down regulate the expression of DC CCR1, a receptor that is involved in leukocyte adhesion and transendothelial migration (Semnani, Mahapatra et al. 2010). Thus, homing of cells to the ldLN maybe influenced by the third stage larvae as well. Furthermore, it would be of interest to analyze whether CCR7, which is involved in homing DCs to secondary lymphoid organs may be altered by skin-migrating L3 as shown in HCMV infection (Varani, Frascaroli et al. 2005). The fact that we observed no cellular increase in the LN eight days after locally restricted infection (data not shown) also suggests the absence of immigration and subsequent cellular responses. Again, this is in strong contrast to the data for s.c. injection (Fig. 12A and (Taylor, van der Werf et al. 2008)).

The data presented here show that the model of the locally restricted natural infection provides evidence that incoming viable *L. sigmodontis* L3 avoid substantial immigration of skin DCs into the ldLN, and as a consequence restrain cellular increase in the ldLN. Since these data are contrary to results from s.c.-injected larvae (which do induce proliferation of T and B cells); the results emphasize the differences between the natural infection and the s.c. injection. The results may be interpreted with regard to the findings that helminths can modulate host immunity and interfere with TLR signaling as discussed in detail in section 4.1.6 (page 63). Future experiments should be carried out to elucidate whether incoming L3 achieve a possible modulation during the early passage through skin and lymphatics, e.g. by analysis of regulatory cells and mediators.

4.1.5 Cellular responses at the site of infection in TLR2$^{-/-}$TLR4^{def}, NOD2$^{-/-}$ and MyD88$^{-/-}$ mice

After *L. sigmodontis* infection the pleural space gets populated by immune cells like MOs, eosinophils and B cells. We therefore extensively studied the cellular content of the pleural lavage and found cellular differences between WT and NOD2$^{-/-}$ mice: whereas the percentage of conventional B2 cells in the pleural space was as expected (about 25% of all B cells (Al Qaoud, Fleischer et al. 1998; Montecino-Rodriguez and Dorshkind 2006)) they were lowered in NOD2$^{-/-}$ mice (Fig. 18).

The B2 cells are a hallmark of adaptive immunity, due to their ability to induce humoral antibody responses and to tailor T cell effector functions (Lund and Randall 2010). These B cells are part of the adaptive arm of immunity and represent the dominant B cell subpopulation. Therefore they are also termed “conventional” B cells and are in contrast to the minor B1 subset that contributes to innate responses (Montecino-Rodriguez and Dorshkind 2006). In helminth infections B cells are

widely known to bias T cell responses to Th2 immunity and to polarize humoral antibody responses towards IgE and IgG1 (Harris and Gause 2011). Also regulatory functions are attributed to B cells, e.g. by secretion of IgG4 (Hoerauf, Satoguina et al. 2005). The exact function of B cells in the murine model of LF seems to be complex because BALB/c mice without B1 cells (Xid mice) have elevated parasite burden (Al Qaoud, Fleischer et al. 1998), whereas mice of the same genetic background without all mature B cells (μMT mice) have similar worm burden (Martin, Saeftel et al. 2001). Furthermore, μMT mice of the semi resistant C57BL/6 background have (as the WT) no parasites on day 60 post infection, indicating that the semi resistance of this mouse strain is not broken by the lack of B cells (Le Goff, Lamb et al. 2002). The observed lower frequency of B2 cells in our experiments may provide an interpretation for the observed impaired larval development in $NOD2^{-/-}$ mice, especially since this B cell compartment has been attributed to larval development during *L. sigmodontis* infection (Martin, Saeftel et al. 2001). Although that study demonstrated that the length of filariae was similar between mice with or without B cells and the observed developmental impairment affected the first (and not the fourth) larval stage, it points towards a development-promoting function of B cells. This is supported by the finding of lower IL-5 level in infected B cell deficient mice (Babayan, Read et al. 2010), since this cytokine also is also considered to be beneficial for larval development. However, we found more IL-5 in $NOD2^{-/-}$ mice (Fig. 19). The different cytokine sources (blood vs. site of infection vs. *ex vivo* restimulated cells) and the different time points of assessment may account for this controversial data. In the above mentioned study of Martin and colleagues it was also observed that significantly more adult worms were surrounded by host cells in the B cell deficient mice sixty days post infection (Martin, Saeftel et al. 2001). Thus, it would be interesting to monitor this effect in our $NOD2^{-/-}$ mice. However, the semi resistant C57BL/6 does not allow the monitoring of those late events, since the parasites are nearly rejected at that time point in this mouse strain (Fig. 16C).

In contrast to the findings for $NOD2^{-/-}$ mice, we did not find any consistent differences in the percentage or absolute cell numbers of DCs, MOs, B and T cells, and eosinophils in the TLR and MyD88-deficient mice (tables 1 and 2) suggesting that the lack of TLR2, TLR4 and MyD88 signaling does not lead to different cellular infiltration and / or proliferation. Using the murine model of LF it has already been documented that even though differences in worm burden and worm length occur, the absolute numbers and percentages of pleural space cells remain similar, e.g. in mice deficient of granzyme A or CCL17 (Hartmann, Marsland et al. 2011; Specht, Frank et al. 2011). However, it has to be considered that not all cell populations of the pleural space were analyzed with our staining protocol. Moreover, non confluent cells of the thoracic cavity were not analyzed, since they are not part of the lavage, e.g. epithelial or lung cells. These cells might contribute to responses against the filarial occupant and future experiments should address this issue.

The data shown here indicate that alterations in the pleural B cell compartment in NOD2$^{-/-}$ mice may be a reason for the altered parasite burden and development, and that IL-5 may play a role in this outcome. Future experiments should address both the role of B cells and IL-5, e.g. by depleting IL-5 or analyzing granuloma formation in NOD2$^{-/-}$ mice. The overall picture of basically similar cellular patterns in the pleural space of *L. sigmodontis*-infected TLR-deficient and (except B cells) in NOD2$^{-/-}$ mice fits in the growing body of literature, which shows that *qualitative* rather than *quantitative* aspects of these cells play a role during responses against the filariae. Future experiments could consider this and analyze markers of cellular activation and regulation, e.g. by flow cytometric analysis of surface markers or Western blot analysis of intracellular mediators.

4.1.6 The role of TLR and MyD88 signaling after *L. sigmodontis* infection

A deficiency in TLR2 or TLR4 signaling could facilitate the establishment of *L. sigmodontis* infection *in vivo*, as many of these cells express TLRs (Kawai and Akira 2010). An example of that kind of *in vivo* relevance due to TLR signaling has already been observed in the murine model of LF: in semi resistant C3H/HeJ mice, which have a natural defect in TLR4 signaling, this lack was associated with higher fertility of female *L. sigmodontis* worms and release of filarial offspring into the pleural space. Interestingly, same study revealed, that the overall worm burden was not different between both groups (Pfarr, Fischer et al. 2003), what fits to our finding of similar parasite burden in C3H WT and TLR2$^{-/-}$TLR4^{def} and MyD88$^{-/-}$ mice (Fig. 15).

Three hypotheses are discussed to present possible explanations for the fact that TLR2 and MyD88 are essential to sense the filarial *Wolbachia in vitro* but do not essentially contribute to rejection of *L. sigmodontis* infection *in vivo*: (1) the general down regulation of host TLR immunity, (2) the spatial separation of response-inducing *Wolbachia* from the host, and (3) the ability of another PRR to compensate for TLR2 signaling. These hypotheses are outlined in figure 36 and discussed as follows.

(1) Immunomodulation is a hallmark of helminth infections (Hoerauf, Satoguina et al. 2005) and can already take place at the site of larval entry (skin): in Semnani's "skin blister" experiments with *B. malayi* L3 the human LCs had diminished surface expression of MHC and activation markers, down-regulated gene expression of genes involved in antigen processing and presentation, and a reduced capacity to activate autologous CD4^{+} T cells, indicating that L3 interfere with LC immunity. Evidence of early suppressive intervention by filarial L3 is also proven by the fact that Treg can already be found in the ldLN seven days after s.c. injection of *L. sigmodontis* L3 (Taylor, van der Werf et al. 2008). Therefore, as several effector arms of adaptive immunity are down-regulated by filarial nematodes, signaling through TLRs (and MyD88) might occur after APCs have encountered the incoming L3 but remain idle with respect to effector mechanisms that could harm the filariae. This theory is supported by our finding using injections of live or dead L3, because cellular increase was

