Concepts of Immune Regulation in Chronic Filarial Infections

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Anna Katharina Filz

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1. Gutachter: Prof. Dr. Marc Hübner

2. Gutachter: Prof. Dr. Walter Witke

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Für Kathi

Die ihre Träume lebt

Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfsmittel angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht.

Es wurden keine vorherigen oder erfolglosen Promotionsversuche unternommen.

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Teile dieser Arbeit wurden vorab in folgender Publikation veröffentlicht:

- **“**The central adaptor molecule TRIF influences *L. sigmodontis* worm development.“ **Wiszniewsky A**, Ritter M, Krupp V, Schulz S, Arndts K, Weighardt H, Wanji S, Hoerauf A, Layland LE. Parasitol Res. 2019 Feb;118(2):539-549. doi: 10.1007/s00436-018-6159-1.

Weitere Teile dieser Arbeit befinden sich aktuell „in preparation“:

- “Evaluating changes in *Litomosoides sigmodontis*-specific $CD4^+$ T cells responses to different filarial antigen preparations.”
  Wiszniewsky A, Neumann A, Arndts K, Wiszniewsky K, Ritter M, Katawa G, Hoerauf A, Hübner MP, Layland LE.

Weitere Beteiligung an folgenden Veröffentlichungen:

- "*Schistosoma mansoni* PCR^+ -infected individuals in the Sudan present elevated systemic levels of chemokines when compared to uninfected and egg^+ cohorts."
  Arndts K, Elfaki TEM, Jelden N, Ritter M, **Wiszniewsky A**, Katawa G, Goreish IA, Atti El Mekki MEYA, Doenhoff MJ, Hoerauf A, Layland LE. Clin Exp Immunol. 2019 Jun;196(3):364-373. doi: 10.1111/cei.13270.

- "Absence of IL-17A in *Litomosoides sigmodontis*-infected mice influences worm development and drives elevated filarial-specific IFN-γ."
  Ritter M, Krupp V, Wiszniewsky K, **Wiszniewsky A**, Katawa G, Tamadaho RSE, Hoerauf A, Layland LE. Parasitol Res. 2018 Aug;117(8):2665-2675. doi: 10.1007/s00436-018-5959-7.

- "Development of patent *Litomosoides sigmodontis* infections in semi-susceptible C57BL/6 mice in the absence of adaptive immune responses."
  Layland LE, Ajendra J, Ritter M, **Wiszniewsky A**, Hoerauf A, Hübner MP. Parasit Vectors. 2015 Jul; 25(8):396. doi: 10.1186/s13071-015-1011-2.

- "Multivariable Regression Analysis in *Schistosoma mansoni*-Infected Individuals in the Sudan Reveals Unique Immunoepidemiological Profiles in Uninfected, egg+ and Non-egg+ Infected Individuals."
  Elfaki TE, Arndts K, **Wiszniewsky A**, Ritter M, Goreish IA, Atti El Mekki Mel Y, Arriens S, Pfarr K, Fimmers R, Doenhoff M, Hoerauf A, Layland LE. PLoS Negl Trop Dis. 2016 May 6;10(5):e0004629. doi: 10.1371/journal.pntd.0004629.

Eingereichte Veröffentlichungen:

- **"**IL4Rα-independent vaginal eosinophil accumulation following helminth infection exacerbates epithelial ulcerative pathology following HSV-2 infection."
  Chetty A, Darby MG, **Filz (nee. Wiszniewsky) A**, Ritter M, McSorley HJ, Masson L, Smith K, Brombacher F, O'Shea MK, Cunningham AF, Ryffel B, Oudhoff MJ, Dewals BG, Layland LE, Horsnell W. Cell Host Microbe. 2020

Summary

Despite worldwide efforts to reduce filariasis, over 100 million individuals still suffer from filarial infections. Filarial infections continue to be a substantial drain at both economic and health levels, especially in rural endemic communities. Part of the problem is the longevity of filariae in man, the different life-stages that the filariae pass through in the host and the number of organs and tissues that are affected. Thus, researchers continue to unravel the complex host:filarial interactions that allow such chronic manifestations. These interactions resulted from an evolutionary process since it is important for the filariae´s own survival to evade the host´s immune responses without killing the host. Using the rodent model of filariasis *Litomosoides sigmodontis*, immune reactions of the host towards the parasite can be analysed in a controlled setting. Interestingly, this nematode can only develop a patent (release of microfilariae (Mf)) infection in immune-component BALB/c mice since in C57BL/6 mice infections are cleared 40 days post infection. These models have been used to generate a plethora of data showing that control of Mf and worm burden depends on $CD4^+$ T and B1 cells, granulocytes, chemokines, granzymes, alternatively activated macrophages, IL-10, Th1 and Th2 cytokines as well as Th17 cytokines indicating that both innate and adaptive immune responses interact to mediate helminth control. Experiments in this thesis work addressed three questions using filarial/ host-specific interactions. The first experiments expanded on previous data showing unique filarial-specific responses from patent and latent *L. sigmodontis*-infected BALB/c mice. Here, cytokine release was monitored by ELISA in cultures of isolated $CD4^+$ T cells from early and late infected mice using soluble or pellet-derived antigens prepared from total, female or male adult filariae. Interestingly, IL-13, IFN-γ and IL-10 responses were absent in $CD4^+$ T cell cultures of early-infected mice regardless of antigen source. Developing adaptive immune responses are strongly influenced by innate signalling pathways since earlier studies have noted toll-like receptor (TLR)-triggering by filarial-derived components and infections in TLR-deficient BALB/c strains presented changed parasitology and host immunity. Investigations in the second part of this work addressed whether lack of TLR-signalling, especially central adaptor molecules Myd88 and TRIF, had an impact on *L. sigmodontis* infections in C57BL/6 mice. Therefore, C57BL/6 mice deficient for TLR3, TLR4, TRIF, MyD88, IRF1 and IRF3 were analysed at different time points of infection. Interestingly, worm numbers in mice deficient for TRIF were significantly increased at day 30 post infection and the recruitment of $CD4^+$ T cells to the site of infection was reduced. Therefore, our results indicate that the adaptor molecule TRIF plays a key role in worm development.

In the final part of this thesis the RAG2/IL-2Rγ-deficient mouse strain (lacks T, B and NK cells) was used to analyse the impact of T cells on worm clearance during *L. sigmodontis* infection and additionally characterise the phenotype of infection-induced T cells using advanced flow

cytometry. Previous studies showed that in contrast to WT mice *L. sigmodontis* infections in RAG2/IL-2Rγ-deficient mice resulted in patent infections with extremely high worm and Mf numbers. Expanding on those data, adoptive transfers of $CD4^+$ or $CD8^+$ T cells from WT C57BL/6 donor mice into RAG2/IL-2Rγ-deficient recipients did not alter worm burden *per se* but did result in reduced adult worm length and in the case of $CD4^+$ T cells reduced fecundity as well. Preliminary findings show that these effects are not mediated by IL-4 or IL-10. However, the transfer of $CD4^+$ T cells from *L. sigmodontis*-infected donor C57BL/6 mice additionally resulted in significantly reduced worm numbers in RAG2/IL-2Rγ-deficient recipients. Thus, these data enhance our understanding of immune networks and interplay during infection since although "educated" $CD4^+$ T cells control worm fecundity, further host components are required to restore the phenotype of *L. sigmodontis*-infected WT C57BL/6 mice.

Zusammenfassung

Trotz der globalen Bemühungen Infektionen mit Filarien einzudämmen, sind noch immer mehr als 100 Millionen Menschen von diesen betroffen und stellen besonders in den ländlichen Endemiegebieten ein enormes Problem dar. Hierbei spielen nicht nur die gesundheitlichen, sondern auch die wirtschaftlichen Aspekte eine große Rolle, aber auch die Langlebigkeit der Filarien im Menschen, die unterschiedlichen Entwicklungsstadien, welche der Parasit im Wirt durchläuft sowie die Anzahl der verschiedenen betroffenen Organe und Gewebe. Aus diesem Grund wird kontinuierlich daran gearbeitet, die komplexe Wirt:Filarien-Interaktion genauer zu untersuchen. Diese ist das Ergebnis eines langen evolutionären Prozesses und ist insbesondere für das Überleben des Parasiten von großer Bedeutung, da dieser auf den Wirt angewiesen ist, gleichzeitig aber versucht dessen Immunantwort auszuweichen. Mit Hilfe der Nagetierfilariae *Litomosoides sigmodontis* können die Immunreaktionen des Wirts während einer Infektion in einem kontrollierten wissenschaftlichen Modell genauer untersucht werden. Interessanterweise entwickelt *L. sigmodontis* nur in BALB/c Mäusen eine patente Infektion (Produktion von Mikrofilarien (Mf)), in C57BL/6 Mäusen hingegen wird die Infektion bis Tag 40 eliminiert. Mittels dieses Tiermodells konnte bereits eine Fülle an Daten gewonnen werden. Hierbei konnte beispielsweise gezeigt werden, dass die Kontrolle der Mf- und der Wurmzahlen von $CD4^+$ T- und B1-Zellen, Granulozyten, Chemokinen, Granzymen, alternativ aktivierten Makrophagen, IL-10, Th1 und Th2 Zytokinen sowie Th17 Zytokinen abhängt. Dies deutet auf eine enge Interaktion zwischen der angeborenen und der erworbenen Immunantwort hin. Die Experimente, die im Rahmen dieser Doktorarbeit durchgeführt wurden, befassen sich mit drei Fragen hinsichtlich der filarienspezifischen Interaktionen: Die ersten Experimente erweitern zuvor gewonnene Daten und zeigen eine jeweils spezifische Immunantwort in patent und latent *L. sigmodontis* infizierten BALB/c Mäusen. Hierfür wurde mittels ELISA die Zytokinproduktion von $CD4^+$ T-Zellen aus Mäusen während eines frühen und eines späten *L. sigmodontis* Infektionsstadiums analysiert. Dazu wurden die Zellen mit löslichem oder pelletiertem Antigen stimuliert, welches aus (i) weiblichen Würmern, (ii) männlichen Würmern oder aus (iii) beiden Wurmgeschlechtern gewonnen wurde. Bei den Zellen aus Mäusen, die sich in einem frühen Stadium der Infektion befanden, konnte interessanterweise weder eine IL-13 oder eine IFN-γ, noch eine IL-10 Produktion beobachtet werden. Die Entwicklung einer adaptierten Immunantwort wird stark von der angeborenen Immunantwort beeinflusst. Frühere Studien konnten bereits zeigen, dass aus Filarien gewonnene Komponenten in der Lage sind Toll-like Rezeptoren (TLR) zu stimulieren. Darüber hinaus konnte beobachtet werden, dass Filarieninfektionen in TLR-defizienten BALB/c Mäusen, verglichen mit Wildtyp BALB/c Mäusen, eine veränderte Parasitologie und Wirtsimmunantwort aufweisen. Aus diesem Grund wurde im zweiten Teil dieser Arbeit untersucht, ob das Fehlen von TLR-Signalen,

insbesondere das Fehlen der zentralen Adaptermoleküle MyD88 und TRIF, einen Einfluss auf den Verlauf einer Filarieninfektion in C57BL/6 Mäusen hat. Dazu wurden *L. sigmodontis*-infizierte TLR3-, TLR4-, TRIF-, MyD88-, IRF1- oder IRF3-defiziente C57BL/6 Mäuse an unterschiedlichen Zeitpunkten der Infektion untersucht. Interessanterweise konnte an Tag 30 der Infektion in Mäusen, denen das TRIF-Molekül fehlt, eine signifikant höhere Wurmlast beobachtet werden. Ebenfalls wurden signifikant weniger $CD4^+$ T Zellen am Ort der Infektion beobachtet. Aus diesem Grund deuten unsere Ergebnisse darauf hin, dass das Adaptermolekül TRIF eine Schlüsselrolle bei der Wurmentwicklung spielt.

Im letzten Teil dieser Doktorarbeit wurde die RAG2/IL-2Rγ-KO Mauslinie (die keine T-, B- oder NK-Zellen besitzen) verwendet, um den Einfluss von T-Zellen auf die Eliminierung der Würmer während einer *L. sigmodontis*-Infektion zu untersuchen. Zusätzlich wurde der Phänotyp, der durch die Infektion aktivierten T-Zellen, mittels Durchflusszytometrie analysiert. In früheren Studien konnte bereits gezeigt werden, dass RAG2/IL-2Rγ-KO Mäuse im Gegensatz zu C57BL/6 Mäuse eine patente *L. sigmodontis* Infektion entwickeln. Diese zeichnet sich durch extrem hohe Wurm- und Mf-Zahlen aus. Aufbauend auf diesen Daten, wurden in der hier beschriebenen Doktorarbeit $CD4^+$ oder $CD8^+$ T-Zellen aus C57BL/6 Mäusen gewonnen und intravenös in RAG2/IL-2Rγ-KO Mäuse injiziert, welche anschließend für 72 Tage mit *L. sigmodontis* infiziert wurden. Es konnte gezeigt werden, dass der Transfer der Zellen keinen direkten Einfluss auf die Wurmzahlen hatte, aber in signifikant kürzeren Würmern resultierte. Zudem konnte bei Würmern, welche aus Mäusen mit transferierten $CD4^+$ T-Zellen stammen, eine verringerte Fertilität beobachtet werden. Vorläufige Daten weisen ebenfalls darauf hin, dass dieser Effekt unabhängig von IL-4 oder IL-10 ist. Interessanterweise wurden in RAG2/IL-2Rγ-KO Mäusen, welchen $CD4^+$ T-Zellen aus zuvor infizierten Donoren injiziert wurden signifikant weniger Würmer beobachtet. Diese Daten erweitern unser Verständnis der komplexen Immunantwort während einer Filarieninfektion, da neben „geprimten“ $CD4^+$ T-Zellen, welche die Fruchtbarkeit der Würmer kontrollieren, weitere Komponenten des Immunsystems den Phänotyp einer *L. sigmodontis*-infizierten WT C57BL/6 Maus beeinflussen.

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1 Introduction

1.1 The immune system

The primary function of the immune system in living organisms is to protect the body from foreign substances, cells, tissues and pathogenic products or organisms. Therefore, there exists a complex system which integrates a broad network of cells, cell products, tissues and organs. This system must be able to distinguish between the body's own cells and proteins ("self") and foreign molecules ("non-self") (1, 2). For the effective protection of the organism the immune system has to respond in the correct manner and intensity since an immune response of an under-stimulated system could allow a pathogen to go undetected. In contrast, an over-stimulation could result in a more dangerous inflammatory response compared to the original stimulus (1-3). Besides physical and biochemical barriers (skin, tears etc.), different cell types are also very important members of the immune system. One of the most important cell types are leukocytes which are also known as white blood cells. These cells originate from hematopoietic stem cells which are located in the bone marrow. Partially, they mature directly in the bone marrow but final maturation and classification occurs in different types of organs (thymus or spleen). Afterwards, cells migrate to peripheral tissues like blood, lymph fluid or secondary lymph organs like lymph nodes or spleen where they carry out protective activities (1, 2). To ensure a correct working of the system a panel of secreted small proteins (chemokines and cytokines) is necessary since they allow cells to communicate with surrounding cells. These signalling molecules are mostly secreted by leucocytes. Another important function of chemokines and cytokines is that they draw other immune cells to different sites of infection, so they create the appropriate environment for the immune response (4). The defence against microbes has been divided into two general types of responses: first the innate immune response and second the adaptive immune response. For an optimal interplay of the whole system both types of reactions need to cooperate and interact with one another (1, 2).

1.1.1 Innate immunity

The innate immune response provides the initial immediate non-specific response and is the first line of defence. Skin and other epithelial surfaces present a physical barrier between the inside of the body and the outside of the world (5). In general, innate immunity is a combination of several defence mechanisms for example, proteins and tissues but also different cell types, including macrophages, granulocytes, mast cells, natural killer cells (NK) or dendritic cells (DCs). Some of these innate mechanisms start acting immediately upon the encounter with infectious agents. Others are activated and amplified during infection and then return to baseline levels after the infection is resolved (1, 2).

1.1.1.1 The Toll-like receptor system

Toll-like receptors (TLRs) belong to an evolutionary ancient recognition and signalling system that was discovered in the fruit fly *Drosophila melanogaster*. They are one of the most well-studied groups of pattern recognition receptors (PRRs) (6). These innate receptors have been identified on macrophages, dendritic cells and some other cells including T cell subsets which recognize pathogens and their products. Recognition stimulates the receptor-bearing cells to produce cytokines and chemokines that help initiate and shape the initial immune response. There are ten TLRs expressed in humans (TLR1-10) and twelve in mice (TLR1-TLR9 and TLR11-13). Each TLR is devoted to recognize a distinct set of "Pathogen Associated Molecular Patterns" (PAMPs) from pathogenic microorganisms or "Damage Associated Molecular Pattern" (DAMPs) (2, 7-11). The stimulation of different TLRs leads to the activation of certain signalling pathways which induce, further downstream, transcription factors, e.g. the nuclear factor-κB (NFκB). The activation of these transcription factors initiates inflammatory responses (2, 6, 9). TLRs act via a domain in their cytoplasmic tail called the Toll/interleukin-1 receptor (TIR) domain which recruits a family of adaptor proteins that also contain a TIR domain. There are four adaptors: MyD88 (myeloid differentiation factor 88), MAL (MyD88 adaptor-like), TRIF (TIR domain-containing adaptor inducing interferon (IFN)-γ) and TRAM (TRIF-related adaptor molecule). Different TLRs interact with different combinations of theses adaptor proteins. If these adaptors activate a serine/threonine kinase (IRAK), this kinase recruits the tumour necrosis factor (TNF) receptor associated factor (TRAF)-6. TRAF-6 stimulates further downstream transforming growth factor beta-activated kinase (TAK)-1 and this phosphorylates and activates the IκB kinase (IKK) complex. The activation of IKK results in the damage of the IκB (inhibitory domain) and the activation of NFκB. Afterwards, NFκB moves into the nucleus and regulates the production of pro-inflammatory cytokines like TNF, IFN-γ, and interleukin (IL)-6 (2, 9).

1.1.1.2 The MyD88-independent/TRIF-dependent pathway

Most of the TLR pathways are MyD88-dependent but there is also a MyD88-independent/TRIF-dependent pathway. In MyD88-deficient macrophages, TLR4 ligand-induced production of inflammatory cytokines does not occur. However, activation of NFκB is observed. This indicates that although the TLR4-mediated production of inflammatory cytokines completely depends on the MyD88-dependent pathway, a MyD88-independent component exists in the TLR4 signalling. In this scenario, TLR4 stimulation leads to the activation of the transcription factor interferon regulatory factor 3 (IRF3), as well as the late phase of NFκB activation in a MyD88-independent manner (9, 12). TLR4-induced activation of IRF3 leads to the production of IFN-γ which in turn induces several IFN-inducible genes. Viral infections or double-stranded RNA were shown to activate IRF3. Accordingly, the TLR3-mediated pathway also activates IRF3 (passed by TRIF) and thereby induces IFN-γ in a

MyD88-independent manner. Hence, TLR3 and TLR4 utilize the MyD88-independent component to induce IFN-γ (Fig. 1.1) (8, 9, 12, 13).

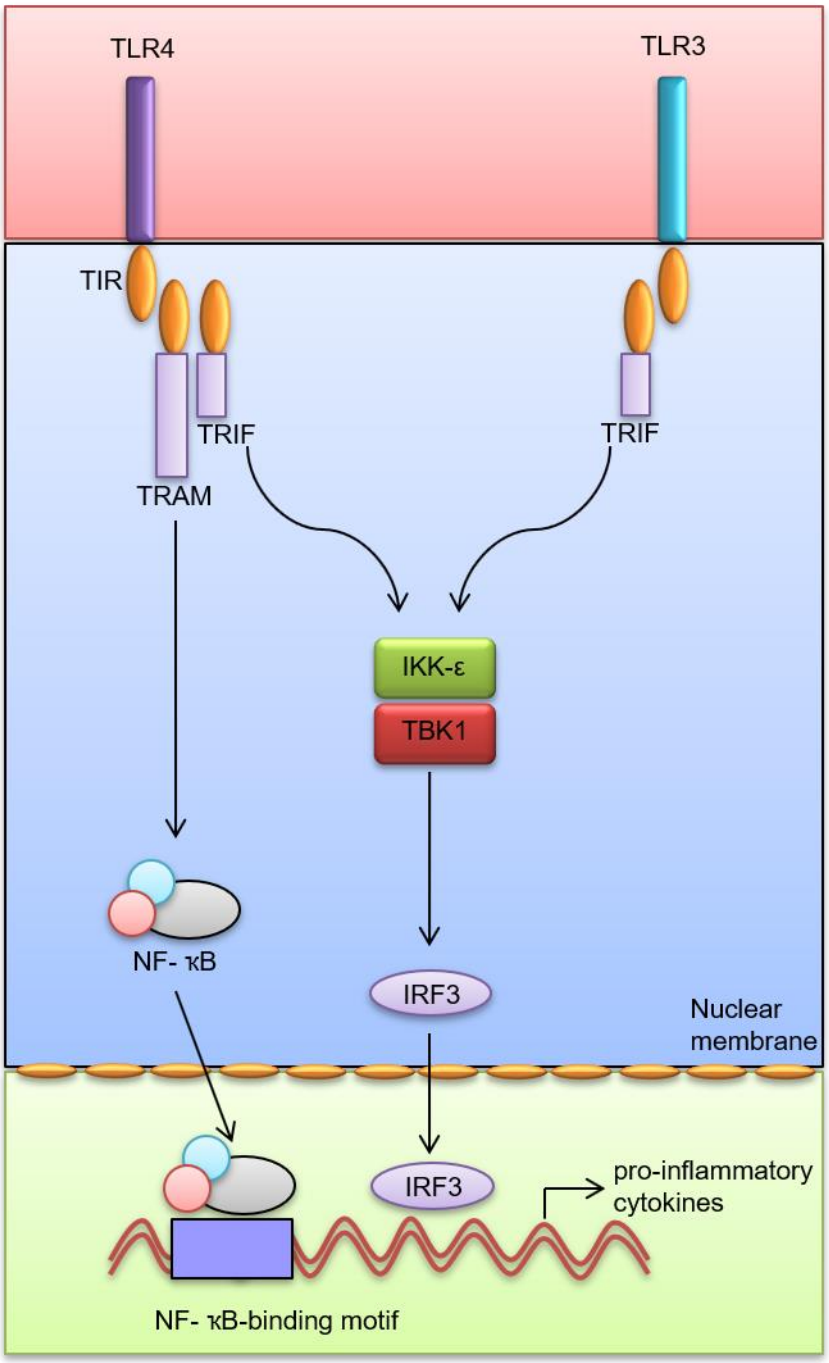


Figure 1.1: The MyD88-independent/TRIF-dependent pathway. TRIF is activated by ligation of TLR3 or TLR4 and leads to further downstream activation of IRF3 which in turn induces several IFN-inducible genes and therefore, the production of pro-inflammatory cytokines like TNF or IFN-γ (adapted from (8)).

1.1.2 Adaptive immunity

When mechanisms of the innate immune system are unsatisfactory and the infection overwhelms these first defence mechanisms of the body, an adaptive immune response is induced. Unlike innate immune responses, adaptive responses are highly specific to the particular pathogen that caused their recruitment and activation and this specificity can provide long-lasting protection through the development of so-called “memory”. The main function of the adaptive immune branch is to destroy invading pathogens and any toxic molecules they

produce. Because these responses are destructive, it is crucial that they are induced only in response to molecules that are foreign to the host and not to the molecules of the host itself. The ability to distinguish what is "foreign" from what is "self" in this way is a fundamental feature of the adaptive immune system. There are two broad classes of such responses: antibody responses and cell-mediated immune responses which are generated by B (B = bone marrow) and T cells (T = thymus) respectively (1, 2).

During an antibody response, B cells detect different antigens (Ag) via B cell receptors (BCRs) and following activation start to secrete antibodies (Ab) which are called immunoglobulins (Ig). Afterwards, Abs circulate in the bloodstream and other body fluids and specifically bind to the foreign antigen that stimulated their production (1, 2, 6). The Abs inactivate viruses and microbial toxins via binding to receptors on host cells. In addition, antibody binding marks pathogens also for phagocytic cells of the innate immune system like neutrophils, eosinophils and monocytes. During cell-mediated immune responses, activated T cells react directly against foreign antigens that are presented to them on the surface of a host cell. Here, they can either kill the infected cell or produce a signal molecule that activates macrophages to destroy the invader via phagocytosis (1, 2, 6).

1.1.2.1 T lymphocytes and their development

T lymphocytes play a major role in cell-mediated responses of the adaptive immunity. Progenitor T cells, derived from the pluripotent hematopoietic stem cells, arise in the bone marrow and migrate to the thymus gland to mature. During positive and negative selection over 90% of the cells are eliminated. In general, successfully developed T cells, reflecting those that do not respond to "self" antigens enter the bloodstream as naïve T cells and circulate between blood and peripheral lymphoid tissues. To participate in an adaptive immune response, naïve T cells must recognise their specific antigen which is presented to them as a peptide within major histocompatibility complex (MHC) molecules by antigen-presenting cells (APCs) such as dendritic cells or B cells (1, 2).

There are two important types of T cells and MHC molecules. Both of these MHCs differ in their structure and in their expression pattern in the tissues of the body. MHC class I and II molecules have distinct distributions among cells which reflects also the different effector functions of the T cells that recognize them. MHC class I molecules present peptides from pathogens (e.g. viruses) to cluster of differentiation (CD)8^+ T cells which attack infected and cancerous cells directly, kill them and play an important role in the defence against intracellular pathogens. Therefore, $CD8^+$ T cells are also called cytotoxic T cells. In contrast, $CD4^+$ T cells recognize peptides presented by MHC class II molecules. These $CD4^+$ T cells activate other effector cells directly and regulate the immune response (T helper cells). Because of this function, MHC class II molecules are mainly found on B cells, DCs and macrophages (1, 2).

There are several types of $CD4^+$ T cells termed T helper (Th) cells 1, Th2, Th17 and regulatory T cells (Treg) which have different functions. Specific cytokines and transcription factors determine their differentiation and expansion, and their differential activation plays a major role in determining whether an immune response will contribute to host protection or pathological inflammation. The Th1 cells (characterised by the transcription factors Stat4 and T-bet) produce cytokines like IL-2, IFN-γ and TNF-α which support inflammation and defence mechanisms against intracellular pathogens. Th2 cells (GATA-3/Stat5) however, produce IL-4, IL-5 or IL-13 and with the support of antibodies from B cells they are involved in parasitic infection or autoimmunity. In contrast, Th17 cells (RORγt/Stat3) secrete IL-17 and are associated with inflammatory responses and defence against extracellular bacteria. Regulatory T cells (Stat5/Foxp3) secrete IL-10 and TGF-β and prevent autoimmune responses whilst maintaining homeostasis (14, 15). An overview of these different T cell subsets is shown in figure 1.2.

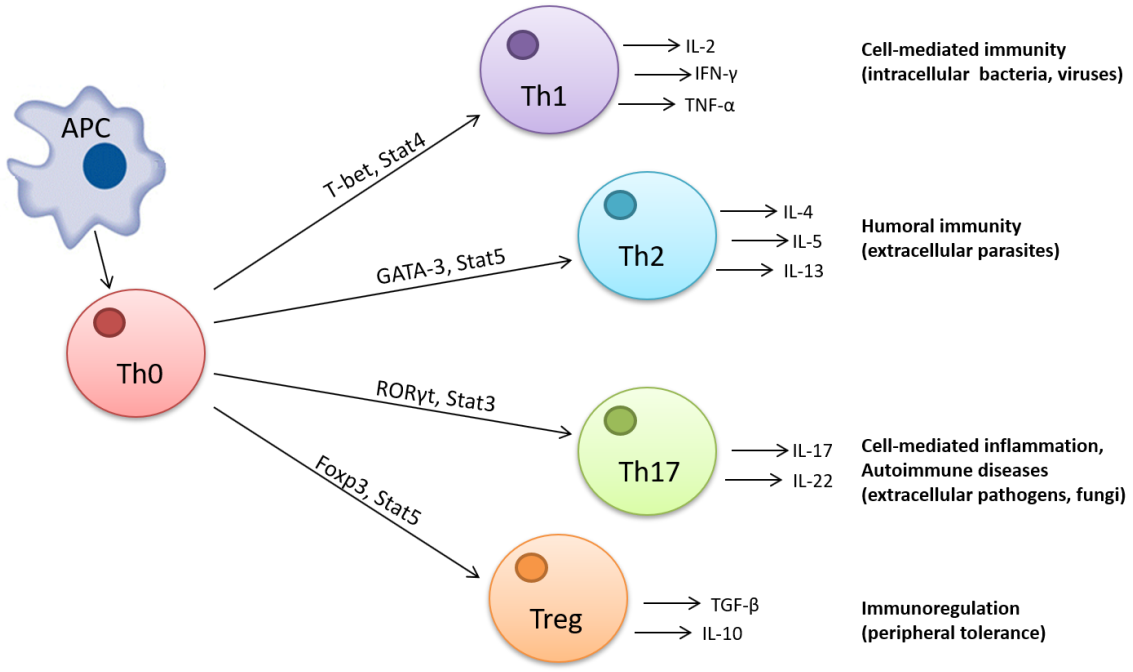


Figure 1.2: Development of naïve T cells into several cell types. Differentiation of naïve $CD4^+$ T cells into different T-helper-cell subsets is dependent on factors present in the local environment, most prominently cytokines. The specific stimulatory conditions influence transcription factor expression which determines the differentiation program of the T cell and thus, the cytokines that it will subsequently produce. The pattern of cytokine expression characterizes the individual T-helper-cell subset and dictates their function in host defences (adapted from (16).

1.2 Filariasis - a major health problem

Filariasis is induced by thread-like parasitic nematodes from the super-family of Filarioidea (family Onchocercidae) which are transmitted by blood feeding vectors. Infections with filarial nematodes are a major health and socioeconomic problem since these infections are chronic

and can persist in humans for many years (17, 18). There are eight different species that can infect man and these are characterised by different clinical manifestations and symptoms (19). The most commonly known filarial diseases are lymphatic filariasis (LF) and onchocerciasis. In general, most of the infections with these filarial parasites are symptomless or show only a weak pathology but, in some cases, the course of infection ends in severe clinical manifestations. Lymphatic filariasis is caused by the parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* which are mainly found in the lymphatic´s of the host. Onchocerciasis (also called river blindness) is caused by *Onchocerca volvulus* and can induce severe dermatitis and visual impairments and adult worms of this species reside in the subcutaneous tissue of the human body. The infection with *Loa loa* (also called eye worm) can be diagnosed by movement of adult worms in the eye. Common clinical manifestations are the Calabar swelling and adult worms can be found in the connective tissue. Mansonelliasis is caused by *Mansonella perstans*, *Mansonella streptocerca* or *Mansonella ozzardi* and can lead to dermatitis and unspecific allergic symptoms. Adult worms are mostly found in the connective tissue of the host (17-20).

1.2.1 Filariasis – life cycle

There are different vectors for the transmission of filarial infections, depending of the species. *W. bancrofti*, *B. malayi* and also *B. timori* are transmitted by different mosquito spp. whereas *O. volvulus* is transmitted by *Simulium* spp. and *L. loa* by different species of *Chrysops*. Mansonelliasis can be transmitted by different *Culicoides* spp. but can also be transmitted via *Simulium* spp.. Figure 1.3 shows a general life cycle of filarial infections in humans.

In general, the infection is initiated by the infective stage of the (L3) larvae which are transferred during the blood meal of the vector. Afterwards, larvae migrate to the site of infection and develop into adult worms. Here, adult worms' mate and the female worms start producing their offspring, the so-called microfilariae (Mf) which are circulating in blood or skin where they will be taken up again by the vector. Interestingly, Mf from *W. bancrofti*, *Brugia* spp. and also *L. loa* have adapted their behaviour to the different vectors since they are found only in blood when vectors are active (only night or only day time). Once in the vector, Mf develop into L2 and finally into the infective L3 stage larvae which can be transmitted during the next blood meal (18, 19).

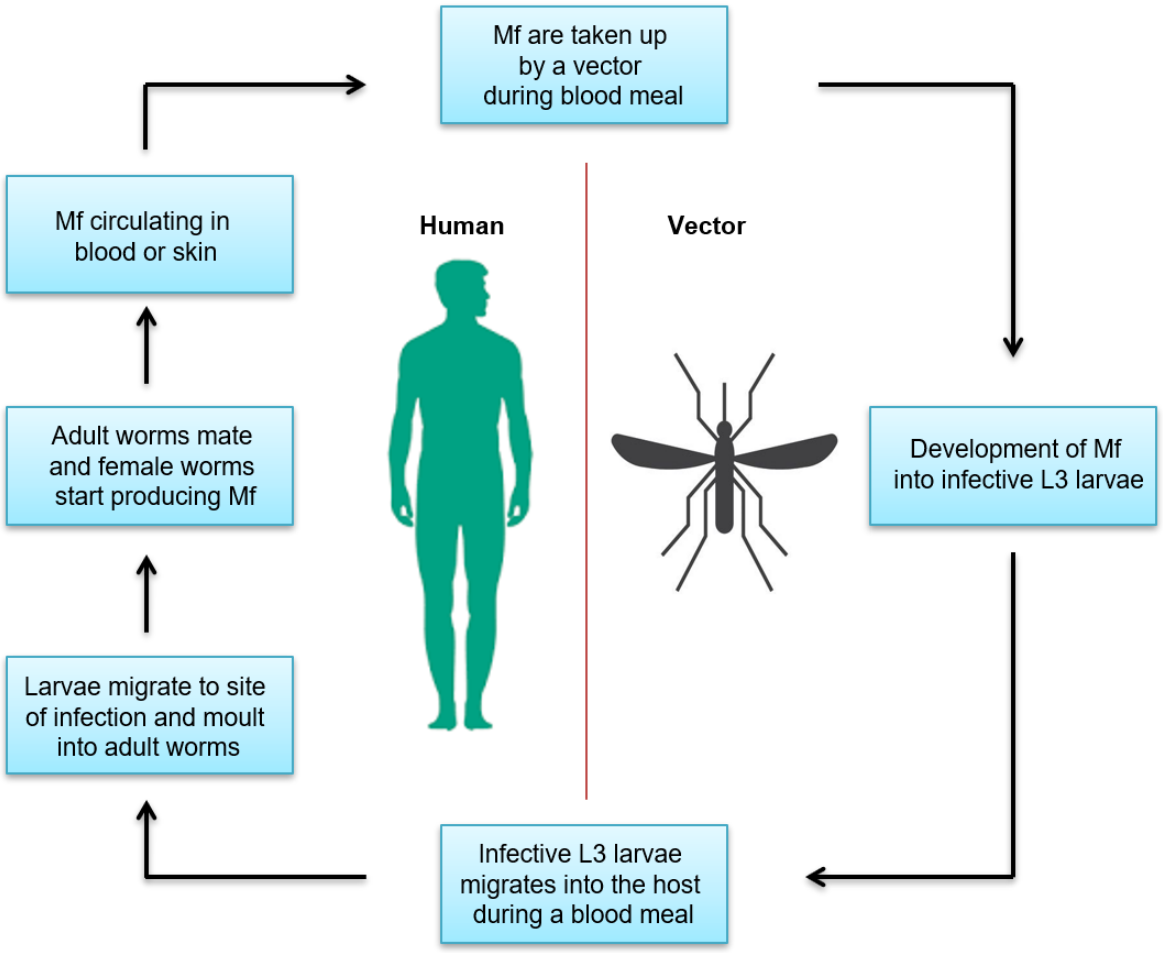


Figure 1.3: Life cycle of filarial infections. During the blood meal of the vector infective L3 stage larvae migrate into the human host. Here they migrate to the site of infection and develop into adult worms. Afterwards adult worms' mate and female worms release their offspring (microfilariae) into the periphery. During the next blood meal microfilariae (Mf) can be taken up again by the vector. Here, they moult into L1, L2 and finally into the infective L3 stage larvae and the transmission cycle is closed (adapted from (19)).

1.2.2 Lymphatic filariasis

As described earlier, LF is one of the most important filarial infections. Therefore in 1997, the World Health assembly resolved to eliminate lymphatic filariasis as a public health problem. As a response, in 2000, the "World Health Organisation" (WHO) set up the "Global Programme to Eliminate Lymphatic Filariasis" (GPELF), with the target to eliminate LF by 2020 (21, 22). Unfortunately, there are still 68 million people infected with lymphatic filariasis (23), across 72 tropical and sub-tropical countries (24) (Fig. 1.4).

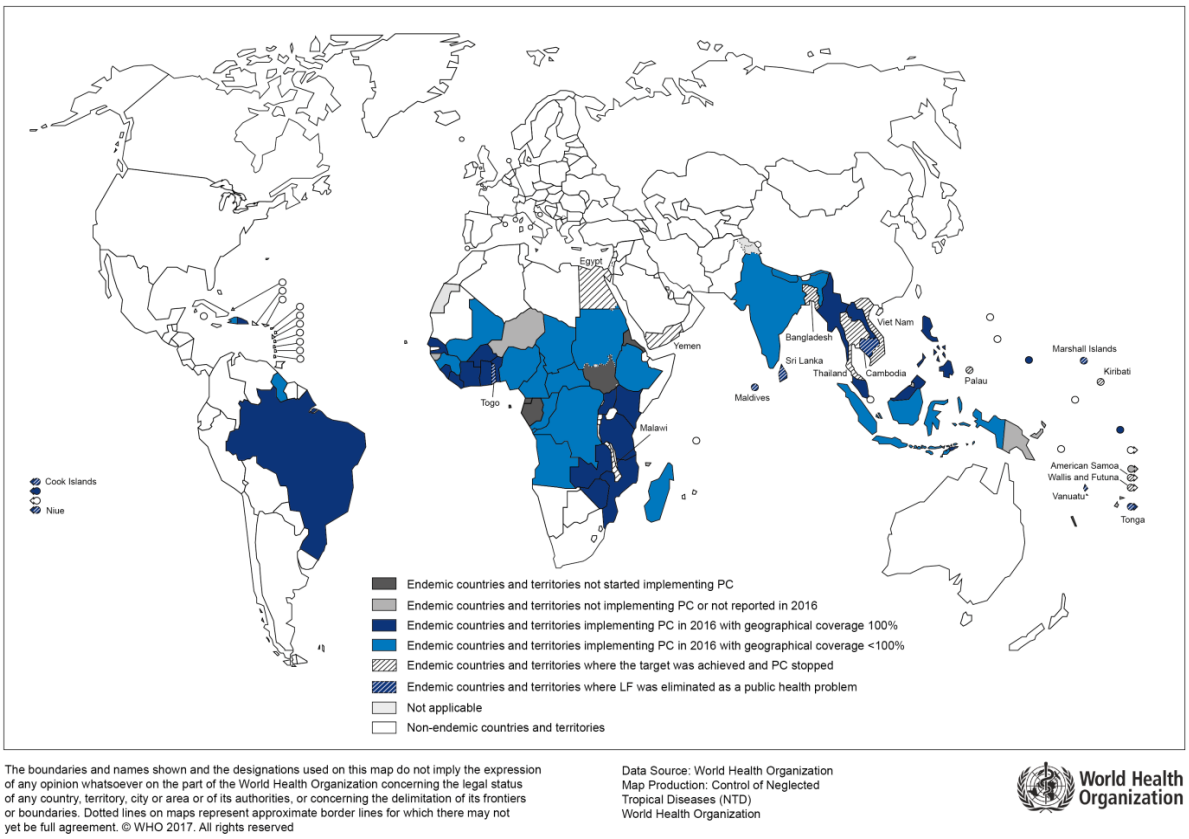
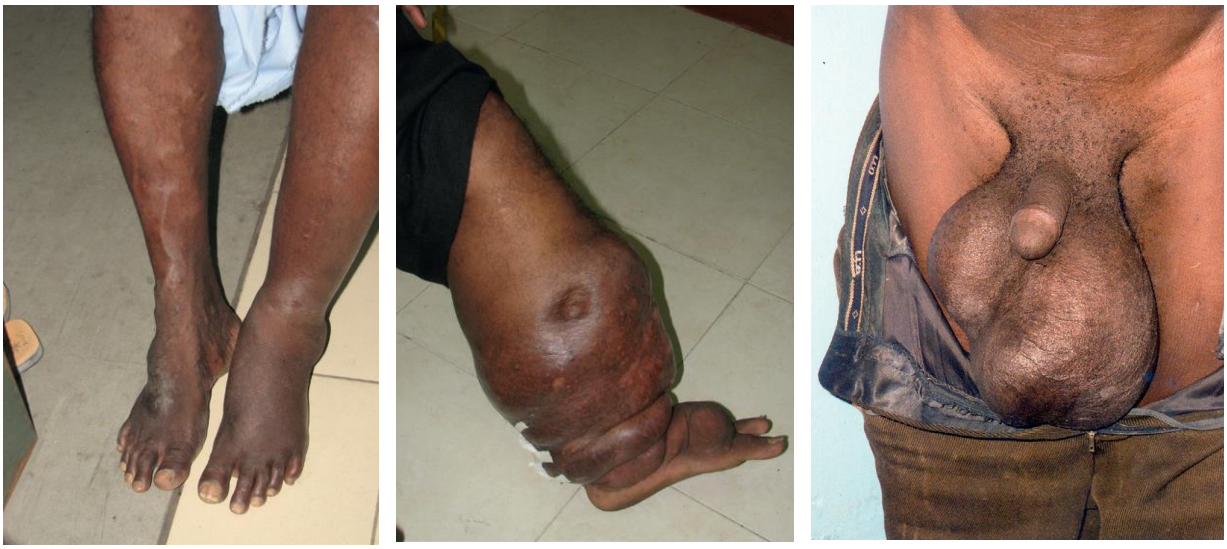


Figure 1.4: Distribution of lymphatic filariasis in endemic countries, 2016 (adapted from https://hdi.no/project/lymphatic-filariasis).

As already described, LF is caused by the helminthic parasites *W. bancrofti* (which accounts for 90% of the infections) and *Brugia* spp.. Both species are transmitted by blood feeding mosquitoes (*Aedes, Anopheles, Coquillettidia, Culex, Mansonia*). After infection with LF, parasites can persist for many years in the lymphatics and bloodstream (6, 19, 25, 26). Most of the infected people remain asymptomatic. However, LF has two major clinical manifestations that occur during chronic conditions: disfigurement of the male genitals (hydrocele) and chronic lymphedema of the legs or arms (Fig. 1.5). These clinical symptoms are mainly caused by adult worms and often lead to a stigmatisation and huge socio-economic problems of affected patients. Furthermore, LF accounts for approximately 2.8 million DALYs (disability-adjusted life years) but this does not include co-morbidity factors such as mental illness which affects both infected individuals and their care givers (18, 19, 23, 27). These impressive numbers highlight the urgent need to eliminate the disease.

