Identification of protective immune responses and the immunomodulatory capacity of *Litomosoides sigmodontis*

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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.

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> "Development of patent *Litomosoides sigmodontis* infections in semi-susceptible C57BL/6 mice in the absence of adaptive immune responses."
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> 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.
>
> "Combination of worm antigen and proinsulin prevents type 1 diabetes in NOD mice after the onset of insulitis."
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Des Weiteren wurden Auszüge auf verschiedenen Konferenzen als Vortrag oder Poster präsentiert.

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Summary

Parasitic helminth infections are a major health problem in the tropical and subtropical regions with diseases like lymphatic filariasis and onchocerciasis still infecting more than 150 Mio people. Although WHO targets the eradication of the aforementioned diseases, successful vaccines or MDA-therapies with a macrofilaricidal effect have not been developed so far. To succeed in the fight against these parasites, a better and detailed knowledge of the induced immune responses is required. Using the mouse model *Litomosoides sigmodontis*, research focusing on the protective immune responses within the host as well as the immunomodulatory capabilities of filarial infections is possible. The immunomodulatory capacities of filariae not only allow the worms to persist for a long time within the host, but also mediate a beneficial effect for the host itself, for example by protecting against autoimmune diseases.

In this thesis, a role for the intracellular pattern recognition receptor NOD2 during infection with *Litomosoides sigmodontis* is demonstrated. NOD2 deficient mice had an increased worm burden at the site of infection compared to wild type (WT) controls during the early phase of infection. However, $NOD2^{-/-}$ mice managed to eliminate the infection at a similar time point as the WT controls. While the immune response within the thoracic cavity, the site of adult worm residence, was not impaired, subcutaneous injection of L3 larvae did not lead to an increased worm burden in $NOD2^{-/-}$ mice. This thesis reveals that the NOD2-dependent mechanism occurs within the skin. We found an impaired neutrophil recruitment to the skin against invading L3 larvae as the decisive mechanism leading to the increased worm burden in $NOD2^{-/-}$ mice. In the skin three hours post injection of crude worm extract as well as in the blood 24h after natural infection, significantly lower frequencies of neutrophils were observed in the $NOD2^{-/-}$ mice compared to WT controls. Gene expression analysis further revealed that genes associated with neutrophils were expressed at lower levels in $NOD2^{-/-}$ mice after L3 injection. Summarizing, a new role for the NOD2 receptor during the skin stage of *L. sigmodontis* infection is shown. The essential role for neutrophils during protective immune responses within the skin was further confirmed using IL-$6^{-/-}$ mice, in which the increased worm burden during early time points of infection was also found to be caused by an impaired neutrophil recruitment to the skin.

Another focus of this thesis was the immunomodulatory abilities of helminth infections on autoimmune diseases. Intraperitoneal injections of a crude worm extract in combination with intra-nasal proinsulin administration prevented the onset of type 1 diabetes in non-obese diabetic (NOD) mice. While each component by itself was not successful when given at a progressed state of disease, our combination therapy protected mice until the age of ten weeks; a time point in NOD mice at which insulitis of the pancreatic islet cells already occurs The protective effect was associated with an increased frequency of regulatory T cells in the pancreatic lymph nodes as well as decreased

frequencies of classically activated macrophages. Our study demonstrates that the inclusion of an antigen-specific treatment to the already known protective effect of helminth antigens improves the protection against type 1 diabetes onset.

Zusammenfassung

Infektionen mit parasitischen Helminthen sind ein großes Gesundheitsproblem in den Tropen und Subtropen. Es wird geschätzt, dass mehr als 150 Mio. Menschen mit den Erregern der lymphatische Filariose oder der Onchozerkose infiziert sind. Obwohl die WHO die Eliminierung dieser Krankheiten bis zum Jahre 2020 anstrebt, gibt es bislang keine Vakzine oder zur Massenbehandlung geeignete Therapie, welche die Adultwürmer abtötet. Um im Kampf gegen diese Parasiten erfolgreich zu sein, wird ein besseres Verständnis und ein detaillierteres Wissen der protektiven Immunantworten benötigt. Mit dem akzeptierten Mausmodell *Litomosoides sigmodontis* können sowohl die protektiven Immunantworten als auch die immunmodulierenden Eigenschaften von Filarieninfektionen untersucht werden. Letztere erlaubt es den Würmern, nicht nur jahrelang im Wirt zu persistieren, sondern auch einen positiven Effekt für den Wirt zu vermitteln, zum Beispiel durch die Vermeidung von Autoimmunerkrankungen.

Im Rahmen dieser Dissertation konnte eine wichtige Rolle für den intrazellulären Mustererkennungsrezeptor NOD2 während der Infektion mit *L. sigmodontis* gezeigt werden. NOD2-defiziente Mäuse hatten eine erhöhte Infektionslast verglichen mit WT Kontrollen während der frühen und mittleren Phase der Infektion. Jedoch gelingt es $NOD2^{-/-}$ Mäusen ähnlich wie WT Kontrollen, die Infektion zu eliminieren. Während in der Pleurahöhle, dem Ort an dem die Würmer persistieren, die Immunantwort nicht beeinträchtigt ist, zeigen Experimente, dass die Wurmlast in $NOD2^{-/-}$ Mäusen nach subkutaner Injektion nicht erhöht war. Diese Studie zeigt, dass der NOD2 abhängige Mechanismus in der Haut stattfindet. Eine beeinträchtigte Rekrutierung von Neutrophilen in die Haut gegen einwandernde L3 Larven war dabei der entscheidende Mechanismus, der zu der erhöhten Wurmlast in den $NOD2^{-/-}$ Mäusen führte. Drei Stunden nach Injektion von Filarienantigenen in die Haut und auch 24h nach natürlicher Infektion im Blut, fanden sich in den $NOD2^{-/-}$ Mäusen signifikant weniger Neutrophile verglichen mit Wildtyp (WT) Kontrollen. Genexpressionsanalysen zeigen, dass Gene assoziiert mit Neutrophilen, signifikant niedriger in $NOD2^{-/-}$ Mäusen exprimiert wurden. Zusammenfassend zeigt diese Arbeit eine neue Rolle für den NOD2 Rezeptor während der Hautphase der Infektion mit *L. sigmodontis* auf. Diese essentielle Rolle für Neutrophile während der protektiven Immunantwort in der Haut wurde darüber hinaus in Experimenten mit $IL\text{-}6^{-/-}$ Mäusen bestätigt. Die erhöhte Wurmlast während der frühen Phase der Infektion wurde auch in diesem Modell durch eine beeinträchtigte Neutrophilen-Rekrutierung in die Haut verursacht.

In dieser Thesis wurden zudem die immunomodulatorischen Fähigkeiten von Filarien während Autoimmunerkrankungen untersucht. Intraperitoneale Injektionen von Filarienextrakt und intranasale Verabreichung von Proinsulin schützten NOD Mäuse vor der Entstehung von Typ 1 Diabetes. Während die Kombination von beiden Komponenten erfolgreich war, war die Verabreichung der einzelnen Komponenten für sich nicht schützend. Mit der hier vorgestellten Kombinationstherapie konnten Mäuse bis zu einem Alter von 10 Wochen geschützt werden, einem Zeitpunkt, zu dem bereits Insulitis vorherrscht. Der protektive Effekt war assoziiert mit einer erhöhten Frequenz von regulatorischen T Zellen in den pankreatischen Lymphknoten sowie einer niedrigeren Frequenz von klassischen Makrophagen. Diese Studie zeigt, dass die Inklusion von einer Antigen-spezifischen Behandlung zu dem schon bekannten protektiven Effekt von Helminthen-Antigenen als Schutz vor Typ 1 Diabetes verbessert.