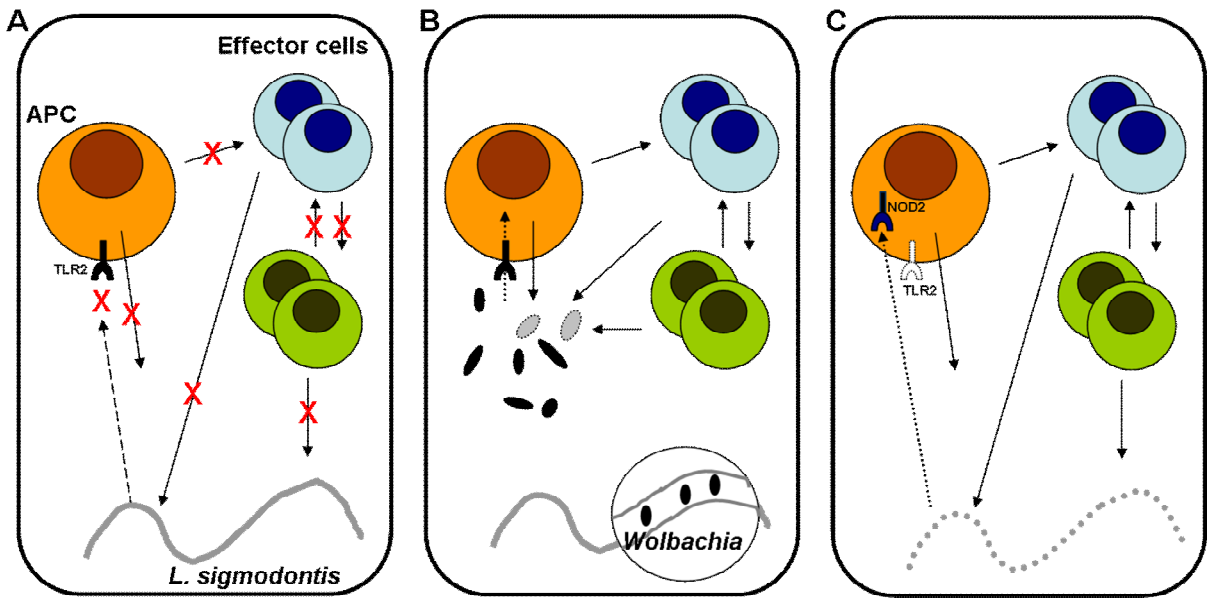


Figure 36. Hypothetical models to explain why the TLR2 capacity to sense *Wolbachia in vitro* is not translated into effective immune responses *in vivo* after *L. sigmodontis* infection. (A) *L. sigmodontis* filariae may variously inhibit (red crosses) host immunity. TLR2-dependent Ag (dashed arrow) sensing might be blocked, as well as communication of APCs to effector cells (e.g. T cells, B cells, NK cells) or communication between effector cells. Furthermore, responses of APCs and effector cells to the filariae might be blocked. (B) Spatial separation of *Wolbachia*. Despite the possible sensing (dotted arrow) and clearance of bacteria outside the filariae (black ellipses intact bacteria, grey ellipses destroyed bacteria), *Wolbachia* within intact worms are separated from the host immune system. (C) NOD2 compensates for missing TLR2-dependent responses. In $TLR2^{-/-}$ mice NOD2 signaling (dotted arrow) may compensate for the missing TLR2 sensing and induces host responses (black arrows), that are finally directed against the filariae.

ower after injection of live L3 compared to injection of dead larvae (Fig. 12A). In line with this, McScorly et al. observed, that *in vivo* expansion of Tregs can be found when live but not when dead *B. malayi* L3 are injected (McSorley, Harcus et al. 2008), suggesting L3-modulated suppression. One way of immunomodulation might be direct impairment of TLR responses. In clinical settings Babu et al. observed diminished baseline expression of TLR1, TLR2, TLR4 and TLR9 on B cells of filarial-infected individuals. Moreover, upon restimulation with filarial antigen the normal up regulation of TLRs is diminished in B cells, T cells and monocytes of these infected individuals, leading to decreased activation and cytokine production in response to TLR ligands (Babu, Blauvelt et al. 2005; Babu, Blauvelt et al. 2006). In another study Mf-exposed monocyte-derived human DCs had diminished TLR3, TLR4 and MyD88 mRNA expression (Semnani, Venugopal et al. 2008). Therefore, in our experiments the missing TLR signaling in $TLR2^{-/-}TLR4^{def}$ and $MyD88^{-/-}$ mice may not be mirrored by increased parasite burden because TLR signaling (and linked possible protective immune responses) is impaired *L. sigmodontis*-mediated immunomodulation also in the TLR-competent WT mice. Thus, WT, $TLR2^{-/-}TLR4^{def}$, and $MyD88^{-/-}$ mice all would not be able to mount TLR-dependent responses against the parasites.

(2) In adult nematodes the *Wolbachia* are mainly located in the hypodermis and the maternal germ line (Bazzocchi, Comazzi et al. 2007) and therefore spatially separated from the host's immune

system. The fact that healthy *B. malayi* adults secrete *Wolbachia* through their excretory / secretory canals into their hosts (thus providing immunogenic material (Landmann, Foster et al. 2010)) indicates that *Wolbachia* are only accessible outside the worms, and only then the bacteria are sensed and eventually cleared by host responses. For *O. volvulus*, another *Wolbachia*-containing filariae, those kind of cellular reactions are already documented: neutrophils accumulate around the filariae only when the endosymbionts are present within the worms (Brattig, Buttner et al. 2001). Maybe TLRs are involved in those responses, as well as soluble PRRs of PGN (Sorbara and Philpott 2011) and membrane channels for PGN sampling (Philpott and Girardin 2010). However, the *Wolbachia* within intact worms remain separated from many (if not most or all) host effector responses. Thus, WT mice may cope with *Wolbachia* outside the filariae, but not with those within healthy filariae, what could be an explanation for the similar parasite burden.

(3) Interactions between both PRR classes can be synergistic as well as antagonistic and TLR signaling can compensate for absent NOD-dependent signaling (Franchi, Warner et al. 2009). However, it was also shown that the NOD signaling axis alone is sufficient enough to reject an *in vivo* infection, even in the absence of MyD88-dependent TLR signaling (Archer, Ader et al. 2010): mice infected with *Legionella pneumophila* are moderately resistant to infection in the absence of MyD88, but become highly susceptible if NOD signaling is additionally defective. Thus, the TLR-deficient mice in our experiments might not have an exacerbated course of *L. sigmodontis* infection, because another PRR has compensated for those absent immune responses (in line with this, we found the $NOD2^{-/-}$ mice to be more susceptible to *L. sigmodontis* infection, as discussed below).

Taken together, our data show that the TLR2- and MyD88-dependent *in vitro* capacity of APCs to sense *Wolbachia* is not translated into an enhanced ability to inhibit establishment and / or to reject filarial infection *in vivo* in the murine model of LF. Reasons for this finding might be (1) a inhibition of TLR-dependent host immune responses, (2) the spatial separation of the endosymbiotic *Wolbachia*, and (3) the ability of another PRR to compensate for missing TLR2 signaling. Our results do not exclude any *in vivo* relevance of TLR signaling, but underlie the necessity of the murine model of LF to confirm and interpret the relevance of results generated with *in vitro* or *in situ* studies. Future experiments should address these issues by further analyzing the possible modes of regulation, the location and accessibility of *Wolbachia* in the pleural space and the interplay of TLR2 and other PRRs. Finally, it should be mentioned that the impact of TLR- and MyD88-signaling on the pathology of the *L. sigmodontis* infection was not investigated, because the pathological manifestations in the murine model of LF do not reflect those of the human disease (see section 1.1) very well. Thus, instead of rejecting the infection *per se*, signaling via TLR2 and MyD88 might play a role in the proinflammatory response-related pathology.

4.1.7 The role of NOD2

As discussed above, *in vitro Wolbachia* sensing requires TLR2 and MyD88, but these receptors did not promote clearance of infection *in vivo*. Interestingly, the situation for NOD2 was *vice versa*: (1) NOD2$^{-/-}$ mice are more susceptible to infection (indicated by increased parasite burden, Fig. 16), suggesting a function of this receptor in the host defense against the filariae, as well (2) filariae in NOD2$^{-/-}$ mice are impaired in development and growth (Fig. 17), suggesting an additional role associated with the development of the parasite. As already exemplified for IL-5, one and the same host factor can be beneficial for the host as well as for the parasite. The functionality of the NOD2 receptor maybe likewise; therefore entities must exist that allow this functional separation.

4.1.7.1 NOD2 in the early phase of infection

Since we found that the NOD2$^{-/-}$ mice are more susceptible to *L. sigmodontis* infection already at day 15 post infection (Fig. 16A), the receptor seems to promote early host responses against the parasites *in vivo*.

NOD2 senses the MDP motif in the bacterial cell; thus, the idea that the endosymbiotic *Wolbachia* provide the ligands for NOD2 stimulation is of beauty - but compromised by the fact, that *Wolbachia* are a cell wall-less genus and no PGN has reliably detected in the endosymbiotic bacteria so far. However, this does not exclude any MDP in *Wolbachia*, since cell wall synthesis pathways have been found in *Wolbachia*, e.g. for Lipid II (Henrichfreise, Schiefer et al. 2009). That the bacteria and not the filariae trigger NOD2 is further supported by the fact that only bacterial pathogens are described as sources of NOD2 ligands so far (Caetano, Biswas et al. 2011; Shaw, Kamada et al. 2011). For example, NOD2 signaling is able to promote immune responses *in vivo* against *Mycobacterium tuberculosis* (Divangahi, Mostowy et al. 2008) or *Staphylococcus aureus* (Hruz, Zinkernagel et al. 2009) bacteria. The latter report showed that NOD2$^{-/-}$ mice suffer from higher bacterial burden and a more severe disease manifestation after *S. aureus* skin inoculation (Hruz, Zinkernagel et al. 2009). The authors could pin down this enhanced susceptibility to reduced IL-1ß-dependent secretion of proinflammatory IL-6 at the site of inoculation in the skin, which in consequence rendered neutrophils less capable to kill the bacteria. Interestingly, in filarial infections it was found that IL-1ß and IL-6 peak six hours after stimulation of murine MOs with recombinant *Wolbachia* surface protein (Porksakorn, Nuchprayoon et al. 2007) and furthermore neutrophils are recruited to the skin within six hours after *L. sigmodontis* skin challenge in mice (Martin, Saeftel et al. 2001). A possible molecular basis for the observed depressed neutrophil response comes from a study from Netea and colleagues (Netea, Kullberg et al. 2004), who found that the mucosal expression of proinflammatory antimicrobial α-defensins is decreased in patients who have a loss-of-function mutation in NOD2, since neutrophils are known to release these host defense peptides against filarial worms in human skin (Brattig, Buttner et al. 2001). In addition to an indirect response (due to recruitment / activation

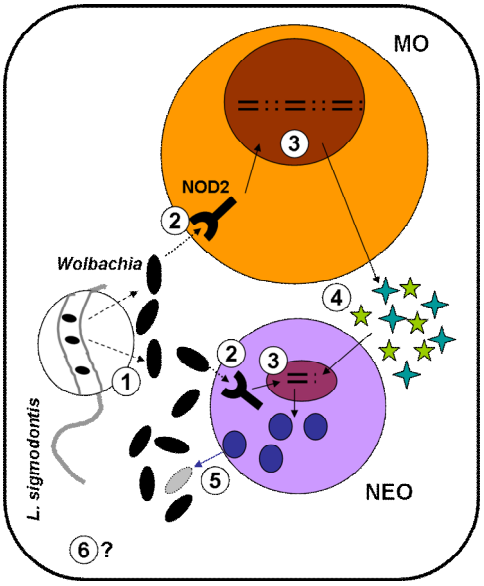


Figure 37. Hypothetic models to explain how NOD2 contributes to effective host immune responses against *Wolbachia in vivo* after *L. sigmodontis* infection. *Wolbachia* (black ellipses) are released (dashed arrows) from *L. sigmodontis* (1) and sensed (dotted arrows) by intracellular sensor NOD2 (2), either by macrophages (MO) or neutrophils (NEO). Sensing by macrophages induces gene expression (3) and leads to secretion of neutrophil recruitment and / or activating mediators (colored stars) (4) with subsequent proinflammatory gene expression (3) and release of antimicrobial defensins (blue arrows) that destroy the bacteria (5). Processes (3) – (5) may also be induced directly by sensing through neutrophil NOD2. How those responses would harm the filariae remains elusive (6).