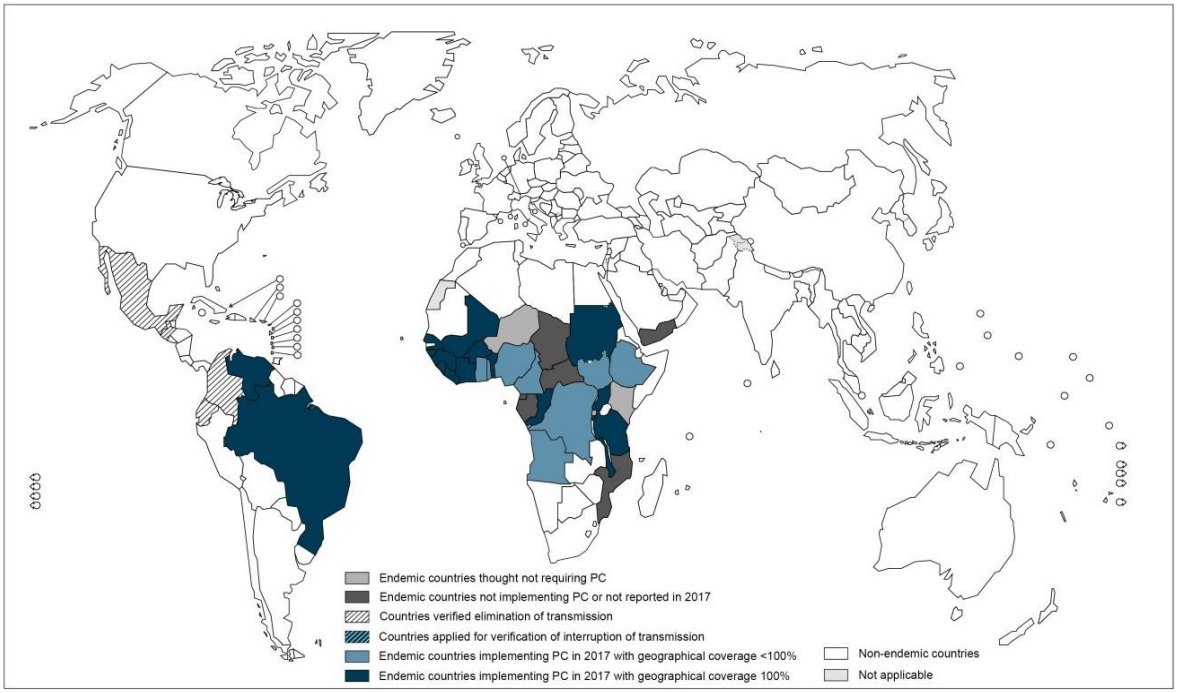


1.5: Clinical manifestations during chronic lymphatic filariasis. Infections with *W. bancrofti*, *B. malayi or B. timori* can cause lymphatic filariasis with different clinical manifestations in the chronic phase of infections. From the left to the right, pictures show lymphedema and elephantiasis of the leg and hydrocele of the male scrotum (adapted from (19)).

1.2.3 Onchocerciasis

Currently, Onchocerciasis is prevalent in 20.9 million infected individuals. More than 99% of these affected people live in one of the 31 sub-Saharan countries but the disease is also prevalent in Latin American countries with small foci in Brazil and the Bolivarian Republic of Venezuela (Fig. 1.6). 205 million people live in areas endemic for Onchocerciasis with around 1 million DALYs (26, 28, 29).

As described in section 1.2, this infection is caused by the filarial parasite *O. volvulus* and transmitted by black flies of the genus *Simulium*. Similar to LF, *O. volvulus* can persist in infected patients over many years if not treated since most individuals present a regulated state and thus asymptomatic symptoms (19, 26). Most prominent clinical manifestations are nodule formation, severe skin pathology and visual impairments up to blindness. Since the highest rates of infections are found in areas with access to rivers the infection is colloquially known as “river blindness“. This is because the flight range of the vector is about 12 km, thus areas further than this range from the river (breeding ground for the *Simulium*) are not usually affected. In contrast to LF, most of the clinical symptoms are cause by the migration of Mf (18, 19, 30).



1.6: Distribution of onchocerciasis in endemic countries 2017 (adapted from https://www.who.int/onchocerciasis/distribution/en/)**.**

In general, infected people can be grouped into two forms (Fig. 1.7): the generalized onchocerciasis (GEO) and hyperresponsive onchocerciasis (HO) which include the so-called Sowda form. Most of the infected patients show a GEO phenotype.

1.7: Clinical manifestation of *Onchocerca volvulus*. From left to right pictures show nodule formation, dermatitis and a sclerosing dermatitis (adapted from (19, 31)).

This form is characterized by high Mf loads (over 10 Mf per mg skin), high IL-10, Foxp3^{+}Treg and nodule formation but a weak pathology. In contrast, patients with HO (approximately 1%) have no or only few Mf but severe skin pathology and elevated IL-4, IL-17A and IgE responses (32-34).

1.2.4 *Wolbachia* bacteria - an endosymbiotic relationship

Most of the human pathogenic filarial species contain obligatory endosymbionts, called *Wolbachia*. The relationship with these gram-negative bacteria which belong to the order *Rickettsiales*, was already discovered in the 1970s (19, 35-38). *Wolbachia* are restricted to the hypodermis of all developmental stages of the parasite and also to the reproductive part of female worms where they are transmitted transovarially (36, 38, 39). *Wolbachia* are not only found in helminths but also in arthropods. In contrast to the arthropod-related *Wolbachia* which live more parasitic, *Wolbachia* related to helminths are mutualistic symbionts since they are essential for filarial survival, fertility and also larval moulting (37, 38, 40-43). Interestingly, *Wolbachia* are potent inducers of an inflammatory immune response via TLR2 and TLR6 and the induction of several Th1 cytokines like TNF, IL-1β or IL-6 whereas they decrease anti-inflammatory responses (44, 45). Additionally, it seems that the *Wolbachia* induced TLR4 responses are related to the immunopathogenesis of *O. volvulus*-induced keratitis (46). Since *Wolbachia* are important for filarial survival and fertility, these bacteria became also an important target for anti-filarial therapies and treatments (36, 38).

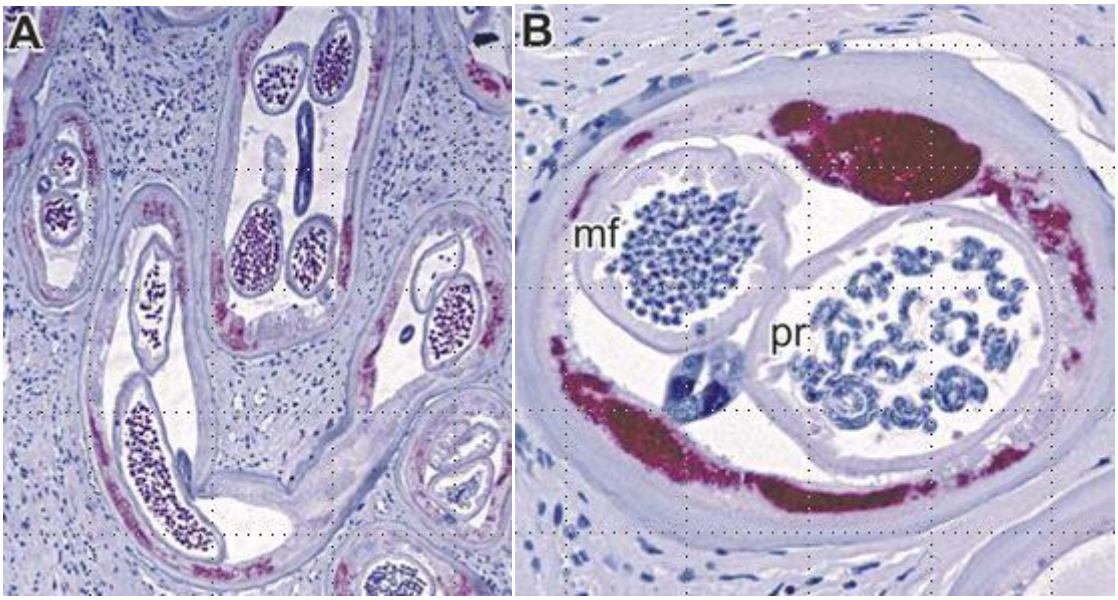


Figure 1.8: *Wolbachia* bacteria in *O. volvulus* female worms. (A and B) Female worms with *Wolbachia* bacteria (red) in the hypodermis (adapted from (42)).

1.2.5 Treatment of filarial infections

As described in the previous sections, millions of people are affected by filarial infections and the different parasites can cause severe pathology, stigmatisation and therefore, also mental illness. Before the onset of pathology, worldwide attempts to eliminate these infections with different chemotherapeutic treatment programs, called mass drug administration (MDA), have been performed over the last decades (e.g. GPELF against LF or the “African Programme of Onchocerciasis Control” (APOC)). In general, there are three chemotherapeutics:

diethylcarbamazine (DEC), ivermectin (IVM) and albendazole (ALB) which all lead to death of blood and skin Mf (18).

Studies have shown that DEC is one of the most effective treatments for filarial infections in humans (18, 19, 26, 47, 48). Diethylcarbamazine is effective in eliminating Mf but it seems also to have a macrofilaricidal effect. It decreases the muscle activity and leads to a hyperpolarization of the membrane. Furthermore, it makes the Mf surface membrane more susceptible for the host immune mechanisms (48). However, it has been reported that the use of DEC in patients with onchocerciasis (especially with high loads of Mf) can lead to irreversible ocular damage (18, 26, 48, 49). Ivermectin is a macrocyclic lactone against helminth and arthropods which was already discovered in the 1970s. Until now, the mode of action of IVM is not completely understood but it is known that it hyperpolarizes the glutamate-sensitive channels of the parasite and thereby prevents the neuronal transmissions which results in paralysis of the nematode musculature. Since it also kills skin and blood Mf but provokes less inflammatory immune reactions, it has become in the 1980s the drug of choice against onchocerciasis (18, 26, 48, 50, 51). Albendazole is a chemotherapeutic with a broad anti-helminthic activity, including soil-transmitted parasites and also filarial helminths. It blocks the microtubule assemble by inhibiting the parasite´s tubulin so that susceptible helminths become paralysed (18, 26, 48). Therefore, in order to fight LF, a combination of ALB and DEC is given for treatment in areas that are not endemic for onchocerciasis whereas in areas endemic for this disease a combination of IVM and ALB is given to LF patients.

The routine two-medicine combination can clear almost all blood Mf in 4-6 years but multiple annual treatments are required since only 8% of the treated patients are Mf free after 24 months. Recent observations have shown that a combination of all three medicines can clear almost 100% of blood Mf at 12 and also 48 months post treatment which indicates that this treatment leads to a permanent sterilisation of adult worms (52). Therefore, in areas non-endemic for onchocerciasis the WHO recommended nowadays triple administrations. Even if a few effects of these drugs on adult worms have been observed (e.g. ALB in LF) all three of them have no reliable macrofilaricidal effect (18, 53). In addition, repeated MDA rounds also increase the risk of upcoming resistance and therefore, new drugs directly targeting adult worms are necessary.

As mentioned in section 1.2.4 most of the human filarial parasites contain the endosymbiotic *Wolbachia* and these bacteria are important for filarial survival and worm fertility. Tetracycline is an effective antibiotic against *Rickettsiales* and studies from Hoerauf and colleagues demonstrated that a depletion of *Wolbachia* with doxycycline leads to a permanent sterilisation of female adult worms and moreover results in worm clearance after time (37, 38, 41, 42). Several studies have demonstrated that repeated treatment with doxycycline for 3 to 8 weeks

can clear Mf completely and also lead to sterility of the female worms and to the death of adult worms (41-43, 54). Due to the fact that doxycycline is already a registered drug and has only minor side effects it has become a promising drug in the fight against filarial infections. However, children under 9 years and also pregnant women cannot be currently treated. For children, this was due to adverse oral health issues but this is currently under debate and approval for short term use in children over 2. Additionally, doxycycline treatment is not convenient in MDA programs because of the length of the required treatment regime (55). Moreover, some of the filariae like *L. loa* do not harbour the *Wolbachia* bacteria and therefore, new drugs with macrofilaricidal effects are still needed (56).

1.3 *Litomosoides sigmodontis* - the rodent model of filariasis

To investigate aspects of filarial infections in more detail, mouse models are useful tools. It was already shown in 1992 that the rodent filariae *Litomosoides sigmodontis* (*L. sigmodontis*) undergoes its complete life cycle in the fully immunocompetent BALB/c mouse strain (57). These rodent filariae belong to the same family as parasites which induce human filarial infections. Its natural host is the cotton rat (*Sigmodon hispidus*) and the L3 larvae are transmitted by mites of the genus *Ornithonyssus bacoti*. The life cycle (Fig. 1.9) is similar to the life cycle of the human pathogen filariae. During the blood meal of the mite, infective L3 stage larvae enter the host through the skin and over the first four days migrate through the lymphatic´s to reach the pleural space of the thoracic cavity (TC) (site of infection) by migrating through the pulmonary capillaries (58, 59). Once in the pleural space, L3 larvae develop into L4 stage larvae (around day 8 post infection (p.i.)) and eventually into adults at day 30 post infection. Male and female adult filariae mate and afterwards filarial embryos develop in the female filariae. Around 40-60% of *L. sigmodontis*-infected BALB/c mice become patent and can result in the circulation of microfilariae in blood after 50 - 55 days post infection (58-60). Here, Mf can be taken up by mites during the next blood meal and develop again into L2 and afterwards in L3 stage larvae. Interestingly, the BALB/c mouse strain is susceptible to *L. sigmodontis* infections and allows the complete parasite development (57, 61-63) whereas other mice strains like the C57BL/6 or C3H mice are semi-susceptible to the full development (61, 62, 64, 65). Therefore, different genetic mouse strains, especially those deficient in a certain cell type or cytokine are indispensable in order to understand how the parasite regulates the host's immune system.

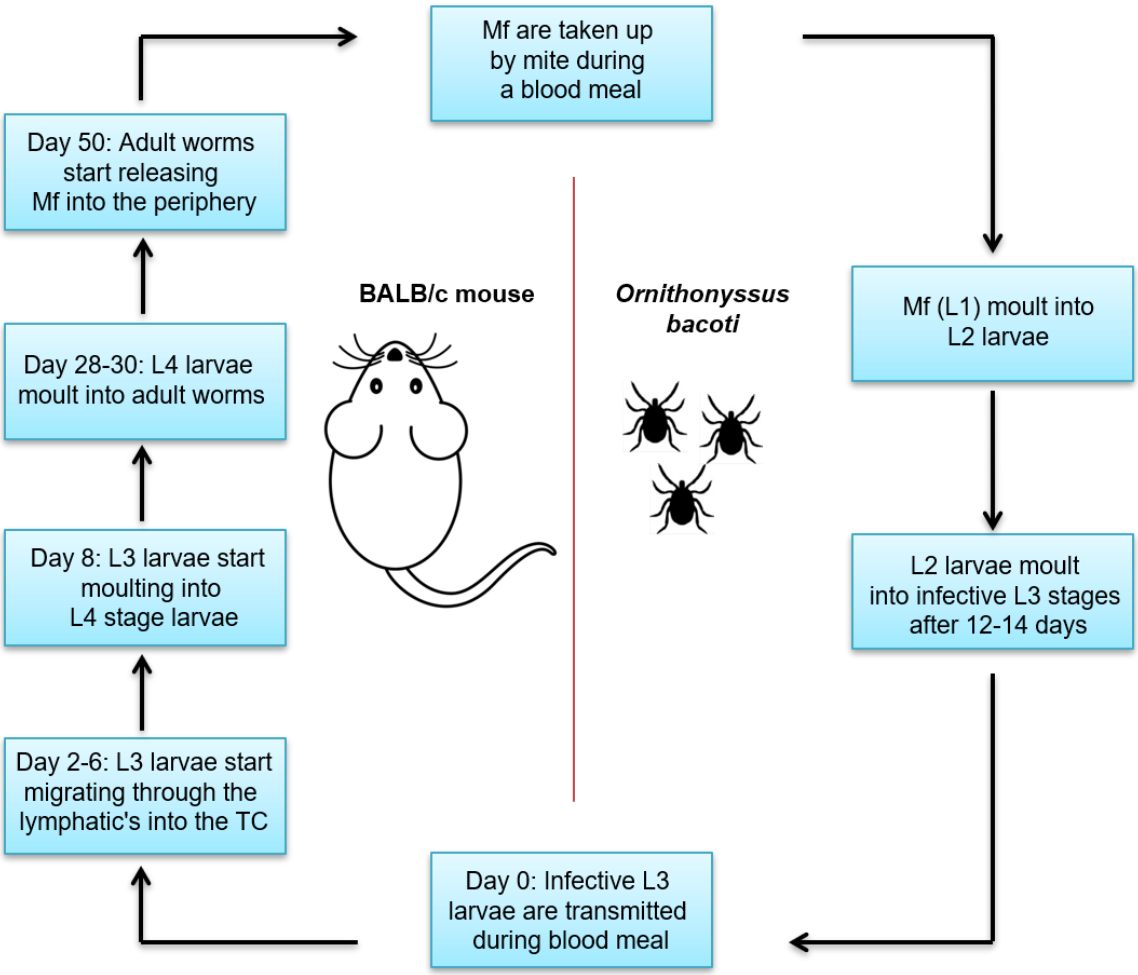


Figure1.9: Laboratory life cycle of *Litomosoides sigmodontis*. During a blood meal, infected mites release infective L3 stage larvae into a host mouse. During the next four days L3 larvae migrate through the lymphatics into the thoracic cavity (TC) the site of infection. Here they moult into L4 stage larvae around day 8 post infection. On day 30 post infection most of the L4 larvae have moulted into adult female and male worms. Following mating, female adult worms start releasing their offspring, the so-called microfilariae (Mf), around day 50 post infection and continue to do so until infections are cleared around day 120 post infection. Microfilariae are released and circulate in the blood where they can be taken up by mites again. Here, the Mf (L1 stage) moult into L2 and furthermore, into infective L3 stage larvae (adapted from (59)).

1.4 Lessons from mouse and man in immunomodulation by filariae

As described in section 1.1, the primary function of the immune system is the protection of the body from foreign substances or organisms and the distinction between the body's own cells and proteins and foreign molecules (1, 2). The infection with filarial helminths and the induction of immune responses is the result of an evolutionary process since it is important for the parasite´s own survival to evade the host's immune responses without killing the host. Otherwise, the immune reaction shouldn't be dampened too much since the host´s immune system must be able to eliminate other pathogens (16). Therefore, helminth are masters of immunoregulation and induce several modified immune responses. Once infected with a filarial

parasite the immune system will be confronted with different stages (L3 and L4 larvae, adult worms and Mf) and therefore, different stage-specific immune reactions are induced. In contrast to infections with protozoans, filarial infections induce a more type 2 biased immune response, including the production of cytokines like IL-4, IL-5, IL-9, IL-10 and IL-13 and the increased production of IgG4 (human) or IgG1 and IgE (human and mouse). Additionally, an accumulation of eosinophils, alternative activated macrophages (AAMs), mast cells and also of innate lymphoid cells (ILCs) could be observed at the infection site (66-70).

Immune reactions during vector-host engagement are not well understood but interestingly almost 80% of the invading larvae do not survive the skin stage of the infection (66) which is mediated by the innate immune response dominated by neutrophils (71). Studies demonstrated that as the vectors are biting the host, during early phase, the contact with invading L3 larvae down-regulates the activity of dendritic cells (17) and the endosymbiotic *Wolbachia* in these L3 larvae induce the degranulation of mast cells which results in an increased vascular permeability (72). Furthermore, NK cells stimulated with L3 larvae but also triggered with Mf secrete pro-inflammatory cytokines like IFN-γ or TNF-α (68, 73). The stimulation of human embryonic kidney 293 (HEK293) cells with Mf from *B. malayi* also activates TLR2 directly but exposure to these Mf can also down-regulate the gene and protein expression of TLRs (e.g. mRNA expression of TLR3, 4, 5 and 7) (6, 74). Innate cells like eosinophils accumulate at the site of infection within the first days and therefore, these cells are also important mediators during the early infection phase (72, 75, 76). Eosinophils are associated with allergic responses, viral infections and helminthiasis. Receptors of these cells are sensitive for cytokines and also chemokines (77, 78). Whereas the development of these cells is mediated by IL-5, IL-13 and GM-CSF (granulocyte macrophage colony-stimulating factor), the recruitment of eosinophils depends on chemokines like eotaxin (2, 79, 80). Therefore, Martin and colleagues demonstrated that an over-expression of IL-5 results in an increased eosinophil number which in turn lead to reduced worm loads (81). Granules of eosinophils mainly consist of four cytotoxic, cationic proteins: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (78) and in this context MBP and EPO seems to play a key role during filarial infections, since lack of these two proteins results in higher worm burden (82).

As mentioned in section 1.3, after 2 to 6 days, L3 larvae reach the thoracic cavity. Here larvae moult into adult worms and a robust Th2 immune response is established including higher IL-5 or IL-13 levels and expanded type 2 innate lymphoid cells (ILC2s) in the TC with a peak during the pre-patent stage (reflecting no Mfs) of infection. However, it seems that these cells have no direct impact on worm numbers (83). By using Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice the importance of the adaptive immune response during filarial infections could be demonstrated

since these mice are deficient for T, B and also NK cells and were able to establish a patent *L. sigmodontis* infection including Mf release (65). Expanded numbers of $CD4^+$ T cells are also part of establishing a robust Th2 immune response. Once in the TC IL-4 triggers the development of $CD4^+$ T cells into Th2 cells which produce more type 2 cytokines and chemokines (2, 76). Furthermore, BALB/c mice lacking $CD4^+$ T cells showed a reduced Th2 cytokine production, eosinophilia and IgE secretion after *L. sigmodontis* infection which results in higher worm and Mf counts (84). Interestingly, the depletion of basophils in mice vaccinated with L3 stage larvae resulted in a significantly decreased Th2 cytokine production by $CD4^+$ T cells (85). This indicates that these cells, even if they can strengthen the Th2 immune response, are not essential for parasite control during a primary infection. However, lacking of $CD8^+$ T cells has no impact on worm numbers in *L. sigmodontis*-infected mice (86) and has also no influence of establishing a resistance against *B. malayi* in infected mice (87).

The major function of Treg cells is to limit pathology by dampening immune inflammation, and more specifically to control autoimmunity (2). Regulatory roles have been ascribed to IL-10 but also to TGF-β (88), two cytokines closely implicated in the activity and induction (89) of Treg cells. This raised the hypothesis that Treg cells mediate helminth-induced immune suppression (90, 91). Two main populations of Treg cells have been well investigated: thymus-derived regulatory T cells (tTreg) which are defined by their production of IL-10 and considered to be 'adaptive' as they develop from naive T cells in the thymus (89, 92, 93) and peripherally induced regulatory T cells (pTreg) (92-94). Both cell types are characterized as $CD4^+CD25^{hi}Foxp3^+$. Since, tTregs express levels of the transcription factor Helios and also the protein receptor Neuropilin-1. Therefore, both can be used as a marker for tTregs. However, studies have also demonstrated that the expression of Helios is higher compared to the expression of Neuropilin-1 (92, 93, 95). Taylor and colleagues could show that $CD4^+CD25^{hi}Foxp3^+$ Treg cells are active in susceptible BALB/c mice during the adult stages of *L. sigmodontis* infection and inhibit protective immune responses resulting in enhanced parasite survival (90, 91). The induction of the immunosuppressive cytokine IL-10 is a common phenomenon found in chronic helminth infections (96, 97) and moreover, during *L. sigmodontis* infections, there is strong evidence that IL-10 plays an essential role in the susceptibility to filariae (61). Earlier studies showed that female *L. sigmodontis* worms, implanted into the TC of mice, promoted the survival of co-injected Mf. The survival was drastically reduced when females were implanted in IL-10-deficient compared to wild type mice (61). Specht and colleagues also demonstrated the requirement for IL-10 in patency and chronic adult worm infections, showing that the susceptibility seen in C57BL/6 IL-4 deficient mice (98), which allows microfilariae to persist, is reverted upon additional IL-10 deficiency using IL-4/IL-10 double deficient mice (99). Later studies from Specht and colleagues suggested that the

induction of high levels of IL-10 is beneficial for the survival of adult worms and Mf in murine filariasis and thus in favour of the worm (100).

Only few studies have been performed so far regarding the role of B cells during filarial infections. One of them has shown that C57BL/6 mice deficient for B cells couldn't establish a patent *L. sigmodontis* infection, which indicates that these cells are not involved in parasite clearance (98). But interestingly, the lack of B cells in BALB/c mice resulted in increased numbers of worms and Mf and also in an impaired Th2 immune response (101). As mentioned earlier, filarial infections in mice are accompanied by an accumulation of IgE (102, 103). Mice lacking IgE showed an increased worm burden after infection with *B. malayi* (69, 104) and also IgM seems to be important for the protective immunity against filarial parasites (69, 105).

As previously mentioned, AAM are also part of the Th2 induced immune response during filarial infections. Taylor and colleagues have demonstrated that a *L. sigmodontis* infection induces the recruitment of AAMs which was restricted to the infection site during the patent phase of the infection (106, 107). A study by Gondorf and colleagues also showed that chronic filarial infections have a beneficial effect on a bacterial induced sepsis by functionally re-programming macrophages. Whereas, the induction of AAM was dispensable for this protective effect (108). However, AAMs are important for helminth expulsion and priming of these cells is also dependent on neutrophils (109). Neutrophils are classically characterised as phagocytic cells (110, 111). Whereas IL-3, IL-6, G-CSF (granulocyte colony-stimulating factor) and also GM-CSF induce neutrophil differentiation, cytokines like IL-8 and TNF-α are important for the recruitment of these cells (110). Studies have demonstrated that neutrophils controlled by IL-5 are important for killing of adult filarial worms and inflammatory nodule formation. Additionally, the lack of IL-5 results in a lower amount of neutrophil promoting cytokines like TNF-α and G-CSF (112). Interestingly, the accumulation of neutrophils could be observed in mice shortly after injection of *O. volvulus* crude antigen into the cornea (113) which is associated with the endosymbiotic *Wolbachia* bacteria (114) since its depletion inhibited neutrophil accumulation and also prevented the onset of ocular pathology (80, 113, 115).

As mentioned earlier, IL-4 and IL-5 are important mediators in filarial infections. Both of them are the dominant cytokines produced during helminth infections (98, 116, 117) and were demonstrated to be important factors for patency in BALB/c mice, as deficiency of either one led to up to a 100-fold higher microfilaremia and prolonged Mf survival. Furthermore, IL-5 appeared also to control adult worm development in primary infections (112) since IL-5-deficient mice had increased parasite burden whereas transgenic mice over-expressing IL-5 were able to more rapidly kill these parasites compared to wild-type littermates (81). Additionally, *L. sigmodontis*-infected mice deficient for IL-4Rα/IL-5 showed a stronger inflammation of pleural diaphragm and lung which also corresponded to increased worm and

Mf numbers (117). As described above, BALB/c mice allow the complete parasite development, but in C57BL/6 mice filariae are progressively encysted and destroyed from day 40 post infection onwards, thus producing no microfilariae (62). However, infections of IL-4-deficient C57BL/6 mice resulted in full parasite development and patency, demonstrating that in fact a Th2 response is the key determinant of susceptibility and resistance in these non-permissive mice (98).

In general, only a few studies analysed the role of TLRs during filarial infections. However, these studies have already demonstrated that immune reactions which induce the induction of filarial-related symptoms, like lymphedema in the legs or arms, are associated with triggering TLR pathways (6, 97). Mediators like angiogenic growth factors which are important for lymphangiogenesis are increased in PBMCs of patients with lymphedema, induced by TLR-ligands (118). As already mentioned in section 1.2.4, *Wolbachia* are also potent inducers of the TLR pathways (39, 44, 45, 119, 120). Studies from Babu et al. have also revealed that in humans with patent filarial infections the expression of TLR1, TLR2 and TLR4 by T cells are diminished (121). Further studies have also shown that the lack of TLR4 hinders the development of a protective immunity against *O. volvulus* in C3H/HeJ mice (122, 123). In addition, lack of this receptor resulted in 93% patent *L. sigmodontis*-infected $TLR4^{-/-}$ BALB/c mice. However, numbers of Mf and female worms and also total worm counts were comparable to WT Mf+ BALB/c mice indicating an increase in fertility. In contrast, the absence of TLR2 in BALB/c mice did not increase patency in infected BALB/c mice but led to an altered cytokine production by $CD4^+$ T cells and a delayed Treg development (63). Further work in this paper then compared LsAg-specific immune responses in latent and patent mice with those observed in individuals infected with *W. bancrofti* (124). Interestingly, in contrast to the human studies with *W. bancrofti*-infected individuals, IL-10 responses to filarial antigens were comparable in both infection groups (Mf+ and Mf- WT BALB/c mice) and IL-5 responses were significantly higher in cultures from Mf+ mice (63, 124). Furthermore, $CD4^+$ T cells from WT BALB/c Mf+ mice secreted significantly higher amounts of cytokines like IL-5 or IL-10 compared to $CD4^+$ T cells from WT BALB/c Mf- mice (63) which lay the foundation for the follow-up study detailed in the results section below.

1.5 Objectives of this thesis

The nature of host-helminth interactions during filarial infections has several levels of complexity due to the length of infection time, the several life-stages that the helminth passes through in the host and the number of organs and tissues that are affected. This provokes a multi-cellular reaction creating substantial adaptive immune responses and depending on the susceptibility of, at least the murine host, leads to either worm clearance (C57BL/6), latent (Mf negative) (C3H, BALB/c) or patent (Mf positive) (BALB/c) infections. The aim of this thesis was to investigate various aspects of such immune-modulation using the rodent model for filariasis, *Litomosoides sigmodontis*.

The first part of the thesis built on previous work from the group showing different immune profiles in latently and patently infected BALB/c mice (63). Since this previous study revealed that filarial-specific cytokine release from $CD4^+$ T cells was only observed from microfilariae-positive (patent) mice, this work now investigated whether $CD4^+$ T cells from patently infected BALB/c mice responded differently to worm extracts prepared from either total, female or male worms and moreover, whether soluble or pellet derived antigens thereof influenced the release of IFN-γ, IL-10, IL-5, IL-13 responses (Wiszniewsky, Neumann et al., in preparation).

Continuing on elucidating why the MHC haplotype changes the outcome of infection, we then performed comparative studies in BALB/c and C57BL/6 mice analysing parasitological and immunological parameters. By considering that perhaps innate signalling pathways shape adaptive host immunity which in turn facilitate helminth clearance, infection studies were then performed in TLR3-, TLR4-, TRIF-, Myd88-, IRF1- and IRF 3-deficient C57BL/6 strains. Interestingly, TRIF-deficient mice presented higher worm burdens revealing a crucial role for this adapter molecule on worm recovery since T cell recruitment into the site of infection was reduced (125).

Remaining with the C57BL/6 model the last part of the thesis investigated whether T cell populations might be responsible for worm clearance. Here the RAG2/IL-2Rγ-deficient mouse strain was used which lacks T, B and NK cell populations. Expanding on previous data, showing that this mouse strain leads to a patent infection with extremely high levels of worms and microfilariae (65), a series of adoptive transfer studies were employed using isolated $CD4^+$ and $CD8^+$ T cells from naïve or infected donor mice into RAG2/IL-2R$\gamma^{-/-}$ recipients prior to infection. Since $CD4^+$ T cells were shown to play a role in reducing female worm size, fecundity and microfilariae release further adoptive transfer experiments were carried out using cytokine-deficient donor cells.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

See appendix A on page 119

2.1.2 Antibodies and Proteins

See appendix B on page 120

2.1.3 Chemicals, Reagents and Kits

See appendix C on page 121

2.1.4 Buffer, Media and Solution

See appendix D on page 122

2.1.5 Software

See appendix E on page 123

2.2 Methods

2.2.1 Animal maintenance

Wildtype (WT) BALB/c and C57BL/6 mice were purchased from Janvier, France. Strains of IRF1-, IRF3-, TLR3-, TLR4- and TRIF-deficient mice on the C57BL/6 background originated from the Institute of Medical Microbiology, Immunology and Hygiene, Munich and were bred at the Institute of Medical Microbiology, Immunology and Parasitology (IMMIP), Bonn. $MyD88^{LSL}$ mice and control littermates were provided by Dr. H. Weighardt (LIMES, Bonn, Germany). B6-$Rag2^{tm1Fwa}$Il2rg^{tm1Wjl} mice were purchased from Taconic Biosciences Inc, Cologne, Germany and bred alongside WT C57BL/6 mice. All mice were bred under SPF conditions in accordance with German animal protection laws and EU guidelines 2010/63/E4 and had access to food and water *ad libitum*. All protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany. Genotyping of the different mouse strains was analysed by polymerase chain reagent (PCR) technique using the following primers: $IRF1^{-/-}$ primer 1, 5´-CAG ACA TCG AGG AAG TGA AGG-3´; primer 2, 5´-TGC TGT GGT CAT CAG GTA GG-3´; primer 3, 5´-CTT GGG TGG AGA GGC TAT TC-3´; primer 4, 5´-AGG TGA GAT GAC AGG AGA TC-3´; $IRF3^{-/-}$ primer 1, 5´-GAA CCT CGG AGT TAT CCC GAA GG-3´; primer 2, 5´-GTT TGA GTT ATC CCT GCA CTT GGG-3´; primer 3, 5´-TCG TGC TTT ACG CTA TCG CCG CTC CCG ATT-3´; $TLR3^{-/-}$ primer 1, 5´-GCC AGA GGC CAC TTG TGT AG-3´; primer 2, 5´-GCA ACC CTT TCA AAA ACC AG-3´; primer 3, 5´-AAT TCA TCA

GTG CCA TGA GTT T-3´; TLR4$^{-/-}$ primer 1; 5´-GCA AGT TTC TAT ATG CAT TCT C-3´, primer 2; 5´-CCT CCA TTT CCA ATA GGT AG-3´, primer3; 5´-ATA TGC ATG ATC AAC ACC ACA G-3´, primer4; 5´-TTT CCA TTG CTG CCC TAT AG-3´; TRIF$^{-/-}$ primer 1, 5'-ACCCTATGAACAGCATGTGTCACAGTG-3'; primer 2, 5'-ACA GTC CCA ATC CTT TCC ATC AGC CTC-3'; primer 3, 5'-CTA AAG CGC ATG CTC CAG ACT GCC TTG-3'; MyD88$^{-/-}$ primer 1; 5´-ACA CTG TAG CTG CCT GCA GAC ACA C-3´, primer 2, 5´-GGA CTC CTG GTT CTG CTG CTT ACC T-3´; primer 3, 5´-CTG AAG AGG AGT TTA CGT CCA G-3´; primer 4, 5´-CTA GCC TTG GCA TAT TAA TCT TG-3´; Rag2IL-2Rγ$^{-/-}$ C57BL/6 primer 1; 5´-CAT TCC AGG AGT GCA GTC ACT ATT TG-3´; primer 2, 5´- GTG CAA TCC ATC TTG TTC AAT GGC CG-3´, primer 3, 5´- CTT TAT TGA TAA CGA TCT ATC CCT CAC CC-3´; primer 4, 5´-CTC CAC TCT GCA GAG TCT ATG GAA TCC-3´.

2.2.2 In house life cycle of *L. sigmodontis*

The life-cycle of *L. sigmodontis* was maintained at the IMMIP using infected cotton rats (*Sigmodon hispidus*) as host. The mites of the genus *Ornithonyssus bacoti* serving as vectors were kept at 28°C and 80% humidity and fed on blood residing microfilaremic (> 2,000 Mf/ml of blood) infected cotton rats. Thereby, L1 larvae were transmitted into the mites. After 10-14 days L1 larvae develop into L3 larvae. During this time mites were fed at least twice a week on 6-8-week-old mice. Mites containing the L3 larvae were used to infect experimental mice.

2.2.3 Experimental infection procedure of jirds or mice with *L. sigmodontis*

Infections with *L. sigmodontis* were performed using a natural method as described earlier (59, 126). In brief, animals were placed over night into a plastic cage in bedding that included the mites. During this blood meal (infection day) infected L3 larvae were transmitted into the mice and migrate through the lymphatics to the thoracic cavity (TC) by days 4–6 p.i.. Here, they molt to the L4 stage between days 8 and 12 p.i., become adults by days 25–30 p.i. and are able to produce microfilariae that circulate in the blood by day 50 p.i. (59, 63, 127). After the blood meal mites return to the bedding. Next day, the bedding was removed. To further remove the mites, mice were transferred for 24h into a wire cage, placed over soap water. During the next 6 days, mice received new bedding each day. Thereafter, mice were relocated into a different room in the animal facility and remained there until the experimental day.

2.2.4 *L. sigmodontis* antigen preparation from jirds

All working steps were performed on ice. For the preparation of *L. sigmodontis* antigen (LsAg), adult worms were isolated from the thoracic cavity of jirds > 120 days post infection, transferred into a petri dish (Greiner bio-one GmbH, Frickenhausen, Germany) and rinsed with endotoxin-free sterile phosphate buffer saline (PBS) (PAA Laboratories GmbH, Pasching, Austria). Worms were divided into three different groups: female worms, male worms or a mixture of both genders. Afterwards, the three different worm groups were transferred separately into a

sterile glass potter (Greiner bio-one GmbH) and 5 ml of sterile PBS was added. Worms were minced until no worm particles were visible anymore. The homogenized worm material from the different groups was then transferred into three different 50 ml falcon tubes (Greiner bio-one GmbH) and the worm material was centrifuged at 1,485g for 10 minutes at 4°C. Afterwards, the supernatant (SN) (contains the soluble material) was transferred into a fresh tube and the pellet was re-suspended in 1 ml sterile PBS. Protein concentrations of SN and pellet were measured via the Bradford Assay (section 2.2.5). Antigen solutions were stored at -80°C until required.

2.2.5 Bradford Assay

First, a bovine serum albumin (BSA) (PAA Laboratories, Pasching, Germany) standard row (diluted in PBS) was prepared with concentrations from 7.8125 µg/ml up to 2000 µg/ml. Next, 3 µl of each standard were pipetted into a separate well of a 96-well flat-bottom ELISA plate (Greiner Bio-One, Solingen, Germany) in duplicates and 3 µl of different sample dilutions (1/50, 1/75 and 1/100) were also transferred into the plate in triplicates. Afterwards, 300 µl of "Advanced Protein Assays" (Cytoskeleton, Denver, USA) were transferred into standard and sample wells and optical density was measured immediately by using the SpectraMAX ELISA reader (Molecular devices, Sunnyvale, USA) with wavelength correction (450 nm and 570 nm). Data were analysed with SOFTmax Pro 3.0 software (Molecular Devices).

2.2.6 Microfilariae monitoring

Peripheral blood (25 µl) was taken from the cheek vain and transferred into 300 µl Hinkelmann solution (Merck, Darmstadt, Germany). On the experimental day also 25 µl of thoracic cavity fluid were transferred into Hinkelmann solution. Thereafter, samples were incubated for 10 minutes at room temperature (RT) and centrifuged for 5 minutes at 1300 rpm. Finally, supernatant was removed and sediment was analysed via microscopy for Mf counts.

2.2.7 Isolation of adult worms and thoracic cavity cells

Infected mice were sacrificed by inhaling Forene® (Piramal Critical Care, West Drayton, UK). Using sterile scissors and tweezers the mice were opened from the abdomen to the sternum. The cut was expanded so that it bordered the deepest ribs without damaging the diaphragm. Holding the tip of the sternum with the tweezers, a small orifice was made in the middle point of the upper border of the diaphragm. Utilizing sterile plastic Pasteur pipettes (Ratiolab, Dreieich, Germany) filled with sterile PBS, the thoracic cavity was washed several times. The collected fluid was passed through a filter into a 15 ml falcon tube (Greiner bio-one GmbH) to collect the worms found in the cavity. To control that all worms were taken, the chest was opened completely and any residual worms were picked out. Additionally, the first 500 µl of the washing procedure was collected for cytokine analysis (stored at -20°C). Worms were

analysed for gender development stage and length, for some studies 5-10 female worms were stored at -20°C for embryogram (section 2.2.8) or PCR-Analysis (section 2.2.9).

2.2.8 Embryogram of female worms

Single female worms were transferred into a 1.5 ml plastic tube (Eppendorf) including 80 µl of sterile PBS and embryonic stages were squeezed out of the worms with a plastic pestle (Greiner bio-one GmbH). Afterwards, 20 µl of Hinkelmann solution (Merck) were transferred into the tube and vortexed. 10 µl of solution was used for analysis by microscopy (128).

2.2.9 LsWFtsZ/ LsActin duplex real-time PCR

Following isolation, worms were separated into single male and female adults and frozen at -20°C. DNA was extracted using the QIAamp DNA Mini Kit for tissue (Qiagen, Hilden, Germany) in accordance to the manufactures´ protocol. In short, individual frozen worms were re-suspended in sterile PBS and homogenized in ACT buffer (Appendix D) by using small scissors. Afterwards, Proteinase K was added followed by 15 seconds of vortexing and an overnight incubation at 56°C in a water bath (VWR, Darmstadt, Germany). Next, worm DNA was eluted with AE buffer (Qiagen). For quantification of *Wolbachia* levels single-cell division protein *Ls-FtsZ* (GenBank Accession No.: AJ010271) was measured (65, 129). The Duplex real-time PCR was performed in a Rotor Gene RG-6000 (Corbett Research, Sydney, Australia) using Qiagen´s Quantitect Multiplex NoROX Kit (Qiagen,) in a 20 µl reaction consisting of: 10 µl master mix, 4.1 µl nuclease free water, 1 µl LsFtsZ forward primer (10 µM) (CGATGAGATTATGGAACATATAA), 1 µl LsFtsZ reverse primer (10 µM) (TTGCAATTACTGGTGCTGC), 0.8 µl LsActin forward primer (10µM) (ATCCAAGCTGTCCTGTCTCT), 0.8 µl LsActin reverse primer (10 µM) TGAGAATTGATTTGAGCTAATG), 0.1 µl LsFtsZ taqman probe (5 µM), 0.2 µl LsActin taqman probe (5 µM) and 2 µl extracted worm DNA. Cycling was performed by acquiring the fluorescence on the FAM channel using the following protocol: 95°C for 15 min, followed by 45 cycles at 95°C for 15 sec and 58°C for 45 sec. Levels of LsFtsZ and LsActin genes were estimated by comparing crossing point data for the test samples to a standard curve obtained from serial dilutions of the plasmid containing the specific genes. LsFtsZ transcription levels were normalized by the signal of LsActin gene to correct for differences in the amount of starting material.

2.2.10 Isolation of sera from murine blood

Blood was taken from the *vena facialis*, collected in 1.5 ml tubes (Eppendorf, Wesseling, Germany) and kept at 4°C over-night to allow coagulation. Afterwards, tubes were centrifuged at 4°C, at 13,200 rpm for 5 minutes. The liquid phase was removed, placed in a new tube and centrifuged again. In the last step, the resulting sera was transferred into a fresh tube and stored at -20°C for further analysis.

2.2.11 Preparation and staining of cytospins

To analyse cell compositions in thoracic cavity fluid cytospins were prepared. In short, cytospin chambers (Hettich, Tuttlingen, Germany) were prepared with a labelled slide (Engelbrecht, Munich, Germany) and filter paper (Hettich). Afterwards, 50 µl of adjusted cells ($5x10^4$/ 50 µl) were pipetted onto the cytospin chamber. Cells were spun down onto the slide via impulse centrifugation up to 300 rpm without break. Appliance and filter paper were removed carefully without damaging the cells on the slide and slides were air dried over-night. In a second step, cytospin preparations were stained using the Diff Quick Staining Set (Medion Diagnostics International, Miami, USA). Here, slides were dipped five times for 1 second in the "Fixative Solution". Afterwards, the slide was dipped 5-7 times for 1 second in the "Stain Solution I". In a last step the slide was dipped 3 times in "Stain Solution II", rinsed with water and air dried. Finally, stained cells were covered with Entellan (Merck, Darmstadt, Germany) and a cover slip (Engelbrecht). To determine frequencies of different cell types, two times 100 cells were counted and the averages was calculated.