1. Introduction

Parasitism is an interspecific non-mutual symbiotic relationship, with one partner, the parasite, benefiting at the expense of the other, the host (1). Metazoans living a parasitic life style are found throughout all systematic clades and some authors claim that more than 50% of all species live parasitic or at least go through a parasitic life stage (2). Because of this number, it comes as no surprise, that also humans are used as hosts by a variety of parasitic organisms.

One of the major groups of human pathogenic parasites is the so-called "helminths". The term "helminths" is a medical term with no real value in taxonomy, combining parasitic worms of not related phyla: mainly the *Nematoda* or roundworms and the *Plathyhelminthes* or flatworms; some authors also include vertebrate-parasitizing members of the phyla *Annelida* and *Acanthocephala* (3). Although not related, all members of the helminths show similarities in behaviour as well as in the immune response they trigger in their respective hosts. While only one subclass within the *Annelida* - the *Hirudinea* or leeches - and only two species of *Acanthocephala* occasionally infect humans, a great number of *Nematoda* and *Plathelminthes* are human pathogenic and represent a major health problem.

1.1 Nematodes as parasites of humans

Nematodes are one of the most successful groups of metazoan on this planet. Although more than 25.000 species of nematodes have already been described, experts have been estimating the total number to be around 1 million species. Nematodes have conquered all kinds of habitats and a remarkable number of the known species evolved a parasitic life style, found in all types of animals and plants. Despite this richness in species, nematodes are displaying a similar phenotype. They are mostly elongated, round, non-segmented and bilaterally symmetrical worms, reaching lengths ranging between a few millimetres and one meter.

Differently than *Plathelminthes*, nematodes are dioecious species with the females being larger than the males (2).

Today around 138 human pathogenic species of nematodes have been described and this is leading to the fact that around 25% of the world population is infected with nematodes. The majority, around 2 billion, are infected with intestinal nematodes and an estimated 150-200 Mio suffer from an infection with extra-intestinal nematodes (4).

The main class of nematodes are the *Secernentea*, which also contain the majority of human pathogenic parasites. Within the *Secernentea* subclass of S*trongylida* or hookworms one can find the two important parasites *Ancylostoma duodenale* and *Necator americanus*, soil-transmitted nematodes living in the gastrointestinal tract of humans and infecting an estimated 576–740 million people globally (5). This high prevalence within the population makes hookworm infections a neglected tropical disease (NTD), a classification by the WHO to prioritize widely spread diseases in the tropical regions of Africa, Asia and America, which still play a minor role in research in high-income western countries (6, 7). Another important soil-transmitted nematode is found in the order *Ascaridida*: *Ascaris lumbricoides* or giant round worm. This worm can grow up to 35 cm and infections are highly common with 1.2 billion infections globally (4, 8). Further members of the order *Ascaridida* causing more infrequent parasitic diseases in humans are *Anisakis simplex*, the causative agent of anisakiasis, caused by the consumption of raw or undercooked seafood (9) as well as *Toxocara canis*/*Toxocara cati* - roundworms of dogs and cats – causing visceral larva migrans leading to headache, fever and in severe cases to blindness (10). The medically most important nematode within the order *Rhabditida* is *Strongyloides stercoralis*, another soil-transmitted helminth, infecting around 30-100 Mio people worldwide (11). While most infections with this nematode are asymptomatic, especially cases of uncontrolled parasite multiplications – so called hyper infections - can have fatal consequences in immune

compromised humans. The helminth with the broadest geographic distribution is arguably *Enterobius vermicularis*, a human infecting pinworm of the order *Oxyurida* (12). Enterobiasis is still common in the western world, with prevalence rates in some communities of as high as 30% (13). An example for a successful eradication program is the guinea worm *Dracunculus medinensis*. Just thirty years ago, 3.5 Mio new cases of Drancunculiasis were reported annually, but thanks to intensive efforts, this disease is on the verge of extinction (14). Two species of the nematode class *Adenophora* are also worth mentioning due to their importance in medicine, namely *Trichinella spiralis* and *Trichuris trichiura*. Infection with *Trichinella spiralis* occurs while consuming uncooked pork, but due to strict inspections of meat and housing conditions in commercial pig farms, the infection rate is especially in European countries very low (15). *Trichuris trichiura*, commonly known as whipworm, is yet another example for soil-transmitted helminths, infecting almost 800 Mio people in the tropics and is therefore classified as an NTD (4).

1.2 Filarial nematodes and filariasis - neglected diseases of the tropics

This thesis is mainly focusing on the immune responses which are caused by infections with helminths of the nematode subfamily *Filarioidea*, which are the most prevalent causative agents of helminths infections within the *Secernentea*. The *Filarioidea*, also known as filariae, consist among others of the families *Filariidae* and *Onchocercidae*, which include several important species known for causing major diseases in humans. Three species of filarial nematodes are causing an infectious disease called lymphatic filariasis (LF), namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*.