by other cells after their NOD2 signaling) neutrophils may also sense the NOD ligands directly since they express the receptor (Ekman and Cardell 2009). Evidence for direct sensing of *Wolbachia* by neutrophils is supported by the finding that a genetic functional impairment of NOD2 is associated with defective neutrophil responses in bacteria-related granulomatous mastitis (Bercot, Kannengiesser et al. 2009). Thus, it can be hypothesized that after *L. sigmodontis* infection neutrophils control, at least in part, the establishment of host responses to the incoming larvae and that the increased susceptibility of NOD2$^{-/-}$ mice in our experiments might be due to impaired neutrophil response to free *Wolbachia*, which may be released from healthy L3 (as seen for *B. malayi* (Landmann, Foster et al. 2010)) or originate from dying larvae. As mentioned above, these neutrophil responses may take place after their directly sensing of *Wolbachia* or in reply to the sensing by other cells, e.g. MOs which in turn secrete proinflammatory (e.g. IL-6) and neutrophil recruiting / activating cytokines. After activation neutrophils could then deposit their bacteria-destroying defensins. This hypothesized model is summarized in figure 37.

Cells other than neutrophils may also participate in NOD2 innate responses at the site of infection. For example, it has been shown that mast cells get activated by PGN in a NOD2-dependent manner (Wu, Feng et al. 2007). Of note, this activation required prior loading of PGN to the mast cells by TLR2, again emphasizing the tight cooperation of these two PRRs. This cooperation might also explain the TLR2-dependency of mast cell-mediated enhanced vascular permeability that is observed in CCL17$^{-/-}$ mice after inoculation with *L .sigmodontis* L3 (Specht, Frank et al. 2011). In addition to neutrophils and mast cells, primary murine intestinal epithelial cells have been shown to respond to NOD2 triggering, namely with secretion of ROS (Lipinski, Till et al. 2009). Since the infective larval stages of *L. sigmodontis* are confronted with epithelial barriers (the squamous epithelium of the skin (Nestle, Di Meglio et al. 2009) and the inner endothelium of the lymph vessels (Scavelli, Weber et al. 2004)) NOD2-dependent responses may take place and account for the lower worm burden in WT mice. However, a pivotal role for epithelial cells in the murine model of LF has not been show so far.

For sure, the manner in which those NOD2-related responses can harm the healthy *L. sigmodontis* filariae remains elusive and should be a main focus on future work in the murine model of LF as further discussed below.

4.1.7.2 NOD2 in the later phase of infection

Whereas NOD2 signaling promotes host immunity in the early phase of infection, the impaired worm development implies a NOD2 function that is beneficial for the parasite. Possible explanations for this phenomenon are discussed in the following section.

Compared to the fully permissive BALB/c mouse strain, adults of the C57BL/6 strain are known to be retarded in development and growth (Babayan, Ungeheuer et al. 2003). This tempted us to speculate that deficiency in NOD2 signaling might enable the parasite to grow and develop better than in NOD2-competent WT mice. For that reason, developmental stage, size and gender balance of parasites were examined. Surprisingly, we found the opposite: $NOD2^{-/-}$ mice had shorter larvae and had an impaired development into the adult stage.

As described in the former section, NOD2 signaling can contribute to proinflammatory host immunity, e.g. by rejecting skin-evading bacteria. However, compared to the overwhelming number of studies that reveal a role for NOD2 in proinflammatory responses *in vitro*, very few studies have addressed the relevance of NOD2 signaling *in vivo*. Moreover, human diseases that are related to a loss-of-function of NOD2 (e.g. Crohn's disease) have features of chronic but not impaired inflammation (Borzutzky, Fried et al. 2010). Thus, it appears that maintaining bacterial homeostasis is a major function of NOD2 signaling (as reviewed in (Biswas, Petnicki-Ocwieja et al. 2012)). Together with the findings that (1) one of the best known targets of NOD2 regulation is TLR2 signaling and (2) the TLR2/6 heterodimer senses *Wolbachia*, one hypothesis to explain the impaired larval development in $NOD2^{-/-}$ mice might be that TLR2 signaling in these mice is not under the control of negative NOD2 regulation any longer. Thus, $NOD2^{-/-}$ mice TLR2-dependent responses might be enhanced and contribute to effector mechanisms that account for the impaired larval development. There are already examples of dampened TLR2 responses in the presence of NOD2 signaling: two studies could show that NOD2 signaling inhibits TLR2 signaling by influencing cRel, a subunit of proinflammatory NFκB that is activated after TLR2 activation (Watanabe, Kitani et al. 2004; Dahiya, Pandey et al. 2011). Interestingly, in one of these studies the deficiency in NOD2 signaling led to a loss of TLR2-driven establishment of Th immune responses, suggesting that NOD2 can act as a negative regulator of this T helper cell axis. This idea has also been endorsed by others: for example it was shown that NOD2 signaling triggers a potent antigen-specific immune response with a Th2-polarized profile (T cell IL-4 and IL-5, as well as systemic IgG1) in C57BL/6 mice (Magalhaes, Fritz et al. 2008). More recently, Yang and colleagues found that the Th2 skew of murine Ana-1 cells, which is observed after stimulation with mCIITA (a mutated form of the transactivator of MHC class II activation (CIITA)), is abolished by the deactivation of the NOD2 with siRNA (Yang, Gu et al. 2012). This Th2 skewing

characteristic trait of NOD2 signaling is of interest with regard to helminth studies, because the Th2 immunity associated with those infections is thought to be a result of long lasting co-evolution which minimizes the overall harm to the infected host by dampening immune responses (Maizels, Pearce et al. 2009; Allen and Maizels 2011). In line with this assumption it is known that Th1 responses limit the success of worm reproduction / transmission, because those responses negatively act on the filarial offspring (see section 1.2.2.3). From that point of view the $NOD2^{-/-}$ mice in our experiments might have suppressed adaptive Th2 responses, what may lead to a milieu that is beneficial for larval development.

Taken together, the data provided here show that NOD2 signaling is part of successful early immune responses against incoming filariae, but is beneficial for larval development in the later phase of infection. The existing knowledge about the role of NOD2 signaling in LF is very limited. However, the results presented here indicate that this receptor may have significant relevance in the onset and manifestation of the disease. Future experiments should elucidate how the NOD2 receptor participates in generating early responses after the larval entry, e.g. by analyzing the immigration of skin DCs into the local draining lymph node in $NOD2^{-/-}$ mice. Special focus should be given to skin-residing neutrophils since these cells have been shown to be relevant for *in vivo* responses to incoming L3, as well as for NOD2 signaling-dependent proinflammatory events. How NOD2 signaling promotes larval development could be elucidated by analyzing the interaction between TLR2 and NOD2 (since NOD2 can inhibit signaling via TLR2) and enlightening the Th1 / Th2 milieu in the $NOD2^{-/-}$ mice (since an altered milieu may be detrimental for larval development). In all this experiments the possible interaction of TLR2 and NOD2 should be elucidated at different time points of infection, because this might allow understanding how NOD2 signaling can be beneficial for both the host (in terms of disordered *L. sigmodontis* development) and the parasite (in terms of increased susceptibility to *L. sigmodontis* infection).

4.2 Immunization of BALB/c Mice against the First Larval Stage of *L. sigmodontis*

Current public health control programs of human filarial infections rely on chemotherapy provided by MDA programs. Antifilarial drug therapy has to be implemented for years with high coverage at rather high logistical costs and the emergence of drug resistance has become a potential threat (Bergquist and Lustigman 2010). Thus, as suggested for other NTDs, a vaccine that results in the reduction of parasite burden would complement the MDA efforts (Hotez 2011).

The present study describes a successful immunization protocol against *L. sigmodontis* Mf in the murine model of LF, which additionally resulted in reduced adult worm burden. Subcutaneous immunization with Mf in alum prevented the onset of microfilaraemia after challenge infection in the majority of mice. Reduced Mf loads were observed in the peripheral blood and at the site of infection in conjunction with intrauterine inhibition of embryogenesis. Protection was further associated with systemic and local Mf-specific IgG and IFN-γ secretion of pleural space exudate cells.

4.2.1 Influence of injection route and adjuvant

In mouse models the adjuvant alum is often administered i.p. and this route has been referred as to be able to establish "systemic" responses in contrast to the "local" s.c. route (Harris, Holloway et al. 2005). Interestingly, we found that neither the systemic i.v. route alone nor the combination of the local s.c. and systemic i.p. and i.v. routes could reduce microfilaraemia (Fig. 22A, C). As systemic immunizations have been reported to be able to induce tolerance rather than immunity (Aebischer, Morris et al. 1994), out of the protocols tested in this study, only the s.c. immunization was able to immunize mice successfully against *L. sigmodontis* Mf. The s.c. immunization would also be one of the routes which would be most applicable to humans.

The injection of IVM after i.v. immunization did not protect mice from microfilaraemia (Fig. 22B), although the injected Mf disappeared within one day from the peripheral blood (Fig. 23B), suggesting that Mf were immobilized as expected (Wolstenholme and Rogers 2005). Together with the finding that immunization (primary s.c., followed by i.p. and i.v. route) with irradiated Mf (leading to paralysis of Mf, Fig. 24) failed as well to establish protection (Fig. 22D), suggests that only the combination of the s.c. immunization route together with an appropriate adjuvant initiates the immunological processes that finally establish protection against microfilaraemia in the immunized host.