2.2.12 Preparation of mediastinal lymph node cells

After isolating worms, as described in section 2.2.7, the thoracic cavity was opened to gain access to the mediastinal lymph nodes (medLN) that were excised using tweezers (Dumont Swissmade, Montignez, Swizerland). Afterwards, medLNs were transferred into a 96-well plate (Greiner bio-one) containing medium and squashed between blunt tweezers heads. Next, cells were transferred through gauze (Labomedic, Bonn, Germany) into a 15 ml falcon tube and centrifuged for 5 minutes at 1300 rpm at 4°C. Supernatants were discarded and the pellet re-suspended in medium plus supplements (Appendix D). Finally, cells were stored on ice until counting (section 2.2.13).

2.2.13 Cell viability and counting

The number of living cells was determined using the trypan blue (Sigma-Aldrich, Munich, Germany) exclusion method. In short, 5 µl of the cell suspension was diluted 1:10 with 0.4% trypan blue. Thereafter, 10 µl of the diluted cells were transferred to a Neubauer counting chamber (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Living cells (non-coloured) were counted and expressed as cell number per ml. Cells were only used if less than 5% of the cells were dead (blue). To determine the cell number, unstained cells from 4 squares were counted and the following formula was applied:

Number of cells in one ml = cell count x 4 x 10^4 x dilution factor.

2.2.14 Lymphocyte preparation from spleens

Mice were sacrificed by inhaling Forene®. Using sterile scissors and tweezers the mice were opened on the left side. Spleens were removed and immediately placed in ice-cold PBS in a

petri dish (Greiner bio-one GmbH) and mashed with a sterile plunger (BD Diagnostics, Heidelberg, Germany). The cell suspension was collected in a 15 ml falcon tube and the cells were centrifuged for 5 minutes at 1300 rpm at 4°C. Supernatants were discarded and the pellet re-suspended in 5 ml of ACT buffer (appendix D). Cell lyses was carried out for 6 minutes at RT with constant shaking. Afterwards, cells were passed through a filter and washed again with ice cold PBS at 1300 rpm for 5 minutes (4°C). Supernatants were discarded and the cells re-suspended in 1 ml culture medium (appendix D), counted (section 2.3.13) and kept on ice until needed.

2.2.15 Cell Sorting Techniques

2.2.15.1 Magnetic separation of CD4 positive or MHC class II negative spleen cells

CD4 positive or MHC Class II negative cells were isolated by using the $CD4^+$ T cell Isolation Kit (mouse) or the Anti-MHC Class II MicroBeads Kit (mouse) from Miltenyi Biotec GmbH, (Bergisch Gladbach, Germany) in accordance with the manufacturer's protocol. Briefly, $1x10^7$ cells were re-suspended in 90 µl of autoMACS buffer (Miltenyi Biotec GmbH). Next, 10 µl of $CD4^+$ T cell Isolation Beads or Anti-MHC Class II Beads (Miltenyi Biotec GmbH) per 10^7 cells were added to the cells, mixed thoroughly and incubated for 15 minutes at 4°C. Afterwards, cells were washed by adding 1-2 ml of autoMACS buffer and centrifuged at 1300 rpm for 5 minutes at 4°C. Supernatants were aspirated completely and up to 10^8 cells were re-suspended in 500 µl of autoMACS buffer. Cells were separated by using LS columns (Miltenyi Biotec GmbH) which were placed in the magnetic field of a suitable MACS separator ((Miltenyi Biotec GmbH) and primed with 3 ml of running buffer. Afterwards, cell suspensions were applied to the columns. Columns were washed three times with 3 ml of autoMACS buffer in each washing step. Labelled $CD4^+$ T cells were held in the column whereas unlabelled MHC Class II negative cells that passed through directly were collected in 15 ml falcon tubes. Finally, columns were removed from the separator and labelled $CD4^+$ T cells were flushed out immediately by firmly pushing the plunger into the column. $CD4^+$ T cells or MHC Class II negative cells were washed and re-suspended in medium before counting (section 2.2.13). Purity of isolated cells was analysed via FACS and revealed a cell purity of 95-97%.

2.2.15.2 Cell sorting of $CD4^+$ and $CD8^+$ T cells

For sorting, $CD4^+$ and $CD8^+$ T cells were re-suspended in 2 ml PBS. Controls and unstained cells were re-suspended in 500 ml PBS. Cells were sorted by using the BD FACSAria III Cell Sorter (BD Bioscience, Heidelberg, Germany), collected in fetal bovine serum (FBS) and stored on ice until needed. For adoptive transfers, determined numbers of sorted $CD4^+$ or $CD8^+$ T cells ($1x10^6$ - $3x10^6$ cells/ 200 µl PBS) were injected intravenously (i.v.) into the tail vain of Rag2IL-2R$\gamma^{-/-}$ C57BL/6 mice. After one day, injected mice and control mice were infected as described in section 2.2.3.

2.2.16 Preparation and cultivation of bone marrow-derived dendritic cells (BMDCs)

Mice were sacrificed by inhaling Forene® and pinned onto a dissection board. Using sterile scissors and tweezers the hind limbs were opened and the muscles were separated from the bones. The bones were cut at hip joint and ankle and divided into lower and upper leg by removing the knee. From these bones the bone marrow was flushed out with endotoxin free sterile PBS using a syringe (BD Diagnostics, Heidelberg, Germany) and cannula (Braun, Melsungen, Germany). The flushed bone marrow was slightly homogenized and then centrifuged at 1200 rpm for 8 minutes (4°C). After centrifugation the pellet was re-suspended in 2 ml of ACT buffer (appendix D) for approximately 6 minutes at RT. Immediately after lysis, the bone marrow suspension was filtered over gauze (Labomedic GmbH, Bonn, Germany), rinsed with 9 ml PBS and centrifuged again at 1200 rpm for 8 minutes (4°C). Afterwards, the supernatant was discarded and the cells were re-suspended in culture medium (appendix D) and counted (see section 2.2.13).

2.2.16.1 Cultivation of bone marrow-derived dendritic cells (BMDCs)

Up to $1x10^7$ bone marrow derived progenitor cells were placed in 10 ml culture media (appendix D) including 20 µl GM-CSF (PreproTech GmbH, Hamburg, Deutschland) and plated onto a 10 cm petri dish. The BMDCs were cultured for 3 days at 37°C. On day 3, petri dishes were filled up with another 10 ml of culture medium including 20 µl GM-CSF. On day 6, the medium was changed, the liquid phase was carefully removed, centrifuged at 1200 rpm for 8 minutes at 4°C, and any remaining cells were re-suspended in another 10 ml fresh culture medium containing GM-CSF and added back to the same petri dish. The cells were than incubated for another day at 37°C. After 7 days of generation, generated BMDC are attached to the bottom of the petri dish, thus the supernatant was discarded and 5 ml PBS was added to each plate. Cells were detached using a cell scraper (Sarstedt, Nümbrecht, Germany) and by flushing the plate several times with PBS. BMDCs were collected in a 50 ml falcon tube and centrifuged for 5 minutes at 1200 rpm at 4°C. Finally, cells were re-suspended in non GM-CSF-containing culture medium and counted as described in section 2.2.13.

2.2.17 Fluorescent activated cell scan (FACS)

For further analysation of cell differentiation, activation and cytokine production in the TC, spleen, medLN and blood, cells were analysed via flow cytometry. This technique was originally developed in the 1960s and utilizes light for cell counting and analysation of expressed cell surface and intracellular molecules, to assess cell purity of isolated cells and also to determine cell size.

2.2.17.1 Extracellular staining for flow cytometry

For extracellular staining, different cell types were stained with antibodies specific for mice with different fluorochromes so that populations could be distinguished from each other

simultaneously (63, 65). First of all, Fc-receptors which are found on different cell types like macrophages, dendritic cells or B cells and bind antibodies via their constant Fc-receptor rather than their antigen specific Fab domain (Neil A, Fc Rceptor; 1998), were blocked with CD16/CD32 antibody (FC-block) (Thermo Fisher Scientific GmbH, Dreieich, Germany), 1/500 diluted in 50 µl PBS, for 15 minutes. Cells were then centrifuged for 5 minutes at 1200 rpm at 4°C and stained with fluorescent-marked monoclonal antibodies. For the different studies a master mix that consisted of desired antibodies (diluted 1/200 or 1/400 in PBS) was prepared (appendix B) and

incubated with cells for 15-20 minutes at 4°C. Single stained controls were stained with only 1 µl of individual antibody and also incubated at 4°C. Afterwards, cells were centrifuged as described previously and supernatant was discarded. Cells were re-suspended in 100 µl PBS and stored at 4°C until measuring.

2.2.17.2 Intracellular cell staining for flow cytometry

For intracellular staining, the Foxp3/ Transcription Factor Staining Buffer Set (Thermo Fisher) was used, in accordance to the manufacturer's protocol. In brief, cells were re-suspended in 1x Foxp3 Fix/Perm solution (appendix C) and incubated for 16 hours at 4°C. Afterwards, samples were centrifuged for 5 minutes at 1700 rpm at 4°C and the SN was removed. Next, the cells were washed twice with 1x Permeabilization buffer (appendix C). Cells were then centrifuged again as previously described and the SN was discarded. Thereafter, cells were stained with the antibody master mix (diluted 1/200 or 1/400 in PBS) (appendix B) for 20-30 minutes at 4°C. Finally, cells were washed twice with 1x Permeabilization buffer and re-suspended in 100 ml PBS. Samples were measured by using the FACSCanto I flow cytometer (BD) (4 colour) or the CytoFlex (BeckmanCoulter Life Science, Krefeld, Germany) (13 colour) and analysed via FlowJo v10 software (FlowJo, Portland, USA).

2.2.18 Cell culture

For co-culture assays, $2.5x10^5$ $CD4^+$ T cells, isolated from the spleen of infected mice, were co-cultured for 72 hours at 37 °C and 5% CO_2, with GM-CSF-derived BMDCs ($1x10^5$), see section 2.2.16, onto 96-well round-bottom plates (Greiner Bio-One) and left either un-stimulated or activated with SN or pellet of different LsAg preparations (40 µg/ml) from total, female or male worms.

For bulk cell assays, $5x10^5$ cells from spleen or medLN were plated out onto 96-well round-bottom plates. The cells were left either un-stimulated or activated either with anti-CD3/anti-CD28 (5 and 1.25 µg/ml respectively) (eBioscience, Frankfurt am Main, Germany) or LsAg (50 µg/ ml). The stimulated cells were cultured for 96 hours at 37 °C and 5% CO_2 and all supernatants were stored at -20°C until further use.

2.2.19 Enzyme-linked Immunosorbent Assay (ELISA)

For further analysis of TC fluid and co-culture SN, sandwich ELISAs were performed, according to the manufacturer´s protocol (R&D Systems, Wiesbaden-Nordenstadt, Germany: CCL17, CCL22, Eotaxin-1, RANTES, IP-10, MIP-2, Granzyme, TGF-β, IL-5; eBiosciences, Heidelberg, Germany: IL-5, IL-10, IL-13, IL-17A, IFN-γ). In short, ELISA plates (Greiner Bio-One) were coated with 50 µl of capture antibody (diluted in the specified buffer) over-night at RT (R&D Systems) or 4°C (eBioscience). Plates were washed 3 times with washing buffer (appendix D) and blocked for one hour with appropriate blocking buffer (appendix D). After repeating the wash step, plates were incubated with 50 µl/ well of standards or samples and incubated for 2 hours at RT. The wash steps were repeated again and 50 µl/ well of detection antibody was added and incubated for 1-2 hours at RT. After a further wash step plates were incubated for 30-45 minutes with 50 µl of Streptavidin-HRP at RT. Next, plates were washed 5 times and incubated with 100 µl developer containing tetramethylbenzidine (TMB) in the dark. After 15 minutes, reaction was stopped with 100 µl/ well 2N H_2SO_4 (Merck) (approximately 15 minutes). Optical density was measured using the SpectraMAX ELISA reader (Molecular devices) with wavelength correction (450 nm and 570 nm). Data were analysed with SOFTmax Pro 3.0 software (Molecular Devices).

2.2.19.1 Mouse Eosinophil Cationic Protein ELISA

To analyse the Eosinophil Cationic Protein (ECP) level within the TC fluid and sera an ELISA was performed using the “Mouse Eosinophil Cationic Protein ELISA KIT” (Abbexa, Cambridge, UK) according to the manufacturer’s protocol. In brief, 50 µl of diluted standards were added into the standard wells of the pre-coated plate. 50 µl of different samples were also added into the test sample wells. After an incubation step plate was washed and 50 µl of HRP conjugate reagent were added to the wells. Following a further incubation and wash step, 50 µl of TMB Substrate A and B was added to each well. After 15 minutes the developmental reaction was stopped with 50 µl of Stop solution and measured at 450nm in a SpectraMax 190 Microplate Reader (Molecular Devices). Data were analysed with SOFTmax Pro 3.0 software (Molecular Devices).

2.2.19.2 *L. sigmodontis*-specific IgG1 and IgE ELISA

To analyse filarial-specific IgG1 and IgE, ELISA plates (Greiner Bio-One) were coated with 50 µl LsAg (10 µg/ml diluted in PBS) and incubated over-night at 4°C. Plates were washed multiple times with washing buffer (appendix D) and blocked with 150 µl PBS/1%BSA (PAA, Pasching, Austria) for 2 hours at RT. After a further washing step, plates were incubated at 4°C for 12 hours with 50 µl of standard or diluted samples (TC fluid 1/100; sera 1/20 in PBS/1%BSA). Plates were washed again 3 times and 50 µl of second antibody (BD 1/250 in PBS/1%BSA) were added and incubated for 90 minutes at RT followed by an additional wash

step. Afterwards, 50 µl of Streptavidin-POD (Roche Diagnostics, Mannheim, Germany) (1/5000 in PBS/1%BSA) were added and incubated for 45 minutes at room temperature. Following a last washing step, 100 µl of development solution (appendix D) was added. When half of the standard had become blue the reaction was stopped with 100 µl/ well 2N H_2SO_4 (Merck). Optical density was measured using the SpectraMAX ELISA reader (Molecular devices) with wavelength correction (450 nm and 570 nm). Data were analysed with SOFTmax Pro 3.0 software (Molecular Devices).

2.2.20 Fluorescent bead immunoassay

To analyse cytokine levels within the supernatants of the cell cultures and the TC fluid, a fluorescent bead-based immunoassay was performed by using the murine $T_H1/T_H2/T_H17/T_H22$ 13plex FlowCytomix Multiple Analyte Detection Kit, the mouse Immunoglobulin Isotyping Panel 6plex FlowCytomix Kit and the mouse IgE FlowCytomix Simplex Kit (all eBioscience) according to the manufacturer's protocol. In brief, a pool of reconstituted standards was prepared and serially diluted. Afterwards, a bead mixture consisting of the different beads and the Biotin-conjugate mixture was prepared. 12.5 µl of bead mixture and Biotin-conjugated mixture were pipetted to the samples, standards and blank controls and incubated for 2 hours at RT in the dark. Afterwards, samples were washed twice and 25 µl of Streptavidin-PE solution (consisting of 0.8 µl concentrated Streptavidin-PE and 24.2 µl 1x assay buffer) was added to each tube and tubes were incubated for another hour. After two further wash steps, samples were re-suspended in 250 µl assay buffer and the individual cytokine concentration was measured using the FACSCanto I flow cytometer (BD). Analysis was performed using the FlowCytomix Pro3.0 software (eBioscience).

2.2.21 Statistical Analysis

Graphs were designed and statistics were performed using the PRISM 6.1 programme (GraphPad Software, Inc., La Jolla, USA). Statistical significances between different groups were tested with ANOVA and in cases of significance followed by a t-test when data were in a Gaussian distribution. If values were non-parametric, significance was first analysed with the Kruskal-Wallis test followed by the Mann-Whitney test. P values of 0.05 or less were considered significant.

3 Results

3.1 Comparison of pre-patent and patent *L. sigmodontis*-infection in wildtype BALB/c mice

As described earlier, *L. sigmodontis* can develop a patent infection in WT BALB/c but not in C57BL/6 mice (57, 61, 98). In the former, the helminth can complete its life cycle, passing from the infective L3 stage into the L4 stage larvae and finally adult worms that begin to produce Mf; the transmission stage (59, 61, 63). Therefore, the immunocompetent BALB/c laboratory strain has become an ideal model to study infections per se. Moreover, since not all mice become patent one can also compare pre-patent (Pp) and patent (P) infections, especially immune pathways and mechanisms. Thus, in the first section of these thesis results, WT BALB/c mice were infected with *L. sigmodontis* for either 42-43 days (pre-patent) or 71-72 days (patent) with the goal of addressing two aims. First, to compare immune profiles in mice prior to fecundity and secondly, to distinguish $CD4^+$ T cell responses in pre-patent and patent periods of infection to different preparations of filarial worm extracts. Expanding on previous studies from the group (63) showing $CD4^+$ T cells from Mf+ mice produced stronger responses that those from Mf- mice, here we show that this observation remains with a) different antigen preparations and b) antigen preparations from different worm genders.

3.1.1 Higher numbers of adult parasites but shorter worms in WT BALB/c mice at the pre-patent stage of a *L. sigmodontis* infection

All BALB/c mice were infected at the same time point and analysed for worm count, sex and worm length either on day 42/43 p.i. or on day 71/72 p.i.. Worm numbers at the pre-patent stage of *L. sigmodontis* infection were significantly increased compared to the patent stage (Fig. 3.1A). Additionally, BALB/c mice with a patent *L. sigmodontis* infection were further analysed with regards to their Mf status and here a significantly higher number of worms could be observed in pre-patent mice, compared to infected BALB/c mice without Mf (Mf-) but not when compared to infected mice with Mf (Mf+) (Fig. 3.1B). However, the numbers of female or male worms were comparable between pre-patent and patent infection stages. The amount of female or male worms was also equal between Mf+ and Mf- mice (Fig.3.1C). Both female and male worm lengths were significantly longer at the patent stage compared to the pre-patent phase of *L. sigmodontis* infection (Fig. 3.1 D and E). With regards to the Mf stage, no differences in worm length were seen between Mf+ and Mf- mice (Fig. 3.1E).

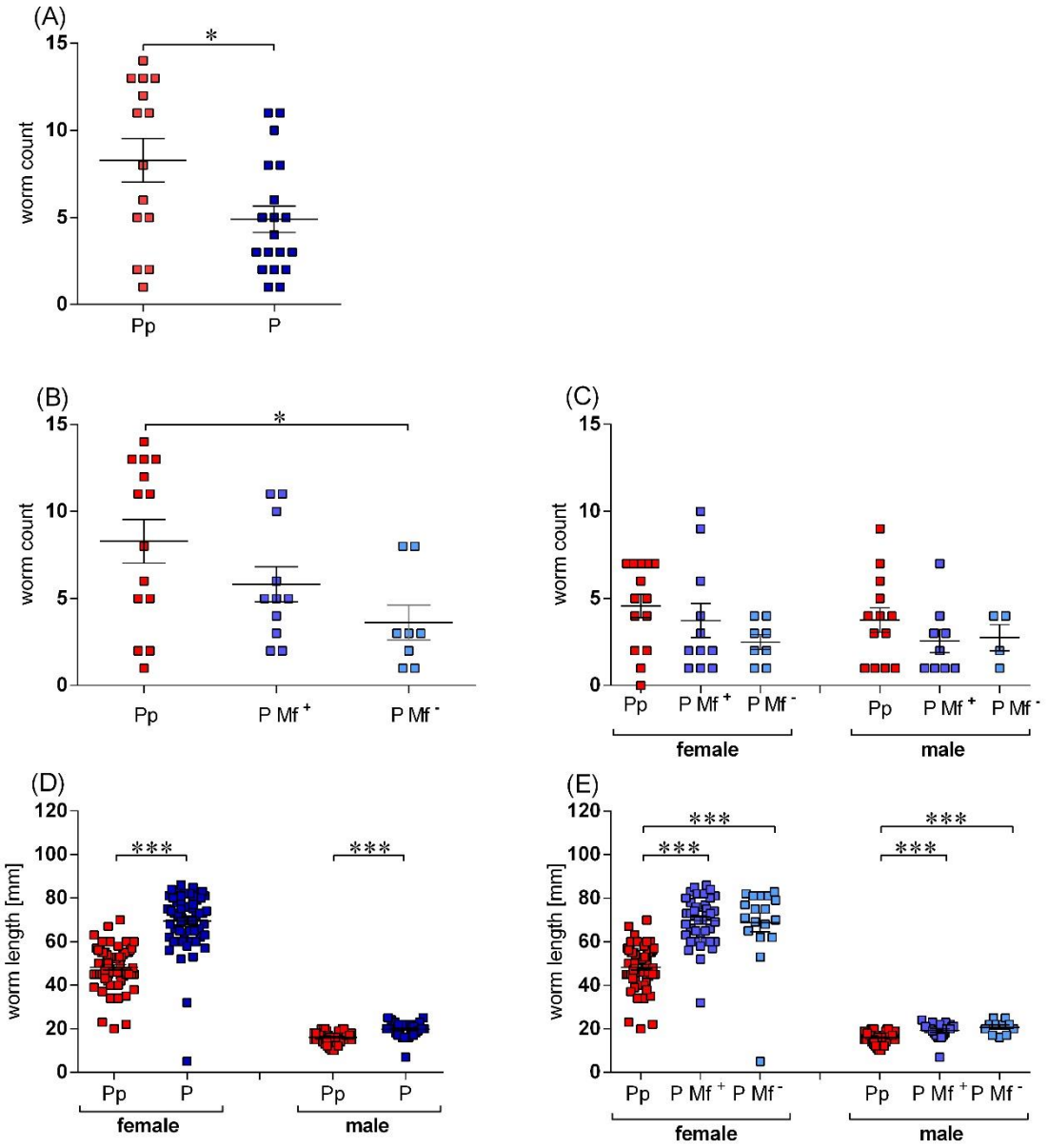


Figure 3.1: Higher numbers of adult parasites but shorter worms in WT BALB/c mice at the pre-patent stage of a *L. sigmodontis* infection. Worms were isolated from the TC of individual *L. sigmodontis*-infected mice at the pre-patent (Pp) stage (d42-43 p.i.) or the patent (P) stage (d71-72 p.i.). (A) Absolute numbers of worms from infected BALB/c mice at the pre-patent (n=14) and at the patent stage (n=19). (B) Comparison of worm numbers at the pre-patent stage and at the patent stage sub-grouped into Mf+ (n=11) or Mf- (n=8) mice. (C) Absolute number of worms separated according to their gender at the pre-patent stage (n=14) and additionally in Mf+ (n=11) or Mf- (n=8) within the patent stage. (D) Comparison of worm length separated according to their gender at the pre-patent stage (n=14) and at the patent stage (n=19). (E) Comparison of worm length separated according to their gender at the pre-patent stage (n=14) and additionally in Mf+ (n=11) or Mf- (n=8) within the patent stage. Symbols represent mean ± SEM of data from individual mice of two independent infection studies. Statistical significances between the indicated groups were either obtained after an unpaired t-test (A) or after Kruskal-Wallis followed by Dunn`s multiple comparison test for further comparison of the groups (B-E). Asterisks show significant differences between the groups indicated by the brackets (*$p < 0.05$ and ***$p < 0.001$).

3.1.2 Significantly higher infiltration of immune cells into the thoracic cavity and mediastinal lymph nodes at the pre-patent stage of an *L. sigmodontis* infection

During *L. sigmodontis* infection L3 larvae migrate through the lymphatics into the TC. Here further moulting into the L4 stage (d8) and adult worm stages (d25-30) takes place. Also, adult female worms start releasing Mf (approximately d50) in the TC which then circulate in peripheral blood (59-61, 65). In this context it's known that filarial infections are associated with an increase of different cell types (e.g. eosinophils) in the TC (32, 130, 131). Therefore, the cell numbers at the site of infection (TC) and draining medLN of pre-patent (d42-43 p.i.) and patent (d71-72 p.i.) infected BALB/c mice were analysed.

Absolute cell numbers in the TC of pre-patent infected mice were significantly higher compared to the naïve (Cont.) group and also compared to the patent group. Moreover, compared to Mf- mice, cell numbers in the TC of pre-patent mice were also significantly increased (Fig. 3.2A). Additionally, cell numbers of medLN were investigated. Again, cell numbers in pre-patent mice were significantly higher compared to the control and patent infected groups. This was also reflected when compared to Mf+ and Mf- mice (Fig. 3.2B). Furthermore, the cell composition of TC fluid was investigated in more detail via cytospins. Here, no statistical differences in numbers of monocytes between the different groups could be observed. However, numbers of monocytes were slightly increased in pre-patent mice compared to naïve controls and patent infected mice (Fig. 3.2C). Lymphocyte (Fig. 3.2D) and neutrophil numbers (Fig. 3.2E) were comparable between naïve controls, pre-patent or patent infected mice. The outliers in figure 3.2E showing higher neutrophil numbers in the Pp group did not correlate to elevated or reduced parasite numbers. Also, no differences between Mf+ and Mf- mice could be observed. In contrast, absolute numbers of eosinophils in pre-patent mice were significantly increased when compared to naïve, patent and furthermore also Mf- mice. Only compared to Mf+ mice, numbers of eosinophils were equal. Also, comparison of eosinophil numbers between Mf+ and Mf- mice showed no differences (Fig. 3.2F). The number of blood Mf did not correlate significantly to the number of eosinophils. Indeed, no cell type correlated with numbers of blood Mf. However, the number of TC Mf did significantly correlate to the number of eosinophils ($r=0.3565$, $p=0.0303$) (data not shown).

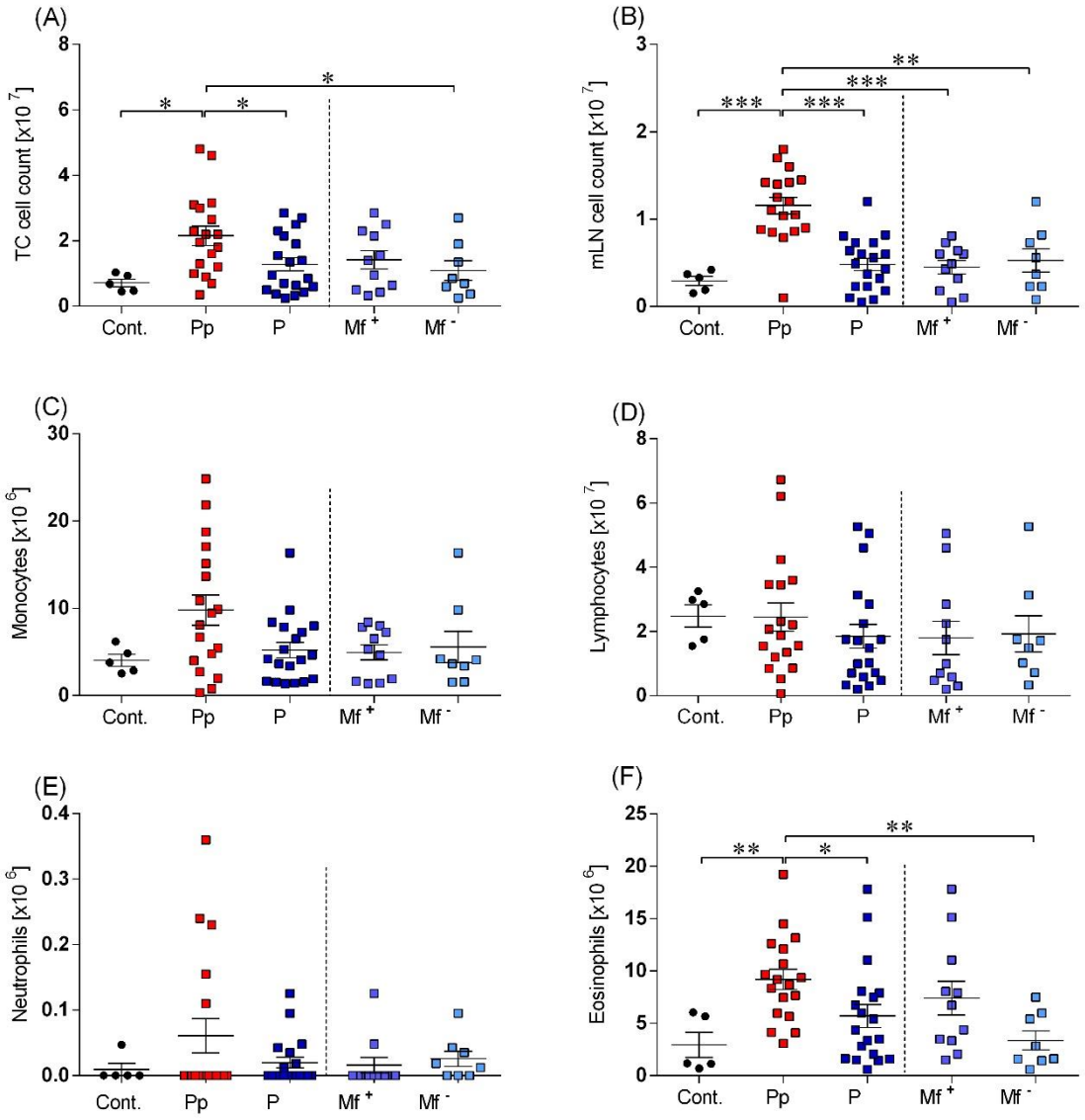


Figure 3.2: Analysis of cell composition at the site of infection. Total cell numbers within the thoracic cavity (TC) (A) and the mediastinal lymph nodes (medLN) (B) of uninfected (Cont.) and individual *L. sigmodontis*-infected mice at the pre-patent (Pp) stage (d42-43 p.i.) or the patent (P) stage (d71-72 p.i.) were counted. The absolute cell number of monocytes (C), lymphocytes (D), neutrophils (E) and eosinophils (F) were also determined in the TC through microscopic assessment of cytospins. Scatter dot plots show the individual cell counts in the described tissues of the control group (Cont.) containing non-infected mice (n=5), pre-patent (n=18) and patent (n=19) infected mice, the latter sub grouped into Mf+ (n=11) or Mf- (n=8) mice. Symbols represent mean ± SEM of individual mice of two independent infection studies. Statistical significances between the indicated groups were either obtained after One-way analysis of variance followed by unpaired t tests (A and B) or after Kruskal-Wallis followed by Dunn`s multiple comparison test for further comparison of the groups (C – F). Asterisks show significant differences between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p< 0.001).

3.1.3 Decreased levels of RANTES but increased levels of CCL17, Eotaxin-1 and Granzyme B in the pre-patent phase

Chemokines and cytokines are important signal transducers and especially chemokines are produced to induce chemotaxis of different cell types. For example, the chemokine Eotaxin-1 is known to be important for eosinophil recruitment and e.g. earlier studies have demonstrated that a chronic helminth infection leads to eosinophilia (117, 131) and cytokines like the Th2 cytokine IL-5 which is also known to be important during *L. sigmodontis*-infection (81, 112, 117, 126). Therefore, different chemokine and cytokine levels in the TC were analysed in pre-patent and patent *L. sigmodontis*-infected wildtype BALB/c mice. The only chemokine which was significantly increased in patent infected mice was RANTES (Fig. 3.3A). Even with regards to the Mf status, RANTES production was significantly higher in both Mf+ and Mf- groups when compared to the pre-patent group. In contrast, levels of CCL17 (Fig. 3.3B), Eotaxin-1 (Fig. 3.3C) and Granzyme B (Fig. 3.3D) were significantly lower in the patent group compared to the pre-patent group. Interestingly, the production of CCL17, which is an important mediator of T cell migration (132), was significantly lower in Mf- mice compared to the naïve control group (Fig. 3.3B). However, Eotaxin-1 is important for eosinophil recruitment and the higher amount of this chemokine during the pre-patent stage is also reflected in the higher eosinophil number in the TC during the pre-patent infection stage (Fig. 3.2F). Moreover, there was a positive correlation in numbers of eosinophils and production of Eotaxin-1 ($r=0.5344$, $p=0.001$). Interestingly, Eotaxin-1 production in Mf- mice was not only significantly lowered compared to the pre-patent group, but also compared to infected Mf+ mice (Fig. 3.3C). However, Granzyme B is mostly produced by cytotoxic T lymphocytes (CTL) or natural killer cells (NK cells) (133) and therefore, it's even more interesting that this chemokine was elevated in the pre-patent stage of infection (Fig. 3.3D). In contrast, no differences could be observed in the production of CCL22 (Fig. 3.3E), MIP-2 (Fig. 3.3F), IP-10 (Fig. 3.3D), and also not in the production of IL-5 (Fig. 3.3H) between controls, pre-patent and patent groups.

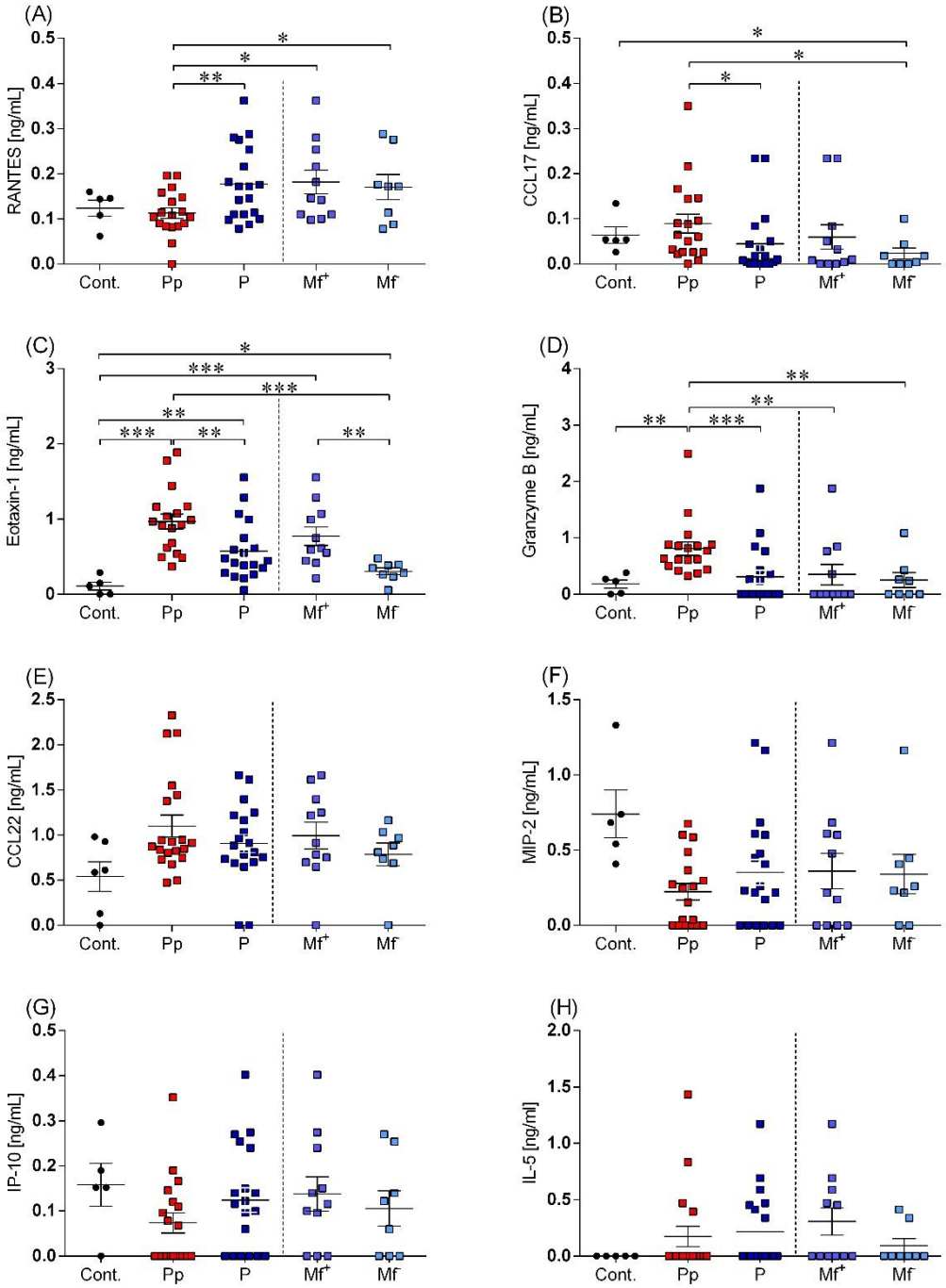


Figure 3.3: Decreased levels of RANTES but increased levels of CCL17, Eotaxin-1 and Granzyme B in the pre-patent phase. Chemokine and cytokine levels of RANTES (A), CCL17 (B), Eotaxin-1 (C), Granzyme B (D), CCL22 (E), MIP-2 (F), IP-10 (G), and IL-5 (H) were measured within the TC of naïve mice (Cont.) (n= 5) and infected mice at the pre-patent (Pp) (n=18) and patent (P) (n=19) stages the latter sub grouped into Mf+ (n=11) or Mf- (n=8) mice. Symbols represent mean ± SEM of individual values from each mouse from two independent infection studies. Statistical significances between the indicated groups were either obtained after One-way analysis of variance or Kruskal-Wallis followed by Dunn`s multiple comparison test for further comparison of the groups or unpaired t test. Asterisks show significant differences between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p< 0.001).

3.1.4 *In vitro* co-cultures of CD4$^+$ T cells from pre-patent or patent infected mice and naïve DCs

Previous studies have already demonstrated that *L. sigmodontis* infections induce biased Th2 immune responses in infected BALB/c mice (61, 63)(. Indeed, signalling of IL-4/IL-5 is important for controlling parasitemia in these mice (117, 131). To investigate how immune reactions are shaped during filarial infections it is useful to measure helminth-specific responses *in vitro* using extracts prepared from the worms. Unfortunately, there is no standardized method for filarial worm antigen preparation which may explain any differences in immune responses that are found by different research groups (62, 63, 98, 116, 134, 135). In short, adult worms are homogenized, centrifuged and the resulting SN is further used for *in vitro* stimulations, but interestingly little is known about immune reactions after stimulation with the worm pellet or stimulations with antigen only from female or male worms.

To analyse immune response after stimulations with the pellet or soluble fractions of different types of LsAg (total, female or male adult worms) cytokine responses in co-culture experiments were performed. Therefore, wildtype BALB/c mice were infected with *L. sigmodontis* for either 42-43 days (pre-patent) or for 71-72 days (patent). CD4$^+$ T cells were then isolated from patent, Mf+ or Mf- mice and co-cultured for 72 hours with naïve GM-CSF-derived DCs and left either un-stimulated or stimulated with the different LsAg preparations

3.1.4.1 Increased Th2 responses in patently infected Mf+ BALB/c mice

The production of Th2 cytokines IL-5 and IL-13 was analysed via ELISA. After stimulation with soluble LsAg, the amount of IL-5 was significantly higher in the co-culture of CD4$^+$ T cells from Mf+ mice compared to the co-culture of CD4$^+$ T cells from pre-patent mice and also compared to co-cultures of CD4$^+$ T cells from Mf- mice. These results appeared independent of worm gender (Fig. 3.4 *c.f*. A, B and C, red symbols). Similar results could be observed after stimulation with LsAg obtained from pellet preparations. However, immune reactions were not as significant as in the stimulation with the soluble LsAg (Fig. 3.4 *c.f.* A, B and C, blue symbols). No differences in IL-5 expression could be observed between the co-culture of CD4$^+$ T cells from Mf+ mice compared to the co-culture of CD4$^+$ T cells from pre-patent mice after stimulation with pellet LsAg from male worms (Fig. 3.4C, blue symbols). Interestingly, direct comparisons of IL-5 secretion induced by different types of LsAg (SN or pellet) showed a significantly higher amount of IL-5 in the co-culture of CD4$^+$ T cells from Mf- mice, stimulated with total or also with female pellet LsAg compared to the stimulation with SNs from total or only female worms (Fig. 3.4A and B).

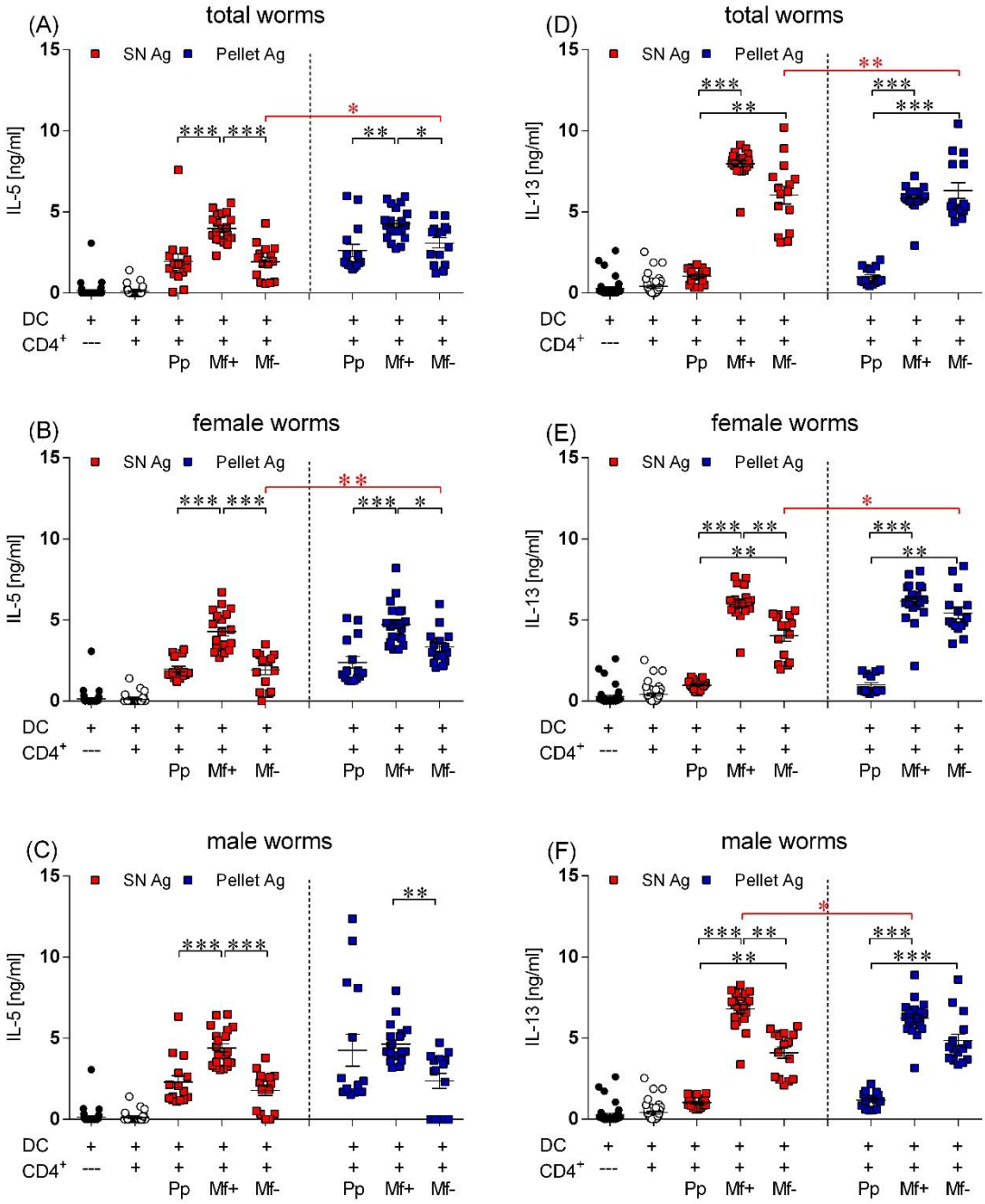


Figure 3.4: Increased release of Th2 cytokines from co-cultures of LsAg preparations and cells from patently infected Mf+ BALB/c mice. Wildtype BALB/c mice were naturally infected with *L. sigmodontis* for 42-43 days (Pp) or 71-72 days (patent). Afterwards, the Th2 cytokine production of *ex vivo* re-stimulated splenic $CD4^+$ T cells from Pp, Mf+ and Mf- mice was determined: $2.5x10^5$ $CD4^+$ T cells were co-cultured for 72 hours with BMDCs ($1x10^5$) and left either un-stimulated or activated with supernatant (SN) or pellet of LsAg (40 µg/ml) from total (A and D), female (B and E) or male (C and F) worms. Symbols represent mean ± SEM of three cell co-culture assays from mice in the pre-patent stage, four assays from mice that were Mf+ and three assays from mice that were Mf- using isolated T cells pooled from 4-5 mice per group. Asterisks show significant differences (Kruskal-Wallis followed by Dunn`s multiple comparison test for further comparison of the groups or unpaired t test) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p<0.001).