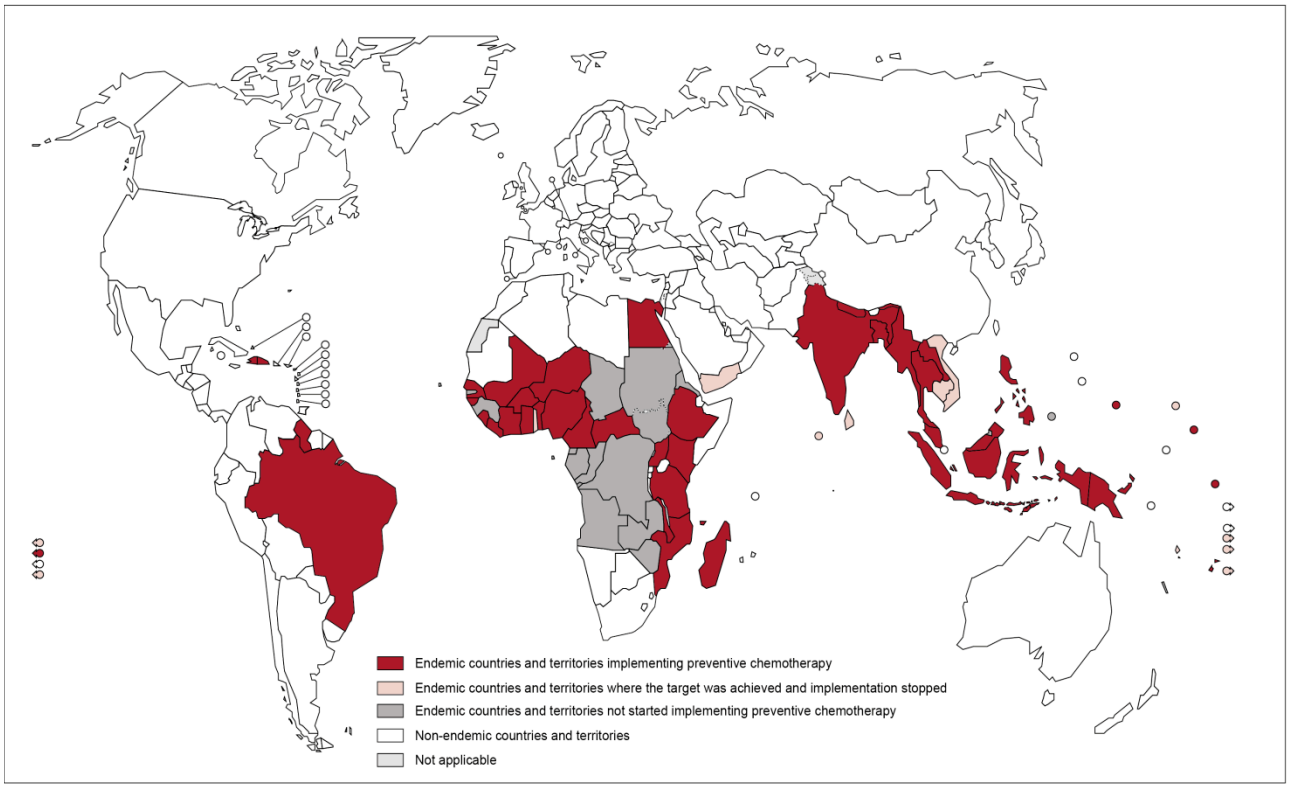
LF, also known as elephantiasis, is still the leading cause of physical disability in the world with more than 120 Mio people currently infected and 40 Mio chronically disabled (16). This disease not only affects people's physical health, it is also a major cause for social isolation and stigma (17). Currently around 1.4 billion people are living in the 73 countries, where this

disease can be transmitted (16). LF can result in significant disfiguration of body parts like genitals (Hydrocele) and extremities (lymphoedema), leading to pain and severe disability. Usually the infection is acquired during childhood, first causing hidden damage to the lymphatics. The visible manifestations occur with advanced age while developing symptoms like hydrocele or lymphoedema and ultimately lead to permanent disability (18). *W. bancrofti* is the most common reason for LF causing more than 90% of the cases, mainly in sub-Saharan Africa (18). *B. malayi* is making up for the remaining 10% of the cases, but is restricted to 16 countries in South and South-East Asia (19). In some regions its distribution overlaps with *W. bancrofti*. The latest identified causative agent for LF is *B. timori* (20), which has been found in the Lesser Sunda Islands of Indonesia (21). Since the last common ancestor of *W. bancrofti* and *B. malayi* occurred around 5-6 Mio years ago, it comes as no surprise that the species are morphologically distinct from each other (22). Nevertheless, they show high similarities in their lifestyle and the life cycle. All three species are transmitted by vectors, namely different types of mosquitoes. In most urban and sub-urban areas the main vector is the *Culex* mosquito, in rural areas LF gets mostly transmitted by *Anopheles* species and in endemic islands of the Pacific region *Aedes* mosquitoes are the main vectors (16). LF has been targeted for elimination as a public health problem by the WHO for 2020 using intensive mass drug administration (MDA) programs as well as vector control which considerably reduced transmission and even got close to eradication in some countries (16, 23).

Another neglected tropical disease is onchocerciasis or river-blindness, caused by the filarial nematode *Onchocerca volvulus*. An estimated 36 Mio people are infected worldwide, with 99% of the infected people living in 31 African countries (24). This parasite can also be found in Latin America (border regions of Brazil and Venezuela) and the Middle East (24). Similar to LF causing nematodes, *O. volvulus* is relying on an arthropod vector for transmission;

black flies of the genus *Simulium*. Usually repeated bites by the insect vector are required before infection occurs (18). As an eye and skin disease, onchocerciasis can lead to severe itching and dermatitis, pathologies associated with the release of the endosymbiotic bacteria *Wolbachia* from dying worms and the subsequent inflammatory response (25, 26). Nodules containing adult worms are found under the skin. If the eye is affected by the infection, it can lead to visual impairment or even blindness. This is caused by the release of *Wolbachia* and their products, leading to initiation of toll-like receptor 2 (TLR2)-driven inflammation, which can then cause blindness, thus the term "River blindness" (26, 27). According to the African Programme for Onchocerciasis Control (APOC), this disease is the second major infectious cause for blindness (17). Onchocerciasis may not only lead to visual loss of infected people, but it also leads to social stigmatisation and supports further poverty in the Third-World countries (17). Due to the intensive work of programs like APOC or OEPA, the Onchocerciasis Elimination Program of the Americas, this disease is on the retreat. For example Colombia and Ecuador as well as Guatemala and Mexico were able to stop transmission in the last decade (24). Nevertheless onchocerciasis and LF still remain a huge problem especially in Africa and until a vaccination or a safe MDA-compatible macrofilaricidal drug is developed, intensive research has to be done. Compared to LF and onchocerciasis, *Loa loa* has a much lower distribution, infecting 12-13 Mio people in 13 countries, especially in Central Africa (18). This disease can manifest initial symptoms like lymphedema or angioedema (Calabar swelling) and is caused by the filarial nematode *Loa loa*, also known as the African eye worm (18). Its vectors are horseflies of the genus *Chrysops*. In contrast to the causative agents for LF and onchocerciasis, *Loa loa* has no *Wolbachia* endosymbionts. Yet another disease which resembles onchocerciasis, but was often underestimated in its epidemiology (18), is streptocerciasis. It is caused by the nematode *Mansonella streptocerca*, a nematode found in the rain forests of western Africa, which needs

midges of the genus *Culicoides* for its transmission. It is playing a minor role in research and therefore no reliable data about its distribution or case numbers are available.



Map 1. Worldwide distribution for lymphatic filariasis according to the WHO. Map shows endemic countries of lymphatic filariasis and onchocerciasis. Source: http://gamapserver.who.int/mapLibrary/Files/Maps/LF_2012.png

1.3 *Litomosoides sigmodontis* – a murine model for LF

For many decades, one of the major obstacles facing filarial research was the lack of a proper animal model. Human pathogenic filariae were either non-permissive to mice or they did not undergo their full life cycle with all stages (28) . For instance, *Brugia* species do not produce microfilariae in mice and are not able to migrate through tissues in their rodent hosts like they do in humans (29).

Known since the late 1940's, the nematode *Litomosoides sigmodontis* was used as a murine model to investigate filarial infections especially since the early 1990's (30-32). *L. sigmodontis* (first description as *L. carinii*) is a parasite naturally found in the hispid cotton rat (*Sigmodon hispidus*; family *Cricitidae*), an up to 30 cm long rodent living in the southern parts of North America and South America. It was found that *L. sigmodontis* undergoes

complete development within the laboratory BALB/c mouse strain causing patent infections and thus allowing researchers to isolate the different life cycle stages as well as to analyse the immunological response this parasite is inducing in its host. Like the natural definitive host, BALB/c mice do not show any symptoms of infection and no pathogenic changes are evident (28, 33). This murine model has led to a deeper understanding of the host-parasite interactions as well as the immunology of a filarial infection. Experimental manipulations provided better insights within the cellular response and the molecular basis of the immunity leading to mechanistic explanations of ongoing responses within the host. The gained knowledge allowed comparisons to human studies, further demonstrating the importance of such a laboratory mouse model. It must be noted, that in some inbred mouse strains, *L. sigmodontis* is not producing a patent infection. For instance, in C57BL/6 mice, the adult filariae never produce microfilariae (28), which further facilitate to decipher protective immune responses.