Our results are in contrast to Wenk and colleagues who where able to render cotton rats protected from peripheral Mf, using the immunization scheme that failed in our experiments (primary immunization s.c., and afterwards i.p and i.v. with untreated Mf; (Wenk and Wegerhof 1982)). The differences between both animal models in the mode of Mf reduction may be due to different susceptibilities of the hosts to *L. sigmodontis*. Both, BALB/c mice and cotton rats develop patent

infections, but BALB/c mice clear the infection after 3-4 months, whereas the cotton rat continues to harbor the filarial parasites for years (Hoffmann, Petit et al. 2000).

To exclude that the adjuvant itself did not influence immunity we also compared the number of peripheral Mf of sham-treated mice, which only received PBS with the numbers of those that received alum alone and did not find any significant differences neither in the course of microfilaraemia, nor in the number of Mf^+ mice (Fig. 26). These data clarify that alum alone does not influence the degree of microfilaraemia.

4.2.2 Immunization reduces microfilarial burden

Inhibition of embryogenesis in immunized mice was indicated by reduced numbers of peripheral and pleural space Mf (Fig. 25) and the presence of embryonic stages that had not developed beyond several divisions of fertilized oocytes (Fig. 27). The latter is again in contrast to Wenk and colleagues who found fully developed Mf in the uterus of female worms and in the pleural space of infected and Mf-immunized cotton rats (Wenk and Wegerhof 1982). However, the already mentioned differences between both animal models again serve as a possible explanation. This is the general downside of using laboratory mice for *L. sigmodontis* infection rather than the natural host; however the advantage is that cytokine responses can be measured and associated with protection, and for future studies cytokine deficient mice may answer further questions about the essentiality of key cytokines for vaccination success.

4.2.3 Immunization affects adult *L. sigmodontis* stages after onset of patency

It is known that immunization with irradiated L3 stages reduces the recovery rate of larvae in the pleural space (Le Goff, Martin et al. 2000). However, immunization with Mf did not render mice less susceptible for infection *per se* as the worm burden did not differ on day 15 post infection (Fig. 28A). The short time between immunization and challenge infection might explain the absence of enhanced immunity against incoming L3. The adult worm burden remained similar in both groups until the onset of patency at day 56 post infection (Fig. 28B), whereas at later time points, namely at days 70 and 90 post infection (thus, after onset of patency), a reduced worm burden in immunized mice was observed (Fig. 28C, D). This suggests that the first accessibility of Mf in the pleural space could be a critical step in initiating the responses that affect the adult filariae. Interestingly, the reduced worm burden was seen both for male and female worms (Fig. 28E), which suggests that effector mechanisms not only act against intrauterine Mf in female worms, but against target structures on all adult worms. In line with this, different developmental stages of filariae share many molecular structures (Moreno and Geary 2008) and cross-reactive immunization effects have been documented in filarial immunization studies (Dabir, Dabir et al. 2008; Sahoo, Sisodia et al. 2009). Importantly, it is unlikely that the lower worm burden, observed on days 70 and 90 post infection, is the reason for reduced microfilaraemia in immunized mice, because all immunized mice contained male and female

worms and it has been shown that only a few fecund females are required to establish peripheral microfilaraemia (Hoffmann, Pfaff et al. 2001).

Taken together we demonstrate the feasibility of an immunization that is directed against the Mf stage, leading to reduced microfilarial burden in the pleural space and the blood, thus protecting against peripheral microfilaraemia with an efficacy of up to 100%. Since it is known for human LF that a threshold of Mf density in the peripheral blood is required to achieve transmission and moreover high numbers of infective bites are needed to produce a patent infection (Bockarie, Taylor et al. 2009), reduction of circulating peripheral Mf at the level we observed might prevent transmission. In the later phase of infection, most likely after onset of patency, immunization further reduces adult worm burden in the host. Future experiments should address the exact roles of the injection route and adjuvant to elucidate the duty of both parameters for successful immunization. Furthermore, time points and mechanisms of responses against the adult filariae should be monitored.

4.2.4 Immunoglobulins and vaccine-induced protection

In our experiments, immunization-induced Mf-specific IgG1 and IgG2, present throughout the whole infection including patency, were associated with protection (Fig. 29). Because protective responses induced by immunization often rely on protective antibodies (Baxter 2007) and the importance of B cells in promoting immune responses against filarial Mf is well documented for the murine model of filariasis (Al Qaoud, Fleischer et al. 1998; Gray and Lawrence 2002), we analyzed the frequency and total number of pleural space B1 and B2 cells by flow cytometry but found no consistent differences between immunized and non-immunized mice (table 4). This suggests that there are differences in B cell activation rather than in total B cell numbers. Interestingly, despite a strong Ab production female worms of immunized mice morphologically resembled those from non-immunized control mice. Furthermore, we did not find any host cells within the female worms. These two findings suggest a blockade of embryonic development, rather than a cell-dependent destruction of the embryonic stages. In our experiments, Mf-specific IgGs may have entered the female worm uterus and bound to the developing early developmental stages hindering their further growth in an Ig-dependent, but cell-independent manner. Indeed, it is known that filarial-infected humans produce filarial-specific Ig, which are able to bind to early intrauterine filarial stages, which was shown using sera from chronic LF patients against isolated intrauterine Mf stages from the filariae *Setaria digitata* (Sahu, Mohapatra et al. 2005). Another possibility for cell-independent but Ig-mediated responses is the activation of the complement cascade resulting in the formation of the membrane attack complex (MAC), due to insertion of complement proteins into a phospholipid bilayer (Dunkelberger and Song 2010). Although there is no evidence for MAC formation in the sheath of adult nematodes so far, earlier developmental stages may be sensitive to MAC formation.

An efficient vaccination integrates the B cell arm of immunity, leading to the production of antigen-specific Ig (Boog 2009). However, adverse IgE reactions after immunization are a major concern after vaccination attempts in countries with endemic Th2-related infections. Such reactions were observed in a phase 1 trial of a hookworm (*Necator americanus*) vaccine in an hookworm-endemic area of Brazil, a single dose of the vaccine resulted in generalized urticarial reactions due to pre-existing IgE (Bethony, Cole et al. 2011). We did not observe a strong IgE response after immunization and infection neither in the blood nor at the site of infection (Fig. 30). However, in our setting we only immunized naïve mice. Thus, immunization of already infected mice should be the next step to determine any possible IgE reactions. In humans, a vaccination may be favorable one month after IVM treatment, when individuals have no skin or blood dwelling Mf, as the risk of urticaria due to adverse IgE reactions on any remaining Mf would be minimum.

4.2.5 T helper cell cytokines may contribute to vaccine-induced protection

The adaptive type 2 response is referred to as "typical" for helminth infections (Allen and Maizels 2011). However, we found that immunization was associated with strong IFN-γ responses, since levels were strongly increased in the pleural space and after antigen-specific restimulation of PLECs (Fig. 31B).

The importance of IFN-γ production in immune responses against Mf in permissive BALB/c mice is underlined by several findings. Interferon-$\gamma^{-/-}$ mice have increased numbers of circulating *L. sigmodontis* Mf compared to the WT littermates (Saeftel, Volkmann et al. 2001). In addition it has been shown that IFN-γ RNA levels of restimulated splenocytes obtained from *L. sigmodontis*-infected BALB/c mice are strongly increased within days after the onset of patency (Taubert and Zahner 2001). These observations may reflect the moderate increase in IFN-γ production seen in non-immunized mice upon natural infection and the less pronounced but still significant differences in IFN-γ between immunized and non-immunized mice during patency (Fig. 32A, B). Furthermore, it is known that injection of *B. malayi* Mf but not implantation of adult stages induces IFN-γ and Th-1-assiociated IgG2a in BALB/c mice (Lawrence, Allen et al. 1994). Since IFN-γ is an inducer of IgG2a (Snapper and Paul 1987) it is most likely that in our experiments, Mf-induced IFN-γ has promoted the secretion of IgG2. Importantly, the induction of IFN-γ is not in conflict with the use of the adjuvant alum, which is generally referred to as a Th2-promoting substance, because recent findings have shown that alum also can influence proliferation and IFN-γ production of $CD8^+$ T cells (Mohr, Cunningham et al. 2010). Furthermore, TLR ligands have been found to bias alum responses towards a mixed Th1/Th2 profile (Duthie, Windish et al. 2011).

Although IL-5 responses did not differ between immunized and control mice (Fig. 31C and Fig. 32C, D) this cytokine may have an overall role during the immunization. It may even be possible that the effect of immunization, although predominated by IFN-γ, may be dependent on at least baseline levels of IL-5, since in earlier reports this cytokine has been shown to be important for both

adult worm and Mf containment during *L. sigmodontis* infection (Martin, Al-Qaoud et al. 2000; Volkmann, Bain et al. 2003). However, the latter study suggests that IL-5 is relevant with respect to the emigration of Mf from the pleural space into the blood rather than with respect to embryogenesis at all, reflected by the similar pleural Mf burden in IL-$5^{-/-}$ mice despite elevated burden in the peripheral blood.

The results presented in this section portrait the association of Mf-specific IgG and a shift of Th immunity from the Th2 bias to a Th1 scenario; that kind of shift (high IgG2a and IFN-γ) has already been observed for a *B. malayi* DNA vaccine in jirds (Thirugnanam, Pandiaraja et al. 2007). The next steps would be to clarify the exact role of specific antibodies in the establishment of immunization-induced protection, e.g. by verification of embryonic stage-bound Mf-specific Ig. Also, a possible IgE reaction to the immunization in already infected host has to be elucidated. At the very best, experiments with mice having a defect in Ig production would give most valid insights in the relevance of Ig for impairment of embryogenesis. The elevated level of IFN-γ that has been induced by the immunization suggests a shift towards a Th1-like milieu in the host that may furthermore promote direct or indirect responses against the Mf during patency, possibly through IFN-γ-promoted IgG2a. Future immunization experiments with BALB/c mice defective for IFN-γ or IL-5 responses should also shed more light on the importance of both Th key cytokines for the inhibition of embryogenesis.

4.2.6 Implications for a human vaccine

The study presented here contributes to the understanding of the immune mechanisms that are required to develop a vaccine against filarial parasites. Whereas the use of Mf recovered from infected humans would be costly and the number of Mf limited, left aside the potential transmission of other infections, our data may serve for a better understanding of the nature of protective Mf vaccination, and future assessments should address the characterization of microfilarial molecular subunits that account for this protection, as the growing field of helminth genomics (Brindley, Mitreva et al. 2009) may predict such potential Mf-related vaccine candidates. Administration of only a subunit vaccine may also avoid vaccination with tolerogenic molecules contained within the Mf and allow more successful of protection.