Similar results could be also seen with IL-13 production, since levels were also higher in co-cultures of $CD4^+$ T cells from Mf+ mice compared to co-cultures of $CD4^+$ T cells from pre-patent mice, independently of the LsAg type (Fig. 3.4 *c.f.* D, E and F) and also compared to co-culture of $CD4^+$ T cells from Mf- mice after stimulation with soluble material (Fig. 3.4E and F, red symbols). Interestingly, compared to the IL-13 secretion in co-cultures of $CD4^+$ T cells from pre-patent mice, levels were also higher in co-cultures of $CD4^+$ T cells obtained from Mf- mice, stimulated with total worm antigen (Fig. 3.4D), female worm antigen (Fig. 3.4E) and also with antigen from male worms (Fig. 3.4F). With regards to the LsAg type, a significantly increased IL-13 release could be observed in pellet LsAg stimulated co-cultures of $CD4^+$ T cells from Mf- mice compared to co-cultures stimulated with SN LsAg (no matter of total, female or male LsAg) (Fig. 3.4*c.f.* D, E and F).

3.1.4.2 Increased IFN-γ response in patent Mf+ mice

In addition, SN of co-cultures were also analysed for the production of the Th1 cytokine IFN-γ. Here the amount of IFN-γ was significantly increased in stimulated co-cultures of $CD4^+$ T cells from Mf+ mice compared with pre-patent and also Mf- mice (Fig 3.5A-C), which was independent from the composition of LsAg (total, female or male worms) and also the type of LsAg (SN or pellet) which were added. Additionally, IFN-γ secretion was also higher in co-cultures of $CD4^+$ T cells from Mf- mice compared to co-cultures with $CD4^+$ T cells from pre-patent mice (also independent of LsAg type) (Fig 3.5A-C). Interestingly, the IFN-γ production was significantly higher in Mf+ and Mf- co-cultures stimulated with the SN of LsAg from total worms and also in Mf+ co-cultures stimulated with SN of LsAg from only female worms, compared to stimulations with the pellet of LsAg (Fig. 3.5A and B).

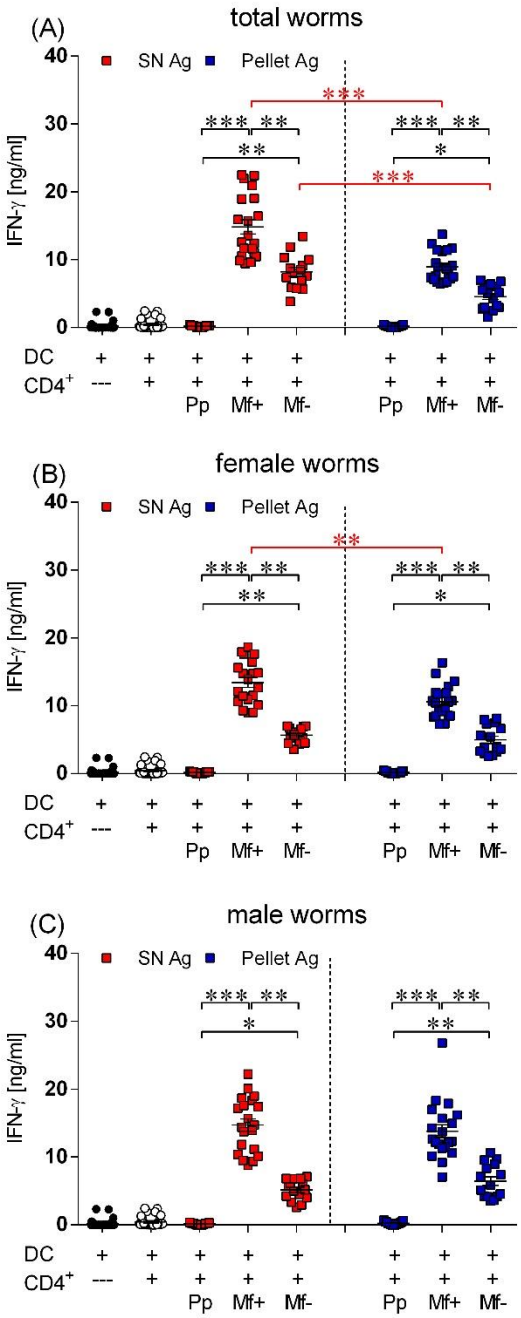


Figure 3.5: Increased filarial-specific IFN-γ release in co-cultures of CD4⁺ T cells from patently infected Mf+ BALB/c mice. Wildtype BALB/c mice were naturally infected with *L. sigmodontis* and the IFN-γ cytokine production of *ex vivo* re-stimulated splenic CD4⁺ T cells from Pp, Mf+ and Mf- mice was determined: 2.5x10^5 CD4⁺ T cells were co-cultured for 72 hours with BMDCs (1x10^5) and left either un-stimulated or activated with supernatant (SN) or pellet of different LsAg preparations (40 µg/ml) from total (A), female (B) or male (C) worms. Symbols represent mean ± SEM of three cell co-culture assays from mice in the pre-patent stage, four assays from mice that were Mf+ and three assays from mice that were Mf- using isolated T cells pooled from 4-5 mice per group. Asterisks show significant differences (Kruskal-Wallis followed by Dunn`s multiple comparison test for further comparison of the groups or unpaired t test) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p<0.001).

3.1.4.3 Lack of filarial-specific IL-10 release by CD4⁺ T cells from pre-patent mice

IL-10 production is associated with regulatory immune responses (136) and can play an important role during filarial infections in C57BL/6 mice (99). Therefore, IL-10 production in filarial-specific T cell co-cultures was also analysed. Again, the highest cytokine production could be observed in cell co-cultures with CD4⁺ T cells from Mf+ BALB/c mice not only compared to cells from pre-patent mice (Fig. 3.6A-C) but also compared to cells from Mf- mice as well (Fig. 3.6B and C). Results were independent of used LsAg (SN or pellet). Interestingly, an increased IL-10 production could also be observed in co-cultures with CD4⁺ T cells from Mf- BALB/c mice compared to CD4⁺ T cells from pre-patent BALB/c mice, but only after

stimulation with pellet Ag from total worms (Fig. 3.6A, blue symbols). Direct comparisons of different Ag types revealed no significant differences in IL-10 secretion.

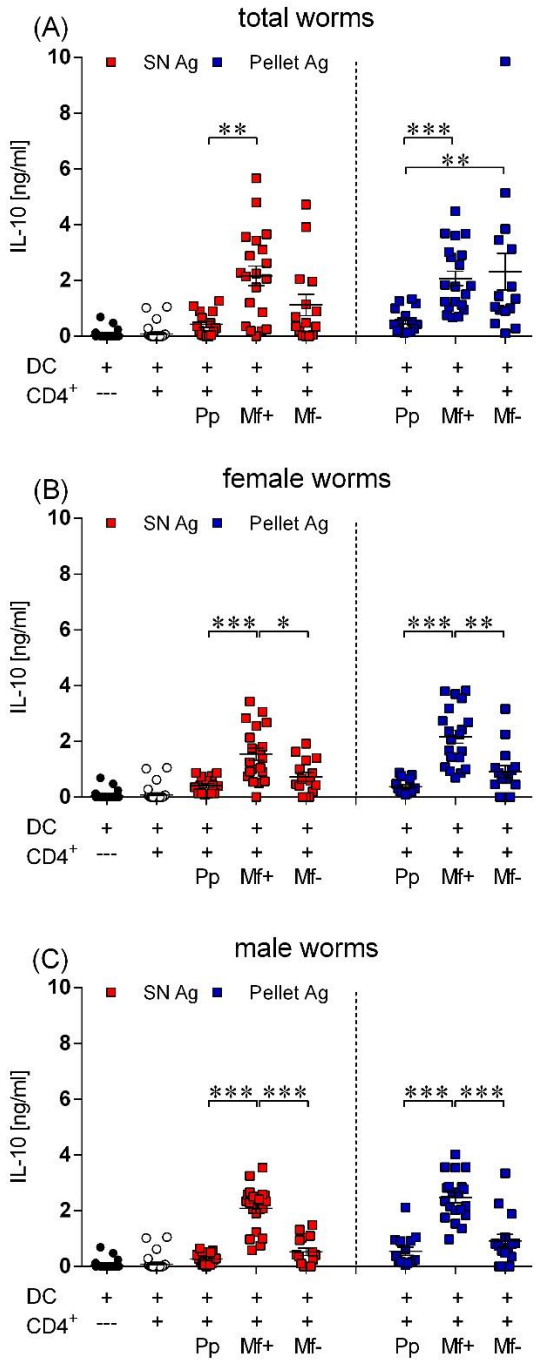


Figure 3.6: Increased IL-10 release by cells from patently infected Mf+ BALB/c mice. Wildtype BALB/c mice were naturally infected with *L. sigmodontis* and IL-10 production of *ex vivo* re-stimulated splenic $CD4^+$ T cells from Pp, Mf+ and Mf- mice was determined. $2.5x10^5$ $CD4^+$ T cells were co-cultured for 72 hours with BMDCs ($1x10^5$) and left either un-stimulated or activated with supernatant (SN) or pellet of different LsAg preparations (40 µg/ml); total (A), female (B) or male (C) adult worms. Symbols represent mean ± SEM of three cell co-culture assays from mice in the pre-patent stage, four assays from mice that were Mf+ and three assays from mice that were Mf- using isolated T cells pooled from 4-5 mice per group. Asterisks show significant differences (Kruskal-Wallis followed by Dunn`s multiple comparison test for further comparison of the groups or unpaired t test) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p<0.001).

In conclusion, worm counts in the pre-patent stage were significantly higher than in the patent stage which was accompanied by increased level of eosinophils, the chemotaxin eotaxin-1 and a lowered Th2 immune response of re-stimulated $CD4^+$ T cells. Moreover, both filarial-specific IFN-γ and IL-10 release by T cells from the pre-patent phase were comparable to control

groups and this was independent of the antigen source. *In vitro* stimulations with filarial antigen induced increased cytokine production in co-cultures with $CD4^+$ T cells from Mf+ mice, compared to co-cultures with $CD4^+$ T cells from pre-patent mice and mostly also compared to co-cultures with $CD4^+$ T cells from Mf- mice. These results were mainly independent of Ag source (total, female or male worms) and type (SN or pellet) revealing 1) that T cells respond more vigorously in Mf+ mice and 2) since responses to female and male worm-based antigens are comparable, these responses are not directed to Mf-derived antigens from fecund female worms.

3.2 *L. sigmodontis* infection in semi-susceptible C57BL/6 mice and fully permissive BALB/c mice

It is already known that *L. sigmodontis* cannot develop a patent and therefore also not a chronic infection in C57BL/6 mice whereas, in BALB/c mice this parasite can complete its life cycle, including Mf release (59, 61, 65). However, it is not completely understood why and how C57BL/6 mice can eradicate the *L. sigmodontis* infection. Since a better understanding of how C57BL/6 mice can clear the *L. sigmodontis* infection is also important for a better understanding of how filarial parasites can trigger the host's immune system, the aim of this results section was to analyse the different progressions of *L. sigmodontis* infections in C57BL/6 and BALB/c mice. Therefore, wildtype C57BL/6 and BALB/c mice were simultaneously infected with *L. sigmodontis* for 37 days, a time point in which most worms have moulted from L4 into adulthood (64). Afterwards, mice were analysed for worm burden, status and stage.

3.2.1 Significantly shorter adult female worms in *L. sigmodontis*-infected C57BL/6 mice

Overall worm burden showed no differences between both mouse strains (Fig. 3.7A). Immune mechanisms against filarial infections include also encapsulation of worms followed by nodule formation (112, 137). With regard to worm status, no differences in the number of worms, encapsulated or nodule stages between C57BL/6 and BALB/c mice could be observed (Fig. 3.7B). Furthermore, as expected both mouse strains had significantly more adult worms compared to L4 stage worms at day 37 p.i. (Fig. 3.7C).

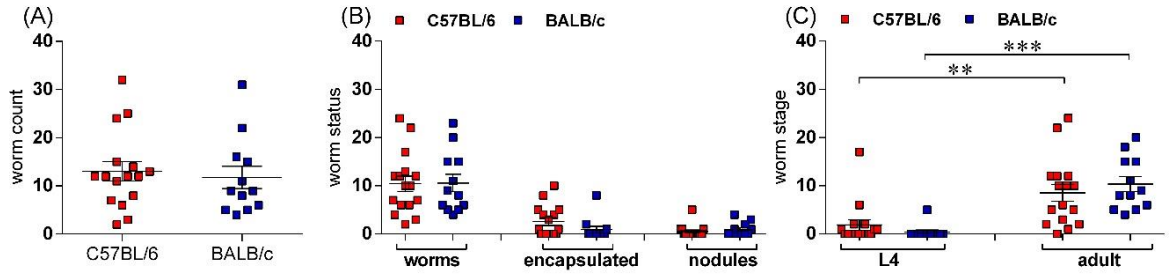


Figure 3.7: Comparisons of *L. sigmodontis* worm burden in C57BL/6 and BALB/c mice. Worms were isolated from the thoracic cavity of individually infected mice on d37 p.i. after infection with the same batch of mites. (A) Shows the absolute numbers of worms, (B) the number of free-living worms, encapsulated worms and nodules recovered from each mouse strain and (C) the number of recovered adult or L4 life-stages. Graphs show mean ± SEM of individually assessed worms from mice from 2 independent infection experiments (n=16 C57BL/6 and n=12 BALB/c mice). Statistical significances between the indicated groups were obtained using Mann-Whitney test (A) or Kruskal-Wallis test followed by Dunn`s multiple comparison test (B and C) for further comparison of the groups. Asterisks indicate significant differences between the groups indicated by the brackets (**p<0.01 and ***p<0. 001).

Additionally, adult worms were investigated with regards to their gender. The amount of female and male worms was similar between C57BL/6 and BALB/c mice (Fig. 3.8A), but female and also male worms in C57BL/6 mice were significantly shorter compared to their counterparts in BALB/c mice (Fig. 3.8B), which indicates that the development of worms in C57BL/6 mice is impaired.

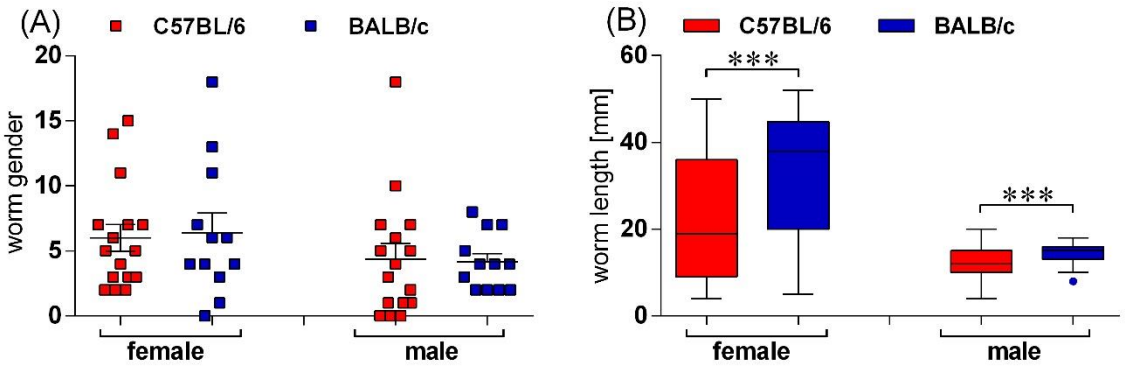


Figure 3.8: Significantly shorter adult worms in *L. sigmodontis*-infected C57BL/6 mice. Adult worms were isolated from the thoracic cavity of individually infected mice on d37 p.i.: a time-point in which most worms have moulted from L4 into adulthood. (A) Number of adult female and male worms. (B) Lengths of individual adult female and male worms. Graphs show (A) mean ± SEM and (B) box whiskers (Tukey) of individually assessed worms from mice from 2 independent infection experiments (n=16 C57BL/6 and n=12 BALB/c mice). Statistical significances between the indicated groups were obtained using Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups. Asterisks indicate significant differences between the groups indicated by the brackets (***p<0.01).

3.2.2 Significantly higher frequencies of macrophages and lymphocytes but not of eosinophils and neutrophils in C57BL/6 mice

Since it is already known that different cell types are involved in worm clearance (82, 98, 99, 112, 117, 131) the cell composition and the cell numbers at the infection site in C57BL/6 and BALB/c mice were investigated via cytospins. Interestingly, the cell numbers in the TC of C57BL/6 mice were significantly higher compared to the BALB/c mouse strain (Fig. 3.9A). In figure 3.9B, it is clear that when comparing the overall frequency of cell populations in *L. sigmodontis*-infected C57BL/6 and BALB/c mice, the former have more macrophages and less eosinophils. In more detail, frequencies of macrophages (Fig. 3.9C) and also of lymphocytes (Fig. 3.9D) were significantly higher in C57BL/6 mice compared to the BALB/c group. In contrast, frequencies of eosinophils (Fig. 3.9E) and neutrophils (Fig. 3.9F) were significantly lower compared to BALB/c mice. Interestingly, even though there was no correlation between worm numbers and frequencies of the different cell types in infected C57BL/6 mice, there was a positive correlation between frequencies of eosinophils (r=0.752, p=0.012) and macrophages

(r=0.700, p=0.024) and worm numbers in BALB/c mice. Note, levels of each cell type in uninfected control mice are negligible (data not shown).

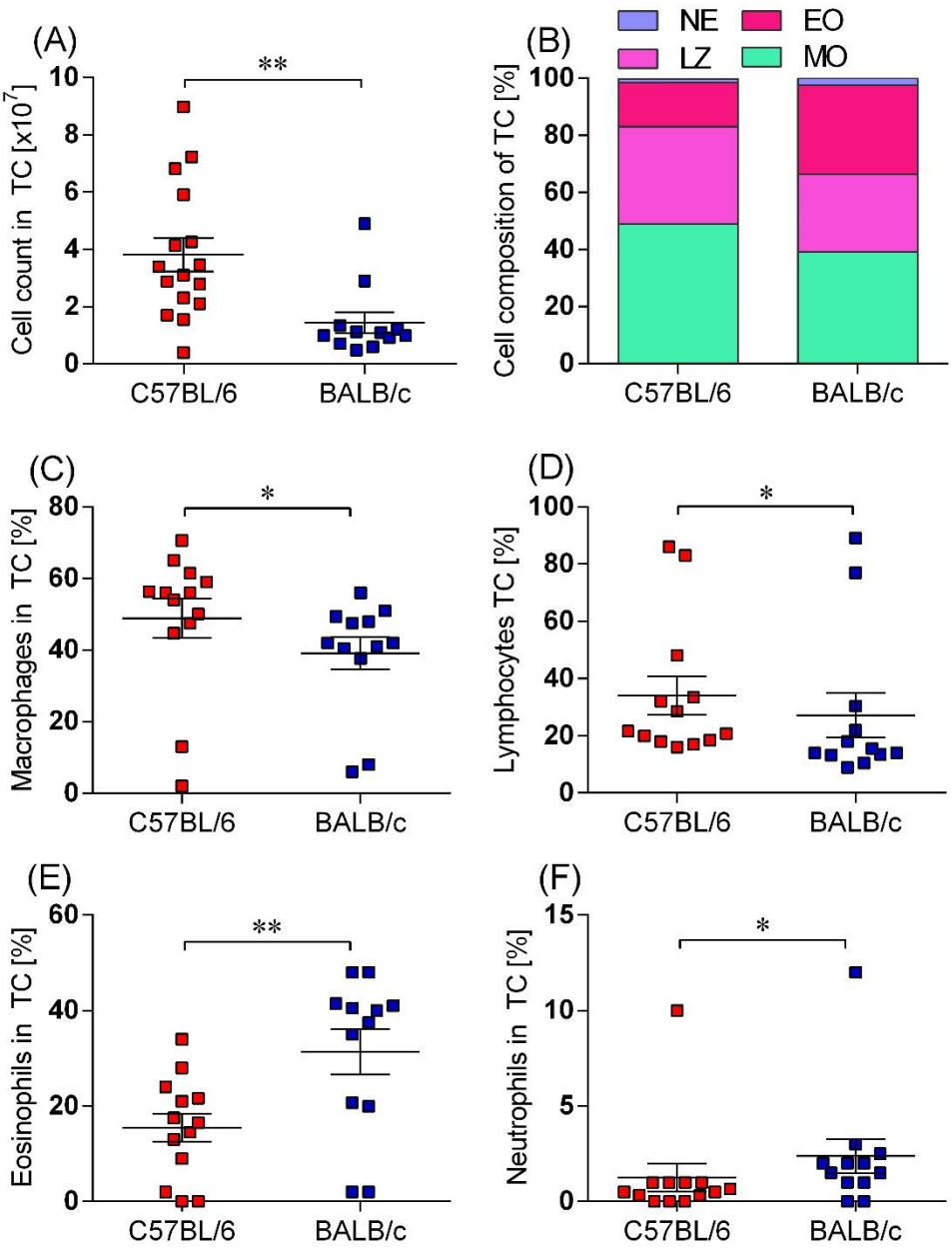


Figure 3.9 Significantly higher frequencies of macrophages and lymphocytes but not of eosinophils and neutrophils in infected C57BL/6 mice. On d37 p.i. the absolute numbers of different immune cells were analysed in the thoracic cavity (TC) of individually infected C57BL/6 (n=10) or BALB/c (n=10) mice. Cell differentiation was determined using stained cytospin preparations. (A) Shows absolute cell count and (B) shows the cell composition of the TC. Additionally, frequencies of (C) macrophages (%), (D) lymphocytes (%), (E) eosinophils (%) and (F) neutrophils (%) were analysed. Graphs show mean ± SEM (A, C-F) or mean (B) of individually values for each mouse. Asterisks show significant differences (Student's t test or Mann Whitney) between groups indicated by the brackets (* p<0.05, ** p<0.01). Data show results from two individual infection studies.

3.2.3 Significantly lower *Wolbachia* levels in *L. sigmodontis* worms of C57BL/6 mice

Wolbachia are endosymbiotic bacteria in *L. sigmodontis* worms. Since female worms of *L. sigmodontis* are not able to produce Mf in C57BL/6 mice and *Wolbachia* are important for female worm fertility (37), levels of these bacteria were also analysed via real time PCR and the ratio of *Wolbachia* Ls-FtsZ/LsActin in total worms (Fig. 3.10A) and copies of Ls-FtsZ in female (Fig. 3.10B) and male worms (Fig. 3.10C) were determined. All ratios were significantly lower in C57BL/6 mice compared to BALB/c mice (Fig.3.10A-C).

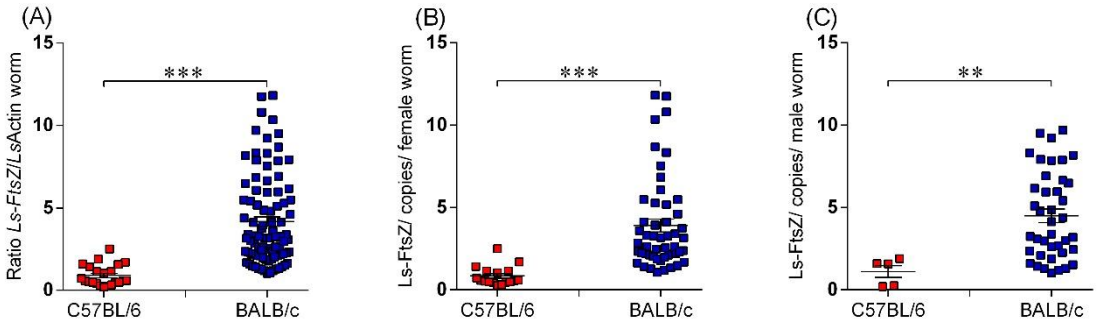


Figure 3.10: Significantly lower *Wolbachia* levels in adult worms from *L. sigmodontis*-infected C57BL/6 mice. On d37 p.i. *L. sigmodontis* worms were recovered from the thoracic cavities of individually infected BALB/c or C57BL/6 mice and separated by gender. DNA was extracted and *Wolbachia* levels were quantified using a duplex real-time PCR. (A) Symbols show the ratio of Ls-FtsZ/LsActin in individual adult worms from BALB/c (n=91) and C57BL/6 mice (n=22). (B) and (C) show ratio levels in female and male worms respectively. Graphs show individual ratio values / worm ± SEM. Asterisks show significant differences (Mann-Whitney tests) between groups indicated by the brackets (** $p<0.01$ and *** $p<0.001$). Data show results from one infection study.

To conclude, these results indicate that worms of C57BL/6 mice are impaired in their development and thus, the different cellular composition in the pleural cavity and the reduced amount of *Wolbachia*, especially in female worms, could be involved in the clearance of *L. sigmodontis* in C57BL/6 mice.

3.3 Deciphering important innate pathways during early filarial worm development

As mentioned above, C57BL/6 mice do not develop a patent *L. sigmodontis* infection and adult worms are eliminated 40 days post infection (61, 62, 64, 65). Interestingly, female worms of C57BL/6 mice are shorter and *Wolbachia* numbers are lower in these mice compared to the susceptible BALB/c mouse strain (see figure 3.8B and 3.10). Since, C57BL/6 mice develop only an acute stage of *L. sigmodontis* it is interesting to investigate the innate immune system in more detail. The Toll-like receptors (TLRs) are important parameters of the innate immune system (8, 138, 139), and the activation of these receptors is also important for immunity against helminthic infections (6, 39, 140). The adapter molecule MyD88 is central to most of these TLRs. In contrast, the adaptor molecule TRIF is specifically triggered by stimulation of TLR3- and/or TLR4 (7-9, 141). Generally, there is a paucity of knowledge about the role of TRIF signalling during helminth infections. Therefore, the aim of this part of the thesis was to investigate the early immune response, especially of the adaptor molecule TRIF during *L. sigmodontis* infection.

3.3.1 Lack of TRIF leads to elevated worm burden and also to a delayed worm development in semi-susceptible TRIF$^{-/-}$ mice 30 days upon *L. sigmodontis* infection

Wild type C57BL/6 mice and also mice deficient for TLR3, TLR4, MyD88, TRIF, IRF1 or IRF3 were naturally infected with *L. sigmodontis* for 30 days. Worms were isolated from the TC and analysed for number, length and gender (Fig. 3.11 – 3.13). Worm counts in TLR3$^{-/-}$ (Fig. 3.11A), TLR4$^{-/-}$ (Fig. 3.11B), MyD88$^{-/-}$ (Fig. 3.11C), IRF1$^{-/-}$ (Fig. 3.11E) and also IRF3$^{-/-}$ (Fig. 3.11F) mice were comparable to WT C57BL/6 mice. In contrast, TRIF$^{-/-}$ mice had a significantly higher worm number (L4 and adult worms combined) compared to their corresponding control group (Fig. 3.11D).

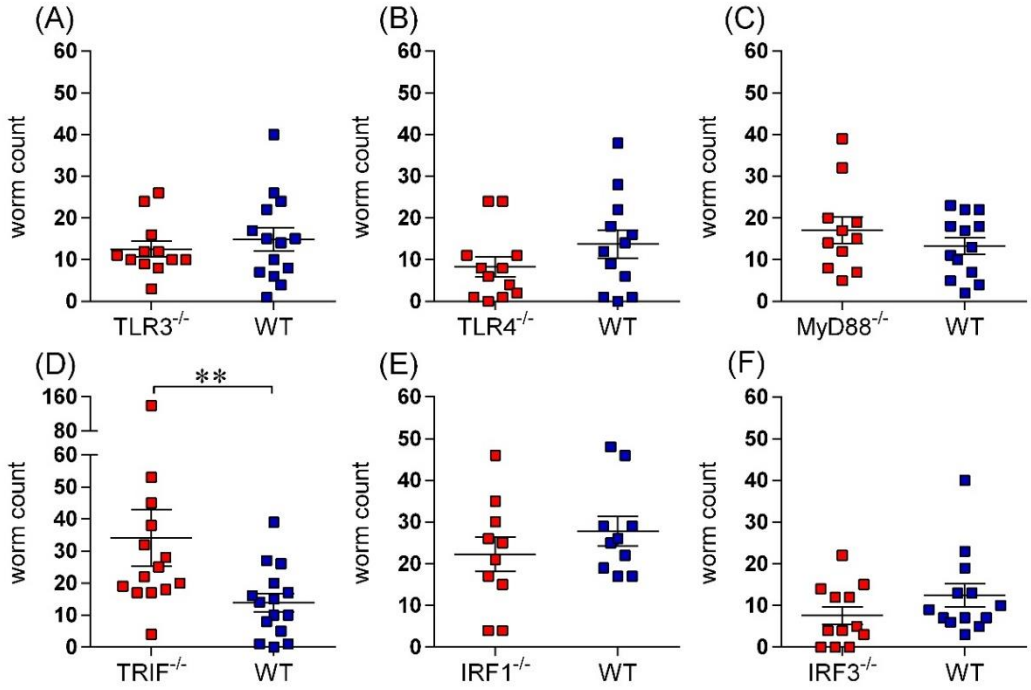


Figure 3.11: Lack of TRIF leads to elevated worm burden in TRIF deficient mice 30 days after *L. sigmodontis* infection. *L. sigmodontis* worm burden was analysed from infected (A) TLR3$^{-/-}$, (B) TLR4$^{-/-}$, (C) MyD88$^{-/-}$, (D) TRIF$^{-/-}$, (E) IRF-1$^{-/-}$ and (F) IRF-3$^{-/-}$ mice and compared to wildtype (WT) C57BL/6 control mice on day 30 p.i.. Graphs show mean ± SEM of individually assessed mice from 2-3 independent infection experiments including (A) n=12 TLR3$^{-/-}$ and n=14 WT, (B) n=12 TLR4$^{-/-}$and n=12 WT, (C) n=11 Myd88$^{-/-}$and n=13 WT, (D) n=14 TRIF$^{-/-}$and n=15 WT, (E) n=11 IRF1$^{-/-}$and n=10 WT and (F) n=12 IRF3$^{-/-}$and n=13 WT individual mice. Statistical significances between the indicated groups were obtained using Mann-Whitney test. Asterisks indicate significant differences between the groups indicated by the brackets (**p<0.01). Data show results from two (A-C; E, F) or three (D) individual infection studies.

Isolated worms were also investigated with regards to their developmental stages. Interestingly, numbers of L4 stage worms in TLR3$^{-/-}$ (Fig. 3.12A) and also in TRIF$^{-/-}$ (Fig. 3.12D) mice were significantly higher than numbers of adult worms. These results were also reflected when compared to the number of L4 worms in WT controls. In contrast the number of adult worms in TLR3$^{-/-}$ mice was significantly lower compared to the control group (Fig. 3.12A). However, no differences could be observed in numbers of L4 and adult worms, between TLR4$^{-/-}$ (Fig. 3.12B), MyD88$^{-/-}$ (Fig. 3.12C), IRF1$^{-/-}$ (Fig. 3.12E) and IRF3$^{-/-}$ (Fig. 3.12F) and their appropriate controls.

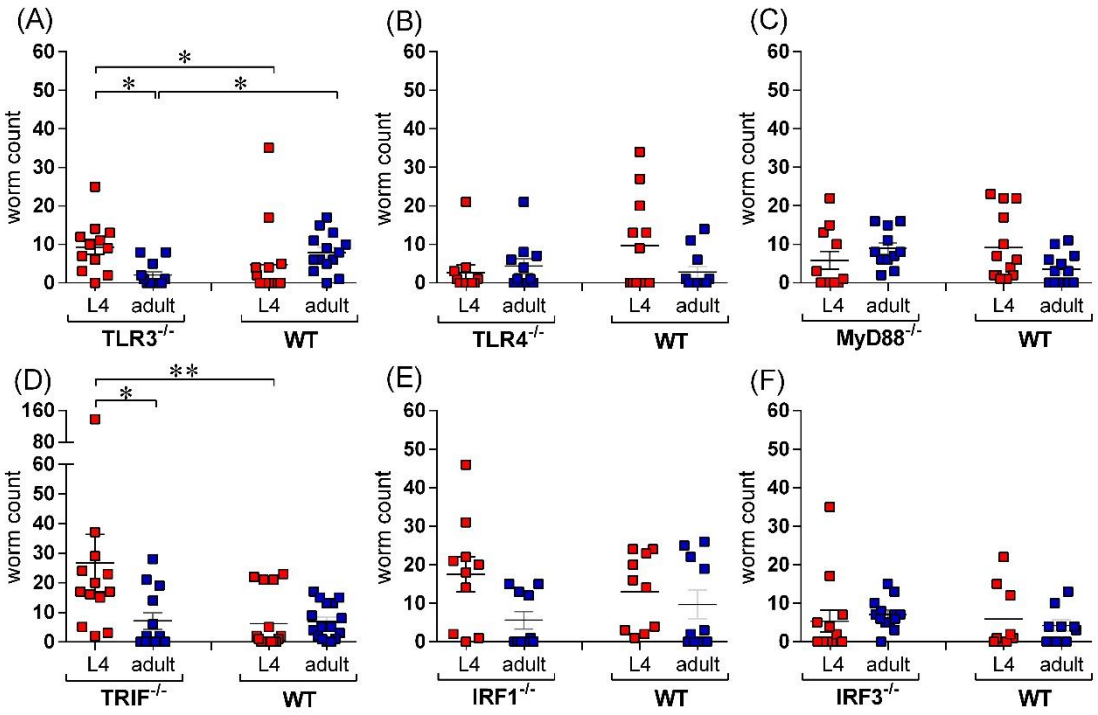


Figure 3.12: Development of *L. sigmodontis* juvenile adult worms is delayed in TRIF- and TLR3-deficient mice on day 30 p.i.. Numbers of *L. sigmodontis* L4 stage larvae and adult worms were analysed in wildtype (WT) and (A) TLR3$^{-/-}$ (n=12 TLR3$^{-/-}$/ n=14 WT), (B) TLR4$^{-/-}$ (n=12 TLR4$^{-/-}$/ n=12 WT), (C) MyD88$^{-/-}$ (n=11 Myd88$^{-/-}$/ n=13 WT), (D) TRIF$^{-/-}$ (n=14 TRIF$^{-/-}$/ n=15 WT), (E) IRF1$^{-/-}$ (n=11 IRF1$^{-/-}$/ n=10 WT) and (F) IRF3$^{-/-}$ (n=12 IRF3$^{-/-}$/ n=13 WT) C57BL/6 mice on 30 day p.i.. Graphs show mean ± SEM of individually assessed mice from 2-3 independent infection experiments. Statistical significances between the indicated groups were obtained using Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups. Asterisks indicate significant differences between the groups indicated by the brackets (*p<0.05 and **p<0.01). Data show results from two (A-C; E, F) or three (D) individual infection studies.

Additionally, the length of female and male worms was analysed. Interestingly, female and also male worms from TLR3$^{-/-}$ mice were significantly shorter compared to the worms from WT mice (Fig. 3.13A). In contrast, no differences could be observed between the length of female worms in TLR4$^{-/-}$ mice and WT controls but the length of male worms was significantly longer in these knockout (KO) mice when compared to their appropriate controls (Fig. 3.13B). Female worms of MyD88$^{-/-}$ mice were significantly longer when compared to WT C57BL/6 mice (Fig. 3.13C) whereas, in TRIF$^{-/-}$ mice the length of female and also male worms was significantly shorter compared to controls (Fig. 3.13D); as with worms developing in TLR3-deficient mice. However, no differences could be observed in female worm length between controls and IFR1$^{-/-}$ (Fig. 3.13E) or IFR3$^{-/-}$ (Fig. 3.13F) mice, but in the latter ones, the length of male worms was significantly shorter (Fig.3.13F).

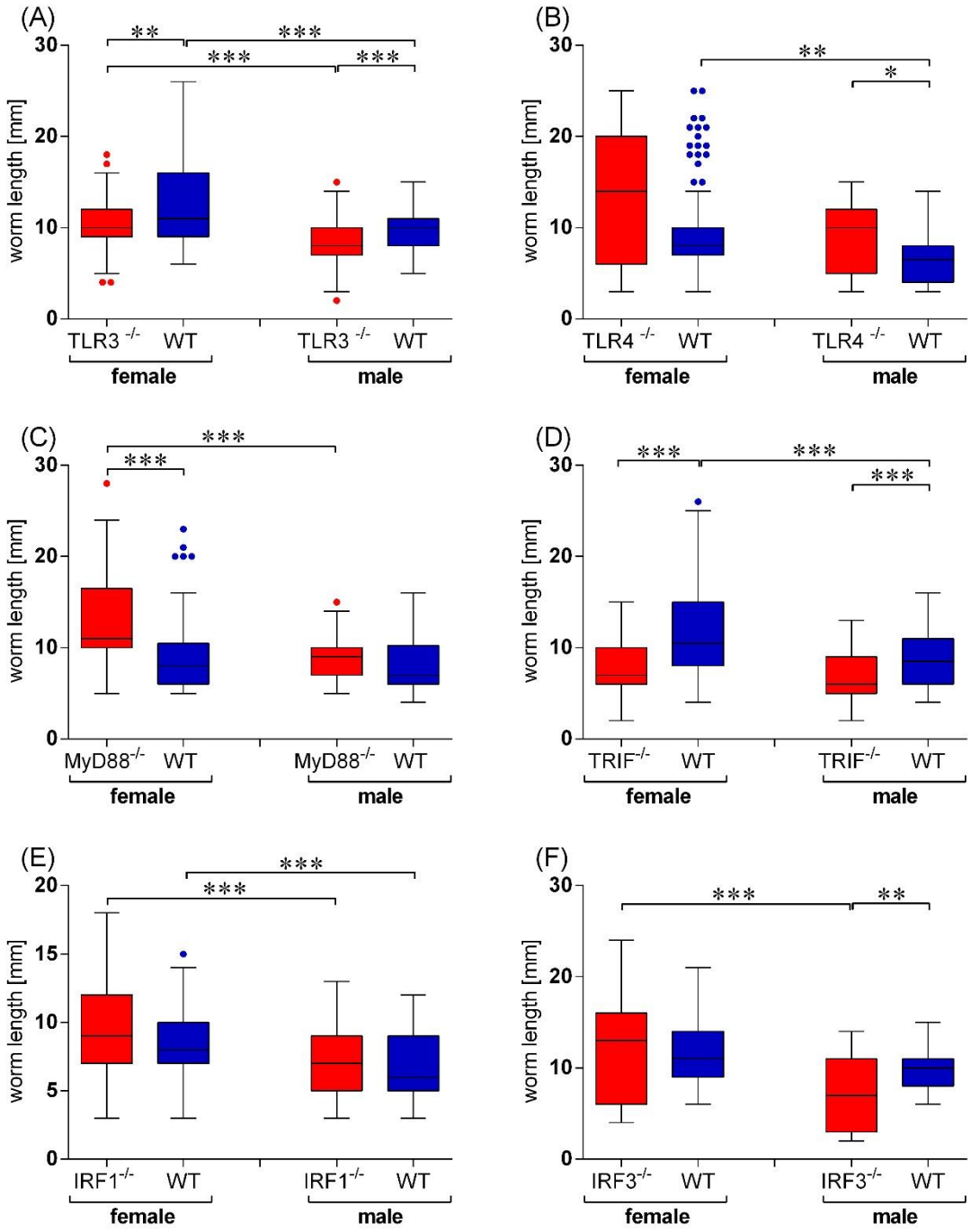


Figure 3.13: Significantly shorter female worms in infected *L. sigmodontis* TLR3$^{-/-}$ and also TRIF$^{-/-}$ mice compared to C57BL/6 control mice. Worms were isolated from the thoracic cavity of individually infected mice on d30 p.i.. Graphs show mean ± SEM from two (A-C; E, F) or three (D) independent infection experiments including (A) n=12 TLR3$^{-/-}$/ n=14 WT, (B) n=12 TLR4$^{-/-}$/ n=12 WT, (C) n=11 Myd88$^{-/-}$/ n=13 WT), (D) n=14 TRIF$^{-/-}$/ n=15 WT, (E) n=11 IRF1$^{-/-}$/ n=10 WT and (F) n=12 IRF3$^{-/-}$/ n=13 WT individual mice. Graph shows box whiskers (Tukey) and statistical significances between the indicated groups were obtained using Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups. Asterisks indicate significant differences between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p<0.01).

3.3.2 Metamorphosis of adult worms in TRIF$^{-/-}$ mice is associated with higher frequencies of monocytes and lymphocytes at the site of infection

Since immune cell infiltration at the site of infection is one important mechanism against pathogens, cell numbers and also cell composition of these cells were analysed. Therefore, cell numbers within the thoracic cavity fluid, mediastinal lymph nodes and also spleens were determined (table 1). Interestingly, there were no differences in the cell numbers of the TC in different KO-strains compared to their WT controls. Only the cell numbers in the TC of TRIF$^{-/-}$ and IRF3$^{-/-}$ mice were significantly lower compared to the C57BL/6 controls (table 1, marked in red). Cell numbers in the medLN are similar in most of the KO-strains compared to WT mice, but in TLR4$^{-/-}$ mice the number was increased compared to the WT group and in contrast in TRIF$^{-/-}$ mice the number was significantly reduced (table1, marked in green). Finally, numbers of spleen cells were also counted and numbers of cells in this organ were significantly reduced in TLR4- and also in TRIF-deficient^{-} mice, compared to WT controls (table1, marked in blue).

Table 1: Local and systemic cell numbers during *L. sigmodontis* infection 30 days p.i..

	TC	p-Value	medLN	p-Value	spleen	p-Value
WT	5.49±0.28	0.4793	0.82±0.09	0.8430	2.08±0.13	0.0329
TLR3$^{-/-}$	5.13±0.45		0.83±0.40		2.99±0.45	
WT	3.18±0.44	0.2187	0.65±0.07	**0.0191 ***	3.84±0.64	**0.0141 ***
TLR4$^{-/-}$	4.05±0.53		1.03±0.13		2.17±0.15	
WT	3.97±0.78	0.8053	0.32±0.04	0.4871	1.34±0.27	0.4070
MyD88$^{-/-}$	3.90±0.55		0.37±0.07		1.05±0.18	
WT	5.19±0.43	**0.0007 *** **	12.69±1.12	**0.0003 *** **	6.41±0.61	**0.0454 ***
TRIF$^{-/-}$	2.71±0.49		6.43±0.92		4.62±0.57	
WT	4.12±0.50	0.7041	0.89±0.19	0.9984	0.91±0.20	0.5491
IRF1$^{-/-}$	4.54±0.94		0.89±0.14		1.04±0.082	
WT	6.33±0.47	**0.0283 ***	3.05±1.72	0.0866	2.04±0.21	0.2857
IRF3$^{-/-}$	4.43±0.68		0.57±0.06		2.36±0.21	

On d30 p.i. the absolute numbers of infiltrated immune cells were analysed in the thoracic cavity, medLNs and spleen of individually infected TLR3$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$, TRIF$^{-/-}$, IRF1$^{-/-}$ and IRF3$^{-/-}$ C57BL/6 and WT C57BL/6 mice. Numbers show mean ± SEM. Asterisks show significant differences (Student's t test) between groups indicated by the asterisks (* p<0.05 and *** p<0.001). Data show results from two or three (TRIF$^{-/-}$) individual infection studies.