1.4 Life cycle of *Litomosoides sigmodontis*

Similar to other filarial nematodes, *L. sigmodontis* is relying on an intermediate host for its transmission. In the case of *L. sigmodontis* the obligate intermediate host is the tropical rat mite *Ornithonyssus bacoti*. The adult stages of *L.sigmodontis* reside within the thoracic cavity of their definite host. There the adults mate and produce their progeny, the microfilariae (MF), which enter the blood stream. The MF (approx. 80 µm of length), the first larval stage of *L. sigmodontis* (L1), can be found in the peripheral blood of infected BALB/c mice from day 50 post infection and are ingested by mites during the blood meal. Inside the mites the L1 stage molts within 6-7 days to the L2 stage and after 10-12 days to the infectious L3 larval stage. When another blood meal occurs, the L3 stage is transferred to the vertebrate host. The L3 larvae actively migrate through the skin and the lymphatic vessels to the thoracic cavity within 5 days and thereafter molt into adult worms around day 30 post infection. The adult worms start mating from day 30 post infection on and embryogenesis takes approximately 18

days (28, 33). Aside from sexual attributes like the vulva or the spiculae, adult worms have a significant sexual dimorphism. Adult females are longer and reach a length of 10-12 cm while male specimen only reach 3 to 5 cm.

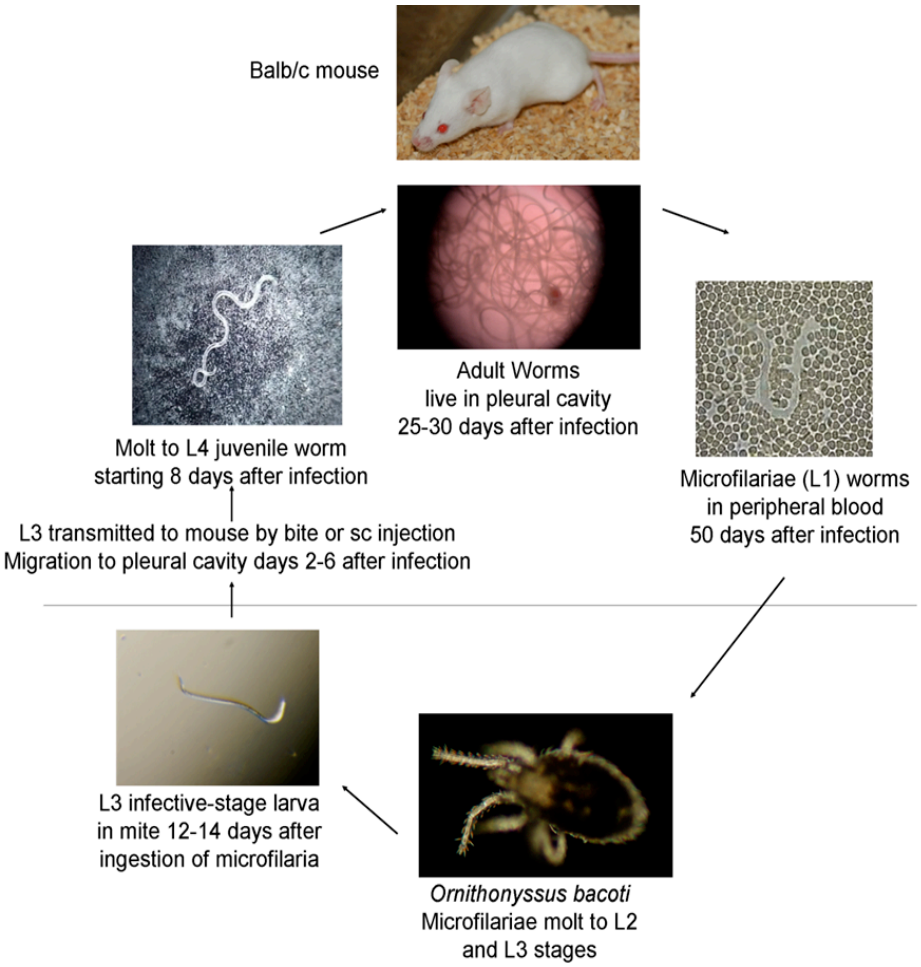


Figure 1. Laboratory life cycle of *Litomosoides sigmodontis*. *L.sigmodontis* is maintained in the host, BALB/c mice or knock-out strains. Infection is initiated by transmission of the infective L3 larvae into the mammalian host by bite trough the intermediate host (*Ornithonyssus bacoti*). Larvae migrate via the lymphatic system to the thoracic cavity by 2-6 dpi, where they molt to L4 stage (8-12 dpi) and to adults (25-30 dpi). Adult worms are able to produce microfilariae, which circulate in the blood by 50 dpi.

1.5 *Wolbachia* – Endosymbiont and drug target

One special feature of most filarial species is the occurrence of endosymbiotic bacteria. Already in 1975 "unusual bodies" were described in hypodermal tissues of larval *Dirofilaria immitis* and *Brugia pahangi*. Later these "bodies" were identified as gram negative bacteria of the genus *Wolbachia* (order *Rickettsiales*) (34). Further studies demonstrated that the *Wolbachia* are found in the hypodermis of not only all larval stages, but also in male and female adult worms as well as embryos and that they are transmitted transovarially (35). *Wolbachia* are the most abundant symbiotic bacteria on earth; they not only have been found within nematodes, but also in the majority of insect species as well as in *arachnidas* and *crustaceans* (36, 37). Interestingly, a minority of filarial species are *Wolbachia*-free, examples are *Loa loa* and *Acanthocheilonema viteae*. It is assumed that *Wolbachia* once infected the common ancestor of all filarial nematodes, but some species independently lost their endosymbionts again (37, 38). The relationship between the filarial nematodes and the bacteria can be defined as an obligate mutual symbiosis, since the endosymbiontic *Wolbachia* are essential for filarial fertility, reproduction, larval molting and the survival in general (39-42). For instance, Hoerauf et al. demonstrated that a tetracycline treatment eliminates *Wolbachia* within *L. sigmodontis* and thus leads to filarial growth retardation and infertility (39). Latest studies are proving that *Wolbachia* are providing metabolites essential for the worms. For instance genes involved in the heme synthesis are lacking in the genome of *B. malayi*, suggesting that *Wolbachia* might deliver the needed compounds to successfully synthesize this essential protein (43). Furthermore, the *Wolbachia* provides riboflavin, Flavin adenine dinucleotides and nucleotides to the host, while the host nematode likely supplies amino acids required for *Wolbachia* growth (43). Beside its vital role in the development of the filarial nematode, *Wolbachia* can also be crucial for the immune response of the infected host (44). Upon death of the worm, *Wolbachia* can get released subsequently providing pro-

inflammatory stimuli. Active release of *Wolbachia* is also known to occur during the different molting phases as well as during MF release.