4.3 The Potential of Flow Cytometry in the Field of Microfilarial Research

Microfilariae were the primary target in the immunization study presented in this thesis. We therefore wanted to know whether the *L. sigmodontis* Mf in the murine model of filarial research can be analyzed with a flow cytometer at standard configuration.

First, we could show for the first time that stretched Mf can pass the flow cell of the cytometer without causing a blockade even at maximum speed of 10,000 events per second. Very likely this must mean that the 80-100 µm long and 4 µm wide larvae align with the laminar flow, thus avoiding blockade of the 10 µm wide flow cell. When Mf were isolated from blood, the resolution was at the single larvae level (Fig. 33). The separation by size and granularity furthermore allowed the depiction of the Mf even in untreated murine blood, albeit with lower resolution quality (Fig. 34). This result approves that flow cytometry is a fast and easy way to count the first larval stage in the murine model of LF.

Finally, we assessed whether the Mf could also be functionally analyzed by flow cytometry. We therefore incubated the larvae with the metabolic marker BCECF and found that metabolically active and inactive larvae could be separated by this method (Fig. 35). To our knowledge, this is the first time that a flow cytometry-based assay has been performed with Mf in the murine model of LF.

Collectively, these results open up a variety of new possibilities in filariasis research: the use of commercially available panels of fluorochrome-conjugated antibodies will allow the search for molecules on the Mf surface that may interact with the host immune system, e.g. analogs to murine ligands of inhibiting immune receptors. Besides staining with antibodies, other commonly used flow cytometry-based methods may be applied, such as staining of DNA or analysis of apoptosis. This would allow new ways of defining the status of Mf, e.g. after drug treatment or irradiation. In line with this, most recently the embryonic stages of the filariae *S. digitata* have been analyzed by using common flow cytometry assays (probidium iodide, Annexin-V, cytochrome-c, ROS, TUNNEL) (Mohapatra, Kumar et al. 2011).

5. REFERENCES

Aebischer, T., L. Morris, et al. (1994). "Intravenous injection of irradiated *Leishmania major* into susceptible BALB/c mice: immunization or protective tolerance." Int Immunol **6**(10): 1535-1543.

Akashi-Takamura, S. and K. Miyake (2008). "TLR accessory molecules." Current opinion in immunology **20**(4): 420-425.

Al Qaoud, K. M., B. Fleischer, et al. (1998). "The Xid defect imparts susceptibility to experimental murine filariosis--association with a lack of antibody and IL-10 production by B cells in response to phosphorylcholine." Int.Immunol. **10**(1): 17-25.

Al Qaoud, K. M., E. Pearlman, et al. (2000). "A new mechanism for IL-5-dependent helminth control: neutrophil accumulation and neutrophil-mediated worm encapsulation in murine filariasis are abolished in the absence of IL-5." Int.Immunol. **12**(6): 899-908.

Al Qaoud, K. M., A. Taubert, et al. (1997). "Infection of BALB/c mice with the filarial nematode *Litomosoides sigmodontis*: role of CD4+ T cells in controlling larval development." Infect.Immun. **65**(6): 2457-2461.

Allen, J. E. and R. M. Maizels (2011). "Diversity and dialogue in immunity to helminths." Nat Rev Immunol **11**(6): 375-388.

Amaral, F., G. Dreyer, et al. (1994). "Live adult worms detected by ultrasonography in human Bancroftian filariasis." The American journal of tropical medicine and hygiene **50**(6): 753-757.

Anand, S. B., V. Murugan, et al. (2008). "Comparison of immunogenicity, protective efficacy of single and cocktail DNA vaccine of *Brugia malayi* abundant larval transcript (ALT-2) and thioredoxin peroxidase (TPX) in mice." Acta Trop. **107**(2): 106-112.

Archer, K. A., F. Ader, et al. (2010). "Cooperation between multiple microbial pattern recognition systems is important for host protection against the intracellular pathogen *Legionella pneumophila*." Infection and immunity **78**(6): 2477-2487.

Arumugam, S., K. M. Pfarr, et al. (2008). "Infection of the intermediate mite host with *Wolbachia*-depleted *Litomosoides sigmodontis* microfilariae: impaired L1 to L3 development and subsequent sex-ratio distortion in adult worms." Int J Parasitol **38**(8-9): 981-987.

Attout, T., C. Martin, et al. (2008). "Pleural cellular reaction to the filarial infection *Litomosoides sigmodontis* is determined by the moulting process, the worm alteration, and the host strain." Parasitology international **57**(2): 201-211.

Babayan, S., T. Attout, et al. (2005). "Increased early local immune responses and altered worm development in high-dose infections of mice susceptible to the filaria *Litomosoides sigmodontis*." Med.Microbiol.Immunol. **194**(3): 151-162.

Babayan, S., M. N. Ungeheuer, et al. (2003). "Resistance and susceptibility to filarial infection with *Litomosoides sigmodontis* are associated with early differences in parasite development and in localized immune reactions." Infect.Immun. **71**(12): 6820-6829.

Babayan, S. A., T. Attout, et al. (2006). "Vaccination against filarial nematodes with irradiated larvae provides long-term protection against the third larval stage but not against subsequent life cycle stages." Int J Parasitol **36**(8): 903-914.

Babayan, S. A., A. F. Read, et al. (2010). "Filarial parasites develop faster and reproduce earlier in response to host immune effectors that determine filarial life expectancy." PLoS Biol **8**(10): e1000525.

Babu, S., R. Anuradha, et al. (2011). "Filarial Lymphatic Pathology Reflects Augmented TLR-mediated, MAPK-mediated Pro-inflammatory Cytokine Production." Infection and immunity.

Babu, S., S. Q. Bhat, et al. (2009). "Filarial lymphedema is characterized by antigen-specific Th1 and th17 proinflammatory responses and a lack of regulatory T cells." PLoS.Negl.Trop.Dis. **3**(4): e420.
Babu, S., C. P. Blauvelt, et al. (2005). "Diminished expression and function of TLR in lymphatic filariasis: a novel mechanism of immune dysregulation." J Immunol **175**(2): 1170-1176.
Babu, S., C. P. Blauvelt, et al. (2006). "Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection." J Immunol **176**(7): 3885-3889.
Bain, O. and S. Babayan (2003). "Behaviour of filariae: morphological and anatomical signatures of their life style within the arthropod and vertebrate hosts." Filaria.J. **2**(1): 16.
Balmer, P. and E. Devaney (2002). "NK T cells are a source of early interleukin-4 following infection with third-stage larvae of the filarial nematode *Brugia pahangi*." Infection and immunity **70**(4): 2215-2219.
Baxter, D. (2007). "Active and passive immunity, vaccine types, excipients and licensing." Occup Med (Lond) **57**(8): 552-556.
Bazzocchi, C., S. Comazzi, et al. (2007). "*Wolbachia* surface protein (WSP) inhibits apoptosis in human neutrophils." Parasite Immunol. **29**(2): 73-79.
Benko, S., D. J. Philpott, et al. (2008). "The microbial and danger signals that activate Nod-like receptors." Cytokine **43**(3): 368-373.
Bercot, B., C. Kannengiesser, et al. (2009). "First description of NOD2 variant associated with defective neutrophil responses in a woman with granulomatous mastitis related to corynebacteria." J.Clin.Microbiol. **47**(9): 3034-3037.
Bergquist, R. and S. Lustigman (2010). "Control of important helminthic infections vaccine development as part of the solution." Advances in parasitology **73**: 297-326.
Bethony, J. M., R. N. Cole, et al. (2011). "Vaccines to combat the neglected tropical diseases." Immunol Rev **239**(1): 237-270.
Biragyn, A., P. A. Ruffini, et al. (2002). "Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2." Science **298**(5595): 1025-1029.
Biswas, A., T. Petnicki-Ocwieja, et al. (2012). "Nod2: a key regulator linking microbiota to intestinal mucosal immunity." Journal of molecular medicine **90**(1): 15-24.
Bockarie, M. J., M. J. Taylor, et al. (2009). "Current practices in the management of lymphatic filariasis." Expert.Rev.Anti.Infect.Ther. **7**(5): 595-605.
Boog, C. J. (2009). "Principles of vaccination and possible development strategies for rational design." Immunol Lett **122**(2): 104-107.
Borzutzky, A., A. Fried, et al. (2010). "NOD2-associated diseases: Bridging innate immunity and autoinflammation." Clin Immunol **134**(3): 251-261.
Bozic, C. R., N. P. Gerard, et al. (1994). "The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization." The Journal of biological chemistry **269**(47): 29355-29358.
Brattig, N. W., C. Bazzocchi, et al. (2004). "The major surface protein of *Wolbachia* endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4." J.Immunol. **173**(1): 437-445.
Brattig, N. W., D. W. Buttner, et al. (2001). "Neutrophil accumulation around Onchocerca worms and chemotaxis of neutrophils are dependent on Wolbachia endobacteria." Microbes.Infect. **3**(6): 439-446.
Brindley, P. J., M. Mitreva, et al. (2009). "Helminth genomics: The implications for human health." PLoS neglected tropical diseases **3**(10): e538.
Caetano, B. C., A. Biswas, et al. (2011). "Intrinsic expression of Nod2 in CD4+ T lymphocytes is not necessary for the development of cell-mediated immunity and host