Additionally, the cell composition in the TC was also analysed using the cytospin method (Fig. 3.14). The frequency of macrophages (MO) was similar in TLR3$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$ and also IRF1$^{-/-}$ mice compared to the WT controls. In contrast, frequency of this cell type was significantly higher in TRIF$^{-/-}$ and also in IRF3$^{-/-}$ mice when compared to their controls (Fig. 3.14 green bars). Frequency of lymphocytes (LZ) was only significantly increased in TRIF$^{-/-}$ mice compared to the WT C57BL/6 mice (Fig. 3.14 light pink bars). Furthermore, frequencies of

eosinophils (EO) in different mouse strains were also analysed and these frequencies were significantly reduced in TRIF$^{-/-}$ and also in IRF3$^{-/-}$ mice compared to their controls (Fig. 3.14 dark pink bars). Finally, frequencies of neutrophils (NE) showed no significant differences between all KO-strains and their appropriate WT controls (Fig. 3.14 blue bars).

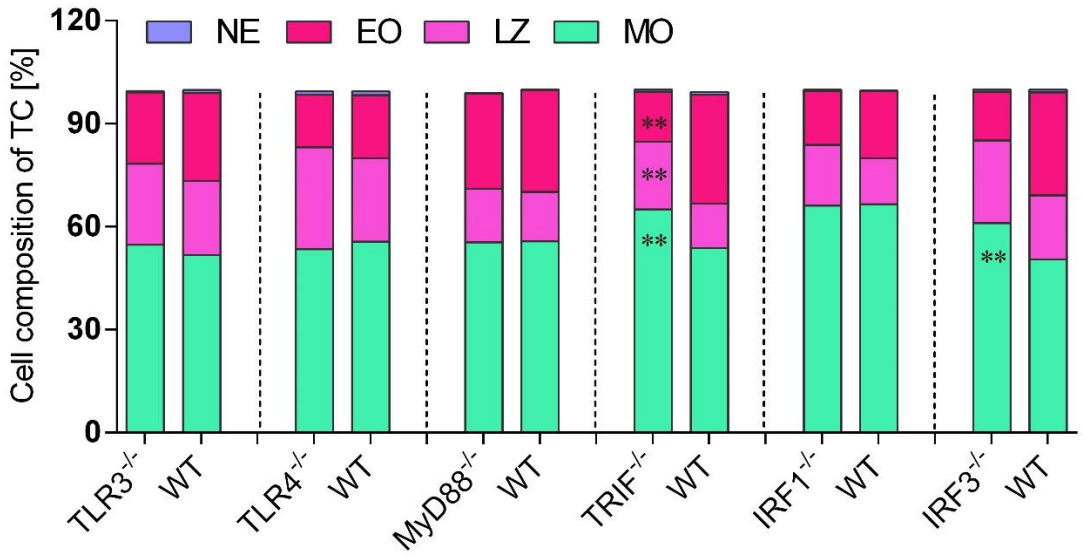


Figure 3.14: Significantly lower frequencies of eosinophils in *L. sigmodontis*-infected TRIF$^{-/-}$ mice 30 days post infection. On d30 p.i. frequencies [%] of macrophages (MO) (green part), lymphocytes (LZ) (light pink part), eosinophils (EO) (dark pink part) and also neutrophils (NE) (blue part) in TLR3$^{-/-}$ (TLR3$^{-/-}$ n= 12 and WT n=14), TLR4$^{-/-}$ (TLR4$^{-/-}$ n=12 and WT n=12), MyD88$^{-/-}$ (MyD88$^{-/-}$ n=5 and WT n=5), TRIF$^{-/-}$ (TRIF$^{-/-}$ n=14 and WT n=15), IRF1$^{-/-}$ (IRF1$^{-/-}$ n=11 and WT n= 10) and IRF3$^{-/-}$ (IRF3$^{-/-}$ n=12 and WT n=13) mice were determined using stained cytospin sections. Bars show mean and statistical significances between the indicated groups and were assessed using the Student's t test or Mann Whitney (**p<0.01). Data show results from one infection study (MyD88$^{-/-}$), from two infection studies (TLR3$^{-/-}$, TLR4$^{-/-}$, IRF1$^{-/-}$, and IRF3$^{-/-}$) or from three (TRIF$^{-/-}$) individual infection studies.

3.3.3 Decreased CD4^{+} and CD8^{+} T cell frequency at the site of infection in TRIF$^{-/-}$ mice

Since, the frequency of lymphocytes was significantly increased in the TC of TRIF$^{-/-}$ mice compared to WT mice (Fig. 3.14), the TC fluid was also analysed for immune cells via flow cytometry. Therefore, TC fluid was stained for populations of CD4, CD8 and CD19. Interestingly, frequencies of CD4^{+} (Fig. 3.15A) and CD8^{+} (Fig. 3.15B) T cells were significantly lower in the TC of mice deficient for TRIF compared to the WT controls: indicating a reduction in T cell migration to the infection site. However, frequencies of CD19^{+} B cells (Fig. 3.15C) were significantly higher at the site of infection (TC) showing that migration per se was not dampened. Frequencies of CD4^{+} T cells in medLNs (Fig. 3.15D) and also in spleens (Fig. 3.15G) were comparable but frequencies of CD8^{+} T cells in this organ were significantly increased compared to their appropriate controls (Fig. 3.15E and H). Finally, no differences

were observed in frequencies of CD19^{+} cells in medLNs (Fig. 3.15F) and also not in spleens (Fig. 3.15I) of TRIF$^{-/-}$ mice and WT controls.

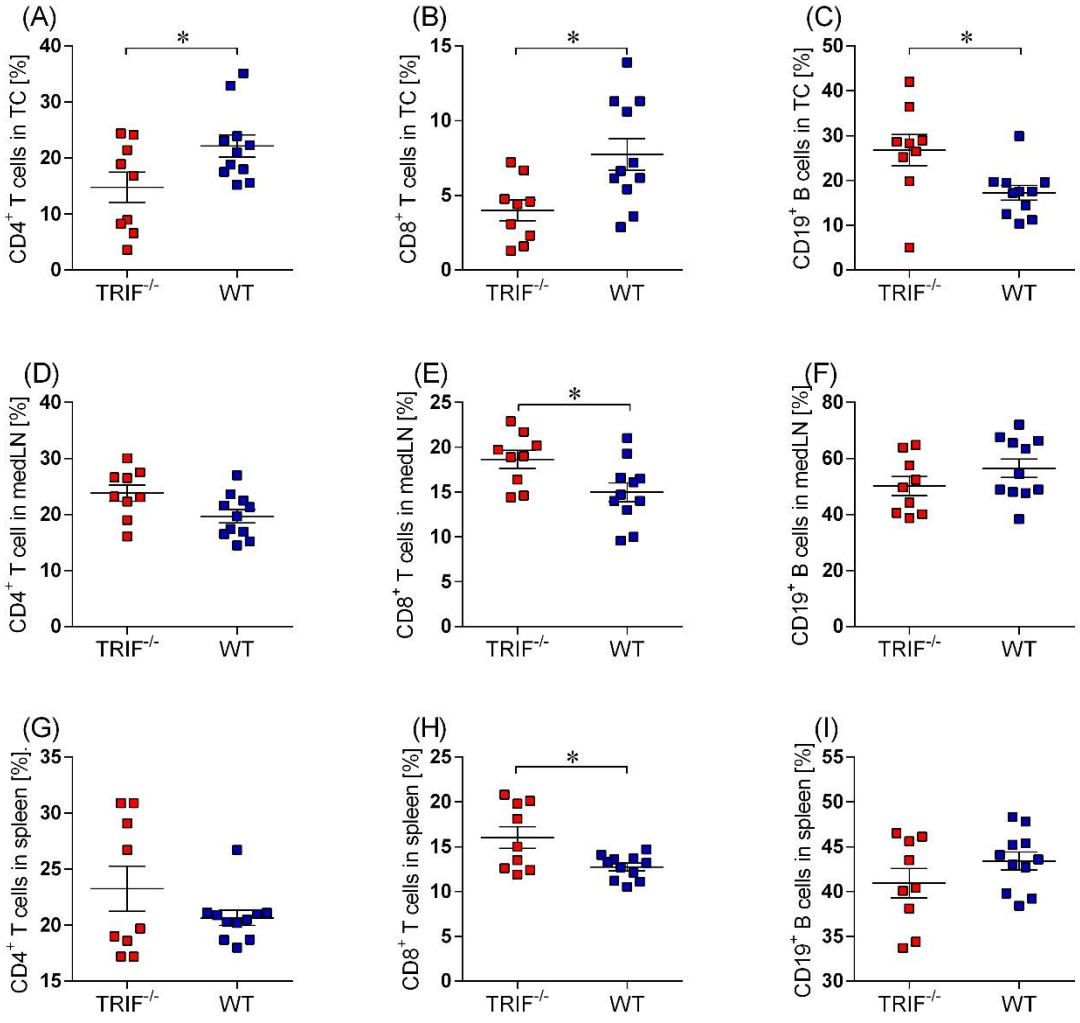


Figure 3.15: Decreased CD4^{+} and CD8^{+} T cell frequencies at the site of infection in TRIF$^{-/-}$ mice on day 30 p.i.. Using flow cytometry, frequencies of (A, D, G) CD4^{+}, (B, E, H) CD8^{+} T cells and (C, F, I) CD19^{+} B cells were analysed in the thoracic cavity (TC A-C), mediastinal lymph nodes (medLN D-F) and spleen (G- I). Graphs show mean ± SEM of individually assessed mice from two independent infection experiments (n=9 TRIF$^{-/-}$/ n=11 WT). Statistical significances between the indicated groups were obtained using an unpaired t-test or Mann Whitney test. Asterisks indicate significant differences between the groups indicated by the brackets (*p<0.05).

Depletion of regulatory T cells results in lower worm numbers and also in reduced fertility of remaining female worms in BALB/c mice (142). Additionally, our group has already observed that *L. sigmodontis*-infected TLR2$^{-/-}$ and TLR4$^{-/-}$ BALB/c mice showed reduced Foxp3^{+} Treg amounts on day 49 and 72 p.i. in the medLN. This was also linked to reduced filarial antigen presentation (63). Since these studies demonstrated that Tregs play a role during *L.*

sigmodontis infection, $CD4^+$ T cells in $TRIF^{-/-}$ mice were further analysed for the expression of regulatory markers like Foxp3, CD25 and CD103.

Frequencies of Foxp3 in $CD4^+$ T cells were similar in the TC (Fig. 3.16A), medLN (Fig. 3.16B) and spleen (Fig. 3.16C) of *L. sigmodontis*-infected $TRIF^{-/-}$ mice compared to infected C57BL/6 mice. Higher frequencies of $CD4^+CD25^{high}$ cells could be only observed only in spleens of mice deficient for TRIF (Fig. 3.16F). Finally, frequencies of CD103 in $CD4^+CD25^{high}$ cells were also analysed but here, no differences in CD103 expression could be observed (Fig. 3.16G-I).

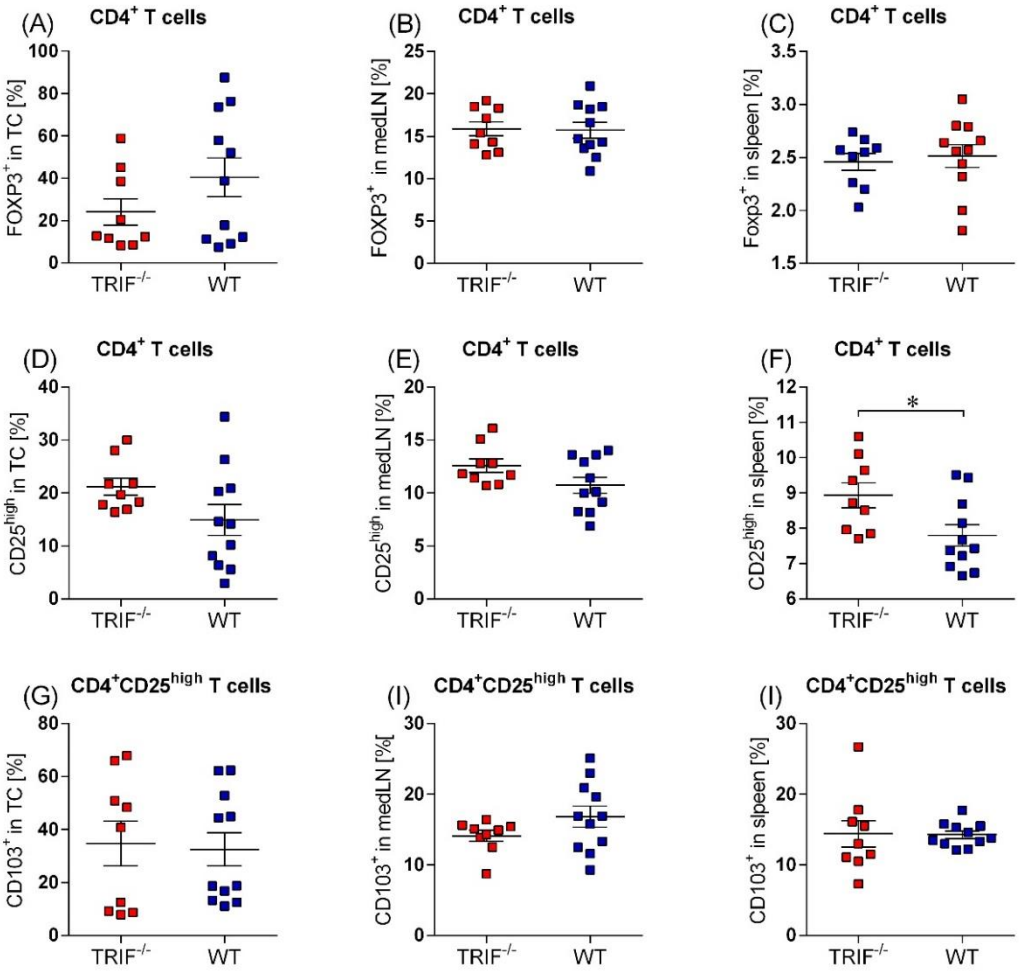


Figure 3.16: Comparable regulatory T cell subset frequencies in TRIF-deficient mice on day 30 p.i.. Using flow cytometry, frequencies of $CD4^+Foxp3^+$ regulatory T cells were analysed in (A) the thoracic cavity (TC), (B) mediastinal lymph nodes (mLN) and (C) spleen and frequencies of $CD4^+CD25^{high}$ regulatory T cells were also analysed in (D) TC, (E) mLN and (F) spleen. Moreover, frequencies of $CD4^+CD25^{high}$ expressing $CD103^+$ were analysed in the (G) TC, (H) mLN and (I) spleen. Graphs show mean ± SEM of individually assessed mice from two independent infection experiments (n=9 $TRIF^{-/-}$/ n=11 WT). Statistical significances were assessed using unpaired t-test or Mann-Whitney-U-tests. Asterisks indicate significant differences between the groups indicated by the brackets (*$p<0.05$).

Overall, these findings indicate that recruitment of $CD4^+$ and $CD8^+$ T cells but not regulatory T cell subsets to the site of infection is influenced by TRIF signalling.

3.3.4 No variations of local and systemic cytokine levels and filarial-specific responses in re-stimulated TRIF-deficient mice on day 30 p.i.

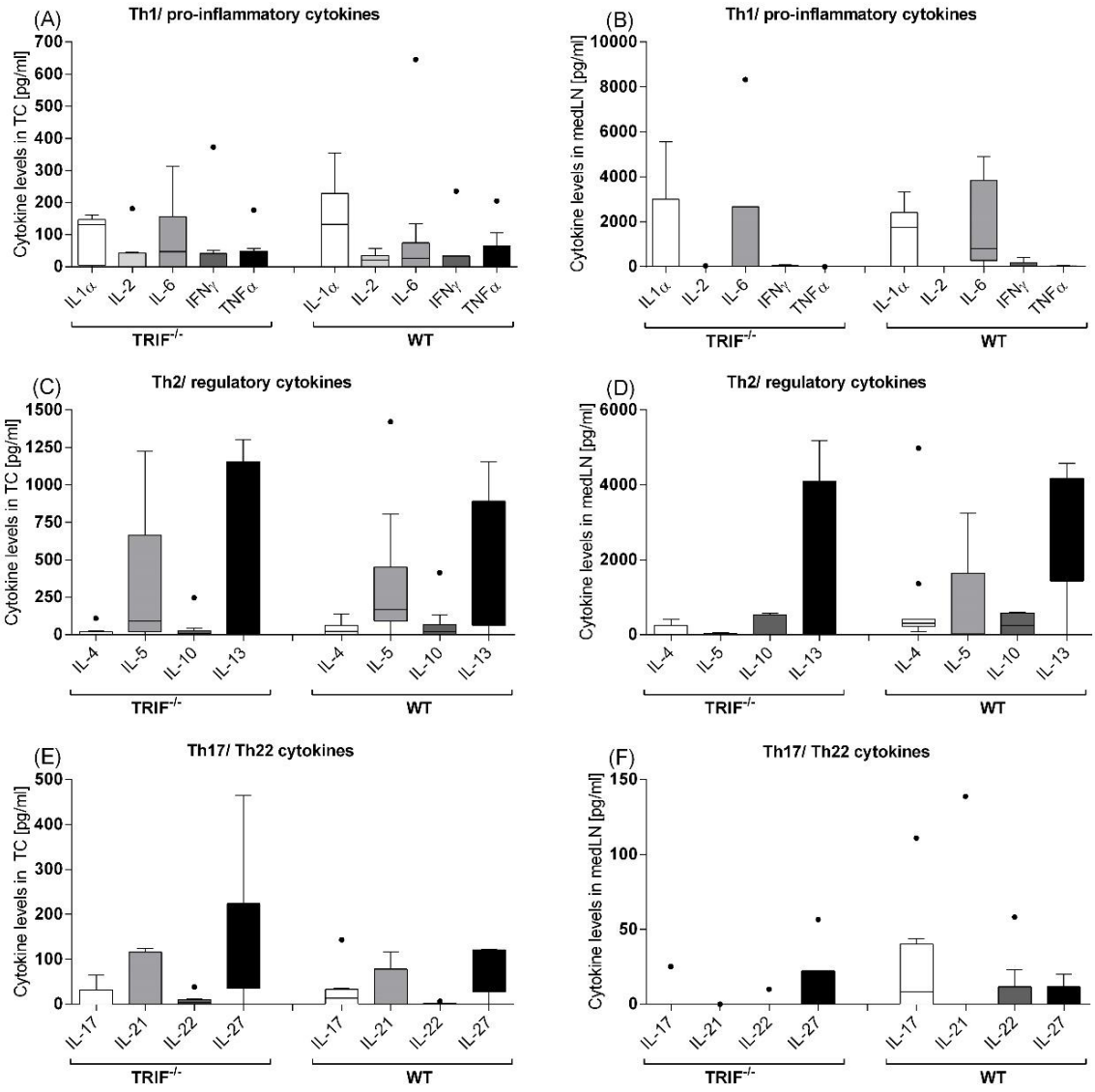


Figure 3.17: Comparable local cytokine levels in infected TRIF$^{-/-}$ and wildtype mice on day 30 p.i.. Levels of in situ cytokines were measured in the thoracic cavity (TC) fluid (A, C and E) of individual mice 30 days p.i.. Released cytokine levels in cell culture supernatants from bulk mLN assays (5×10^5/well) following stimulation with LsAg (72h) were also determined in individual TRIF$^{-/-}$ and wildtype mice (B, D and F) using the murine TH1/TH2/TH17/TH22 13-plex FlowCytomix Multiple Analyte Detection Kit. Graph shows box whiskers (Tukey) of assessed mice from two independent infection experiments A, C and E n=9 TRIF$^{-/-}$/ n=11 WT and B, D and F n=7 TRIF$^{-/-}$/ n=11 WT. Statistical significances between the indicated groups were assessed using the Kruskal-Wallis test followed by Dunn's multiple comparison test for further comparison of the groups: no significant differences were found.

Since the immune cell composition was altered and $CD4^+$ and $CD8^+$ T cells were reduced in the TC of *L. sigmodontis*-infected $TRIF^{-/-}$ mice on day 30 p.i., we further analysed cytokine levels in the TC as well as filarial-specific recall responses from medLN by using the murine TH1/TH2/TH17/TH22 13-plex FlowCytomix Multiple Analyte Detection Kit. However, cytokine levels in the TC (Fig. 3.17A-C) as well as immune responses from medLN cells upon *L. sigmodontis* antigen recall (Fig. 3.17D-F) were comparable between $TRIF^{-/-}$ and wildtype C57BL/6 mice on day 30 p.i..

Additionally, systemic cytokine levels in sera and also re-stimulated spleen cells were analysed using the murine TH1/TH2/TH17/TH22 13-plex FlowCytomix Multiple Analyte Detection Kit (Fig. 3.18). However, no differences could be observed in cytokine production in sera of *L. sigmodontis*-infected $TRIF^{-/-}$ and WT mice (Fig. 3.18A-C) and also not in LsAg re-stimulated spleen cells of both mouse strains (Fig. 3.18D-F).

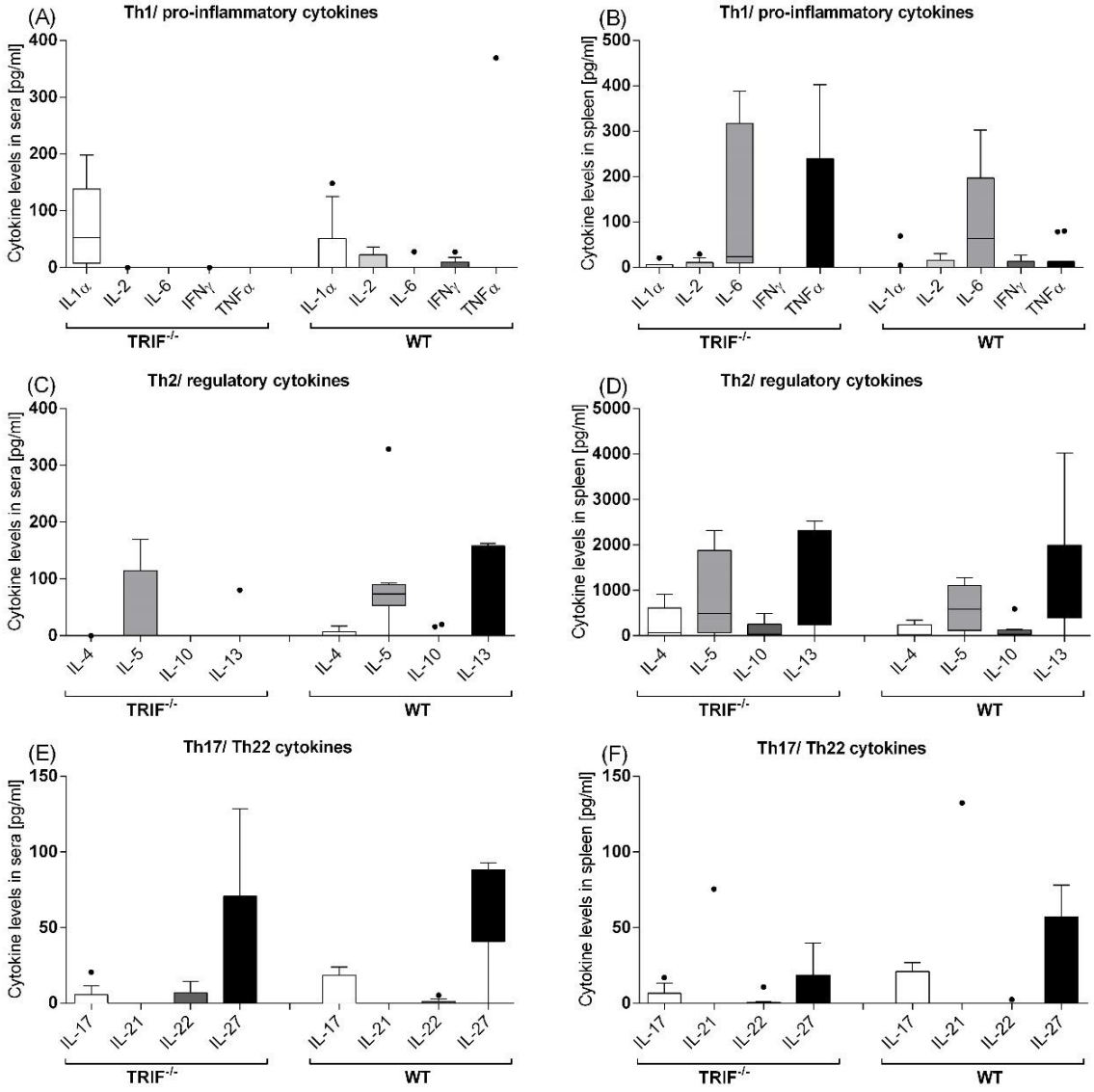


Figure 3.18: Comparable systemic cytokine levels in infected TRIF$^{-/-}$ and wildtype mice on day 30 p.i.. Levels of *in situ* cytokines were measured in the sera (A, C and E) of individual mice 30 days p.i.. Released cytokine levels in cell culture supernatants from bulk spleen assays (5×10^5/well) following stimulation with LsAg (72h) were also determined in individual TRIF$^{-/-}$ and wildtype mice (B, D and F) using the murine TH1/TH2/TH17/TH22 13-plex FlowCytomix Multiple Analyte Detection Kit. Graph shows box whiskers (Tukey) of assessed mice from two independent infection experiments A, C and E (n=9 TRIF$^{-/-}$/ n=11 WT) and B, D and F (n=7 TRIF$^{-/-}$/ n=11 WT). Statistical significances between the indicated groups were assessed using the Kruskal-Wallis test followed by Dunn's multiple comparison test for further comparison of the groups: no significant differences were found.

3.3.5 Significantly reduced levels of IgM in the TC but not sera of *L. sigmodontis*-infected TRIF$^{-/-}$ mice

Since the frequency of CD19^{+} cells was significantly higher in TRIF$^{-/-}$ mice the production of total immunoglobulins (Ig) in the TC and sera was also analysed using the "Mouse Immunoglobulin Isotyping Panel 6plex FlowCytomix Kit" or the "Mouse IgE FlowCytomix Simplex Kit".

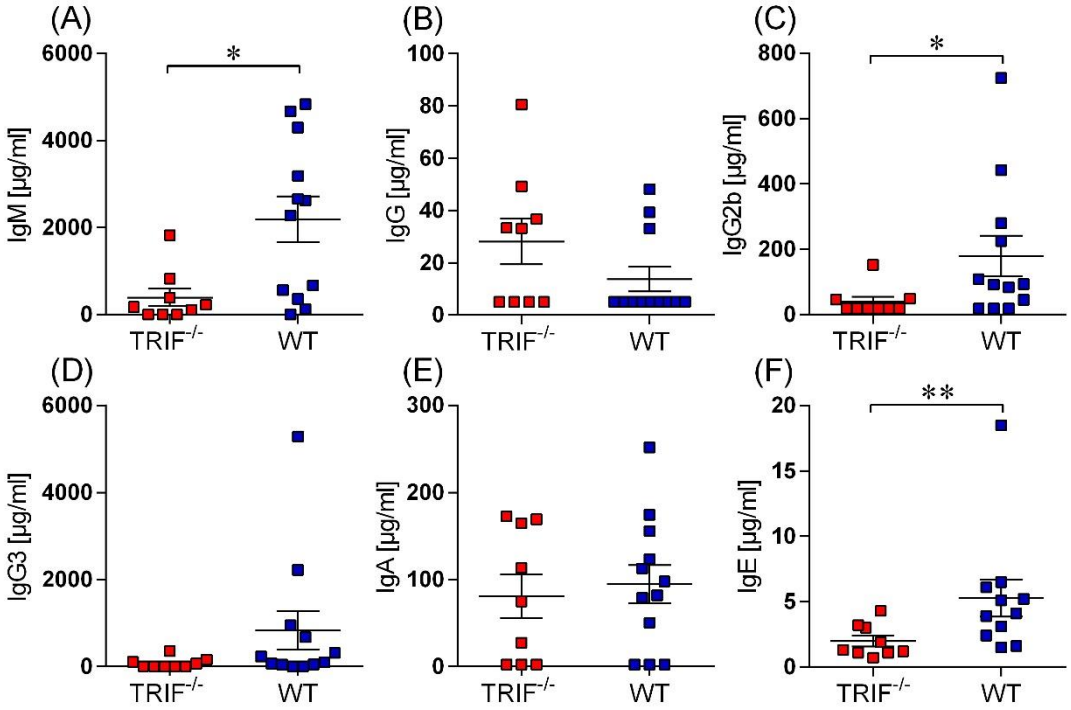


Figure 3.19: Reduced IgM, IgG2b and also IgE production the thoracic cavity fluid of infected TRIF$^{-/-}$ mice on day 30 p.i.. Levels of (A) IgM, (B) IgG, (C) IgG2b, (D) IgG3, (E) IgA and (F) IgE were measured in the thoracic cavity (TC) fluid of *L. sigmodontis*-infected mice 30 days p.i. by using the Mouse Immunoglobulin Isotyping Panel 6plex FlowCytomix Kit (A-E), or the Mouse IgE FlowCytomix Simplex Kit (F). Graphs show mean ± SEM of assessed mice from two independent infection experiments A–F (n=9 TRIF$^{-/-}$/ n=12 WT). Statistical significances between the indicated groups were obtained using an unpaired t-test or Mann Whitney test. Asterisks indicate significant differences between the groups indicated by the brackets (*p<0.05 and **p<0.01).

The amount of IgM, IgG2b and also IgE in the TC of *L. sigmodontis*-infected TRIF-deficient mice was significantly reduced compared to infected WT controls (Fig. 3.19A, C and F). In contrast, no differences could be observed in the amount of IgG (Fig. 3.19B), IgG3 (Fig. 3.19D) and also IgA (Fig. 3.19E).

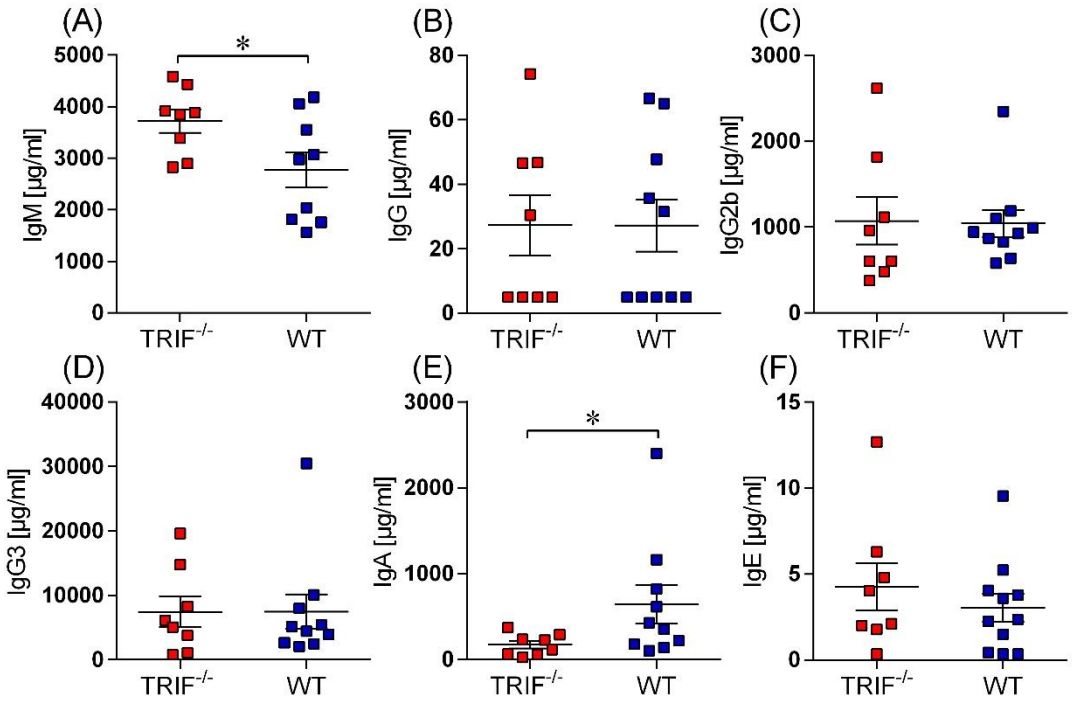


Figure 3.20: Reduced IgG2a and IgA production the sera of *L. sigmodontis*-infected TRIF$^{-/-}$ mice on day 30 p.i.. Levels of (A) IgM, (B) IgG, (C) IgG2b, (D) IgG3, (E) IgA and (F) IgE were measured in the sera of *L. sigmodontis*-infected mice 30 days p.i. by using the Mouse Immunoglobulin Isotyping Panel 6plex FlowCytomix Kit (A-E), or the Mouse IgE FlowCytomix Simplex Kit (F). Graphs show mean ± SEM of assessed mice from two independent infection experiments A–F (n=8 TRIF$^{-/-}$/ n=10 WT). Statistical significances between the indicated groups were obtained using an unpaired t-test or Mann Whitney test. Asterisks indicate significant differences between the groups indicated by the brackets (*p<0.05).

In contrast, in sera the amount of IgM was significantly increased in TRIF-deficient mice compared to WT controls (Fig. 3.20A) whereas, the level of IgA (Fig. 3.20E) was significantly reduced in TRIF$^{-/-}$ mice. No differences were observed in levels of IgG (Fig. 3.20B), IgG2b (Fig. 3.20C), IgG3 (Fig. 3.20D) and IgE (Fig. 3.20F).

3.3.6 Increased levels of *L. sigmodontis*-specific IgG1 in TRIF$^{-/-}$ mice

Finally, the levels of *L. sigmodontis*-specific immunoglobulins were also analysed. The OD of *L. sigmodontis*-specific IgE (Fig. 3.21A) and also IgG1 (Fig. 3.21B) in TC were similar between infected TRIF-deficient and WT mice. However, *L. sigmodontis* IgE (Fig. 3.21C) and levels of specific IgG1 (Fig. 3.21D) were significantly increased in sera of infected TRIF-deficient mice compared to controls.

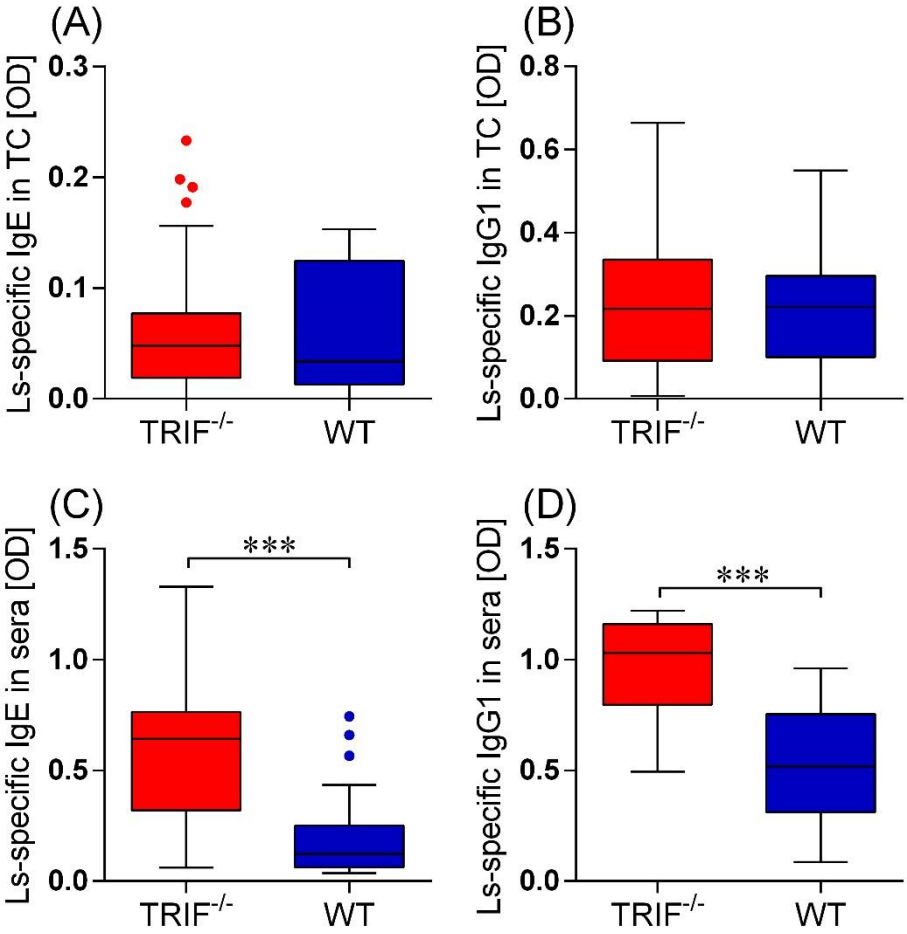


Figure 3.21: Significantly increased *L. sigmodontis*-specific IgE and IgG1 production in sera of *L. sigmodontis*-infected TRIF$^{-/-}$ mice on day 30 p.i.. Levels of *L. sigmodontis*- specific IgE (A and C) and IgG1 (B and D) were measured in the thoracic cavity (TC) (A and B) and sera (C and D). Graph shows box whiskers (Tukey) of assessed mice from two independent infection experiments (A and B n=9 TRIF$^{-/-}$/ n=11 WT; C n=4 TRIF$^{-/-}$/ n=11 WT; D n=5 TRIF$^{-/-}$/ n=11 WT). Statistical significances between the indicated groups were obtained using an unpaired t-test or Mann Whitney test. Asterisks indicate significant differences between the groups indicated by the brackets (***p<0.001).

3.3.7 Worm counts and cell compositions in mice deficient for TRIF and mice deficient for TLR3 at day 40 and day 60 post *L. sigmodontis* infection

Additionally, mice deficient for TLR3 and also deficient for TRIF were infected with *L. sigmodontis* for 40 or 60 days. No differences were observed in worm burden in TLR3$^{-/-}$ mice on day 40 p.i. whereas, worm counts in TRIF$^{-/-}$ mice were significantly increased compared to WT controls (Table 2, red). When worms were further subdivided into L4 stage and adult worms, the amount of both developmental stages was comparable between KO-mice and their appropriate controls (table 2, upper part). Furthermore, cell numbers in the TC were also

comparable in TLR3$^{-/-}$ or TRIF$^{-/-}$ compared to WT controls and also composition of TC cells showed no significantly differences between genetic modified mice and WT controls (table 2, upper part).

Table 2: Worm counts and immune cell infiltration in *L. sigmodontis*-infected TLR3$^{-/-}$ and TRIF$^{-/-}$ C57BL/6 mice on day 40 and 60 post infection

	Parameter	WT	TLR3	p-Value	WT	TRIF	p-Value
day 40 p.i.	worm count	9.71±1.71	7.30±2.05	0.3498	3.62±0.98	9.92±2.76	**0.0277 ***
	L4	0.00±1.22	1.50±0.87	>0.9999	0.00±0.00	3.67±2.27	0.4522
	adult	2.43±0.37	4.38±2.13	>0.9999	3.90±0.99	3.50±0.97	>0.9999
	cell number TC (x10^7)	3.79±0.50	3.57±0.46	0.7489	2.50±0.35	2.21±0.39	0.5854
	Monocytes [%]	63.58±2.10	64.88±2.05	0.6636	66.51±1.67	66.36±1.85	0.9515
	Lymphocytes [%]	17.90±1.88	20.81±2.39	0.3506	18.98±2.47	17.31±1.24	0.9787
	Neutrophils [%]	0.53±0.28	0.25±0.071	0.6028	0.82±0.21	1.04±0.15	0.3992
	Eosinophils [%]	18.24±2.48	14.21±2.30	0.2487	13.14±1.35	15.10±1.89	0.4018
day 60 p.i.	worm count	1.73±0.47	1.54±0.42	0.7659	2.30±0.68	5.00±1.56	0.1932
	L4	0.22±0.22	0.375±0.183	>0.9999	0.22±0.22	0.33±0.33	>0.9999
	adult	1.00±0.37	0.125±0.125	>0.9999	1.00±0.37	2.33±1.03	0.2100
	cell number TC (x10^7)	2.37±0.28	1.838±0.335	0.0637	2.95±0.45	3.20±0.81	0.7816
	Monocytes [%]	72.40±1.52	70.75±2.52	0.9298	73.45±1.29	63.37±2.62	**0.0040 ****
	Lymphocytes [%]	17.36±1.73	16.96±2.91	0.8864	18.00±1.49	17.58±3.22	0.9078
	Neutrophils [%]	0.32±0.12	0.769±0.20	0.0805	0.45±0.17	0.72±0.18	0.2999
	Eosinophils [%]	9.32±2.49	12.15±3.31	0.2958	7.95±1.37	18.38±4.53	**0.0405 ***

C57BL/6 mice deficient for TLR3 or, TRIF and also WT controls were infected with *L. sigmodontis* for 40 or 60 days. Afterwards worm counts, worm differentiation, absolute numbers of infiltrated immune cells in the TC and frequencies of monocytes, lymphocytes, neutrophils and eosinophils in the TC were. Numbers show mean ± SEM. Asterisks show significant differences (Student's t test) between groups indicated by the brackets (* $p<0.05$ and ** $p<0.01$). Data show results from two individual infection studies (d40: n=10 TLR3$^{-/-}$, n=7 WT / n=12 TRIF$^{-/-}$, n=13 WT and d60: n=13 TLR3$^{-/-}$, n=11 WT / n=10 TRIF$^{-/-}$, n=10 WT.

Next, worm counts in TLR3$^{-/-}$ and TRIF$^{-/-}$ mice on day 60 p.i. were also analysed. At both time-points no significant differences could be observed (table2, lower part). Interestingly, approximately a mean of 5 worms could be detected in TRIF mice, even if the infection in C57BL/6 mice is normally cleared about day 40 post infection. The number of L4 stage larvae and adult worms was also comparable and moreover, cell numbers in the TC showed no differences between KO-mice and WT controls. No differences could be observed in

frequencies of different cell types in the TC of TLR3$^{-/-}$ and WT controls. However, frequencies of monocytes were significantly reduced and frequencies of eosinophils were significantly increased in TRIF$^{-/-}$ mice compared to C57BL/6 WT mice (table 2, marked in green and red).

Collectively, there were an increased number of worms in TRIF$^{-/-}$ mice. Interestingly the number of L4 staged worms in this KO-strain was also significantly higher when compared to WT controls. This was accompanied by an increased number of monocytes, eosinophils and B cells but also by a reduced number of CD4^{+}and CD8^{+} T cells at the site of infection. Therefore, these results indicate that the adapter molecule TRIF are involved in worm recovery and immune cell recruitment into the site of infection 30 days upon *L. sigmodontis* infection in C57BL/6 mice.

3.4 The role of the adaptive immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice

The final section of this thesis, aimed to clarify how *L. sigmodontis*-infected C57BL/6 mice can clear a *L. sigmodontis* infection and the immunological mechanisms which are important for such clearance. Earlier studies from our group demonstrated that infections with Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice (absence of T, B and also NK cells) were fully permissive to *L. sigmodontis* infections resulting in high worm burden and Mf counts (100% fecundity) (65). Thus, it was anticipated that these mice are a useful tool for analysing immune mechanisms that support or hinder worm development. Therefore, the following set of adoptive transfer studies were designed to determine the influence of different T cells on the development and survival of worms in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice.