In the widely spread filarial diseases, *Wolbachia* presents a promising target for drug administration and antibiotic therapy (45-47). The antibiotic doxycycline provides macrofilaricidal activity and is effective as mono-therapy (45, 48-50). Nevertheless doxycycline is not suited for a mass drug administration (MDA) program because of the long treatment period of four to six weeks and since its side effects make it not suitable for treating children below the age of 8 and pregnant or lactating women (51, 52). Further drugs to treat filariasis include ivermectin, and the combination of diethylcarbamazine (DEC) and albendazole, to reduce microfilariae in blood and skin of LF and onchocerciasis patients, respectively, which prevents the transmission of the disease (53). A new approach is a triple administration of DEC, albendazole and ivermectin, which was currently tested in Papua-New Guinea in *W. bancrofti* infected adults. As this pilot study was successful, there is hope that this triple therapy has the potential to accelerate elimination of lymphatic filariasis (54). Still several challenges are hampering the efficacy of a successful drug against filarial diseases. Long-lived adult worms are not efficiently killed by this treatment, reports of drug resistance and suboptimal responses are increasing, and areas of co-endemicity of *Loa loa* and *O. volvulus* harbor the risk of severe adverse events after treatment with ivermectin and DEC (52, 53, 55-58). On top of that, logistical problems within the third-world countries, which are often thrilled by instable political situations, civil wars and migration, are an underestimated factor, which further complicate the already difficult circumstances of fighting these diseases, especially in Sub-Saharan Africa (53)

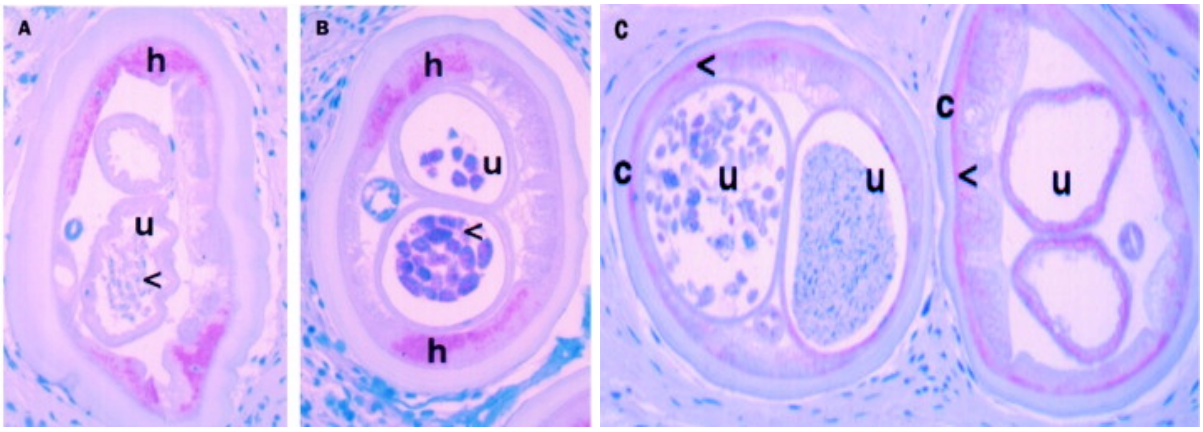


Figure 2. The endosymbiotic *Wolbachia* bacteria of filarial nematodes. (A+B) Histology of midbody cross-sections of *Onchocerca volvulus* in skin nodules. *Wolbachia* bacteria are stained red within the hypodermis (h). (C) Worm from a doxycycline plus ivermectin treated patient, nodule removed 5 months after ivermectin treatment. No bacteria are seen in the hypodermis. Depicted are worms uterine (u) containing early embryonic stages as well as the cuticle (c). From Hoerauf et al. 2001 (45).

1.6 Immune mechanisms against helminth infections

The immune system of vertebrates developed during the evolution several ways to cope with pathogens, since every pathogen displays a different challenge for the host (59). While infections with bacteria or protozoans elicit a protective type 1 immune response, helminth infections trigger a type 2 immune response (60). This type 2 immune response favours antibody mediated responses while down regulating inflammation. It is characterized by high levels of type 2 cytokines like IL-4, IL-5, and IL-13 as well as increased numbers of eosinophils, basophils and mast cells (61). Macrophages switch to an alternatively activated phenotype (AAM, M2) and antibodies of the types IgE and IgG1 (mouse) or IgG4 (human) get increasingly produced.

First immune responses are mounting against the infective L3 larvae of *L. sigmodontis*, when they enter the host during the blood meal of the vector and migrate to site of infection (62). While this first phase of infection is less well studied than the chronic phase of infection, it is known that especially innate immune cells within skin and lymphatics are driving the first response. *Wolbachia* provide a stimulus by getting recognized by mast cells in the skin,

leading to mast cell degranulation and increased vascular permeability controlled by CCL17, a chemokine produced by dendritic cells (DCs) (63). L3 migration occurs rapidly and as experiments using *B. pahangi* demonstrated, inflammatory responses are getting stimulated within 3 hours after infection, mainly IL-6, TNF and neutrophils. Other typical type 2 associated cells like eosinophils play a pivotal role during primary infections after 7 dpi, a time point were the L3 larvae already reached the site of infection (64, 65). Of note, almost 80 % of the larvae do not survive the skin stage of infection, allowing only the strongest larvae to survive and migrate to the pleural cavity (64). The processes along the passage from skin through the lymphatics to the pleural cavity are largely unknown. With the use of knock-out mice, roles for granzyme A (66) has been suggested, while the deficiency of IFNγ (67) and IL-5 (68) did not influence early parasite burden.

After reaching the site of infection, the pleural cavity, *L. sigmodontis* induces a variety of immune responses. The initial points for the type 2 immunity against helminths are type 2 innate lymphoid cells (ILC2s). They respond to IL-25 and IL-33, which are induced by epithelial cells when worms are migrating to the pleural cavity. An expansion of ILC2 results in increased levels of IL-5 and IL-13, leading to a robust Th2 response (69). ILC2s expand locally, but were not expanded in the spleen or blood, suggesting that ILC2s function primarily at the site of infection (69). The induction of a type 2 immune response in the thoracic cavity leads to an increased $CD4^+$ T cell population, which further strengthens the immune response against the residing worms. Under the influence of IL-4, which are also known to be produced by the ILC2s, the $CD4^+$ T cells differentiate to type 2 effector cells, which not only produce more type 2 cytokines (70), but also important chemokines like CCL11 (Eotaxin 1) (71). The key role of $CD4^+$ T cells in helminth infections was already shown in several studies using various helminths. Mice deficient for $CD4^+$ T cells were not able to establish protective immunity against *S. mansoni* (72) and could not expulse intestinal

N. brasiliensis (73). During *L. sigmodontis* infection depletion of $CD4^+$ T cells leads to a significantly increased microfilariae load and adult worm burden as was shown by Al-Qouad et al. (74). In this particular study the depletion went along with diminished type 2 cytokine production like IL-4 and IL-5 and filarial specific IgE as well as a lower frequency of eosinophils. In contrast, depletion of CD8+ T cells did not influence the adult parasite burden (75). As demonstrated in the complimentary project D, infection with *L. sigmodontis* in Rag2IL-2R$\gamma^{-/-}$ mice, mice which are deficient in T, B and NK cell populations, resulted in a comparable adult worm burden between C57BL/6 WT and Rag2IL-2R$\gamma^{-/-}$ mice by 30 dpi. By 72 dpi worms were still abundant in the KO-mice and high quantities of microfilaria were found both in the thoracic cavity and in the peripheral blood of the KO-mice, but not in the WT controls. Production of cytokines like IL-4, IL-6 or TNF were lower in the Rag2IL-2R$\gamma^{-/-}$ mice and eosinophil and macrophage populations were significantly lower in the knock-outs (76).