resistance to Toxoplasma gondii." European journal of immunology **41**(12): 3627-3631.
CDC (2011). "Epidemiology and Prevention of Vaccine-Preventable Diseases." U.S. Department of Health and Human Services **12th Edition**.
Chandrashekar, R., U. R. Rao, et al. (1986). "*Brugia malayi*: rat cell interactions with infective larvae mediated by complement." Exp Parasitol **62**(3): 362-369.
Cox, F. E. (2002). "History of human parasitology." Clinical microbiology reviews **15**(4): 595-612.
Dabir, S., P. Dabir, et al. (2008). "Prophylactic evaluation of recombinant extracellular superoxide dismutase of *Brugia malayi* in jird model." Vaccine **26**(29-30): 3705-3710.
Daehnel, K., I. Gillette-Ferguson, et al. (2007). "Filaria/*Wolbachia* activation of dendritic cells and development of Th1-associated responses is dependent on Toll-like receptor 2 in a mouse model of ocular onchocerciasis (river blindness)." Parasite Immunol. **29**(9): 455-465.
Dahiya, Y., R. K. Pandey, et al. (2011). "Nod2 downregulates TLR2/1 mediated IL1beta gene expression in mouse peritoneal macrophages." PloS one **6**(11): e27828.
de Veer, M. J., J. M. Kemp, et al. (2007). "The innate host defence against nematode parasites." Parasite Immunol. **29**(1): 1-9.
Debrah, A. Y., S. Mand, et al. (2007). "Macrofilaricidal effect of 4 weeks of treatment with doxycycline on Wuchereria bancrofti." Tropical medicine & international health : TM & IH **12**(12): 1433-1441.
Debrah, A. Y., S. Mand, et al. (2007). "Plasma vascular endothelial growth Factor-A (VEGF-A) and VEGF-A gene polymorphism are associated with hydrocele development in lymphatic filariasis." The American journal of tropical medicine and hygiene **77**(4): 601-608.
Demeure, C. E., K. Brahimi, et al. (2005). "*Anopheles* mosquito bites activate cutaneous mast cells leading to a local inflammatory response and lymph node hyperplasia." J.Immunol. **174**(7): 3932-3940.
Depinay, N., F. Hacini, et al. (2006). "Mast cell-dependent down-regulation of antigen-specific immune responses by mosquito bites." J.Immunol. **176**(7): 4141-4146.
Dittrich, A. M., A. Erbacher, et al. (2008). "Helminth infection with *Litomosoides sigmodontis* induces regulatory T cells and inhibits allergic sensitization, airway inflammation, and hyperreactivity in a murine asthma model." J Immunol **180**(3): 1792-1799.
Divangahi, M., S. Mostowy, et al. (2008). "NOD2-deficient mice have impaired resistance to Mycobacterium tuberculosis infection through defective innate and adaptive immunity." Journal of immunology **181**(10): 7157-7165.
Dixit, S., R. L. Gaur, et al. (2006). "Protection against L3 induced *Brugia malayi* infection in *Mastomys coucha* pre-immunized with BmAFII fraction of the filarial adult worm." Vaccine **24**(31-32): 5824-5831.
Dunkelberger, J. R. and W. C. Song (2010). "Complement and its role in innate and adaptive immune responses." Cell Res **20**(1): 34-50.
Duthie, M. S., H. P. Windish, et al. (2011). "Use of defined TLR ligands as adjuvants within human vaccines." Immunol Rev **239**(1): 178-196.
Dziarski, R. and D. Gupta (2010). "Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity." Innate immunity **16**(3): 168-174.
Ekman, A. K. and L. O. Cardell (2009). "The expression and function of Nod-like receptors in neutrophils." Immunology.
Fenn, K. and M. Blaxter (2006). "Wolbachia genomes: revealing the biology of parasitism and mutualism." Trends Parasitol **22**(2): 60-65.

Fernandez, R. D., B. Dubben, et al. (2009). "Filarial infection induces protection against P. berghei liver stages in mice." Microbes.Infect. **11**(2): 172-180.
Franchi, L., N. Warner, et al. (2009). "Function of Nod-like receptors in microbial recognition and host defense." Immunol.Rev. **227**(1): 106-128.
Gao, Q., L. Qi, et al. (2012). "*Clostridium butyricum* activates TLR2-mediated MyD88-independent signaling pathway in HT-29 cells." Molecular and cellular biochemistry **361**(1-2): 31-37.
Goldstein, B. (2001). "On the evolution of early development in the Nematoda." Philosophical transactions of the Royal Society of London. Series B, Biological sciences **356**(1414): 1521-1531.
Goodridge, H. S. and D. M. Underhill (2008). "Fungal Recognition by TLR2 and Dectin-1." Handbook of experimental pharmacology(183): 87-109.
Gray, C. A. and R. A. Lawrence (2002). "A role for antibody and Fc receptor in the clearance of *Brugia malayi* microfilariae." Eur J Immunol **32**(4): 1114-1120.
Haas, B. and P. Wenk (1981). "Elimination of microfilariae (*Litomosoides carinii* Filarioidea) in the patent and in the immunized cotton-rat." Trans.R.Soc.Trop.Med.Hyg. **75**(1): 143-144.
Habedank, B. (2002). "Die Tropische Rattenmilbe *Ornithonyssus bacoti* und andere Raubmilben - seltene Parasiten des Menschen in Mitteleuropa." Denisia **6**(184): 447 - 460.
Harris, N. and W. C. Gause (2011). "To B or not to B: B cells and the Th2-type immune response to helminths." Trends in immunology **32**(2): 80-88.
Harris, N. L. (2011). "Advances in helminth immunology: optimism for future vaccine design?" Trends Parasitol.
Harris, N. L., J. Holloway, et al. (2005). "Tissue localization and frequency of antigen-specific effector CD4 T cells determines the development of allergic airway inflammation." Immunology and cell biology **83**(5): 490-497.
Hartmann, W., I. Haben, et al. (2011). "Pathogenic nematodes suppress humoral responses to third-party antigens in vivo by IL-10-mediated interference with Th cell function." Journal of immunology **187**(8): 4088-4099.
Hartmann, W., B. J. Marsland, et al. (2011). "A novel and divergent role of granzyme a and B in resistance to helminth infection." J Immunol **186**(4): 2472-2481.
Hedl, M. and C. Abraham (2011). "Secretory mediators regulate Nod2-induced tolerance in human macrophages." Gastroenterology **140**(1): 231-241.
Henrichfreise, B., A. Schiefer, et al. (2009). "Functional conservation of the lipid II biosynthesis pathway in the cell wall-less bacteria Chlamydia and Wolbachia: why is lipid II needed?" Mol Microbiol **73**(5): 913-923.
Hise, A. G., K. Daehnel, et al. (2007). "Innate immune responses to endosymbiotic *Wolbachia* bacteria in *Brugia malayi* and *Onchocerca volvulus* are dependent on TLR2, TLR6, MyD88, and Mal, but not TLR4, TRIF, or TRAM." J.Immunol. **178**(2): 1068-1076.
Hoebe, K., P. Georgel, et al. (2005). "CD36 is a sensor of diacylglycerides." Nature **433**(7025): 523-527.
Hoerauf, A. (2008). "Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis." Curr.Opin.Infect.Dis. **21**(6): 673-681.
Hoerauf, A., S. Mand, et al. (2001). "Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment." Lancet **357**(9266): 1415-1416.
Hoerauf, A., S. Mand, et al. (2003). "Doxycycline in the treatment of human onchocerciasis: Kinetics of Wolbachia endobacteria reduction and of inhibition of embryogenesis in female Onchocerca worms." Microbes Infect **5**(4): 261-273.

Hoerauf, A., K. Nissen-Pahle, et al. (1999). "Tetracycline therapy targets intracellular bacteria in the filarial nematode Litomosoides sigmodontis and results in filarial infertility." J Clin Invest **103**(1): 11-18.
Hoerauf, A., K. Pfarr, et al. (2011). "Filariasis in Africa-treatment challenges and prospects." Clin Microbiol Infect **17**(7): 977-985.
Hoerauf, A., J. Satoguina, et al. (2005). "Immunomodulation by filarial nematodes." Parasite Immunol. **27**(10-11): 417-429.
Hoerauf, A., S. Specht, et al. (2008). "*Wolbachia* endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study." Med.Microbiol.Immunol. **197**(3): 295-311.
Hoerauf, A., L. Volkmann, et al. (2000). "Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis." Lancet **355**(9211): 1242-1243.
Hoffmann, W., G. Petit, et al. (2000). "*Litomosoides sigmodontis* in mice: reappraisal of an old model for filarial research." Parasitol.Today **16**(9): 387-389.
Hoffmann, W. H., A. W. Pfaff, et al. (2001). "Determinants for resistance and susceptibility to microfilaraemia in *Litomosoides sigmodontis* filariasis." Parasitology **122**(Pt 6): 641-649.
Hotez, P. (2011). "Enlarging the "Audacious Goal": Elimination of the World's high prevalence neglected tropical diseases." Vaccine: Epub ahead of print.
Hotez, P. J., P. J. Brindley, et al. (2008). "Helminth infections: the great neglected tropical diseases." J.Clin.Invest **118**(4): 1311-1321.
Hruz, P., A. S. Zinkernagel, et al. (2009). "NOD2 contributes to cutaneous defense against Staphylococcus aureus through alpha-toxin-dependent innate immune activation." Proc Natl Acad Sci U S A **106**(31): 12873-12878.
Jenkins, S. J., D. Ruckerl, et al. (2011). "Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation." Science **332**(6035): 1284-1288.
Jin, M. S., S. E. Kim, et al. (2007). "Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide." Cell **130**(6): 1071-1082.
Jordan, J. M., M. E. Woods, et al. (2008). "The Absence of Toll-Like Receptor 4 Signaling in C3H/HeJ Mice Predisposes Them to Overwhelming Rickettsial Infection and Decreased Protective Th1 Responses." Infect.Immun. **76**(8): 3717-3724.
Junpee, A., T. Tencomnao, et al. (2010). "Association between Toll-like receptor 2 (TLR2) polymorphisms and asymptomatic bancroftian filariasis." Parasitol Res **107**(4): 807-816.
Kang, J. Y., X. Nan, et al. (2009). "Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer." Immunity **31**(6): 873-884.
Kawai, T. and S. Akira (2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol **11**(5): 373-384.
Kimmig, P. and P. Wenk (1982). "[Suppression of parasitemia in rodent filariasis (*Litomosoides carinii*) by immunization with BCG and microfilaria. I. Intracutaneous inoculation of BCG]." Z.Parasitenkd. **67**(3): 317-327.
Kissenpfennig, A., S. Henri, et al. (2005). "Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells." Immunity **22**(5): 643-654.
Korten, S., L. Volkmann, et al. (2002). "Expansion of NK cells with reduction of their inhibitory Ly-49A, Ly-49C, and Ly-49G2 receptor-expressing subsets in a murine helminth infection: contribution to parasite control." J.Immunol. **168**(10): 5199-5206.
Kozek, W. J. (1977). "Transovarially-transmitted intracellular microorganisms in adult and larval stages of *Brugia malayi*." The Journal of parasitology **63**(6): 992-1000.