3.4.1 Ongoing *L. sigmodontis* infection beyond day 120 p.i..

In the initial study, our group compared worm burden in *L. sigmodontis*-infected in WT C57BL/6 mice and Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 30 and 72 p.i., since the latter time-point corresponded to peak Mf count in infected BALB/c mice (63). Additionally, Mf loads in blood were measured over time (d50 – d72). Whereas no Mf are observed in WT C57BL/6 mice, Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice became patent and Mf loads increased over time (65) comparable and even exceeding *L. sigmodontis*-infected WT BALB/c mice. Since no data were available on how long infections and patency lasted in infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice, a kinetic experiment determining Mf loads in blood were analysed over time (Fig. 3.22).

Interestingly, the normal Mf peak seen on day 72 p.i. in BALB/c mice was not observed in infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice. Indeed, compared to the normal Mf counts observed in BALB/c mice at this time-point, infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice presented a 30-fold increase. Furthermore, Mf were still circulating in the blood of infected mice on day 120 p.i. at high levels. Even that most levels had begun to decline at this time-point, Mf are still detectable at day 190 p.i. (Fig. 3.22). Since the average life-span of C57BL/6 mice is 2 years, the mice in this infection had been infected for nearly 30% of their life-span.

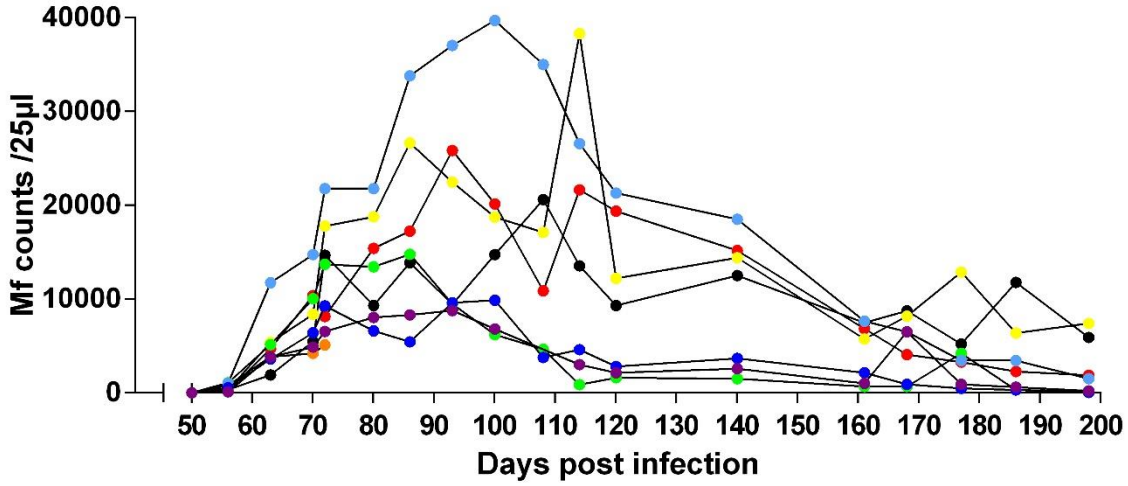


Figure 3.22: Ongoing *L. sigmodontis* infection beyond day 190 p.i.. Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice were naturally infected with *L. sigmodontis*. From day 50 p.i. Mf loads in blood of individual mice were analysed at the depicted time points. Symbols represent Mf loads of individual mice (n=7) within the study.

3.4.2 Adoptive transfer of CD4^{+} T cells leads to significantly reduced length of female worms and Mf numbers in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i.

To determine the impact of T cells on clearing Ls infection in Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice, a series of adoptive transfer studies were performed. The first was to assess the effects on worm burden following the reconstitution of T cells prior to infection, that is naïve T cells. Thus, sorted CD4^{+} or CD8^{+} T cell populations were adoptively transferred (a.t.) into Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice. After one day, animals with a.t. cells and control groups without a cell transfer were infected with *L. sigmodontis* for 72 days. The amount of Mf was detected in blood on days 50, 56, 63, 70 and on analysis day. Additionally, on day 72 p.i. mice were analysed for worm numbers, gender and length. Furthermore, Mf were detected in the TC of all groups since studies have shown that mice can be patent in the TC but not periphery (5/100 *L. sigmodontis*-infected BALB/c mice (unpublished data AG Layland)).

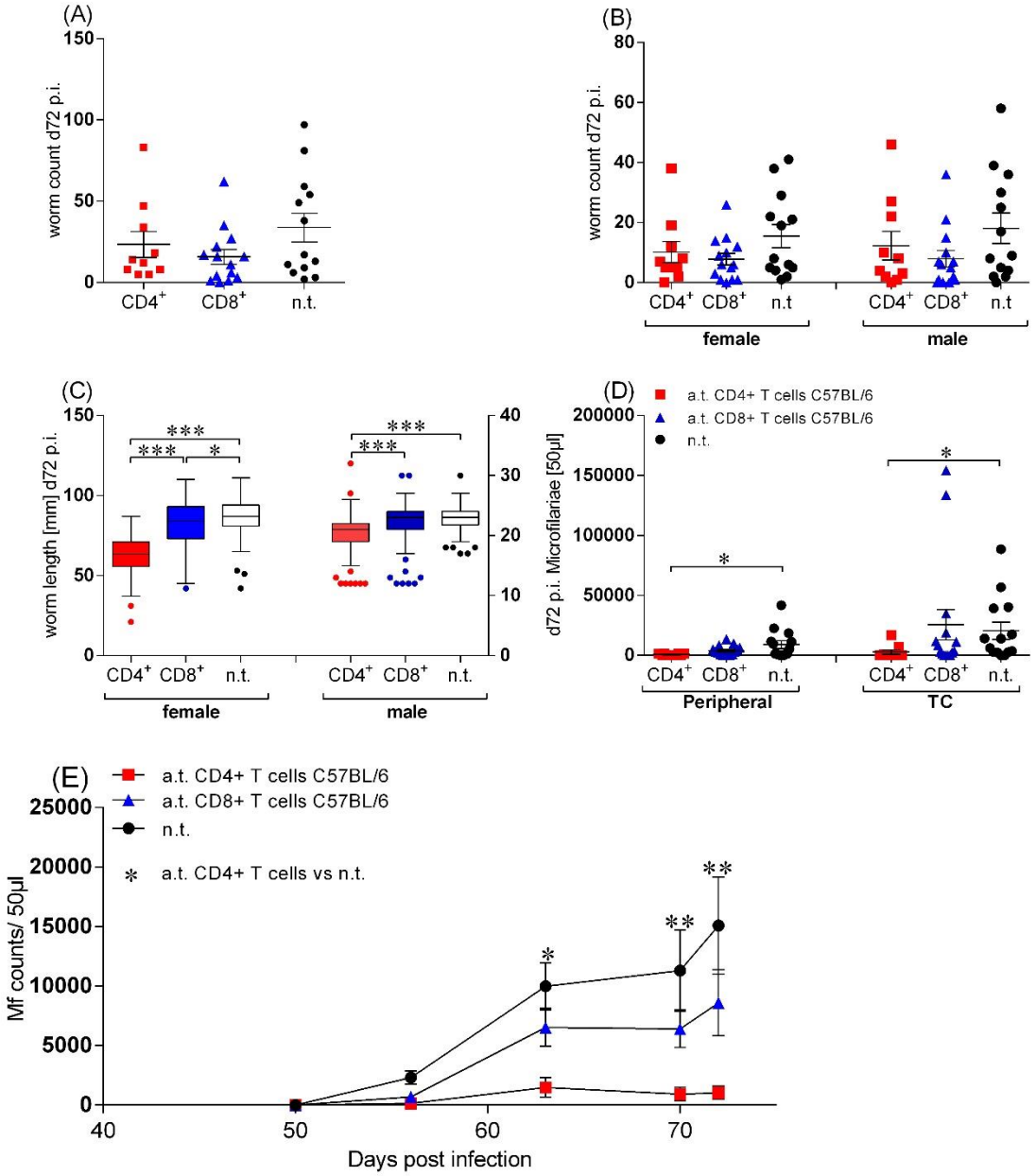


Figure 3.23: Adoptive transfer of naïve CD4⁺ T cells leads to significantly shorter female worms and numbers of Mf in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i.. Naïve CD4⁺ or CD8⁺ T cells were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. After 72 days mice were assessed for absolute worm counts (A and B) and worm length from individual worms (C). Levels of microfilariae (Mf) were detected on d72 p.i. in blood and thoracic cavity (TC) fluid (D) and additionally over time in the periphery (E) from each mouse. Data represents mean ± SEM of individual mice from two different adoptive transfer studies in two individual independent infection experiments (n=10 a.t. CD4⁺ T cells, n=14 a.t. CD8⁺ T cells, n=13 n.t.). Asterisks denote significant differences (Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups (A-D; F) or (E) 2way ANOVA) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p < 0.001).

No significant differences could be seen in the overall worm burden between an a.t. of $CD4^+$ T cells, an a.t. of $CD8^+$ T cells and no transfer (n.t.) (Fig. 3.23A). Worms were further analysed with regards to their different gender and here also no differences could be observed in the amount of female or male worms between the three different groups (Fig. 3.23B). Next female and also male worms were analysed for their length. Interestingly, worms of both genders were significantly shorter in mice following an a.t. with naïve $CD4^+$ T cells compared to controls and also compared to mice after an a.t. of naïve $CD8^+$ T cells. Female worms of the $CD8^+$ group were also significantly shorter, compared to controls (Fig. 3.23C). No differences could be observed with regards to the status of the worms since there were no significant differences in numbers of capsulated worms or nodule formation between the groups (data not shown). Numbers of blood and TC derived Mf on day 72 were significantly lower in the $CD4^+$ T cell a.t. group compared to controls whereas, the a.t. of $CD8^+$ T cells had no effect (Fig. 3.23D). Microfilariae numbers in blood were also analysed over time. Interestingly, from day 62 post infection the number of Mf in the group following an a.t. of $CD4^+$ T cells were significantly reduced compared the controls (Fig. 3.23E).

Additionally, embryograms of female worms were performed which shows the different embryonic stages (Oo – oocyte; De – divided egg; Pr – pretzel and Mf – microfilariae) in *L. sigmodontis* female worms (Fig. 3.24A-D). Regardless of the embryo stage, significantly reduced numbers were observed in mice following an a.t. of $CD4^+$ T cells when compared to the n.t. group but also to the a.t. $CD8^+$ group as well (Fig. 3.24E).

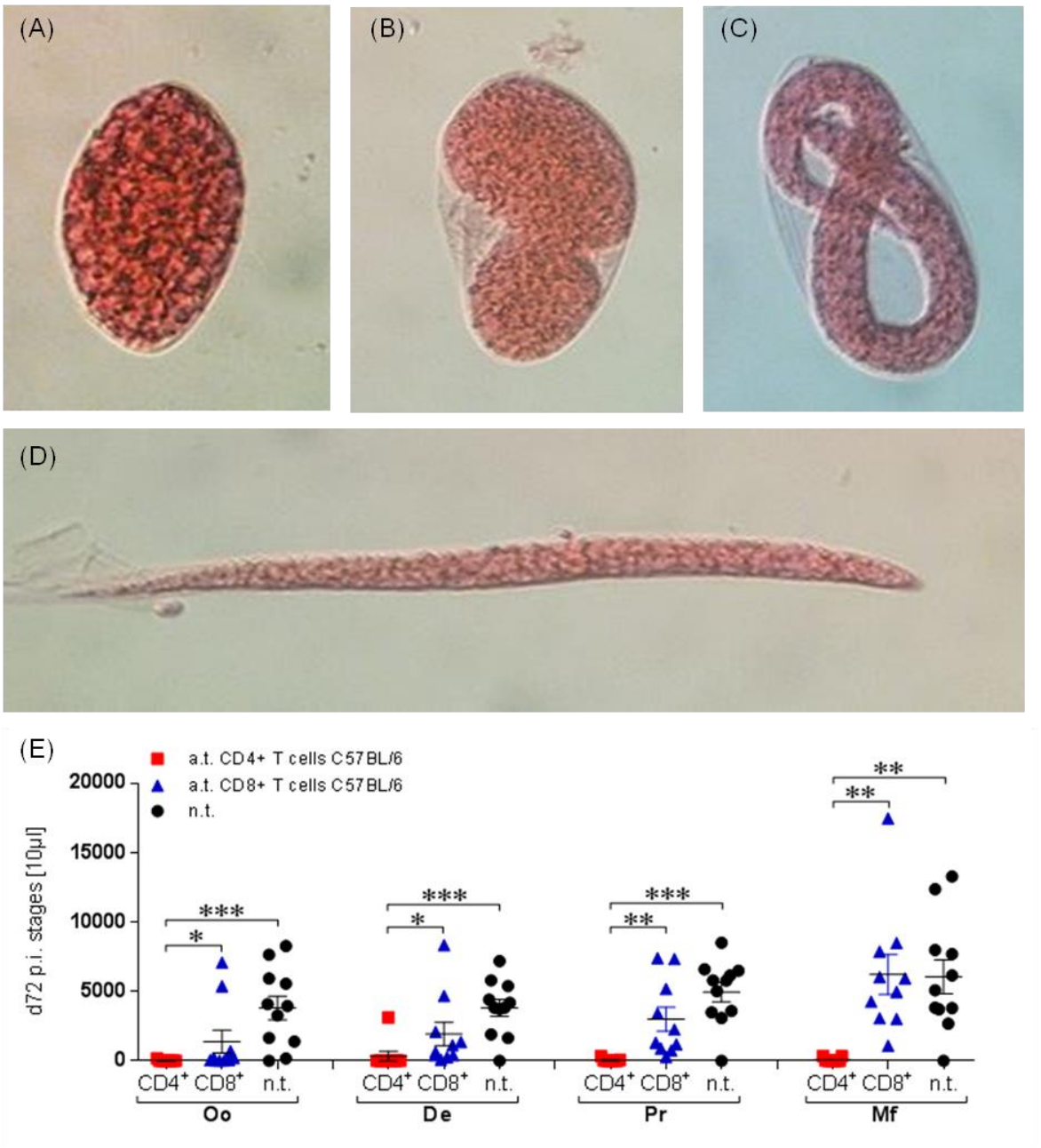


Figure 3.24: Significantly reduced numbers of the different embryonic stages in female worms of *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with adoptively transferred CD4$^+$ T cells. The embryogram shows the composition of embryonic stages (A) Oo – oocyte, (B) De – divided egg, (C) Pr – pretzel and (D) Mf – microfilariae in female worms of the different group (E). Data represents mean ± SEM of individual mice from two different adoptive transfer studies in two individual independent infection experiments (n=10 a.t. CD4$^+$ T cells, n=14 a.t. CD8$^+$ T cells, n=13 n.t.). Asterisks denote significant differences (Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups (A-D; F) or (E) 2way ANOVA between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p < 0.001).

3.4.2.1 More Th1 biased immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice following an a.t. of naïve CD4$^+$ or CD8$^+$ T cells

On day 72 p.i. the cytokine expression of CD4$^+$ and also CD8$^+$ T cells derived from the TC, was determined via FACS using a 9-parameter based panel. As expected, frequencies of CD4$^+$

and also CD8⁺ T cells were significantly increased compared to the control group (Fig. 3.25A) although the percentage of surviving CD4⁺ T cells (average: 6.141%) was higher than the CD8 subset (average: 2.353%). Interestingly, both groups (a.t. of CD4⁺ and of CD8⁺ T cells) showed a more Th1 biased immune response (Fig. 3.25B and C, green part) followed by a Th2/IL-10 immune response (Fig. 3.25B and C, red part).

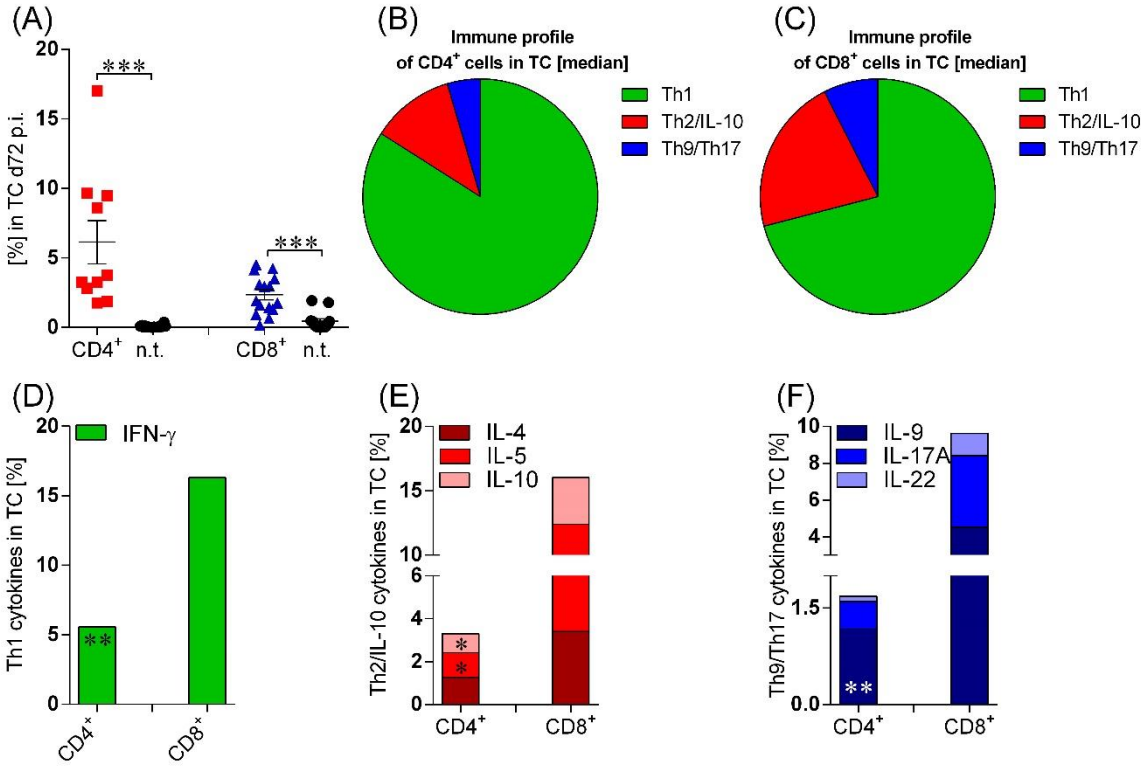


Figure 3.25: More Th1 biased immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with following an a.t. of either CD4⁺ or CD8⁺ T cells. CD4⁺ or CD8⁺ T cells were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipient mice one day before *L. sigmodontis* infection. Thoracic cavity (TC) cells, isolated from individual mice on day 72 p.i. were assessed for frequencies of CD4 and CD8 (A) via FACS. Immune profiles of CD4⁺ (B) and CD8⁺ T cells (C) were also analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 (F) cytokines were determined. Symbols represent mean ± SEM of individual mice (n=13 a.t. CD4⁺ T cells, n=14 n.t., n=15 a.t. CD8⁺ T cells, n=13 n.t.) (A) or mean of frequencies (D-F). Pie charts show median of Th1, Th2/IL-10 and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (*p<0.05, **p<0.01, ***p<0.001). Data show results from two individual infection studies.

With regards to the Th1 immune response, the frequency of IFN-γ production was significantly lower in CD4⁺ T cells when compared to CD8⁺ T cells (Fig. 3.25D). Further analysis of the Th2/IL-10 immune profile showed that IL-4 production of CD4⁺ T cells was reduced compared to CD8⁺ T cells, albeit not significantly. Furthermore, IL-5 and IL-10 expression was significantly lower by CD4⁺ T cells compared to CD8⁺ T cells (Fig. 3.25E). Finally, the Th9/Th17 immune response was analysed in more detail. Here only frequencies of IL-9 expression

revealed statistical differences since the frequency of IL-9 expression by CD4$^+$ T cells was lower. However, the portion of CD4$^+$ T cells producing IL-9 is much higher than the portion of CD4$^+$ T cells producing IL-17A or IL-22. Frequencies of IL-17A and IL-22 expression were comparable between both groups (Fig. 3.25F).

3.4.2.2 More Th1 biased immune response in blood of *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with following an a.t. of CD4$^+$ or CD8$^+$ T cells

Next, the immune profile of *L. sigmodontis*-infected mice was also analysed in blood 72 days post infection. Frequencies of CD4$^+$ and CD8$^+$ T cells were also significantly higher compared to levels in n.t. groups (Fig. 3.26A). The immune profile, of CD4$^+$ and also of CD8$^+$ T cells in blood, was also more Th1 biased (Fig. 3.26B and C, blue part), followed by the Th9/Th17 immune response in the CD4$^+$ groups (Fig. 3.26B blue part), but not in the CD8$^+$ T cell a.t. group. Here the Th2/Treg immune response was the second dominant parameter (Fig. 3.26C, green part).

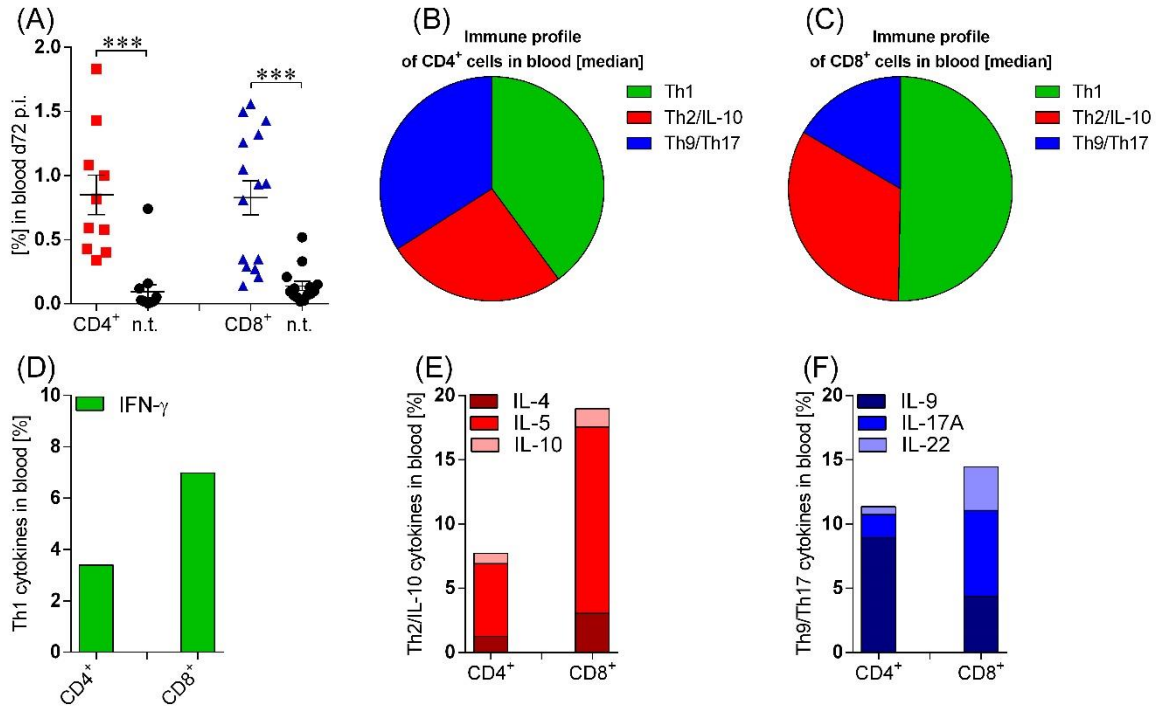


Figure 3.26: Th1 biased immune response in blood of *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice following an a.t. of naïve CD4$^+$ or CD8$^+$ T cells. CD4$^+$ or CD8$^+$ T cells were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57Bl/6 recipients one day before *L. sigmodontis* infection. Blood cells, isolated from individual mice on day 72 p.i. were assessed for frequencies of CD4 and CD8 (A) by FACS. Immune profiles of CD4$^+$ (B) and CD8$^+$ T cells (C) were also analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 cytokines were analysed. Symbols represent mean ± SEM of individual mice (n=13 a.t. CD4$^+$ T cells, n=14 n.t., n=15 a.t. CD8$^+$ T cells, n=13 n.t.) (A) or only mean (D-E). Pie charts show median of Th1, Th2/Treg and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (***p<0.001). Data show results from two individual infection studies.

In more detail, there were no statistically significant differences in the production of the Th1 cytokine IFN-γ between $CD4^+$ and $CD8^+$ T cells (Fig. 3.26D). Also, no statistically relevant differences could be observed in the expression of the Th2/IL-10 cytokines IL-4, IL-5 and IL-10. However, the IL-5 expression by $CD4^+$ and $CD8^+$ T cells did dominate within the Th2/IL-10 immune response (Fig. 3.26E). With regard to the Th9/Th17 immune response, expression levels of IL-9, IL17A and IL-22 showed also no significant differences between both groups. Even though, frequency of IL-9 expression by $CD4^+$ T cells was slightly increased and frequencies of IL-17A and IL-22 expression were faintly decreased (Fig. 3.26F).

To conclude, the a.t. of naïve $CD4^+$ T cells from WT C57/BL/6 mice into Rag2IL-2Rγ$^{-/-}$ C57BL/6 prior to infection lead to significantly shorter worm lengths, significantly lower Mf load in blood and TC and also to significantly reduced developmental embryonic stages in female worms. The a.t. of $CD8^+$ T cells had no clear effect on worm development. The immune profile of the a.t. $CD4^+$ and also $CD8^+$ T cells in TC and also blood is a Th9/Th17 biased immune response. Furthermore, these data also show that the transfer i) is valid, since cells are retained in the mouse, become activated and expand and ii) that their ability to produce cytokines is not impaired.

3.4.3 Adoptive transfer of $CD4^+$ T cells from IL-$10^{-/-}$ C57BL/6 donor mice leads to significantly reduced worm length and numbers of Mf in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i..

Interleukin (IL)-10 was first described as a Th2 cytokine but further investigations have shown that because of its immunosuppressive effect the production of IL-10 is more associated with a regulatory immune response (99, 143). Today it´s known that most of the cells of the innate and also of the adaptive part of the immune system can produce IL-10 (143) and that during a *L. sigmodontis* infection this cytokine has an antagonistic effect to IL-4 with regards to parasite loads (99). In this experiment sorted $CD4^+$ T cells from naive C57BL/6 or IL-$10^{-/-}$ C57BL/6 mice were adoptively transferred into Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice. After one day, animals with an a.t. of cells and untreated mice were infected with *L. sigmodontis* for 72 days. Amounts of Mf were detected in blood on days 50, 56, 63, 70 and on the final analysis day. Additionally, on day 72 p.i. mice were analysed for worm numbers, gender and length. Furthermore, Mf were detected in the TC of all groups.

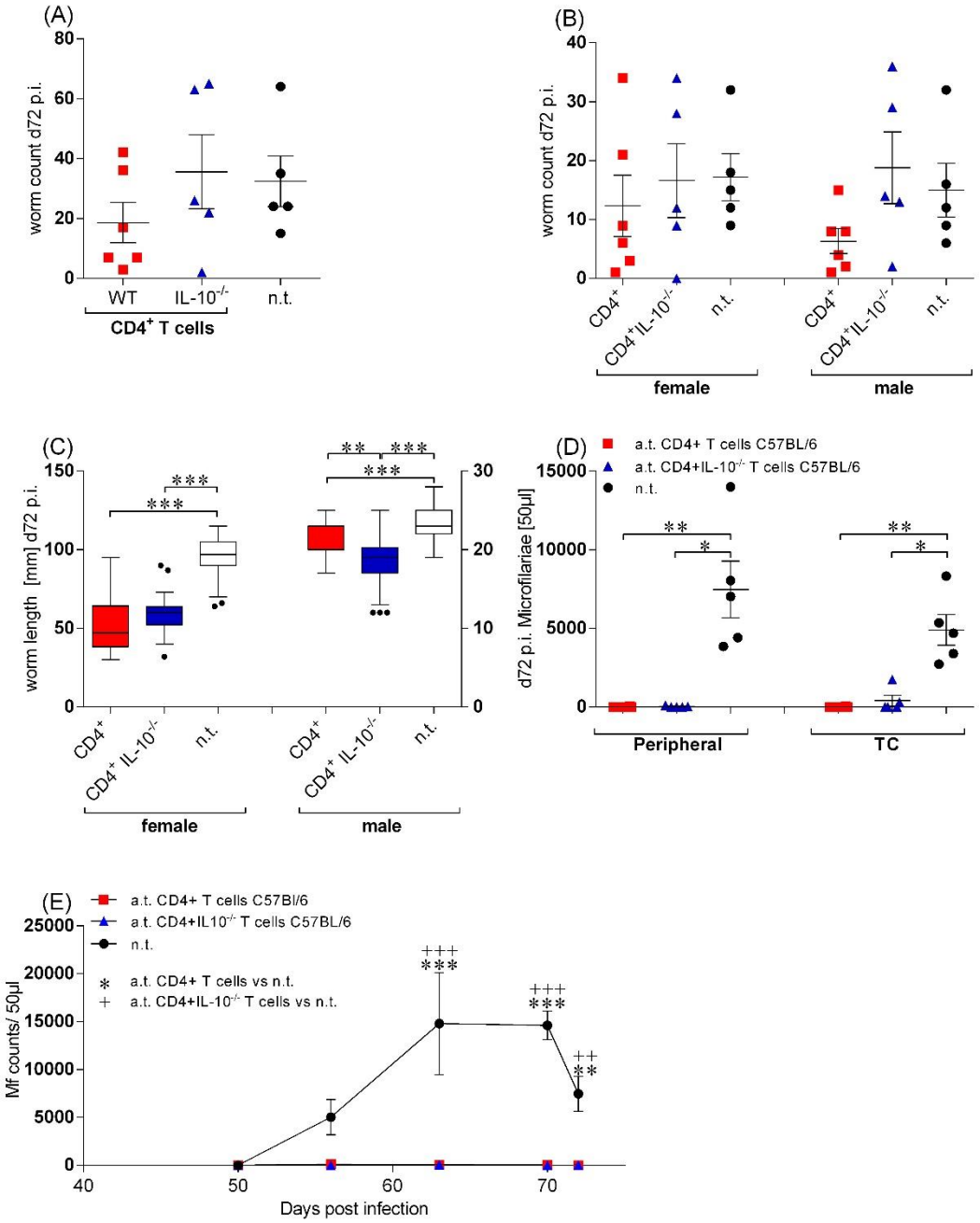


Figure 3.27: Adoptive transfers of CD4⁺ T cells from IL-10$^{-/-}$ C57BL/6 donor mice leads to significantly reduced worm length and numbers of Mf in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i.. CD4⁺ T cells from naïve WT C57BL/6 or IL-10$^{-/-}$C57BL/6 mice were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. After 72 days mice were assessed for absolute worm counts (A and B) and worm length (C) from individual worms. Levels of microfilariae (Mf) were detected on d72 p.i. in blood and thoracic cavity (TC) fluid (D) and additionally over time in the periphery. (E). Data show values from one individual infection experiment (n=6 a.t. CD4⁺ T cells, n=5 a.t. CD4⁺IL-10$^{-/-}$ T cells, n=6 n.t.) ± SEM. Asterisks denote significant differences (Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups (A-D) or (E) 2way ANOVA) between the groups indicated by the brackets or crosses (*p<0.05, **p<0.01 and ***p < 0.001).

As depicted in Fig. 3.27A, no significant differences could be seen in the overall worm counts between all three groups and this was independent of IL-10. Worms were again analysed with regards to their different gender and here also no differences could be observed in the amount of female or male worms between different groups (Fig. 3.27B). Interestingly, and in correlation with the previous a.t. data set, the length of female and also male worms were significantly reduced in mice following an a.t. with $CD4^+$ T cells from WT but additionally so with cells from IL-$10^{-/-}$ mice too. Interestingly, male worms from mice of the $CD4^+$IL-$10^{-/-}$ group were also significantly shorter compared to the worms from the $CD4^+$ WT group (Fig. 3.27C). Numbers of Mf on the experimental day in blood and also TC were significantly lower in both adoptively transferred groups compared to controls (Fig. 3.27D). Additionally, Mf numbers in blood were analysed over time and from day 63 p.i. Mf numbers in both a.t. groups were significantly reduced compared the normal infected group (Fig. 3.27E). Thus, IL-10-release from $CD4^+$ T cells has no impact on the development of *L. sigmodontis* worms and patency.

3.4.3.1 Prominent Th2/IL-10 immune response of $CD4^+$ T cells in the TC after a.t. of $CD4^+$ T cells from IL-$10^{-/-}$C57BL/6 mice in *L. sigmodontis*-infected Rag2IL-2R$\gamma^{-/-}$ C57BL/6 mice on day 72 p.i.

In line with the previous experiments, cytokine expressions were determined via FACS in Rag2IL-2R$\gamma^{-/-}$ C57BL/6 mice adoptively transferred with $CD4^+$ or $CD4^+$IL-$10^{-/-}$ T cells on day 72 post *L. sigmodontis* infection. First of all, to assess the adoptive transfer, frequencies of $CD4^+$ T cells from WT and KO-mice were analysed. Cell frequencies of both groups were significantly higher compared to the control group (Fig. 3.28A). Interestingly, the immune profile in the group receiving an adoptive transfer of $CD4^+$ T cells from WT and IL-10-deficient C57BL/6 mice showed a dominant Th2/IL-10 immune response (Fig. 3.28B and C, red part). With regard to the Th1 immune response, slight but not statistically significant higher frequencies of IFN-γ were measured in the $CD4^+$ T cell a.t group compared to the $CD4^+$IL-$10^{-/-}$ group (Fig. 3.28D). Also, no statistical differences could be observed in the expression of IL-4, IL-5 and also IL-10 production (Fig. 3.28E). Finally, expression of Th9/Th17 cytokines, more precisely of IL-9, IL-17A and IL-22 was also comparable between the groups (Fig. 3.28F).

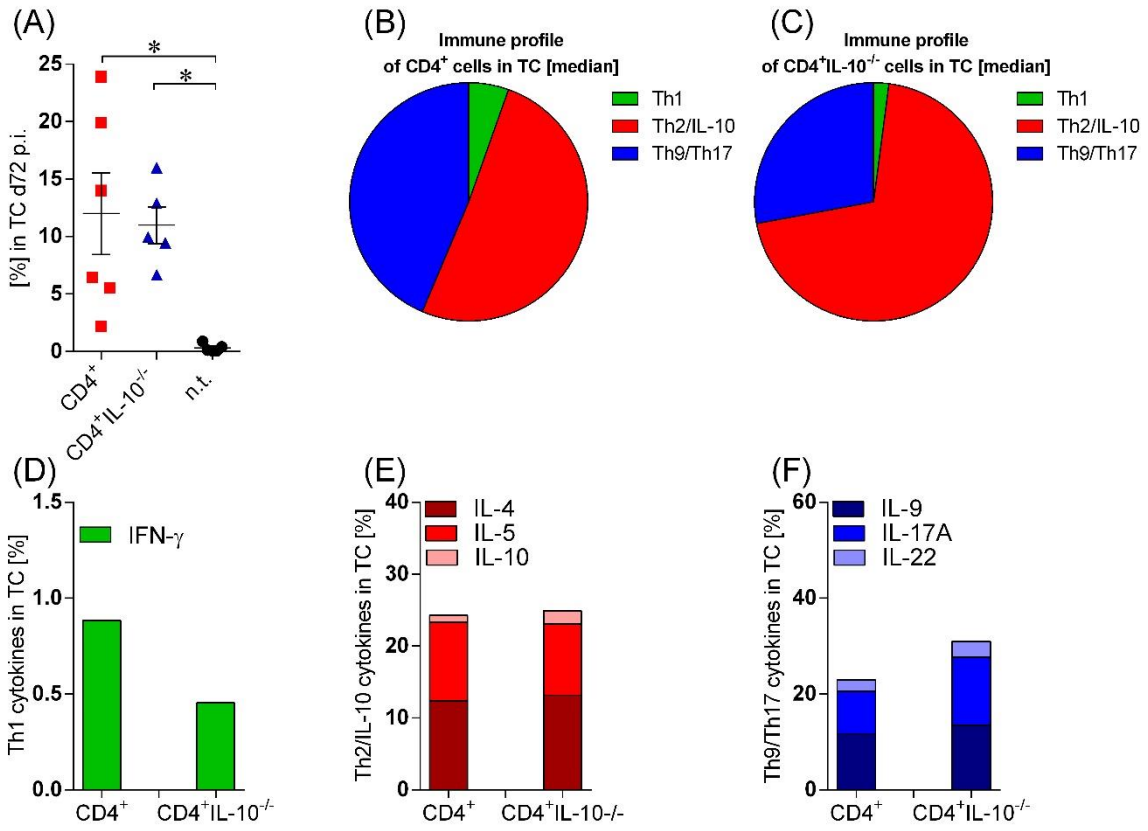


Figure 3.28: Prominent Th2/Treg immune response in TC after a.t. of CD4⁺ T cells from IL-10$^{-/-}$C57BL/6 mice in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i.. CD4⁺ T cells from C57BL/6 WT or IL-10$^{-/-}$ mice were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. Thoracic cavity (TC) cells isolated from individual mice on day 72 p.i. were assessed for frequencies of CD4 (A) by FACS. Immune profiles of residing CD4⁺ (B) and CD4⁺IL-10$^{-/-}$ T cells (C) in the TC were also analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 cytokines was analysed. Symbols show mean ± SEM for individual mice (n=6 a.t. CD4⁺ T cells, n=5 a.t. CD4⁺IL-10$^{-/-}$ T cells, n=6 n.t.) (A) or mean alone (D-E). Pie charts show median of Th1, Th2/Treg and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (*p<0.05). Data show results from one infection study.

3.4.3.2 Comparable Th1 biased immune response in blood of *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with CD4⁺ T cells from WT C57BL/6 or IL-10$^{-/-}$C57BL/6 mice at day 72 p.i.

Finally, the immune profile of *L. sigmodontis*-infected mice was analysed in blood 72 days post *L. sigmodontis* infection. First of all, frequencies of CD4⁺ T cells from WT C57BL/6 mice and also from IL-10$^{-/-}$C57BL/6 mice were statistically higher compared to controls (Fig. 3.29A). The Th1 immune response was the dominant one in the blood of both groups (Fig. 3.29B and C, green part). Whereas, mice transferred with CD4⁺ T cells from WT C57BL/6 mice showed comparable median values of Th2/IL-10 and Th9/Th17 immune responses (Fig. 3.29B, blue and red part), was the Th2/IL-10 immune response the second dominant part in mice with

CD4$^+$ T cells from IL-10$^{-/-}$C57BL/6 mice (Fig. 3.29C, red part). In more detail, expression of the Th1 cytokine IFN-γ was slightly but not significantly increased in the mice with CD4$^+$ T cells from WT C57BL/6 mice (Fig. 3.29D). Also, no statistically significant differences could be observed in the expression of the Th2/IL-10 cytokines IL-4, IL-5 and IL-10 (Fig. 3.29E). With regards to the Th9/Th17 immune responses, expression of IL-9, IL17A and IL-22 showed also no significant differences between both groups. However, the expression of IL-9 by CD4$^+$ T cells from IL-10$^{-/-}$C57BL/6 mice was slightly higher compared to the expression by CD4$^+$ T cells from WT C57BL/6 mice (Fig. 3.29F).

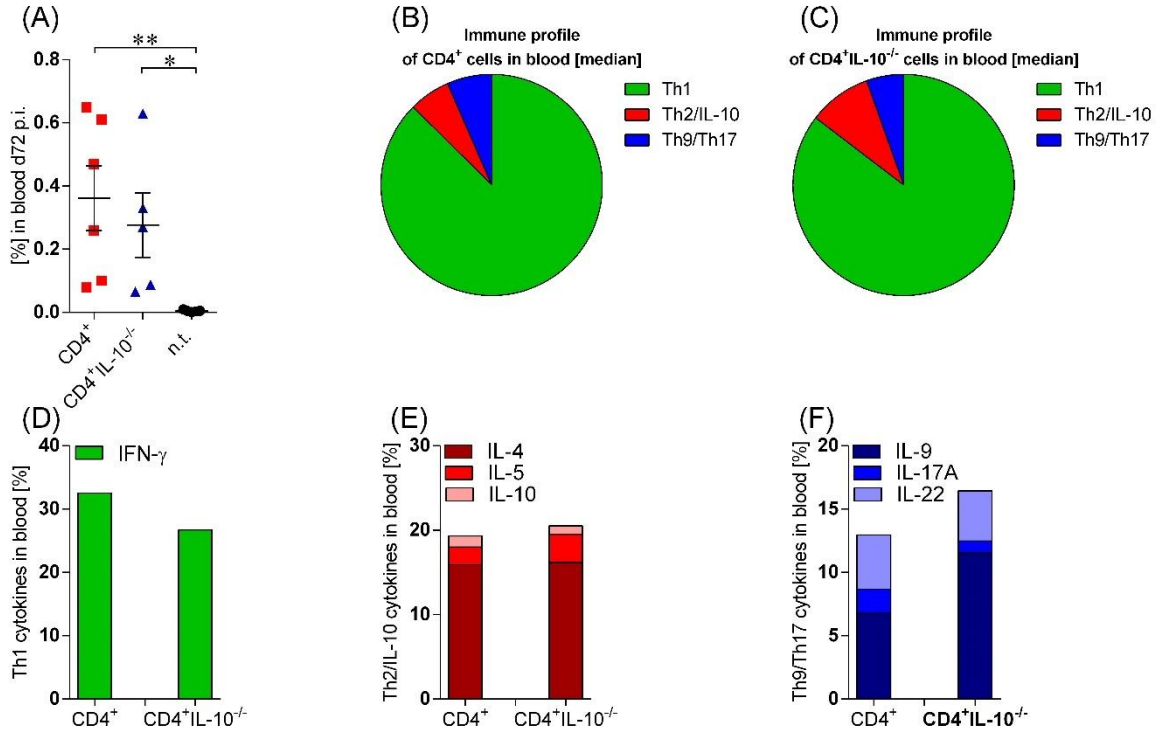


Figure 3.29: Reflective Th1 biased immune response in blood of *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with CD4$^+$ T cells from WT C57BL/6 and IL-10$^{-/-}$C57BL/6 mice at day 72 p.i.. CD4$^+$ T cells from C57BL/6 WT or IL-10$^{-/-}$ C57BL/6 mice were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. Blood cells isolated from individual mice on day 72 p.i. were assessed for frequencies of CD4 (A) by FACS. Immune profiles of CD4$^+$ (B) and CD4$^+$IL-10$^{-/-}$ T cells (C) were also analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 cytokines were analysed. Symbols show mean ± SEM for individual mice (n=6 a.t. CD4$^+$ T cells, n=5 a.t. CD4$^+$IL-10$^{-/-}$ T cells, n=6 n.t.) (A) or mean alone (D-E). Pie charts show median of Th1, Th2/Treg and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (*p<0.05 and **p<0.01). Data show results from one infection study.

In conclusion, the absence of IL-10 has no significant influence on the role of CD4$^+$ T cells in altering worm development and Mf control. Also, the immune profile of these cells in the TC and blood showed no statistically significant differences between CD4$^+$ T cells from WT

C57BL/6 and IL-10$^{-/-}$ C57BL/6 mice following a.t. and activation thereafter. The next step would be to differentiate T cell populations further into naïve T cells, central memory T cells, effector memory T cells and Tregs and whether the Tregs influence the balance of cytokine production.