The role of B cells on the other hand is still not sufficiently described. Due to IL-4 and IL-5, B cells proliferate and differentiate to antibody producing plasma cells. The antibodies bind to the surface of pathogens and this leads to the opsonisation of the pathogen (70). XID-deficient mice, which lack B1-cells, harbour more MF and adult worms, while type 2 immune responses are impaired (77). Similarly, experiments using μMT mice, which lack mature B cells, demonstrated an impaired killing of injected *B. malayi* MF (78). Interestingly, μMT mice infected with *L. sigmodontis* showed no circulating MF, suggesting that antibodies may be present to stimulate embryogenesis by adult worms (79). Due to the controversial data, more studies focusing on B cells have to be done to clarify their role in immunity against helminths.

Granulocytes, i.e. neutrophils, basophils and especially eosinophils are well addressed in the context of filarial infections and are known to have major importance. Eosinophils are a

hallmark of the type 2 immune response. The maturation of eosinophils and their infiltration into tissues is depending on IL-5 (80). Eosinophils are accumulating a few days after *L. sigmodontis* infection in the thoracic cavity and stay there over the course of infection in an IL-5 depending manner (68). Using IL-5 transgenic mice, which produce more IL-5, it was shown that increased eosinophil numbers leads to a decreased adult worm burden (81). In this context the production of the two eosinophilic granule proteins EPO (eosinophil-peroxidase) and MBP (major basic protein 1) is from major importance, as mice lacking these two proteins exhibit a significantly increased worm burden compared to WT controls (82). Basophils are the granulocytes with the lowest frequency in the human host, but they are potent effector cells producing high amounts of IL-4 during helminth infections (83). Studies using *N. brasiliensis* also showed their impact during encapsulation and killing of helminths (84). In the *L. sigmodontis* model, depletion of basophils subsequently leads to a significant decrease in IgE and eosinophils as well as parasite specific T cell-proliferation (85). However, adult worm burden and microfilaremia were not altered in this study. Thus, basophils can strengthen the type 2 immune response against helminths, but are not essential for protection against *L. sigmodontis*. Torrero et al. evaluated in their study from 2013 the role of basophils in vaccine-mediated protection against filariae. While depletion of basophils during the primary infection did not alter the protective immune responses, the vaccine-induced cellular immune response was substantially decreased in basophil-depleted mice. They especially found the type 2 cytokine production by CD4+ T cells to be significantly decreased (86).

Neutrophils are known to be one of the first immune cells at infection sites, but are mainly associated with bacterial infections. Nevertheless they are implemented in helminth infections as more and more studies reveal. Chen et al. for instance demonstrated that neutrophils are essential for the priming of AAMs in an IL-13 dependent manner, which accelerate helminth expulsion (87). While they used infections with *N. brasiliensis*, a similar function for

neutrophils may exist during infection with *L. sigmodontis*. Neutrophils can produce both IL-4 and IL-5 in response to helminths (59). In *L. sigmodontis*-infected BALB/c mice neutrophils are essential for killing of adult worms (68). Al-Qaoud et al. demonstrated that the cell population essential for inflammatory nodule formation are IL-5 controlled neutrophils. Neutralization of IL-5 led to a failure of both eosinophil and neutrophil accumulation at the site of infection resulting in the lack of inflammatory nodule formation around worms. The study further proved that production of neutrophil promoting cytokines like TNF, G-CSF and KC (CXCL1) were found to be strongly reduced after anti-IL-5 treatment, demonstrating an indirect effect of IL-5 on neutrophils (68). Endosymbiotic *Wolbachia* are crucial for the neutrophil-depending immune response. For instance in human patients with onchocerciasis, *Wolbachia* are decisive for neutrophil-accumulation (88) and results from Pearlman et al. indicate an essential role for *Wolbachia* in the corneal pathology. Innate inflammatory responses are initiated by the release of *Wolbachia* and *Wolbachia* endotoxin-like molecules from dead and degenerating worms, which then activate TLR2 on resident corneal cells. This facilitates neutrophil recruitment to the corneal stroma and neutrophil activation, subsequently leading to the corneal haze (26, 89).

As already mentioned for the different cell types of the immune system, IL-4 and IL-5 are broadly implemented in the immunity against helminths. IL-5 is thereby more important for containment of parasitemia than IL-4. IL-$5^{-/-}$ mice allowed development of more larvae into adult worms and exhibited an increased adult worm burden compared to WT controls and prolonged worm survival (90). In contrast, in IL-$4^{-/-}$ mice *L. sigmodontis* adult worm survival and nodule formation did not differ between IL-$4^{-/-}$ and BALB/c WT controls. In both IL-$4^{-/-}$ and IL-$5^{-/-}$ microfilaremia was greatly enhanced and prolonged compared to WT mice (91, 92). IL-5 transgenic mice producing high amounts of IL-5 were proven to be more efficient in encapsulation and killing of *L. sigmodontis* adult worms compared to WT controls (81), while

anti-IL-5 treatment during *L. sigmodontis* infection leads to an increased worm burden (68). On the other hand, IL-4 seems to be more involved in protection against microfilariae, as IL-$4^{-/-}$ mice have an increased and prolonged MF load compared to WT controls (91). The study also showed that absence of IL-4 leads to longer adult worms 77 dpi. Interestingly, the genetic background impacts the role of IL-4 on adult worm burden, as it does not influence adult worm burden in BALB/c mice, whereas semi-resistant C57BL/6 mice lacking IL-4 had an increased adult worm burden (93).

As one of the main antigen-presenting cells, macrophages are also involved in the protective immunity against helminths. It was assumed that macrophages are recruited to the pleural cavity upon infection (94), but newer studies proved that after *L. sigmodontis* reach the thoracic cavity, local proliferation of AAM is occurs (95). These AAMs are appearing quickly after infection and remain in the pleural cavity at least until 60 dpi (96). AAMs are a characteristic part of the type 2 immune response and have regulatory capacities. While the role of the AAMs during *L. sigmodontis* infection is not fully elucidated, Chen et al. demonstrated in their study from 2014, that AAMs are primed by neutrophils and mediate expulsion during infection with *N. brasiliensis* (87).

Interestingly, several studies demonstrated that even characteristic features of a type 1 immune response can be involved in the protection against helminths. For example, Saeftel et al. proved that IFNγ deficient mice have a reduced neutrophil recruitment and accumulation compared to WT controls, suggesting that IFNγ is important for a proper neutrophil migration. The neutrophils are then essential for then encapsulation processes in the thoracic cavity, thus directly controlling worm load and nodule formation (67).