Krueger, G. G. and M. Emam (1984). "Biology of Langerhans cells: analysis by experiments to deplete Langerhans cells from human skin." J.Invest Dermatol. **82**(6): 613-617.
Kufer, T. A. and P. J. Sansonetti (2011). "NLR functions beyond pathogen recognition." Nature immunology **12**(2): 121-128.
Kupper, T. S. and R. C. Fuhlbrigge (2004). "Immune surveillance in the skin: mechanisms and clinical consequences." Nat Rev Immunol **4**(3): 211-222.
Kwissa, M., S. P. Kasturi, et al. (2007). "The science of adjuvants." Expert review of vaccines **6**(5): 673-684.
Landmann, F., J. M. Foster, et al. (2010). "Asymmetric Wolbachia Segregation during Early *Brugia malayi* Embryogenesis Determines Its Distribution in Adult Host Tissues." PLoS Negl Trop Dis **4**(7): e758.
Landmann, F., D. Voronin, et al. (2011). "Anti-filarial activity of antibiotic therapy is due to extensive apoptosis after Wolbachia depletion from filarial nematodes." PLoS pathogens **7**(11): e1002351.
Lawrence, R. A. (2001). "Immunity to filarial nematodes." Vet Parasitol **100**(1-2): 33-44.
Lawrence, R. A., J. E. Allen, et al. (1994). "Adult and microfilarial stages of the filarial parasite *Brugia malayi* stimulate contrasting cytokine and Ig isotype responses in BALB/c mice." J Immunol **153**(3): 1216-1224.
Lawrence, R. A. and E. Devaney (2001). "Lymphatic filariasis: parallels between the immunology of infection in humans and mice." Parasite Immunol **23**(7): 353-361.
Le Goff, L., T. J. Lamb, et al. (2002). "IL-4 is required to prevent filarial nematode development in resistant but not susceptible strains of mice." International journal for parasitology **32**(10): 1277-1284.
Le Goff, L., C. Martin, et al. (2000). "Parasitology and immunology of mice vaccinated with irradiated *Litomosoides sigmodontis* larvae." Parasitology **120 (Pt 3)**: 271-280.
Lipinski, S., A. Till, et al. (2009). "DUOX2-derived reactive oxygen species are effectors of NOD2-mediated antibacterial responses." Journal of cell science **122**(Pt 19): 3522-3530.
Lund, F. E. and T. D. Randall (2010). "Effector and regulatory B cells: modulators of CD4(+) T cell immunity." Nat Rev Immunol **10**(4): 236-247.
Magalhaes, J. G., J. H. Fritz, et al. (2008). "Nod2-dependent Th2 polarization of antigen-specific immunity." J.Immunol. **181**(11): 7925-7935.
Maizels, R. M., E. J. Pearce, et al. (2009). "Regulation of pathogenesis and immunity in helminth infections." J.Exp.Med. **206**(10): 2059-2066.
Makepeace, B. L., S. A. Jensen, et al. (2009). "Immunisation with a multivalent, subunit vaccine reduces patent infection in a natural bovine model of onchocerciasis during intense field exposure." PLoS.Negl.Trop.Dis. **3**(11): e544.
Mand, S., A. Y. Debrah, et al. (2012). "Doxycycline Improves Filarial Lymphedema Independent of Active Filarial Infection: A Randomized Controlled Trial." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.
Mand, S., K. Pfarr, et al. (2009). "Macrofilaricidal activity and amelioration of lymphatic pathology in bancroftian filariasis after 3 weeks of doxycycline followed by single-dose diethylcarbamazine." Am J Trop Med Hyg **81**(4): 702-711.
Marechal, P., L. Le Goff, et al. (1997). "Immune response to the filaria *Litomosoides sigmodontis* in susceptible and resistant mice." Parasite Immunol. **19**(6): 273-279.
Martin, C., K. M. Al-Qaoud, et al. (2000). "IL-5 is essential for vaccine-induced protection and for resolution of primary infection in murine filariasis." Med Microbiol Immunol **189**(2): 67-74.
Martin, C., L. Le Goff, et al. (2000). "Drastic reduction of a filarial infection in eosinophilic interleukin-5 transgenic mice." Infect.Immun. **68**(6): 3651-3656.

Martin, C., M. Saeftel, et al. (2001). "B-cell deficiency suppresses vaccine-induced protection against murine filariasis but does not increase the recovery rate for primary infection." Infect.Immun. **69**(11): 7067-7073.

Martinez, F. O. (2011). "Regulators of macrophage activation." European journal of immunology **41**(6): 1531-1534.

McLaren, D. J., M. J. Worms, et al. (1975). "Micro-organisms in filarial larvae (Nematoda)." Transactions of the Royal Society of Tropical Medicine and Hygiene **69**(5-6): 509-514.

McSorley, H. J., Y. M. Harcus, et al. (2008). "Expansion of Foxp3+ regulatory T cells in mice infected with the filarial parasite *Brugia malayi*." J.Immunol. **181**(9): 6456-6466.

Mohapatra, A. D., S. Kumar, et al. (2011). "Caspase Dependent Programmed Cell Death in Developing Embryos: A Potential Target for Therapeutic Intervention against Pathogenic Nematodes." PLoS neglected tropical diseases **5**(9): e1306.

Mohr, E., A. F. Cunningham, et al. (2010). "IFN-gamma produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine." Proc Natl Acad Sci U S A **107**(40): 17292-17297.

Moll, H., H. Fuchs, et al. (1993). "Langerhans cells transport *Leishmania major* from the infected skin to the draining lymph node for presentation to antigen-specific T cells." Eur J Immunol **23**(7): 1595-1601.

Montecino-Rodriguez, E. and K. Dorshkind (2006). "New perspectives in B-1 B cell development and function." Trends Immunol **27**(9): 428-433.

Moreno, Y. and T. G. Geary (2008). "Stage- and Gender-Specific Proteomic Analysis of *Brugia malayi* Excretory-Secretory Products." PLoS.Negl.Trop.Dis. **2**(10): e326.

Moreno, Y., J. F. Nabhan, et al. (2010). "Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*." Proc Natl Acad Sci U S A **107**(46): 20120-20125.

Nair, M. G., I. J. Gallagher, et al. (2005). "Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells." Infection and immunity **73**(1): 385-394.

Nakamura, Y., N. Kambe, et al. (2009). "Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria." J Exp Med **206**(5): 1037-1046.

Nestle, F. O., P. Di Meglio, et al. (2009). "Skin immune sentinels in health and disease." Nat Rev Immunol **9**(10): 679-691.

Netea, M. G., G. Ferwerda, et al. (2005). "Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release." Journal of immunology **174**(10): 6518-6523.

Netea, M. G., B. J. Kullberg, et al. (2004). "NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease." European journal of immunology **34**(7): 2052-2059.

O'Neill, H. C. and H. L. Wilson (2004). "Limitations with in vitro production of dendritic cells using cytokines." J.Leukoc.Biol. **75**(4): 600-603.

O'Neill, L. A. and A. G. Bowie (2007). "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling." Nat.Rev.Immunol. **7**(5): 353-364.

Osei-Atweneboana, M. Y., K. Awadzi, et al. (2011). "Phenotypic evidence of emerging ivermectin resistance in Onchocerca volvulus." PLoS neglected tropical diseases **5**(3): e998.

Petit, G., M. Diagne, et al. (1992). "Maturation of the filaria *Litomosoides sigmodontis* in BALB/c mice; comparative susceptibility of nine other inbred strains." Ann Parasitol Hum Comp **67**(5): 144-150.

Petterson, T., J. Jendholm, et al. (2011). "Effects of NOD-like receptors in human B lymphocytes and crosstalk between NOD1/NOD2 and Toll-like receptors." Journal of leukocyte biology **89**(2): 177-187.
Pfaff, A. W., H. Schulz-Key, et al. (2000). "The role of nitric oxide in the innate resistance to microfilariae of *Litomosoides sigmodontis* in mice." Parasite Immunol. **22**(8): 397-405.
Pfaff, A. W., H. Schulz-Key, et al. (2002). "Litomosoides sigmodontis cystatin acts as an immunomodulator during experimental filariasis." Int.J.Parasitol. **32**(2): 171-178.
Pfarr, K. M., A. Y. Debrah, et al. (2009). "Filariasis and lymphoedema." Parasite Immunol. **31**(11): 664-672.
Pfarr, K. M., K. Fischer, et al. (2003). "Involvement of Toll-like receptor 4 in the embryogenesis of the rodent filaria *Litomosoides sigmodontis*." Med.Microbiol.Immunol. **192**(1): 53-56.
Philpott, D. J. and S. E. Girardin (2010). "Nod-like receptors: sentinels at host membranes." Current opinion in immunology **22**(4): 428-434.
Porksakorn, C., S. Nuchprayoon, et al. (2007). "Proinflammatory cytokine gene expression by murine macrophages in response to Brugia malayi Wolbachia surface protein." Mediators.Inflamm. **2007**: 84318.
Rathaur, S., M. Yadav, et al. (2008). "Filarial glutathione-S-transferase: a potential vaccine candidate against lymphatic filariasis." Vaccine **26**(32): 4094-4100.
Saeftel, M., M. Arndt, et al. (2003). "Synergism of gamma interferon and interleukin-5 in the control of murine filariasis." Infect.Immun. **71**(12): 6978-6985.
Saeftel, M., L. Volkmann, et al. (2001). "Lack of interferon-gamma confers impaired neutrophil granulocyte function and imparts prolonged survival of adult filarial worms in murine filariasis." Microbes Infect **3**(3): 203-213.
Sahoo, M. K., B. S. Sisodia, et al. (2009). "Immunization with inflammatory proteome of *Brugia malayi* adult worm induces a Th1/Th2-immune response and confers protection against the filarial infection." Vaccine **27**(32): 4263-4271.
Sahu, B. R., A. D. Mohapatra, et al. (2005). "A flow cytometry based method for studying embryogenesis and immune reactivity to embryogenic stages in filarial parasites." Filaria.J. **4**(1): 11.
Saleh, M. (2011). "The machinery of Nod-like receptors: refining the paths to immunity and cell death." Immunological reviews **243**(1): 235-246.
Scavelli, C., E. Weber, et al. (2004). "Lymphatics at the crossroads of angiogenesis and lymphangiogenesis." J.Anat. **204**(6): 433-449.
Semnani, R. T., M. Law, et al. (2004). "Filaria-induced immune evasion: suppression by the infective stage of *Brugia malayi* at the earliest host-parasite interface." J.Immunol. **172**(10): 6229-6238.
Semnani, R. T., L. Mahapatra, et al. (2010). "Expanded Numbers of Circulating Myeloid Dendritic Cells in Patent Human Filarial Infection Reflect Lower CCR1 Expression." J Immunol **185**(10): 6364-6372.
Semnani, R. T., P. G. Venugopal, et al. (2008). "Inhibition of TLR3 and TLR4 function and expression in human dendritic cells by helminth parasites." Blood **112**(4): 1290-1298.
Shapiro, H. (2003). "Practical Flow Cytometry 4th Edition." John Wiley & Sons.
Shaw, M. H., N. Kamada, et al. (2011). "The ever-expanding function of NOD2: autophagy, viral recognition, and T cell activation." Trends in immunology **32**(2): 73-79.
Shaw, P. J., M. Lamkanfi, et al. (2010). "NOD-like receptor (NLR) signaling beyond the inflammasome." European journal of immunology **40**(3): 624-627.
Smith, H. L. and T. V. Rajan (2000). "Tetracycline inhibits development of the infective-stage larvae of filarial nematodes in vitro." Experimental parasitology **95**(4): 265-270.