3.4.4 Adoptive transfer of CD4^{+} T cells from IL-4$^{-/-}$ C57BL/6 donor mice leads to significantly reduced female worm length in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i..

It is well documented that Th2 cytokines are important mediators during a filarial infection and previous studies with both C57BL/6 and BALB/c filarial mouse models have noted alterations in worm pathology and host responses (67, 99, 117, 131). IL-4 is known to be part of the Th2 cytokine family and is an important parameter during *L. sigmodontis* infection since it was already shown that the lack of this cytokine leads to the development of a 33% higher patency in *L. sigmodontis*-infected C57BL/6 mice (98).

Therefore, in the next experiment CD4^{+} T cells from WT C57BL/6 mice and IL-4$^{-/-}$C57BL/6 mice were adoptively transferred into Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice one day prior to *L. sigmodontis* infection. After one day, animals with an a.t. of CD4^{+} T cells and also a group of mice with no transfer were infected with *L. sigmodontis* for 72 days. Numbers of Mf were detected during the course of infection and on the analysis day. Additionally, on day 72 p.i. mice were analysed for worm numbers, gender and length. Furthermore, Mf were detected in the TC of all groups.

No differences in worm count could be observed between the groups that received the transfer of CD4^{+} T cells and the control group without these cells (Fig. 3.30A). Additionally, worms were further subdivided according to their gender and here also no differences could be seen in the amount of female or male worms in all three groups (Fig. 3.30B). Moreover, worms were analysed for their length. As shown previously (see section 3.4.2), female worms from mice with an a.t. of CD4^{+} T cells from WT mice were significantly shorter compared to the n.t. control group. However, worms from mice that had received IL-4$^{-/-}$ CD4^{+} T cells were also significantly shorter compared to worms from the n.t. group (Fig. 3.30C). No difference in male worm length could be observed (Fig. 3.30C). On the experimental day, mice were also analysed for Mf in blood and the TC fluid. Here, Mf numbers in groups of mice that had received CD4^{+} T cells were low but not statistically significant reduced when compared to the control group (Fig. 3.30D). However, one does observe that Mf production during the course of infection was significantly reduced in both a.t. groups compared to the control group (Fig. 3.30E), which indicates that also IL-4 is not involved in the infection clearance. It should be noted here that in comparison to the other experiments (Figs. 3.23 and 3.27), infection rates were much lower than normal; this occasionally happens when dealing with natural infections.

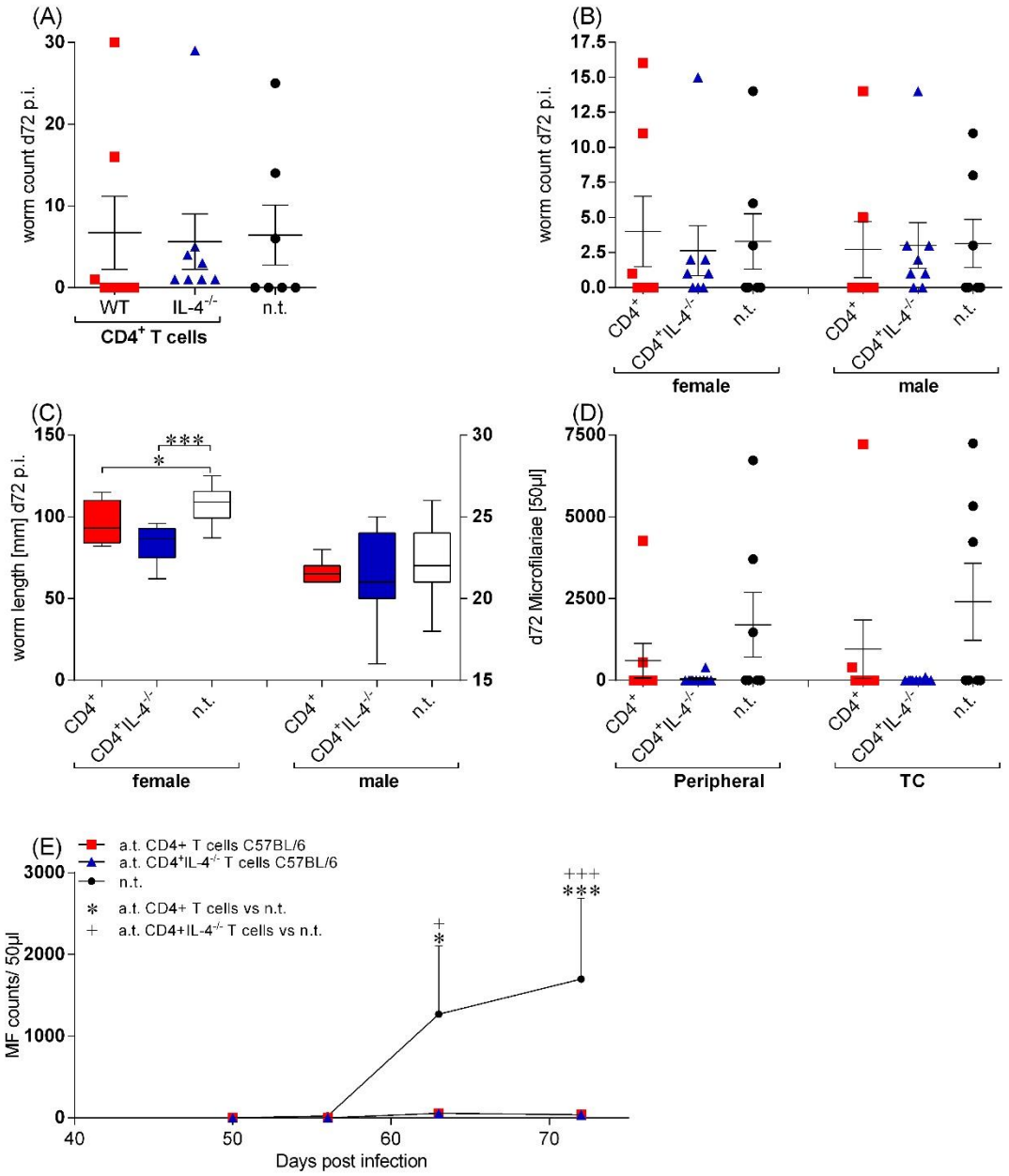


Figure 3.30: Adoptive transfer of CD4⁺ T cells from IL-4$^{-/-}$ C57BL/6 donor mice leads to significantly reduced female worm length in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i.. CD4⁺ T cells from naïve WT C57BL/6 or IL-4$^{-/-}$C57BL/6 mice were adoptively transferred (a.t.) into Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. After 72 days mice were assessed for absolute worm counts (A and B) and worm length (C) from individual worms. Levels of microfilariae (Mf) were detected on d72 p.i. in blood and thoracic cavity (TC) fluid (D) and additionally over time in the periphery. (E). Values show mean ± SEM of individual mice (n=7 a.t. CD4⁺ T cells, n=8 a.t. CD4⁺IL-4$^{-/-}$ T cells, n=7 n.t.). Figure shows representative data from one of two individual infection experiments. Asterisks denote significant differences (Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups (A-D) or (E) 2way ANOVA) between the groups indicated by the brackets (*p<0.05 and ***p < 0.001).

3.4.4.1 More Th1 biased immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with CD4^{+} T cells from IL-4$^{-/-}$ C57BL/6 mice at day 72 p.i.

Additionally, expression of different cytokines in the TC was analysed via FACS at day 72 p.i.. First of all, frequencies of CD4^{+} T cells from WT and KO-mice were analysed. The frequency of CD4^{+} T cells within the group that received IL-4$^{-/-}$ T cells, but not from the group following transfer of WT cells was significantly higher compared to the control group (Fig. 3.31A). The Th9/Th17 immune response was the most prominent response in the CD4^{+} WT group (Fig. 3.31B, blue part) whereas, in the CD4^{+}IL-4$^{-/-}$ group the immune response was more Th1 biased (Fig. 3.31C, green part).

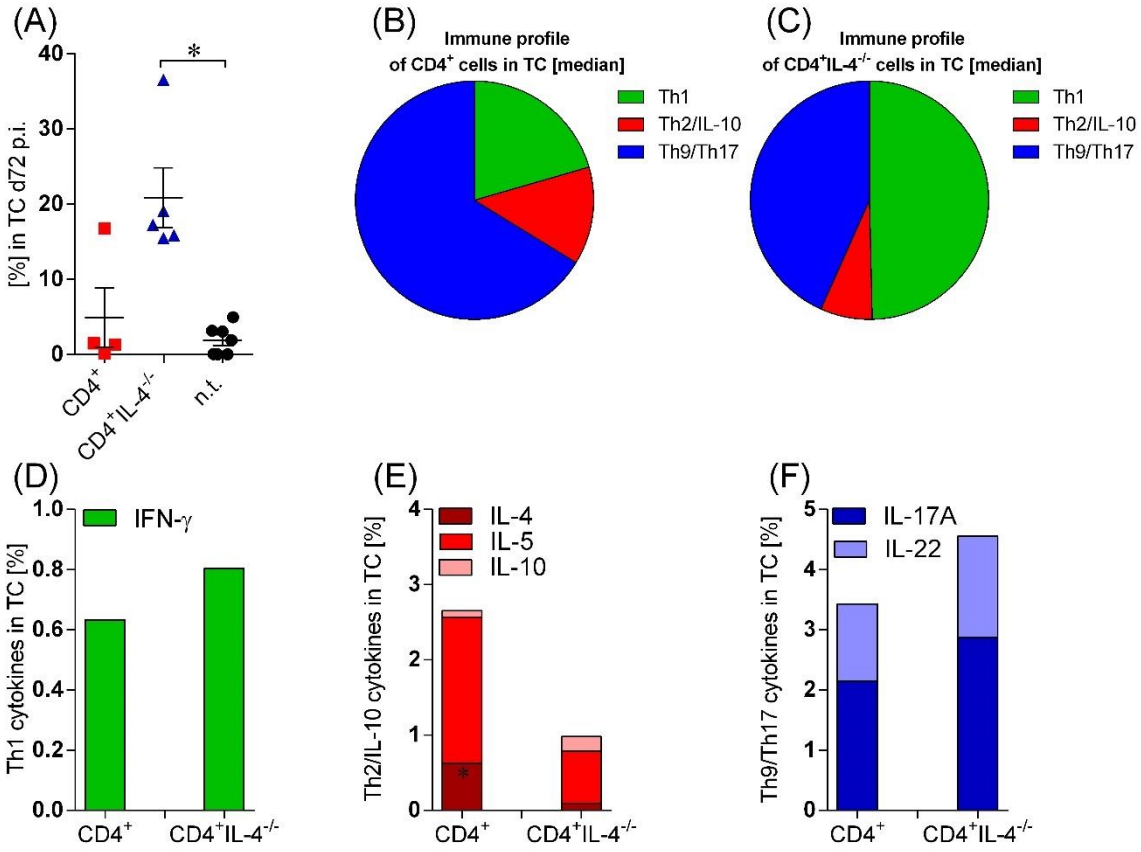


Figure 3.31: More Th1 biased immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with CD4^{+} T cells from IL-4$^{-/-}$ C57BL/6 mice at day 72 p.i.. CD4^{+} T cells from C57BL/6 WT or IL-4$^{-/-}$ C57BL/6 mice were adoptively transferred (a.t.) into Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. Thoracic cavity (TC) cells, isolated from individual mice on day 72 p.i. were assessed for frequencies of CD4^{+} (A) by FACS. Immune profiles of CD4^{+} (B) and CD4^{+}IL-4$^{-/-}$ T cells (C) were also analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 cytokines were analysed. Symbols show values for each mouse (n=4 a.t. CD4^{+} T cells, n=5 a.t. CD4^{+}IL-4$^{-/-}$ T cells, n=7 n.t.) ± SEM (A) or mean alone (D-E). Pie charts show median of Th1, Th2/Treg and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (*p<0.05). Data shown are from one of two individual infection studies.

With regard to the Th1 immune response, only IFN-γ was measured and no significant differences could be seen (Fig. 3.31D). In contrast, IL-4 expression was significantly reduced in mice with $CD4^+$ T cells from IL-$4^{-/-}$ mice whereas, no statistical differences could be observed in IL-5 and IL-10 secretion (Fig. 3.31E). Finally, there was also no statistical differences between Th9/Th17 immune responses (expression of IL-17A and IL-22) between both experimental groups (Fig. 3.31F).

To sum up, no direct influence of IL-4 on worm burden or Mf counts could be observed since results were comparable. The immune profile of $CD4^+$ T cells from IL-4 mice was more Th1 biased whereas the profile of $CD4^+$ T cells from WT C57BL/6 mice was more Th9/Th17 biased but here also no statistically significant differences could be determined. Here a further follow-up study would be required since the second a.t. infection lead to poor worm burden making the overall parasite and immune response data section difficult to interpretate.

3.4.5 Adoptive transfer of $CD4^+$ T cells from infected donor mice leads to significantly reduced worm burden, length of worms and number of Mf in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 72 p.i..

For the next experiment $CD4^+$ or $CD8^+$ T cells were sorted from *L. sigmodontis*-infected C57BL/6 mice 28 days p.i.. Cells from infected donors were than a.t. into Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice and one day later, animals with a.t. $CD4^+$ and $CD8^+$T cells and also a group of untreated mice were infected with *L. sigmodontis* for 72 days. Amount of Mf was detected in blood at days 50, 56, 63, 70 and on analysis day. Additionally, on day 72 p.i. mice were analysed for worm numbers, gender and length. Furthermore, Mf were detected in the TC of all groups.

In contrast to an a.t. of $CD4^+$ T cells from naïve C57BL/6 mice (Fig. 3.23), the adoptive transfer of $CD4^+$ T cells from infected donor mice lead to a significant reduction of worm numbers in these mice compared to the controls but also to mice with an a.t. of $CD8^+$ T cells from infected donors (Fig. 3.32A). Similar results could be also observed when worms were subdivided into female and male worms (Fig. 3.32B). Moreover, worm length of female worms was statistically shorter in the group following an a.t. of $^{inf}CD4^+$ group compared to the $^{inf}CD8^+$ T cells transfer group and control mice. Interestingly, female worms from mice with ^{inf}CD8 T cells were also significantly shorter compared to the control group (Fig. 3.32C). Additionally, male worms following the transfer of $^{inf}CD4^+$ but not $^{inf}CD8^+$ mice were also significantly shorter compared to the control group and again also to the $CD8^+$ group (Fig. 3.32C).

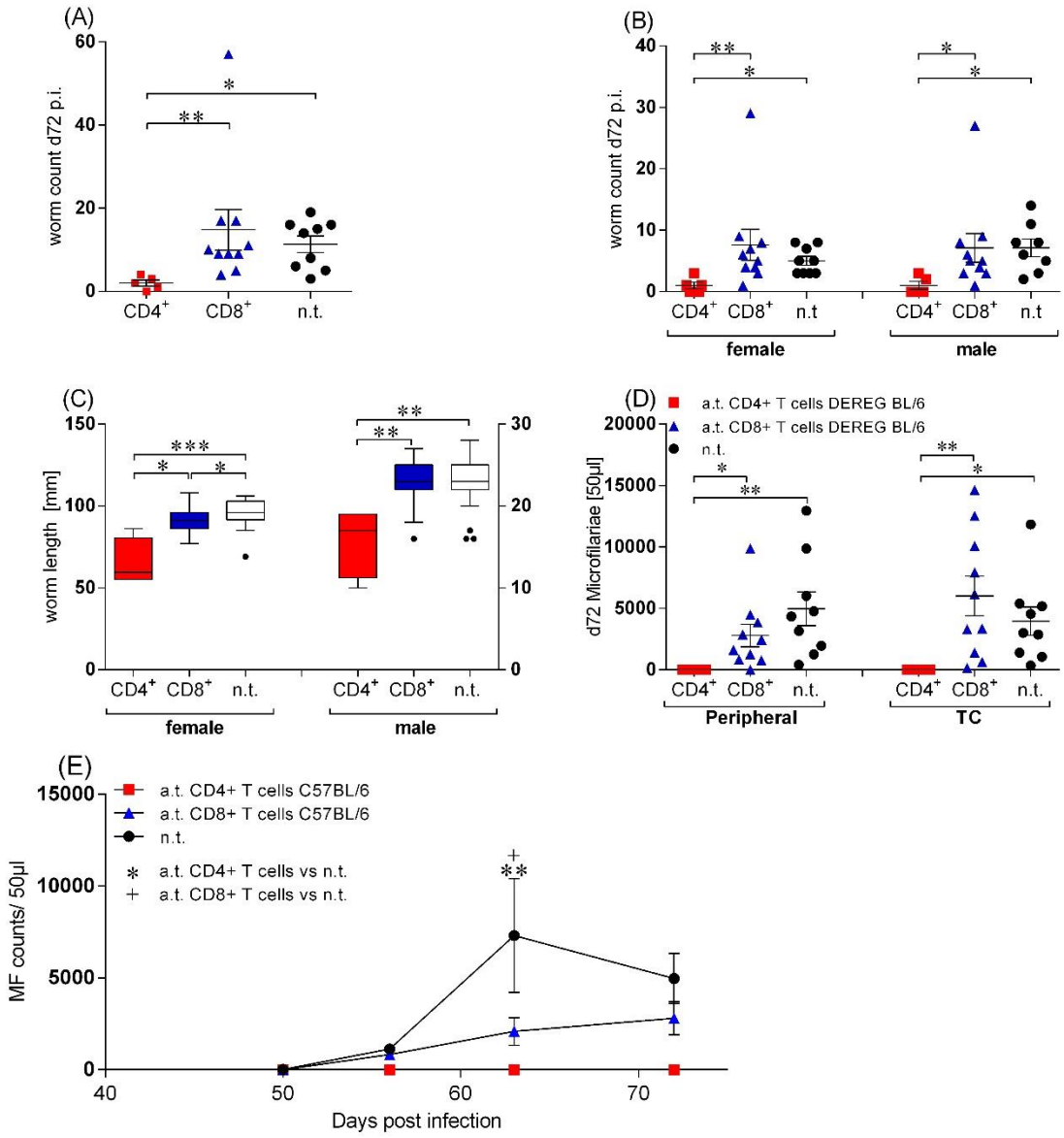


Figure 3.32: Adoptive transfer of CD4⁺ T cells from infected donor mice leads to significantly reduced parasitology in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients. Donor C57BL/6 mice were infected with *L. sigmodontis*. After 28 days CD4⁺ or CD8⁺ T cells were isolated from these mice via FACS sorting and adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. After 72 days mice were assessed for absolute worm counts (A and B) and worm length (C) from individual worms. Levels of microfilariae (Mf) were detected on d72 p.i. in blood and thoracic cavity (TC) fluid (D) and additionally over time in the periphery (E). Figure shows representative data from one of three individual infection experiments (n=5 a.t. CD4⁺ T cells, n=10 a.t. CD8⁺ T cells, n=9 n.t.) ± SEM with comparable data sets. Asterisks denote significant differences (Kruskal-Wallis test followed by Dunn`s multiple comparison test for further comparison of the groups (A-D) or (E) 2way ANOVA) between the groups indicated by the brackets (*p<0.05, **p<0.01 and ***p < 0.001).

As described earlier, Mf numbers were determined in the periphery and also in the TC on day 72 p.i.. Similar to the mice with a transfer of $CD4^+$ T cells from naïve C57BL/6 mice (Fig. 3.23), Mf counts were reduced in mice following $^{inf}CD4^+$ T cell transfers; indeed, almost no Mf could be detected throughout infection and upon analysis (Figs. 3.32D and E). Peripheral Mf counts following a transfer of $^{inf}CD8^+$ T cells was also observed but levels were not as drastically reduced as those seen in the group with $^{inf}CD4^+$ T cells (Figs. 3.32D and E). An interesting aspect does arise though here since levels of Mf in the TC of mice with $^{inf}CD8^+$ T cells were equally high as the control group indicating that the "release" order into the periphery for Mf was absent in this group (Fig. 3.32D).

3.4.5.1 More Th1 biased immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with a.t. $CD4^+$ or $CD8^+$ T cells from *L. sigmodontis*-infected C57BL/6 mice at day 72 p.i..

$CD4^+$ and $CD8^+$ T cells of TC were further analysed via FACS for cytokine expression at day 72 p.i.. In general, $CD4^+$ and also $CD8^+$ T cells are present at the site of infection (Fig. 3.33A). Interestingly, immune profiles in the TC of mice with $CD4^+$ and mice with $CD8^+$ T cells were comparable. Both of them showed a dominant Th1 biased immune response (Fig. 3.33B and C).

With regard to the Th1 immune response, only IFN-γ was measured. Frequencies of this cytokine were significantly lower in mice with $^{inf}CD4^+$ T cells compared to the group with $^{inf}CD8^+$ T cells (Fig. 3.33D). No statistically relevant differences were observed in IL-4, IL-.5 and IL-10 expression between both groups (Fig. 3.33E). Frequencies of IL-9 and also IL-22 were significantly higher in the group with a.t. $CD8^+$ T cells whereas, the level of IL-17A was reduced (although not statistically significant) in this group when compared to the controls (Fig. 3.33F).

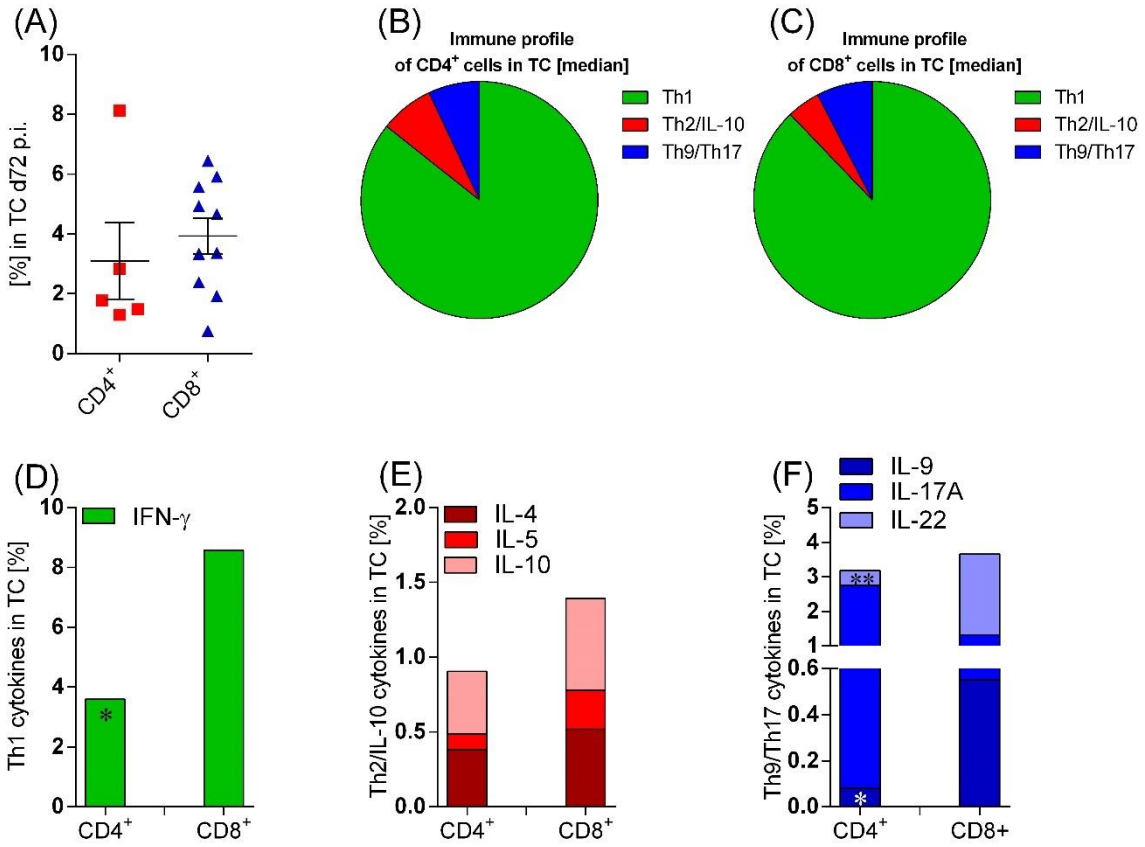


Figure 3.33: More Th1 biased immune response in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with a.t. CD4$^+$ or CD8$^+$ T cells from *L. sigmodontis*-infected C57BL/6 mice at day 72 p.i.. CD4$^+$ or CD8$^+$ T cells from *L. sigmodontis*-infected (d28) C57BL/6 donor mice were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. After 72 days, thoracic cavity (TC) cells were isolated from individual mice and assessed for frequencies of CD4$^+$ and CD8$^+$ T cells (A). Immune profiles of CD4$^+$ (B) and CD8$^+$ T cells (C) were also analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 cytokines was analysed. Symbols show frequencies for each mouse (n=5 a.t. CD4$^+$ T cells, n=10 a.t. CD8$^+$ T cells) ± SEM (A) or mean alone (D-E). Pie charts show median of Th1, Th2/Treg and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (*p<0.05). Data show representative results from one of three infection studies.

3.4.5.2 Th1 biased immune response in blood of in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice with a.t. of CD4+ T cells from infected donors

Finally, the immune profile of *L. sigmodontis*-infected mice was analysed in blood 72 days post *L. sigmodontis* infection. First of all, small frequencies of ^{inf}CD4$^+$ T cells and ^{inf}CD8$^+$ T cells could be detected in blood at this time point (Fig. 3.34A). The Th1 immune response was the dominant phenotype in the blood of mice with ^{inf}CD4$^+$ donor T cells. However, the Th9/Th17 immune response was also quite strong (Fig. 3.34B). The immune profile of CD8$^+$ T cells from infected donor mice is more Th9/Th17 biased, but here also the Th1 response was a strongly present (Fig. 3.34C).

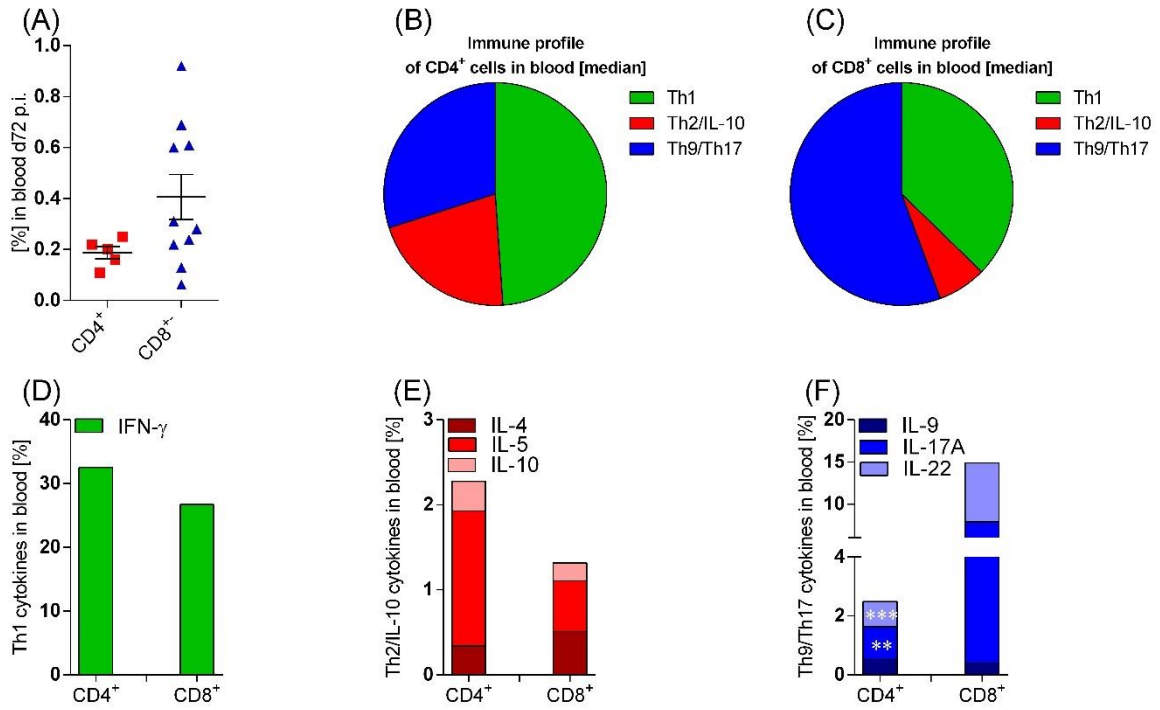


Figure 3.34: Immune responses in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients following an a.t. of ^{inf}CD4$^+$ or ^{inf}CD8$^+$ donor T cells. CD4$^+$ or CD8$^+$ T cells from *L. sigmodontis*-infected (d28) C57BL/6 donor mice were adoptively transferred (a.t.) in Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients one day before *L. sigmodontis* infection. Blood cells isolated from individual recipients on day 72 p.i. were assessed for frequencies of CD4$^+$ and CD8$^+$ T cells (A). Immune profiles of CD4$^+$ (B) and CD8$^+$ T cells (C) were analysed via FACS. Furthermore, expression of Th1 (D), Th2/IL-10 (E) and Th9/Th17 cytokines were analysed. Symbols show frequencies for each mouse (n=5 a.t. CD4$^+$ T cells, n=10 a.t. CD8$^+$ T cells) ± SEM (A) or mean alone (D-E). Pie charts show median of Th1, Th2/IL-10 and Th9/Th17 cytokine expression (B and C). Asterisks show significant differences (Mann Whitney test) between groups indicated by the brackets (*p<0.05). Data show representative results from one of three infection studies.

In more detail, expression of the Th1 cytokine IFN-γ was comparable between both groups (Fig. 3.34D). Also, no significant differences could be observed in the expression of the Th2/IL-10 cytokines IL-4, IL-5 and IL-10 (Fig. 3.34E). With regard to the Th9/Th17 immune response, expression of IL-9 showed also no significantly differences between both groups. However, the expression of IL17A and IL-22 mice was significantly lower in mice with CD4$^+$ T cells compared to mice with CD8$^+$ T cells from infected C57BL/6 donor mice (Fig. 3.34F).

To summarize, the a.t. of ^{inf}CD4$^+$ T cells and not ^{inf}CD8$^+$ donor T cells into Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipients, results in an overall reduction of parasitology, especially following transfer of ^{inf}CD4$^+$ T cells and also on their development since there was reduced worm length. These results are accompanied by a strong Th1 immune response in TC.

4 Discussion

The aims of this thesis focused on enhancing our knowledge about host:filarial interactions and moreover, deciphering the immune mechanisms and networks responsible for eliminating adult worm stages in *L. sigmodontis*-infected C57BL/6 mice. With regards to the former, we compared how $CD4^+$ T cells from early and late *L. sigmodontis*-infected BALB/c mice responded to soluble and pellet-derived antigens prepared from different worm genders. This study resolved two aspects that are important for the broader community of filarial researchers. Namely, cytokine release by $CD4^+$ T cells from *L. sigmodontis*-infected mice was similar to all worm antigen preparations and also, prior to Mf release, $CD4^+$ T cells failed to mount filarial-specific IL-13, IL-10 and IFN-γ responses (Wiszniewsky and Neumann et al., in preparation). Interestingly, IL-17 release was extremely low in these assays (data not shown) although a further study during this thesis period showed that Th17 signalling during *L. sigmodontis*-infection in IL-$17A^{-/-}$ C57BL/6 mice was important for host immune responses against filarial infection and appears to facilitate worm growth in those that reach the TC (117). These data exemplify how refined the balance is between host and filariae and that different immune networks are at play in varying susceptible hosts. The latter studies in this work delved into identifying these networks and determined links between innate and adaptive immunity since their interaction often dictates how adaptive immune responses are going to develop (144). First, we investigated how lack of TLR signalling components altered worm development and adaptive responses in semi-susceptible C57BL/6 mice. Interestingly, TRIF- but not TLR2-, TLR4-, IRF-1-, IRF-3- and Myd88-deficient C57BL/6 strains presented higher worm burden and reduced overall absolute cell numbers in the TC . *L. sigmodontis*-infected $TRIF^{-/-}$ C57BL/6 mice also presented changed cellular profiles with reduced levels of T cells but elevated macrophages indicating a crucial role of this adapter molecule for worm recovery and immune cell recruitment (125). The role of T cells, especially $CD4^+$ T cells has been a recurring research aspect in both rodent and human filarial infection settings (63, 84, 117, 142, 145-149). Our earlier studies showed that Rag2IL-2Rγ-deficient C57BL/6 mice allowed a full patent infection with high worm burden and Mf counts; indeed 30-fold higher than in *L. sigmodontis*-infected BALB/c mice in general (63, 65, 117). Therefore, we employed this infection system to begin identifying components that would revert the phenotype to a wildtype C57BL/6 state and thus discover how and why emerging adults (not larvae) are cleared. Interestingly, although the adoptive transfer of $CD4^+$ T cells had an effect on worm development and fecundity, they were not responsible for worm number *per se*. This is the first evidence that a multitude of factors are involved in worm clearance and this model provides an exciting platform to test numerous theories ranging from cell interaction during worm development to novel drug testing. The

sections below expand on the scientific findings in these studies and the conceptual advances these have made to the filarial research field.

4.1 Clearance of *L. sigmodontis* infection in C57BL/6 mice is accompanied by reduced *Wolbachia* loads

As already described, the rodent filariae *L. sigmodontis* is an important tool for understanding how host immune reactions during filariasis shape the outcome of infection. One of the most intriguing facets of this model is that worms can complete their life cycle in BALB/c mice (57, 63) but not in C57BL/6 mice, where infections are cleared as soon as adult worms have developed (61, 62, 64, 65). This is also relevant in man since many individuals reside in close proximity to transmitting vectors yet do not become infected, e.g. putative immunity in *O. volvulus* endemic communities (150-154). Until now, the mechanisms how *L. sigmodontis* infections in C57BL/6 are cleared before becoming established, is not understood. Several studies have compared *L. sigmodontis* infections in various immunocompetent mouse strains (57, 61-65, 125) trying to pinpoint a MHC-haplotype basis but many have missed an essential facet of filarial infections: *Wolbachia*. Thus, our studies here compared not only immune and parasitology parameters in BALB/c and C57BL/6 mice but *Wolbachia* levels as well since this third-party player is essential for worm fecundity and survival (37, 38, 40, 41, 43, 155).

Interestingly, on day 37 p.i. no significant differences in worm burden and development were observed in either *L. sigmodontis*-infected BALB/c or C57BL/6 mice. Female but not male worms from BALB/c mice were significantly longer when compared to counterparts from C57BL/6 mice. These findings support earlier studies from Babayan and colleagues and show that worm development in both strains is independent of how infections are performed: injection vs natural (62). This was also supported by the fact that C57BL/6 mice presented no significant differences in nodule formation or the number of encapsulated worms when compared to susceptible BALB/c mice. Earlier studies have already demonstrated that *Wolbachia* bacteria are obligatory endosymbionts in human filarial species but also in many other mammalian-associated species including *L. sigmodontis* (19, 35-38) and are essential for filarial survival, fertility and also larval moulting (37, 38, 40-43). Therefore, *Wolbachia* levels were also analysed in worms from BALB/c and C57BL/6 mice on day 37 p.i.. Interestingly, *Wolbachia* levels in the analysed worms (total worms, female worms or male worms) were significantly lower in worms from C57BL/6 mice when compared to those from the BALB/c group. These results were in line with earlier findings from our group which showed that *Wolbachia* levels in *L. sigmodontis*-infected C57BL/6 mice were also significantly lower than in worms recovered from Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice on day 30 p.i.. As mentioned earlier, in these deficient mice, *L. sigmodontis* parasites could also develop a patent infection including Mf production (65). However, the relationship between healthy worms, worm length and

Wolbachia amounts is still unclear. For example, another study showed that female worms recovered from IL-4Rα/IL-5$^{-/-}$ BALB/c mice were longer and produced more Mf than those from WT mice on day 72 p.i. but *Wolbachia* levels were comparable (117), indicating that worm size does not influence *Wolbachia* levels. Until now little is known about the mechanistic interactions between *Wolbachia* and filarial nematodes but female adult worms carry higher amounts of these bacteria than male worms and it appears that the bacteria are concentrated in the reproductive organs which tallies with the requirement of *Wolbachia* for worm fertility (36, 156). However, a recent study from Foray and colleagues has revealed that in filarial nematodes the presence of *Wolbachia* is important for the female germline development and therefore, also for the production of viable eggs and embryos since the depletion of these bacteria results in the loss of quiescence of a pool of germline stem cells and furthermore in the disorganization of the distal germline (157). The depletion of *Wolbachia* via Doxycycline results in the reduction of Mf production and has a macrofilaricidial effect too (41-43, 54, 129). In recent years, research has focused on other potential drug candidates for their elevated efficiency to target this filarial infection (158-163). Therefore, it seems that the reduced levels of *Wolbachia* are involved in preventing a chronic infection state, including Mf release in C57BL/6 mice, since all worms are carrying the *Wolbachia* bacteria and these bacteria are also important for the survival of the worms (43). Thus, the reduced *Wolbachia* loads in C57BL/6 mice may be an important mediator for the clearance of the *L. sigmodontis* infection in these mice although data does indicate that they are more likely to effect fecundity. So, the next step is to discover what is causing the reduction in *Wolbachia* levels. The location of this final moulting stage (L4-adult) does not change and since worm death is rapid (2-3 days) a hypothetical scenario emerges: in which the moulting step could trigger the expression or loss of a worm-specific factor that a) could cause natural death to the worm (not related to host responses at all) or b) the immediate attack of worms by host immunity. Without specific targeted experiments these intricate biological associations cannot be concluded. Nevertheless, two tools were available during this thesis, the Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice and comparison of infected C57BL/6 and BALB/c mice and both were studied to determine potential (immune) factors that may instigate reduced worm development and thus *Wolbachia* levels too.

4.2 Neutrophils, eosinophils and macrophages: lead role or understudies?

During helminth infections, neutrophils are one of the first cells at the site of infection (75, 164, 165). Studies from Saeftel and colleagues showed that neutrophils are also directly involved in worm recovery rates. Moreover, disturbed neutrophil recruitment results in more than twofold higher worm numbers and significantly reduced nodule formation in mice on the BALB/c background (137). However, in the studies performed here, frequencies of neutrophils were significantly reduced in infected C57BL/6 mice compared to the BALB/c mice on day 37 p.i.

regardless of the fact, that the overall cell number was increased, which indicates that at that time-point, neutrophils are not the key cell population for infection clearance in the semi-susceptible C57BL/6 strain. Furthermore, neutrophils are also mediators for worm encapsulation and subsequent inflammatory nodule formation (112, 137). So, it is more than interesting that even though the BALB/c mice showed significantly higher frequencies of neutrophils, numbers of encapsulated worms and also nodules were comparable between both mice strains on day 37 p.i.. Several earlier studies have described that the neutrophil chemotactic activity and the neutrophil accumulation around adult filarial worms are dependent on the presence of *Wolbachia* bacteria, in an IFN-γ dependent manner (62, 114, 137). These findings underline our data, since the reduced neutrophil frequency in the semi-susceptible C57BL/6 mice was also accompanied by significantly lower *Wolbachia* levels in the worms.

Frequencies of eosinophils are also significantly reduced in the infected semi-susceptible C57BL/6 mice compared to BALB/c mice at this developmental stage and the importance of these cells has already been described (75, 76, 81, 82, 84). For example, it is known that eosinophils and their associated products like eosinophil peroxidase (EPO) or the major basic protein (MBP) and chemokines or cytokines like eotaxin-1 or IL-5 are also important parameters for the fight against *L. sigmodontis* infections (76, 82, 112, 116). In association, *L. sigmodontis*-infected BALB/c mice deficient for eosinophils or IL-5, showed higher worm loads during the patent phase of infection (131) and mice over-expressing IL-5 presented higher eosinophilia which results in faster clearance of *L. sigmodontis* infection (81). In C57BL/6 mice, lack of IL-5 results in impaired parasite development during the early phase of infection (166). Additionally, an artificially induced eosinophilia results in an increased Mf release by adult female worms (166). Therefore, the fact that we observed lower frequencies of these cells in the C57BL/6 strain and not in the BALB/c mice is even more interesting since the latter do not clear the infection. Thus, hypothetically, eosinophils could be more important during the early (moulting and development of larvae) or late phase (Mf release) of the infection.

Macrophages, next to DCs are important antigen presenting cells (2). During *L. sigmodontis* infections these cells are one of the most prominent cell populations in the TC (108, 167, 168)). There are various types of macrophages and only a few investigations have analysed the different compositions of macrophages in the TC of *L. sigmodontis*-infected C57BL/6 mice and BALB/c mice, during the different infection stages. In the present study, frequencies of macrophages were significantly increased in the *L. sigmodontis*-infected C57BL/6 mice compared to the infected BALB/c mice. Campbell and colleagues have demonstrated that a special subset of macrophages (F4/80^{hi} Mϕ) accumulates in the TC of *L. sigmodontis*-infected C57BL/6 mice but not in infected BALB/c mice. Additionally, they also showed that macrophages in the TC of the latter ones displayed enhanced programmed death ligand 2

(PD-L2) expression. The expression of PD-L2 also strongly correlates with the worm recovery rate in BALB/c mice (169). The ligand of PD-L2 is expressed on Th2 cells during chronic *L. sigmodontis* infections in BALB/c mice which in turn lead to a reduced IL-4 and also IL-5 production (170) and studies from Le Goff and colleagues have demonstrated that the production of IL-4 is important for the prevention of a chronic infection in C57BL/6 mice but not in susceptible BALB/c mice (98). Alternative activated macrophages (AAM) are also induced during filarial infections (69, 106, 108, 171, 172). Studies with C57BL/6 mice have demonstrated that there is a rapid proliferation of local AAM, during the first 10 to 15 days post *L. sigmodontis* infection (173). Since it is already known that AAM act in a more suppressive manner and as mediators of specific immune responses during filarial infections (16, 174) these cells together with PD-L2 ligand expressing macrophages, are probably involved in establishing a chronic *L. sigmodontis* infection in BALB/c mice. The performed cytospin method used here is limited in detecting the actual phenotype of the macrophages. However, in our earlier studies using Rag2IL-2Rγ$^{-/-}$-deficient C57BL/6 mice we performed flow cytometry to determine the frequency of neutrophils, eosinophils, macrophages and AAM in cells from the TC. Two aspects could be ascertained; 1) all cell types were detectable in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$-deficient C57BL/6 mice, albeit levels of AAM were low and thus 2) this cell type does not seem to directly affect worm growth and development (but actions could be prompted by other cells e.g. T cells). Therefore, these data revealed that adaptive immunity plays a key role in the prevention of chronic *L. sigmodontis* infections in semi-susceptible C57BL/6 mice (65). Thus, as consistently observed with chronic host:helminth interactions there are a multitude of players and factors which drive these responses (175) and the following section discusses how innate signalling may help shape them.

4.3 Expanding on innate-adaptive interplay, TRIF becomes a new partner in deciphering host immunity to filariae

Macrophages and DCs have an essential role in innate signalling too; specifically responding to stimuli that lay the foundation for adaptive responses. The central adaptor molecule MyD88 is associated with most TLR signalling pathways with the exception of TLR3, whereas TRIF is only related to TLR3 and TLR4 (7-9, 141). Several studies have demonstrated that TLRs are important for immune responses against filarial infection (6, 39, 63, 140, 175). Moreover, the endosymbiotic *Wolbachia* bacteria can induce innate and also adaptive immune responses via TLR2 and also TLR6 during filarial infections (44, 120). The activation of pattern recognition receptors, like TLRs are more associated with innate immunity but they are also expressed on adaptive immune cells (8, 138, 139). Indeed, previous studies from our group using susceptible BALB/c mice demonstrated that lack of TLR2 had a negative impact on filarial specific CD4^{+} T cell cytokine production and lack of TLR4 resulted in up to 93% patency compared to 40-60% in WT BALB/c mice (63). These findings were also supported by an earlier study from Pfarr

and colleagues which demonstrated that TLR4 is necessary for the release of Mf from adult female worms at the site of infection (122). In general, only two *in vitro* based studies have investigated the role of the TRIF pathway during filarial infections and detailed no direct influence on these infections (120, 176). Therefore, in the present study the role of TRIF (and its associated receptors and molecules) and also MyD88 during *L. sigmodontis* infection was analysed *in vivo* using knockout strains on the C57BL/6 background.