Neutrophils, which are essential for the encapsulation process in the thoracic cavity, where the adult worms reside, were diminished at this location in $IFN\gamma^{-/-}$ mice infected with *L.*

sigmodontis compared to infected WT mice; they also displayed strongly reduced chemotactic and phagocytic activity compared to neutrophils of controls (67).

Other than protective immune responses, helminths are able to induce a regulatory immune milieu within the host to secure their long-term survival. These immunomodulatory abilities of helminths and especially *L. sigmodontis* are discussed in section 1.9.

1.7 Nod-like-receptors

To cope with the crucial task of recognizing and differentiating between self and non-self, especially in the context of pathogen recognition, the immune system of metazoans evolved two major classes of germ-line encoded patter-recognition-receptors (PRR).

The best known group of PRRs are the Toll-Like-Receptors (TLRs). As part of the innate immune system they recognize conserved structures of pathogens, called pathogen-associated molecular patterns (PAMPs). Depending on the type of TLR, they sense a variety of PAMPS like lipopolysaccaride (LPS, by TLR4), bacterial lipopeptides and peptidoglycans (via TLR2, TLR6) or single stranded RNA (via TLR7) (reviewed in (97)). The 11 functional described TLRs in humans are widely spread in several cell types and are often assisted in their function by other membrane-standing proteins like CD14 (98) or CD36 (99).

While TLRs are - due to their importance in immunity - subject of a lot of studies in several disease models and basic research (100) another group of PPRs, the nucleotide-binding and oligomerization domain containing receptors (short: NOD-like receptors or NLRs) were for a long time underestimated in their role and importance in immunity, but lately gained increased attention. The cytosolic, intracellular NLRs have emerged as one of the central players in innate and adaptive immunity with a broad range of functions for the organism (101, 102). NLRs are found among both plants and animals, with to date 22 described NLRs in humans as well as 34 in mice and even larger numbers described in invertebrates like in sea

urchins, which contain more than 200 receptors (103, 104). As sensors of intracellular events, NLRs not only respond to pathogens, but moreover to general signals of stress and danger (DAMPs = danger associated molecular patterns, (105)). The activation of the inflammasome is one of the main tasks of many NLRs (102). Studies also demonstrated that NLRs and TLRs can act synergistically, which can be crucial for the establishment of effective immune responses (97). All members of the NLRs are characterized by a central NACHT nucleotide binding domain (NBD) and – with one exception (NLRP10) - a C-terminal leucine rich repeat (LRR) for sensing ligands and probably mediating ligand binding (106). As seen with the nomenclature established by Ting and colleagues, the domain architecture can be used to classify all 22 human NLRs into subgroups (103). The N-terminal of the NLRs is designated by heterogeneity since they vary in the NLR subgroups and specify the function of the receptor (reviewed in (107)). Most NLRs contain a pyrin domain (PYD) or, like for NOD1 and NOD2, a caspase-activation and recruitment domain (CARD) (107).

Although NLRs were first described to be an intracellular sensor for pathogens, NLRs have been shown to play important roles in distinct biological processes ranging from regulation of antigen presentation, sensing metabolic changes in cells, modulation of inflammation, cell death, and differentiation of the adaptive immune response (108). While proteins similar to human NLRs have been found in plants and all types of invertebrates, it is pretty interesting that they are apparently missing in insects and nematodes, the two big phyla containing endosymbiotic *Wolbachia* (107).

1.8 The NOD2 receptor

The NOD2 receptor is an intracellular PRR, which is activated after recognition of the muramyl dipeptide (MDP) motif within the cell wall component peptidogyclan (PGN) in gram-negative bacteria (105). Just recently the first biochemical evidence for a direct, high-affinity interaction between NOD2 and MDP was reported by Grimes et al. (109). After its

activation, NOD2 induces a signal cascade that mounts in NFκB-induced pro-inflammatory responses, which includes the production of pro-inflammatory cytokines like IL-1ß or IL-18 as well as other mediators of inflammation like ß-defensins or reactive oxygen species (ROS) (110). Further downstream effects include the induction of autophagy, antiviral responses and contributions to the maintenance of the intestinal microbiota composition (111). The complexity of NOD2 signalling is highlighted by the fact that over 30 cellular proteins interact directly with NOD2 and influence its functional activity (112). Polymorphisms of the NOD2 gene are the cause of the inflammatory disorder Blau syndrome or the inflammatory bowel disease Crohn's. Recent studies also linked the NOD2 receptor to wound healing processes (113).

Similar to other NLRs, NOD2 contains a central NACHT domain and C-terminal LRR, which act as a sensor domain and mediate protein-protein interactions (114). As an N-terminal effector domain, NOD2 contains two CARD domains (115). The close relative of NOD2, NOD1, contains one CARD domain. In contrast to NOD2, NOD1 is activated by the ligand D-glutamyl-meso-diaminopimelic acid (meso-DAP), which is mainly present in Gram-negative bacteria (116). Both NOD2 and NOD1 interact after their respective activation with the well-studied adaptor protein Rip2 (117). Questions still remain about the interaction between NOD2 and Rip2 as well as its role in the downstream signalling like the MAP-kinase pathways (118). Further studies also have to investigate the mechanisms underlying the internalization of the ligands (MDP) into the cytosol, since this process also remains poorly understood. One study showed that MDP reaches the cytosol by endocytosis in a clathrin-dependent manner with the cytosolic internalization of the ligands being pH-dependent and occurring prior to the acidification mediated by vacuolar ATPase (119). From special importance in that context could be the finding that NOD2 partially localizes to the plasma and endosomal membrane of cells in which they are endogenously expressed. Using

overexpression techniques, this characteristic was also confirmed in vitro (120). The exact mechanisms remain unclear, but it is known that NOD2 is positioning itself to the site of ligand entry (120). The NOD2 receptor can bind to peripheral membrane proteins, anchoring itself to the membrane (121). This whole interaction is most probably mediated by the LRR domains of NOD2. Membrane recruitment of NOD2 within the cells not only seems to have a positive effect on NOD2 signalling as it enhances NF-κB signalling (121, 122), but also can directly improve bacterial killing.

Initially, the NOD2 receptor was described to be mainly found in classical antigen presenting cells (APCs) like macrophages (123) or dendritic cells (124). However, the distribution is much more prevalent with studies reporting NOD2 receptor expression in eosinophils (125), neutrophils (126), intestinal epithelial cells (127), paneth cells (128), keratinocytes (129), lung and oral epithelial cells (130, 131), and osteoblasts (132).