Snapper, C. M. and W. E. Paul (1987). "Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production." Science **236**(4804): 944-947.
Sorbara, M. T. and D. J. Philpott (2011). "Peptidoglycan: a critical activator of the mammalian immune system during infection and homeostasis." Immunological reviews **243**(1): 40-60.
Specht, S., J. K. Frank, et al. (2011). "CCL17 Controls Mast Cells for the Defense against Filarial Larval Entry." J Immunol **186**(8): 4845-4852.
Specht, S. and A. Hoerauf (2009). "Filarial parasites in the postgenomic era." Expert.Rev.Anti.Infect.Ther. **7**(2): 189-192.
Specht, S., M. Saeftel, et al. (2006). "Lack of eosinophil peroxidase or major basic protein impairs defense against murine filarial infection." Infect.Immun. **74**(9): 5236-5243.
Specht, S., M. D. Taylor, et al. (2011). "Over expression of IL-10 by macrophages overcomes resistance to murine filariasis." Experimental parasitology.
Specht, S., L. Volkmann, et al. (2004). "Interleukin-10 (IL-10) counterregulates IL-4-dependent effector mechanisms in Murine Filariasis." Infect.Immun. **72**(11): 6287-6293.
Stutte, S., B. Jux, et al. (2008). "CD24a expression levels discriminate Langerhans cells from dermal dendritic cells in murine skin and lymph nodes." J.Invest Dermatol. **128**(6): 1470-1475.
Tagliabue, A. and R. Rappuoli (2008). "Vaccine adjuvants: the dream becomes real." Hum Vaccin **4**(5): 347-349.
Takahashi, H. and M. Shibuya (2005). "The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions." Clinical science **109**(3): 227-241.
Taubert, A. and H. Zahner (2001). "Cellular immune responses of filaria (*Litomosoides sigmodontis*) infected BALB/c mice detected on the level of cytokine transcription." Parasite Immunol. **23**(8): 453-462.
Taylor, M. D., A. Harris, et al. (2006). "F4/80+ alternatively activated macrophages control CD4+ T cell hyporesponsiveness at sites peripheral to filarial infection." J.Immunol. **176**(11): 6918-6927.
Taylor, M. D., N. van der Werf, et al. (2008). "Early recruitment of natural CD4(+)Foxp3(+) Treg cells by infective larvae determines the outcome of filarial infection." Eur.J.Immunol. **39**(1): 192-206.
Taylor, M. J., H. F. Cross, et al. (2000). "Inflammatory responses induced by the filarial nematode *Brugia malayi* are mediated by lipopolysaccharide-like activity from endosymbiotic *Wolbachia* bacteria." J.Exp.Med. **191**(8): 1429-1436.
Taylor, M. J. and A. Hoerauf (1999). "*Wolbachia* bacteria of filarial nematodes." Parasitol Today **15**(11): 437-442.
Taylor, M. J., A. Hoerauf, et al. (2010). "Lymphatic filariasis and onchocerciasis." Lancet **376**(9747): 1175-1185.
Thirugnanam, S., P. Pandiaraja, et al. (2007). "Brugia malayi: comparison of protective immune responses induced by Bm-alt-2 DNA, recombinant Bm-ALT-2 protein and prime-boost vaccine regimens in a jird model." Experimental parasitology **116**(4): 483-491.
Torrero, M. N., M. P. Hubner, et al. (2010). "Basophils Amplify Type 2 Immune Responses, but Do Not Serve a Protective Role, during Chronic Infection of Mice with the Filarial Nematode *Litomosoides sigmodontis*." J Immunol.
Tsai, W. H., D. Y. Huang, et al. (2011). "Dual roles of NOD2 in TLR4-mediated signal transduction and -induced inflammatory gene expression in macrophages." Cell Microbiol **13**(5): 717-730.

Turner, J. D., R. S. Langley, et al. (2009). "*Wolbachia* lipoprotein stimulates innate and adaptive immunity through toll-like receptors 2 and 6 (TLR2/6) to induce disease manifestations of filariasis." J.Biol.Chem. **284**(33): 22364-22378.

Varani, S., G. Frascaroli, et al. (2005). "Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5." J Leukoc Biol **77**(2): 219-228.

Vedi, S., A. Dangi, et al. (2008). "Vaccination with 73kDa recombinant heavy chain myosin generates high level of protection against *Brugia malayi* challenge in jird and mastomys models." Vaccine **26**(47): 5997-6005.

Venugopal, P. G., T. B. Nutman, et al. (2008). "Activation and regulation of Toll-Like Receptors (TLRs) by helminth parasites." Immunol.Res.

Volkmann, L., O. Bain, et al. (2003). "Murine filariasis: interleukin 4 and interleukin 5 lead to containment of different worm developmental stages." Med Microbiol Immunol **192**(1): 23-31.

Volkmann, L., M. Saeftel, et al. (2001). "Interleukin-4 is essential for the control of microfilariae in murine infection with the filaria *Litomosoides sigmodontis*." Infect.Immun. **69**(5): 2950-2956.

Watanabe, T., A. Kitani, et al. (2004). "NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses." Nature immunology **5**(8): 800-808.

Wenk, P. and P. H. Wegerhof (1982). "Studies on acquired resistance of the cotton rat against microfilariae of *Litomosoides carinii*. 2. Injection of microfilariae during prepatency." Z.Parasitenkd. **68**(3): 321-329.

WHO (2004). "The Global Burden of Disease - 2004 update."

WHO (2011). "Global Programme to Eliminate Lymphatic Filariasis: progress report on mass drug administration." Wkly Epidemiol Rec **86**(35): 377-387.

Wolstenholme, A. J. and A. T. Rogers (2005). "Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics." Parasitology **131 Suppl**: S85-95.

Wu, L., B. S. Feng, et al. (2007). "Bacterial peptidoglycan breaks down intestinal tolerance via mast cell activation: the role of TLR2 and NOD2." Immunology and cell biology **85**(7): 538-545.

Yang, Z., P. Gu, et al. (2012). "Mutated MHC class II transactivator upregulates IL-33-dependent differentiation of Th2 subset through Nod2 binding for NLR signaling initiation." The Journal of biological chemistry.

Zurek, B., H. Bielig, et al. (2011). "Cell-based reporter assay to analyze activation of Nod1 and Nod2." Methods in molecular biology **748**: 107-119.

Erklärung

An Eides statt versichere ich, dass die vorgelegte Arbeit – abgesehen von den ausdrücklich bezeichneten Hilfsmitteln – persönlich, selbständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt wurde, die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte unter Angabe der Quelle kenntlich gemacht sind, die vorgelegte Arbeit oder ähnliche Arbeiten nicht bereits anderweitig als Dissertation eingereicht worden ist bzw. sind, für die inhaltlich-materielle Erstellung der vorgelegten Arbeit und/oder die Gelegenheit zur Promotion keine fremde Hilfe, insbesondere keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberatern/-vermittlern oder anderen Personen) in Anspruch genommen wurde, sowie keinerlei Dritte vom Doktoranden unmittelbar oder mittelbar geldwerte Leistungen für Tätigkeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Arbeit stehen.

_____

Sebastian Ziewer Bonn, den 03.09.2012

Danksagung

Ich danke Prof. Dr. Achim Hörauf für die Möglichkeit, meine Dissertation im Institut für medizinische Mikrobiologie, Immunologie und Parasitologie anfertigen zu dürfen.

Ebenso danke ich auch Prof. Dr. Norbert Koch, Prof. Dr. Jörg Höhfeld sowie PD Dr. Gerhild von Echten-Deckert für Ihr Engagement bei der Bewertung der Arbeit und der Prüfung.

Natürlich gilt PD Dr. Sabine Specht mein Dank für die intensive Betreuung während dieser Zeit.

Zugleich gilt mein Dank den Kollegen im Institut, insbesondere Bettina Dubben, die während all der Jahre stets hilfreich zur Verfügung stand.

Ich danke Kathrin Arndts, die mich während der schwierigen Phasen der Promotion aufgebaut und motiviert hat.

Dank gebührt auch meinen Eltern, die mich in den entscheidenden Phasen weiter unterstützt haben.

Zuletzt und vor allem danke ich Martin Bischof, der mir durch seinen Optimismus, aber auch seine Beharrlichkeit geholfen hat, das Projekt Promotion erfolgreich anzugehen und durchzustehen.

Bonn, 03.09.2012