Interestingly, mice deficient for TLR3 but not TLR4, showed a delayed moulting process of L4 larvae into adult worms resulting also in shorter worms compared to the WT group. This phenotype was also reflected in *L. sigmodontis*-infected C57BL/6 mice deficient for TRIF. Furthermore, in $TRIF^{-/-}$ mice, worm numbers were also significantly higher compared to the controls, which indicates that a disruption of the TRIF pathway results in a delayed moulting process but higher worm recovery (125). Since $TLR3^{-/-}$ and WT mice had comparable worm numbers, it seems that in this context only the adapter molecule TRIF is responsible for infection control and the TRIF pathway could be at least partly restored via TLR4 in the *L. sigmodontis*-infected $TLR3^{-/-}$ mice. Interestingly, an injection of the TLR3 related trigger polyinosinic-polycytidylic acid (poly(I:C)) into *Brugia malayi*-infected NOD/LtSz-scid/scid mice resulted in an increased worm burden (177), which underlines our findings that the TRIF-dependent pathway, including TLR3, plays an important role for worm development and therefore also for parasite specific immune response. The phenotype in infected $TRIF^{-/-}$ mice was accompanied by increased frequencies of macrophages in the TC of the infected $TRIF^{-/-}$ mice. As already mentioned, AAMs with an immune suppressive phenotype are recruited into the TC during *L. sigmodontis* infections (69, 106, 108, 171, 172) and recent studies from Turner and colleagues have demonstrated that AAMs are activated via IL-4/IL-4Rα, induced eosinophil recruitment to the infected tissue and granuloma formation by these cells during filarial infections (178). However, more studies are needed to reveal the exact monocyte subset in the TC and also in addition, if AAMs are responsible for parasite survival and higher worm numbers in $TRIF^{-/-}$ C57BL/6 mice.

Several studies have demonstrated that reduced frequencies of $CD4^+$ T cells and also eosinophils support worm development (81, 84, 131, 179, 180) and therefore support our results, since higher worm numbers in mice deficient for TRIF were accompanied by reduced frequencies of eosinophils and also $CD4^+$ T cells at the site of infection. Interestingly, frequencies of $CD19^+$ B cells were increased although levels of filarial-specific IgM, IgGb and IgE antibodies were all lower than those observed in WT mice. However, to reveal a possible correlation between increased B cell recruitment and the lack of TRIF, further studies need to be performed. Collectively, these results underline that the interaction of filarial parasites and TLRs is important and that the TRIF-pathway is involved in worm development during the final

moulting process of *L. sigmodontis*-infected mice on the C57BL/6 background. Moreover, the overwhelming evidence that filarial infections provoke action and responses from so many different cell types and factors prompted us to simplify the model further and in the following section, the findings by using Rag2IL-2Rγ-deficient C57BL/6 mice are discussed.

4.4 The Rag2IL-2Rγ$^{-/-}$ C57BL/6 mouse model - A new tool to investigate human filarial infections

As already mentioned, millions of people in the tropics and subtropics are at risk for infections with human pathogenic filarial parasites. Clinical studies of these helminth infections are difficult since studies must comply with ethical requirements and different ethnical and religious settings can hinder the recruitment of patients and sufficient sample collections. Furthermore, accurate diagnostics of the different filariae pose other issues since a community of exposed individuals may present active (e.g. Mf+), non-active (e.g. residual antibodies), resolved (e.g. dying worms) or newly treated stages of infection. Thus, an array of methods (e.g. PCR, ELISA, microscopy) is required to conclusively determine the infected state and additional epidemiological data are needed to support the findings (e.g. last MDA intake). These are all time consuming and require trained personnel. To complicate matters further, there are several groups of infected individuals beginning with those that never show signs of infection or exposure. Moreover, it is known that *W. bancrofti*-infected individuals can develop patent infections, which means that adult female worms release Mf or they can have a latent state, adult worms but no Mf and this latter group was only confirmed through the use of ultrasound which detected moving adult worms (124, 181-183). Until now it is also not known if *M. perstans* can develop latent (amicrofilaremic) infections in humans and therefore, it is more than likely that the estimated infection rate of 114 million people (25, 184) is even higher. In terms of clinical manifestations, infections can result in hydrocele or chronic lymphedema of the legs and arms (*W. bancrofti*) or severe skin disorders during onchocerciasis (18, 19, 32-34) In contrast, infections with *M. perstans* result in mild clinical manifestations and therefore, often remain undiscovered. (19, 25, 184). Obtaining worms for study or analysis is also impaired since, with the exception of *O. volvulus* that hides in nodules under the skin, others are hidden deep in the body. This includes, *M. perstans* worms which are mostly found in body cavities like the pericardium or the mesentery and the perirenal connective tissues thus obtaining worms is near impossible without an autopsy. Therefore, other systems are required to more readily investigate these worms especially their immune-provoking or regulatory actions.

4.4.1 *In vitro* versus *in vivo*, the necessity of both?

In vitro models are useful tools when investigating any filarial interactions in more detail especially drug development (59). Recent studies have also revealed techniques to finally

grow worms *in vitro* which has led to both biological discoveries (185-189) and also provided material to prepare specific filarial worm extracts for culturing with cells derived from infected individuals (148, 189). However, when using only *in vitro* models it is hard to reveal the complex immune mechanisms and cellular components which are induced during the different developmental stages of the parasites life and are also involved in infection clearance. The discovery that the rodent filariae *L. sigmodontis* undergoes its complete life cycle in WT BALB/c mice by Petit and colleagues in 1992 (57) was a milestone in investigations of filarial infections. This model offered the opportunity to analyse different parameters of the innate and adaptive immune response during the different developmental stages of a filarial infection. By using this model, the importance of e.g. IL-4, IL-5 and IFN-γ but also of $CD4^+$ T cells and B cells could be revealed (84, 98, 126, 190). Knowing whether these parameters are important to all filariae pathogenic to man is difficult since the helminths do not develop well or at all in BALB/c mice. However, some of them can be investigated *in vivo* with other mammalian animal models. *B. malayi* is one of the human pathogenic parasites which cause lymphatic filariasis and (6, 19, 26, 103) there are some animal models where this parasite can develop its complete life cycle. These include for example ferrets, rhesus monkeys and jirds, the latter being the major source of worms for antigen preparation and research (191-193). Additionally, similar clinical manifestations to those that arise in infected humans can also be observed. However, they are also inconsistent to one another, for example, whereas, 85-90% of the ferrets develop patency after 3 months, in rhesus monkeys the development of a patent infection is highly variable (194, 195). Chimpanzees are permissive for the human pathogenic parasite *O. volvulus*. In most of these infected animals, the helminth can also develop a patent infection although some produce Mf only temporarily or remain amicrofilaridermic (192, 196). They also develop nodules (including adult worms) in the skin but in contrast to humans no eye lesions were observed (192, 197, 198). Baboons and also *Mandrillus* spec. are susceptible for *L. loa* and the course of infection is comparable to humans, since they can also be amicrofilaremic, transiently microfilaremic, or stably microfilaremic (199, 200). In contrast, in WT BALB/c or Swiss mice, *L. loa* larvae survived only one week (201, 202). Thus, there is a lack of murine models that can host these human infections (192) which focused research on strains deficient in certain pathways (IL-4/5 signalling) (117) or more severe immune-deficient mice such as the Rag2IL-2R$\gamma^{-/-}$ C57BL/6 mice (65).

4.5 Extended *L. sigmodontis* infection times in Rag2IL-2R$\gamma^{-/-}$ C57BL/6 mice reveal just how important adaptive cells are in controlling filariasis

In *L. sigmodontis*-infected C57BL/6 mice, no Mf are produced and therefore transmission of the parasite would be interrupted leading to a break in the cycle and consequential extinction of the helminth. It may also reflect the occurrence of non-infections in endemic communities which may never present a viable infection. On the opposite end of the spectrum and

expanding on the earlier 2015 studies (65), we demonstrated that in Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice, patency reached 100% with exceedingly high Mf levels (30-fold over BALB/c mice, unpublished findings) that were still detectable after 190 days post *L. sigmodontis* infection, even though these mice are on the C57BL/6 background. This strain is comparable and seems more viable for infection than RAG$^{-/-}$/IL-4$^{-/-}$ C57BL/6 mice, since here adult worms had developed by d30 p.i. and on day 60 p.i. Mf were detectable and reportable higher than those found in BALB/c mice (166). As mentioned earlier, fecundity in female worms can be measured following analysis of reproductive stages (128, 131). Analyzation of the embryonic stages in worms isolated from these infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice at day 72 post infection showed a typical Mf development over all stages (oocytes, divided eggs, pretzels and microfilariae). Interestingly, when comparing to previous studies performed with worms recovered from day 72 infected BALB/c mice, parameters measured for embryogenesis were approximately 20–30 fold higher highlighting that that environment in Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice facilitated worm development to a greater extent (Layland, unpublished findings). This is the first data on embryogenesis in this setting and alongside the fact that all worms were "free-living", that is, no host cell attachment, it underlies the fact that adaptive cells (or potentially NK cells) are the most important factors in eliminating worms in WT C57BL/6 mice. The following sections discuss the effects of reconstituting the mice with either CD4^{+} or CD8^{+} T cell compartments.

In this thesis work, the role of NK cells, also deficient in Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice was not studied, neither was the role of B cell populations. Very few studies have addressed the role of NK or NKT cells that are important in fighting bacterial infections (203). A study on *O. volvulus* showed that individuals without clinical symptoms presented reduced numbers of NK and NKT cells. Hypothetically, since these individuals have higher worm burdens there may be increased amounts of filarial-derived glycolipids and glycoproteins too, which may cause these populations to migrate to the skin, resulting in decreased numbers in blood (34). Using an *in vitro* based system with PBMCs and live Mf from *Brugia* species parasites, Babu and colleagues identified that filariae induced NK cell activation and cytokine secretion leading to apoptosis indicating that such mechanisms could down-regulate host immune responses (73). Studies from Korten and colleagues have demonstrated that the depletion of NK cells in *L. sigmodontis*-infected BALB/c mice resulted in an increased parasite load and increased Th2 cytokines in plasma in the chronic infection stage (86). They also demonstrated that this was not observed with NKT cell deficient mice. However, no data are available about the role of NK cells in C57BL/6 mice during *L. sigmodontis* infection. As mentioned previously, findings from Le Goff and colleagues demonstrated that B cells (μMT-deficient) do not facilitate the development of a chronic infection in C57BL/6 mice (98) even though in BALB/c mice lacking B cells leads to higher Mf loads (101). Thus, in the next section it will be discussed that this

thesis presents evidence that CD4+ T cells appear essential for a reduced fecundity, but they are not responsible for worm elimination. Therefore, it is not a one keylock scenario and future studies need to address combinations of NK, B and T cells to decipher the full mechanism of host elimination.

4.5.1 CD4+ T cells are essential for the prevention of Mf release in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice

As described earlier, CD4+ T cells are important for worm load in BALB/c mice since the depletion of these cells results in increased worm loads at day 28 p.i. and also increased Mf loads over time. Additionally, diminished Th2 cytokine and IgE production was observed (84). In humans, filarial infections are associated with increased parasite specific CD4+ T cell proliferation (145, 204) and also increased secretions of pro-inflammatory Th1 and Th17 cytokines (34, 205). *In vitro* studies with human CD4+ T cells showed that the early T cell response is dominated by pro-inflammatory Th1 cytokine production which in turn is dependent on the presence of APCs (68). However, asymptomatic LF in humans is associated with more down regulated CD4+ T cell response (32, 145), whereas steady state, patent LF is associated with IL-10 expressing Tregs (206, 207). Following an adoptive transfer of donor WT C57BL/6 CD4+ T cells into Rag2IL-2Rγ$^{-/-}$ C57BL/6 recipient mice no direct influence on worm load was observed. However, both female and male worms, were significantly shorter when compared to the control group (no adoptive transfer) and more interestingly, almost no Mf were detectable in blood and the TC in these mice indicating difficulties with embryogenesis. Further inspection showed that the development of Mf in the female worms was impaired, since only few numbers of different embryonic stages were detectable following CD4+ T cell transfer; correlating with the observed decrease in Mf counts. Indeed, levels were on average 95 fold lower than in worms received from Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice without cell transfer and moreover, became more comparable to those normally observed in worms from BALB/c infected mice (Layland unpublished findings).

Comparative studies were also performed using isolated CD8+ T cells from naïve C57BL/6 donor mice that were adoptively transferred into Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice one day before *L. sigmodontis* infection. Interestingly, the adoptive transfer of CD8+ T cells has almost no impact on *L. sigmodontis* infection in the Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice. Only the average length of female worms was reduced when compared to the length of female worms in the control group. In general, CD4+ T cells are helper cells with a more regulatory phenotype, which activates other effector cells like CD8+ T cells. In contrast, CD8+ T cells have a cytotoxic phenotype and attack infected cells directly and play an important role in the defence against intracellular pathogens (1, 2). Even if CD8+ T cells are characterized as cells directly responsible for the elimination of pathogens, these cells are obviously not responsible for worm

clearance and the suppression of Mf release in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice. Findings of Korten and colleagues support our findings, since their studies showed also that in *L. sigmodontis*-infected BALB/c mice depletion of CD8^{+} T cells had no impact on worm counts or Mf numbers (86), although here no embryogenesis parameters were looked at. However, naïve CD4^{+} T cells alone do appear able to eliminate the infection and since Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice lack T, B and NK cells the data indicate that at least B or NK cells may be involved in this process. Therefore, it would be interesting to transfer CD4^{+} T cells in combination with B or also NK cells to analyse if these cells work together to eradicate the infection.

4.5.2 Actions of CD4^{+} T cells on fecundity and Mf secretion appears independent of IL-4 and IL-10.

Humans and mice, susceptible for filarial infections and without severe pathology, present a strong Th2 immune response with a dominant regulatory phenotype (90, 91, 208). The anti-inflammatory cytokine IL-10 is known to be important for Treg mediated suppression of Th17 dependent inflammatory immune responses during filariasis (34, 88, 209). Moreover, the lack of IL-10 results in the reversal of the susceptibility in C57BL/6 mice deficient for IL-4 (99). However, here, lack of IL-10 on CD4^{+} T cells had no significant impact on the ability of the transferred cells to reduce the anticipated fecundity and Mf counts. Both, Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice recipients of donor CD4^{+} T cells from IL-10$^{-/-}$ or WT C57BL/6 mice had similar worm loads and reduced Mf counts. Only the length of the male worms was significantly shorter in the latter group. Therefore, lack of IL-10 on CD4^{+} T cells alone doesn't result in the production of Mf. Hoffmann and colleagues injected Mf directly in the TC and here the depletion of IL-10 supported the Mf survival but also only in the presence of at least one female worm (61). Additionally, the overexpression of IL-10 by macrophages results in full patency in *L. sigmodontis*-infected mice on the C57BL/6 background (100).

Several studies have identified that IL-4 plays a role in filarial infections. For example, depletion of IL-4 resulted in enhanced susceptibility in *L. sigmodontis*-infected C57BL/6 WT mice, comparable to infected BALB/c mice (98, 99). Moreover, lack of IL-4 in RAG$^{-/-}$IL-4$^{-/-}$ C57BL/6 mice also results in significantly higher Mf loads compared to infected WT BALB/c mice (166). However, Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice a.t. with CD4^{+}IL-4$^{-/-}$ T cells showed no differences in worm load and Mf numbers compared to those mice a.t. with CD4^{+} T cells from WT donors. This is in contrast to the study of Le Goff and colleagues, which showed that lack of IL-4 resulted in Mf secretion by female *L. sigmodontis* worms in C57BL/6 mice (98). Here, our experiments were refined to IL-4 release from T cells but IL-4 can also be secreted by macrophages, mast cells, basophils, eosinophils etc. (2, 210) which suggests that IL-4 secretion by the different innate cells may be sufficient enough to suppress the Mf secretion

by the female worms. Nevertheless, the data show that the ability of intrinsic IL-4 release by $CD4^+$ T cells does not hinder their ability to reduce fecundity.

4.5.3 Primed *L. sigmodontis*-specific $CD4^+$ T cells are more effective than naïve populations in reducing worm burden and fecundity.

An essential part of our long-term immunity is the ability of $CD4^+$ T cells to develop "memory". When naïve T cells have a first contact to pathogens, they start proliferation and develop into different types of effector T cells that are specific to that pathogen. As the infection resolves, these developed memory T cells become part of the hosts T cell repertoire and are able to mount a faster response should the individuals have another infection with the same pathogen (211). Research on helminths has shown memory and specific responses upon recall (16, 63, 212-215). Regarding filarial infections in man, immune profiling has been identified using recall assays with *W. bancrofti*, *O. volvulus* and *M. perstans* infected cohorts (34, 88, 124, 148, 153, 216, 217). Taubert and colleagues also described the effect of increased parasite numbers, on the cytokine production (mRNA levels) of re-stimulated $CD4^+$ T cells. These effects were stronger during the pre-patent stage of infection (134). These findings support our own previous observations, since $CD4^+$ T cells isolated from *L. sigmodontis*-infected but not naïve BALB/c mice respond specifically to LsAg or Mf in recall assays. Indeed, this is further complicated since responses were significantly stronger in cells from patent (Mf+) mice (63). Expanding on those data, research in this thesis showed that Th1, Th2 and Treg immune responses by $CD4^+$ T cells were increased in Mf+ mice compared to mice in the prepatent phase and mostly also to Mf- mice. Therefore, in the next set of adoptive transfer studies, $CD4^+$ T cells from *L. sigmodontis*-infected C57BL/6 donor mice (28 days p.i.) were transferred into Rag2IL-2R$\gamma^{-/-}$ C57BL/6 recipients and resulted not only in significantly reduced Mf counts but significantly lower worm numbers (for the first time) and shorter worms. This effectiveness may be the result of an enlarged population of filarial-specific T cells upon transfer. Identifying the phenotype of this population would significantly enhance research into understanding the mediators involved in the elimination of this helminth.

In addition to observing the effects on worm load and development, transferred cell populations were analysed by flow cytometry for the expression of Th1 (IFN-γ), Th2/IL-10 (IL-4, IL5) and Th9/Th17 cell populations. In the initial experiment using T cells from WT C57BL/6 mice, transferred $CD4^+$ T cell populations that infiltrated the TC showed higher frequencies of Th1 cells than Th2, albeit not all Th1 (TNF-α) nor Th2 cytokines (IL-13) were measured. Levels of Th9/Th17 cells were much lower than those observed in analysed $CD8^+$ T cells. Indeed, the frequency of cytokine expressing $CD8^+$ T cells was generally higher than those observed following the transfer of $CD4^+$ T cells. This pattern was also reflected when characterising T cell subsets found in blood. Here however, there was a much stronger release of IL-5 by both

subsets and of IL-9 and IL-17A by $CD4^+$ T cells and $CD8^+$ T cells respectively. Interestingly, loss of IL-10 secretion by $CD4^+$ T cells resulted in a reduced number of IFN-γ producing cells but not other populations. This effect was strongly observed in the cells recovered from the TC and to a smaller extent in peripheral blood, where more IL-9-releasing cells were observed in IL-$10^{-/-}$ $CD4^+$ T cells. In contrast, lower IFN-γ secreting TC-derived populations was not observed following the transfer of IL-$4^{-/-}$ $CD4^+$ T cells; here Th2 responses were much lower as well with IL-5 being the primary release. Following transfer of primed T cells resulted again in a much higher frequency of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells but here there was a larger portion of IL-10^+ cells within the Th2/IL-10 compartment. Moreover, in the blood there was a higher frequency of IFN-γ producing $CD4^+$ T cells than $CD8^+$ T cells; a characteristic not observed after the transfer of naïve cells. Interestingly, studies using adoptive transfers of memory $CD4^+$ T cells in a murine model of malaria showed that they were more protective (slowed parasite growth, reduced peak parasitemia and mice had less pathology compared). These chronically stimulated $CD4^+$ T cells contained more effector cells and a greater proportion of $TNF\alpha^+IFN\text{-}\gamma^+IL2-$ double-producing cells than resting memory cells (218). Furthermore, in a study on *Salmonella* specific $CD4^+$ T cells, McSorley et al., found that a transfer of naive T cells showed signs of activation the Peyer´s patches 3 hours after oral infection producing IL-2 and proliferating therein. In contract, *Salmonella* specific $CD4^+$ T cells were not activated in the spleen and thus antigen-specific T cells may migrate preferentially to sites of antigen deposition (219). This readiness to home into the site of infection may explain why the transfer of *L. sigmodontis*-specific T cells was more effective than naïve ones. Of course, this is just a snapshot of cell responses after 72 days of infection; it is not clear what function or phenotype the cells would have had during earlier time-points nor is it a reflection of their response to *L. sigmodontis*-specifically. Further experiments would also need to be performed to observe how much polyclonal spontaneous proliferation (SP), that is seen in $RAG^{-/-}$ strains, was dependent on the infection or the environment (cells in $RAG^{-/-}$ mice kept in SFP undergo SP whereas in germ-free conditions they do not) (220). Moreover, Kim and colleagues observed that a transfer of $CD4^+$ and $CD8^+$ T cells resulted in positive cross-talk between the two subsets in which the SP response of one compartment is facilitated by those in the other in SPF $RAG^{-/-}$ hosts (221). Thus, future experiments should also combine T cell fractions.

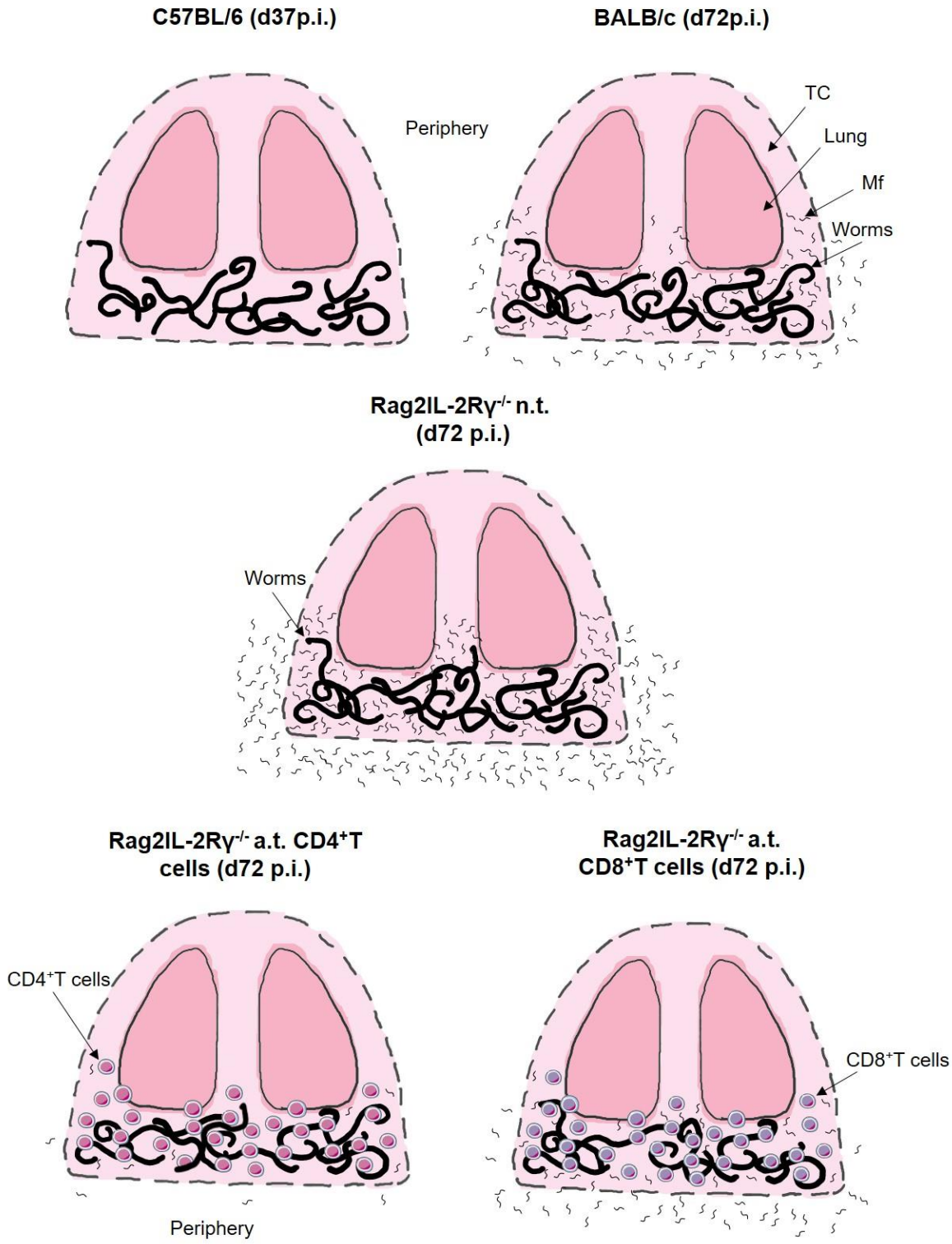


Figure 4.1: Adoptive transfer of CD4$^+$ T cells, but not CD8$^+$ T cells, into Rag2IL-2Rγ$^{-/-}$ C57BL/6 results in a disturbed embryogenesis of adult female worms.

4.6 Elevating the model to the bigger picture

Our findings demonstrated that the Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice could be a useful tool also for the investigation of human filarial parasites *in vivo*. Recently, a collaboration of Prof. Wanji

and our group demonstrated already that the human filariae *L. loa* can complete their life cycle in infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice, including the release of Mf (188, 222). This opens the door to investigate how other filaria like *O. volvulus* or *W. bancrofti* may develop in the host and obtaining specific material (e.g. worms) for assays and immune profiling. Indeed, in ongoing collaborations with Prof. Wanji we will establish the *M. perstans* life cycle in Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice in Cameroon since the biology of this parasite remains relatively unknown and therefore, it would be more than useful to investigate the whole life cycle in a mouse model. Different life stages and moulting processes could be analysed in detail and the resulting antigen/extracts of those worms or the different stages could be prepared for *in vivo* and *in vitro* experiments. Indeed, when investigating filarial specific responses, antigens or extracts prepared from the same helminth are an important factor for the analyzation of immune mechanisms and *in vitro* experiments. Indeed, in a study assessing the cytokine release of PBMCs from *O. volvulus*-infected individuals, data revealed varying responses when comparing *O. volvulus*- or *B. malayi*-derived antigens (223). The Rag2IL-2Rγ$^{-/-}$ C57BL/6 mouse model could therefore be a potential platform for obtaining material for antigen preparation of the different human pathogenic filariae. Even though, antigens/extracts have been used by several researchers for *in vitro* stimulation assays (76, 90, 91, 106, 116, 120, 142) or also *in vivo* studies (reviewed in Hübner et al., 2009), there is no standardized method for the preparation. This includes the source of the worms, which stages of the parasite and the process: mashed verses sonicated, filtered verses non-filtered, soluble or pellet, centrifuged or ultracentrifuged and levels of endotoxin (62) (Wiszniewsky, Neumann in preparation). In most cases, antigens were prepared from gender mixed adult worms (62, 63, 65, 98, 116, 135). Research performed in this thesis showed that DCs co-cultured with CD4^{+} T cells from Ls-infected mice and stimulated with LsAg preparations containing only female, male or mixed genders resulted in similar Th1, Th2 and Treg responses. Comparisons between pellet and SN of LsAg also showed only small differences. Therefore, for the activation of T cells in recall assays, the preparation method of our LsAg offers a robust extract that activates in a repeated manner and is independent of worm gender or soluble and non-soluble extracts. However, further tests need to be performed, including innate responses or specific antibody titres.

Thus, this mouse model and the preliminary findings described here, show that CD4^{+} T cells play such a crucial role in fecundity which may help clarify some of the outstanding questions about immune mechanisms during filariasis. Another important field where the Rag2IL-2Rγ$^{-/-}$ C57BL/6 mouse model could be helpful, is drug testing and here especially the development of vaccine-based treatments which would circumvent initial ethical complications which occur with clinical trials or using samples from human. The development of effective vaccines against

the different filarial parasites could be the scientific breakthrough in the fight against these parasites.

4.7 Concluding remarks

The picture of filarial interactions with man have been steadily emerging for the last decades but only since the development of new technologies has host immunity really began to appear. Nevertheless, despite intensive research, the reasons why C57BL/6 mice can clear *L. sigmodontis* infections remains unknown and understanding this principal is key to breaking transmission and thus eradicating this helminth in endemic communities. Therefore, our group established a useful tool, which offers the opportunity to identify cell types and pathways that are involved in eliminating *L. sigmodontis* infections. Through a series of adoptive transfer studies, the research in this thesis showed that naïve $CD4^+$ T cells but not $CD8^+$ T cells from WT donor mice dampened fecundity, embryogenesis and thus Mf loads in Rag2IL-2R$\gamma^{-/-}$ C57BL/6 recipients which was independent of IL-4 or IL-10. These effects were strengthened by the adoptive transfer of *L. sigmodontis* infection derived $CD4^+$ T cells and here worm burden was reduced too. One interesting fact is also that the transferred un-primed T cells find their way through the spleen to the site of infection. Since B cells are missing in Rag2IL-2R$\gamma^{-/-}$ C57BL/6 mice, other APCs expressing MHCII must compensate their function. Therefore, our findings underline the complexity of the immune mechanisms, induced during filarial infections. Moreover, it demonstrates that not only one cell population is responsible for worm clearance and therefore, this is more dependent of the interaction of two or more cell types. Further studies now concentrate on reconstituting a combination of different cell types so that an analysis of cell-cell interactions during filarial infections can be identified further.

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List of abbreviations

AAM	alternative activated macrophages
Ab	antibody
Ag	antigen
ALB	albendazole
APC	antigen-presenting cell
APOC	African Programme for Onchocerciasis Control
a.t.	adoptive transfer
BCR	B cell receptor
BMDC	bone marrow derived cell
BSA	bovine serum albumin
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
DALY	disability-adjusted life years
DAMP	damage-associated molecule pattern
DC	dendritic cell
DEC	diethylcarbamazine
DNA	deoxyribonucleic acid
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidise
FBS	fetal bovine serum
Fig	figure
G-CSF	granulocyte colony-stimulating factor
GEO	generalized onchocerciasis
GM-CSF	granulocyte macrophage colony-stimulating factor
GPELP	Global Programme to Eliminate Lymphatic Filariasis
HEK293	human embryonic kidney 293
HO	hyperreactive onchocerciasis
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
ILC	innate lymphoid cells
IRF	interferon regulatory factor

IVM	ivermectin
KO	knockout
L3	third stage larval
LF	lymphatic filariasis
LsAg	*Litomosoides sigmodontis* antigen
L. sigmodontis	*Litomosoides sigmodontis*
µg	micro gram
µl	micro litre
µM	micro molar
MACS	magnetic activated cell sorting
MAL	MyD88-adapter-like
MBP	major basic protein
MDA	mass drug administration
Mf	microfilariae
MHC	major histocompatibility complex
ml	millilitre
MyD88	myeloid differentiation primary response 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NK cells	natural killer cells
P	patent
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-L2	programmed death ligand 2
p.i.	post infection
poly(I:C	polyinosinic-polycytidylic acid
Pp	pre-patent
PRR	pattern recognition receptor
pTreg	peripheral regulatory T cells
RT	room temperature
SN	supernatant
SP	spontaneous proliferation
TAK1	transforming growth factor-β-activated kinase 1
TC	thoracic cavity
Th cell	T helper cell
TIR	toll/interleukin-1 receptor
TLR	toll like receptor

TMB	3,3 ', 5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cell
tTreg	thymus derived regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
WHO	world health organisation
WT	wild type

Appendix

Appendix A: Equipment

Material	Company
Automatic pipettes (10-1000 µl)	Eppendorf AG, Hamburg, Germany
BD FACSAria III Cell Sorter	BD™ Biosciences, Heidelberg, Germany
BD FACSCanto I™ flow cytometer	BD™ Biosciences, Heidelberg, Germany
Cannula	B.Braun Meslungen AG, Melsungen, Germany
Cell scraper	Corning B.V. Life science, Lowell, USA
CELLSTAR Tubes (15 ml and 50 ml)	Greiner bio-one, Frickenhausen, Germany
Centrifuge (Eppendorf 5415 R)	Eppendorf AG, Hamburg, Germany
Centrifuge (Multifuge 4 KR)	Fischer Scientific GmbH, Schwerte, Germany
Centrifuge (Varifuge 3.OR)	Fischer Scientific GmbH, Schwerte, Germany
Cover slip	Engelbrecht, Munich, Germany
Culture plates (6, 12 and 96 well)	Greiner GmbH, Frickenhausen, Germany
CytoFLEX S	Beckman Coulter Life Science, Krefeld, Germany
Cytospin chamber	Hettich, Tuttlingen, Germany
ELISA plates (96 well)	Greiner GmbH, Frickenhausen, Germany
Eppendorf tubes (0.5-2ml)	Eppendorf AG, Wesseling, Germany
FACS tubes	BD™ Biosciences, Heidelberg, Germany
Freezer (-20°C)	Bosch GmbH; Stuttgart, Germany
Freezer (-80°C)	Heraeus Holding GmbH, Hanau, Germany
Fridge	Bosch GmbH; Stuttgart, Germany
Filter	BD™ Biosciences, Heidelberg, Germany
Filter paper	Hettich, Tuttlingen, Germany
Gauze	Labomedic GmbH, Bonn, Germany
Glass potter	Greiner GmbH, Frickenhausen, Germany
Glass slides	Engelbrecht, Munich, Germany
Glassware	Schott AG, Mainz, Germany
Gloves	Ansell Healthcare, Bruessel, Belgian
Heating Block	Eppendorf AG, Hamburg, Germany
HERAsafe	Kendro, Langenselbold, Germany
Ice machine (Scotsman AF 80)	Gastro Handel GmbH. Wien, Austria
Incubator (Hera cell 240)	Fischer Scientific GmbH, Schwerte, Germany
MACS® columns (LS)	Miltenyi Biotech, Bergisch Gladbach, Germany
MACS® seperator	Miltenyi Biotech, Bergisch Gladbach, Germany
Microscope (Leica DM IL)	Leica Microsystems GmbH, Wetzlar, Germany
Microscope (Diavert)	Leica Microsystems GmbH, Wetzlar, Germany
"Neubauer“ counting chamber	Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Pasteru pipette (sterile)	Ratiolab, Dreieich, Germany
Petri dishes	Greiner GmbH, Frickenhausen, Germany
pH meter	Mettler Toldo GmbH, Giessen, Germany
Pipetboy (pipetus®-akku)	Hirschmann Laborgeräte, Eberstadt, Germany
Pipet tips (10-1000 µl)	Eppendorf AG, Hamburg, Germany
Plastic pestile	Greiner GmbH, Frickenhausen, Germany

Plunger	BD™ Biosciences, Heidelberg, Germany
Refuse bags	Brand GmbH & Co.KG, Wertheim, Germany
Rotot Gene RG-6000	Cirbett Research, Sydney, Australia
SpectraMAX ELISA reader	Molecular Devices, Sunnyvale, USA
Syringes (2-10 ml)	B.Braun Meslungen AG, Melsungen, Germany
Thermocycler	Biometra GmbH, Göttingen, Germany
Tweezer	Dumont Swissmade, Montignez, Switzerland
Vortex mixer (Minishaker)	IKA® GmbH & Co.KG, Staufen, Germany
Water bath	VWR, Darmstadt, Germany
Water purifier Milli-Q plus	Millipore, Schwalbach, Germany

Appendix B: Antibodies and Proteins

Antibody	**Company**
Mouse MACS Beads	
Anti-CD4$^+$ T cell MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
Anti-MHC ClassII MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
Other mouse antibodies	
CD3 Monoclonal Antibody	eBiosience, Frankfurt, Germany
CD Monoclonal Antibody	eBiosience, Frankfurt, Germany
APC conjugated anti-mouse CD4 mAb	Biolegend, Fell, Germany
APC conjugated anti-mouse CD4 mAb	eBiosciences Inc., San Diego, USA
FITC conjugated anti-mouse CD4 mAb	Biolegend, Fell, Germany
PerCP.Cy5.5 conjugated anti-mouse CD8 mAb	Biolegend, Fell, Germany
PerCP-Cy5.5 conjugated anti-mouse CD8 mAb	eBiosciences Inc., San Diego, USA
PE-Cy7 conjugated anti-mouse CD25 mAb	eBiosciences Inc., San Diego, USA
FITC conjugated anti-mouse CD11b mAb	Biolegend, Fell, Germany
PE conjugated anti-mouse CD103 mAb	eBiosciences Inc., San Diego, USA
PE conjugated anti-mouse Foxp3 mAb	eBiosciences Inc., San Diego, USA
APC conjugated anti-mouse IL-4 mAb	Biolegend, Fell, Germany
PE conjugated anti-mouse IL-5 mAb	Biolegend, Fell, Germany
PE conjugated anti-mouse IL-9 mAb	Biolegend, Fell, Germany
PE-Cy7 conjugated anti-mouse IL-10 mAb	Biolegend, Fell, Germany
PE conjugated anti-mouse IL-17A mAb	Biolegend, Fell, Germany
PerCP.Cy5.5 conjugated anti-mouse IL-22 mAb	Biolegend, Fell, Germany
APC conjugated anti-mouse IFNγ	Biolegend, Fell, Germany
APC conjugated anti-mouse F4/80	Biolegend, Fell, Germany
PE-Cy7 conjugated anti-mouse Ly6c mAb	Biolegend, Fell, Germany
PerCP.Cy5.5 conjugated anti-mouse Siglec F mAb	Biolegend, Fell, Germany
Proteins	
Ls-FtsZ protein	GenBank Accession No.: AJ010271

Appendix C: Chemicals, Reagents and Kits

Reagent	Company
Advanced Poteine Assay	Cytoskeleton, Denver, USA
AE buffer	Qiagen, Hilden, Germany
Ammonium chloride	Sigma-Aldrich GmbH, Munich, Germany
autoMACS buffer	Miltenyi Biotech, Bergisch Gladbach, Germany
BD FACS™ Clean BD	BD™ Biosciences, Heidelberg, Germany
BD FACS™ Rinse	BD™ Biosciences, Heidelberg, Germany
Bovine serum albumin (BSA)	PAA Laboratories, Pasching, Austria
Diff Quick Staining Set	Medion Diagnostics International, Miami, USA
ELISA kits Ready-SET-Go (IL-5, IL-10, IL-13, IL-17A, IFN-γ)	eBiosience, Frankfurt, Germany
Entellan	Merck, Darmstadt, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Ethylendiamintetraacetat (EDTA)	Merck KGaA, Darmstadt, Germany
Fetal calf serum (FCS)	PAA Laboratories, Pasching, Austria
Forene	Piramal Critical Care, West Drayron, UK
Foxp3/Transcription Factor Staining Buffer Set	Fischer Scientific GmbH, Schwerte, Germany
Gentamycin	PAA Laboratories GmbH, Pasching, Austria
GM-CSF	PreproTech GmbH, Hamburg, Germany
IMDM	Fischer Scientific GmbH, Schwerte, Germany
L-glutamine	PAA Laboratories GmbH, Pasching, Austria
LPS	Sigma-Aldrich GmbH, Munich, Germany
murine IgE FlowCytomix Simplex Analyte Detection Kit	eBiosience, Frankfurt, Germany
murine Immunoglobulin Isotyping Panel 6plex FlowCytomix Multiplex Analyte Detection Kit	eBiosience, Frankfurt, Germany
murine Th1/Th2/TH17/TH22 13plex FlowCytomix Multiplex Analyte Detection Kit	eBiosience, Frankfurt, Germany
Non essentielle amino acid (NEAA)	PAA Laboratories GmbH, Pasching, Austria
Phosphate buffer saline (PBS)	PAA Laboratories GmbH, Pasching, Austria
Penicillin/Streptomycin	PAA Laboratories GmbH, Pasching, Austria
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany
Quantitect Multiplex NoROX Kit	Qiagen, Hilden, Germany
RPMI	Fischer Scientific GmbH, Schwerte, Germany
R&D Systems ELISA Kits (CCL17, CCL22, Eotaxin-1, RANTES, IP-10, MIP-2, Granzyme, TGF-β)	R&D Systems, Wiesbaden-Nordenstadt, Germany
Schwefelsäure (H_2SO_4)	Merck KGaA, Darmstadt, Germany

Sodium bicabonate	PAA Laboratories GmbH, Pasching, Austria
Sodium Pyruvate	PAA Laboratories GmbH, Pasching, Austria
Streptavidin-POD	Roche Diagnostics, Mannheim, Germany
Trypan blue	Sigma-Aldrich GmbH, Munich, Germany
Tween20	Sigma-Aldrich GmbH, Munich, Germany

Appendix D: Buffer, Media and Solution

Hinkelmann solution
0.5% Eosin yellow
0.5% Phenol
0.185% Formaldehyd

Fetal calf serum (FCS)
FCS used for medium supplementations was heated for 30 minutes at 56°C to inactivate the complement factors. Aliquots were the stored a

Cells culture reagents

Complete medium

IMDM
10% Fetal Calf Sera
1% 2 mM L-glutamine
1% 50 µg/ml penicillin/streptomycin
1% 50 µg/ml gentamicin
1% NEAA
1% Sodium pyrovate
1% Sodium bicarbinate

RPMI
10% Fetal Calf Sera
1% 2 mM L-glutamine
1% 50 µg/ml penicillin/streptomycin
1% 50 µg/ml gentamicin

ACT Buffer
1 litre: 2.06g Tris
8.99g ammonium chloride
Fill up with water to 1 litre.

FACS
1X Foxp3 Fix/Perm Solution
1 part Foxp3 Fixation/Permeabilisation Concentrate
3 parts Fixation/Permeabilisation Diluent

1x Permeabilisation Buffer
10x Perm Buffer Diluent in PBS

ELISA

eBioscience
Coating solution
10x coating buffer diluted with a. dest to 1x coating buffer
Blocking solution
1x Assay diluents
Enzyme
Arvidin-HRP (diluted 1:250 with a. dest)
Substrate
3,3', 5,5' Tetramethylbenzidine (TMB)
Stop solution
2N H2SO4

R&D duo set
Coating buffer
PBS
Blocking solution
1x PBS
1% BSA
Enzyme
Streptavidin-HRP (diluted 1:200 with 1% PBS/ 1% BSA)

Lösung4

1L = 15.6g NaH_2PO_4 in A.dest, pH5.5

Development solution

100ml Lösung4, 200µl TMB, 2µl $H2SO_4$

Washing buffer
5L = 47.75g PBS in A.dest + 2.5ml Tween, pH 7.2-7.4

Appendix E: Software

Software	Company
FlowJo v10 software	FlowJo, Portland, USA
GraphPad Prism 6.1	GraphPad Softwaer, Inc., La Jolla, USA
SOFTmax Pro 3.0 Software	Molecular devices, Sunnyvale, USA
EndNote X7	Clarivate Analytics, Jersey, USA

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