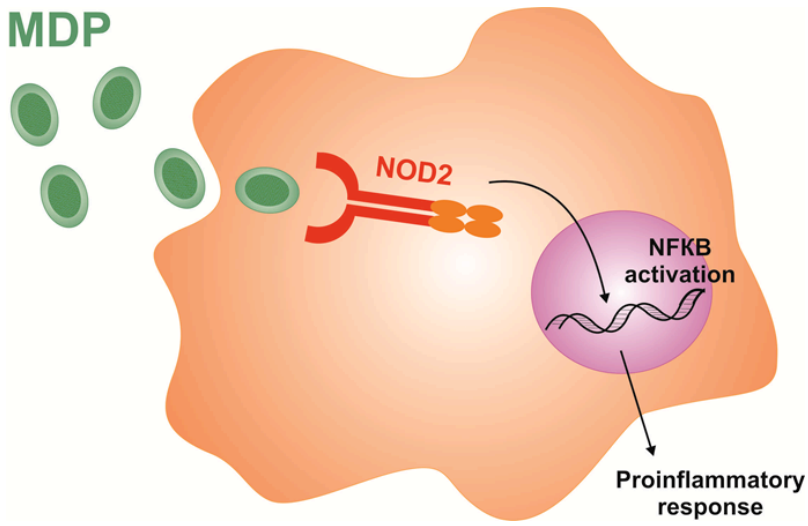


Figure 3. NOD2 receptor. The NOD2 receptor recognizes the MDP motif from cell walls of bacteria. Binding and activation of the NOD2 receptor leads to activation of the NFκB pathway and ultimately to a pro-inflammatory response.

1.9 The NOD2 receptor in the context of parasite infections

The NOD2 receptor is mainly known for its recognition of bacterial pathogens. This role is confirmed by a broad range of studies, e.g. during infections with bacterial pathogens like *Listeria monocytogenes* (133, 134), *Chlamydophila pneumoniae* (135), *Bacillus anthracis* (136), *Legionella pneumophila* (137) or *Citrobacter rodentium* (138). Additionally, NOD2 also seems to have an important role in recognition of viruses in experimental infections (139).

Due to the fact that most parasites do not offer a peptidoglycan structure as a ligand for NOD2, only a handful of studies were conducted to investigate the role of NOD2 on parasite infections. A study by Shaw et al. showed that $NOD2^{-/-}$ mice failed to clear infection with the protozoan parasite *Toxoplasma gondii*. In their study, lack of NOD2 led to an impaired IFNγ production and Th1 lymphocyte differentiation (140). However, these results were rebutted by three independent groups (141). Those studies could not find any influence of the NOD2 receptor during *T. gondii* infection. The reason for this significant discrepancy may be related to variations in the bacterial microbiota of the used NOD2-deficient mice. Studies were also performed using other intracellular protozoan parasites such as *Plasmodium berghei* ANKA, a mouse model for malaria (142), and *Trypanosoma cruzi*, the causative agent of the Chagas disease (143). While NOD1 was found to be associated with host resistance against *T. cruzi*, NOD2 was neither required for protective responses against *T. cruzi* nor *P. berghei* ANKA.

The idea of the intracellular NOD2 receptor controlling or having an influence during the infection with large multicellular pathogens like helminths may appear farfetched. However, due to the *Wolbachia* endosymbionts within most parasitic filarial nematodes, the NOD receptors could have an impact on filarial infections. *Wolbachia* may provide ligands for NOD2 stimulation with their MDP motif within their cell wall. Of note, *Wolbachia* are a cell-wall less genus, and a peptidoglycan (PGN) structure has not reliably been detected yet in

these bacteria. However, *Wolbachia* do own the complete enzymatic machinery to synthesize cell wall components like Lipid II, from which MDP is derived (144). A further hint for NOD2 being involved in the immunity against parasitic helminths is the fact that *Wolbachia* are only contained as endosymbionts by arthropods and nematodes (145), the exact two genera lacking the NOD2 receptor (146).

1.10 Immunmodulatory ability of helminths

As mentioned in section 1.5, helminths are able to down regulate host immune responses to establish a hyporesponsive, regulatory milieu within the host to facilitate their long-term survival. This ability to modulate the host immunological environment is a key part of the evolutionary success of helminths. Inflammatory processes are suppressed and pathogenic damages of the host are reduced or even subclinical. This ultimately allows the parasite to persist within the host for years to decades (60). Mechanisms which down regulate type 1 and type 2 immune responses include the induction of regulatory T cells (147), AAM (95), the suppression of antigen-presentation by DCs (148, 149), as well as increased production of regulatory cytokines like IL-10 and TGF-ß (150, 151). The immunomodulatory abilities of helminths impact the course of co-infections and the efficacy of vaccinations (152, 153). For example in the co-infection model of *L. sigmodontis* and malaria, *L. sigmodontis*-derived IL-10 protects mice from malaria-associated pathology (154). In asthma mouse models, *L. sigmodontis* infection suppressed asthmatic symptoms like Ag-specific Ig and pulmonary eosinophilia (155). Furthermore, Hübner et al. demonstrated that chronic filarial infections do not exacerbate *Mycobacterium tuberculosis* infection (156) and a study by Gondorf et al. showed that chronic filarial infection provides protection against bacterial sepsis by functionally re-programming macrophages and reducing proinflammatory immune responses (157). Especially in the field of autoimmune diseases, helminth infections and products excreted/secreted by helminths proved to be a promising therapeutic tool. In humans,

infections with diverse intestinal helminths suppressed the progression of multiple sclerosis (158). Administration of *T. suis* eggs is furthermore an efficiently used experimental therapy in human patients (159, 160). Similarly, helminth-derived molecules have been described with potential immunosuppressive effects, which could be used to counter inflammatory diseases (161). One such molecule is ES-62, a glycoprotein from the nematode *Acanthoceilonema viteae*, which modulates cellular activity via TLR4 (162). This molecule was shown to promote type 2 immune responses through TLR4-dependent activation of APCs and it successfully prevented the development of collagen-induced arthritis, oxazolone-induced contact sensitivity and ovalbumin-induced airway hypersensitivity in mice (163). Cystatin, a cysteine protease inhibitor (CPI), is another well studied immunomodulatory molecule, shown to attenuate Ovalbumin airway hypersensitivity as well as Dextran sulfate sodium (DSS)-induced colitis (164). Nematode cystatins inhibit proteases involved in antigen processing and presentation and therefore reduce T cell responses. Nematode cystatins were further described to promote IL-10 and Th2 cytokine secretion by macrophages (165). In the case of ES-62, small molecule analogues are now synthesized which mimic the immunomodulatory functions of the helminth molecules, but are better suited for clinical administrations as they lack antigenic properties.

Recent research is dealing with the effects induced by helminths suppressing autoimmune diseases, especially regarding the ever spreading diseases of industrial countries - type 1 and type 2 diabetes (166).

1.11 Type 1 Diabetes and helminth infections

Diabetes is a chronic metabolic disease characterized by elevated blood glucose levels due to impaired insulin production by pancreatic ß-islet cells or an insufficient insulin-sensitivity of insulin target organs (167). In 2014, around 387 Mio people were living with diabetes (168), with a worldwide increasing incidence in both industrial and developing countries. Estimations for 2035 assume an increase to 592 Mio diabetes cases (168). Around 5 Mio

people die every year from diabetes, as the chronic disease causes blood vessel damages and affects organs such as eyes, brain, kidney, and heart. Due to the rapid increase in the incidence of diabetes, its chronic nature and associated co-morbidities, as well as high health care costs, diabetes represents one of the biggest global challenges for public health.

While type 2 diabetes is the result of insulin resistance of organs and impaired glucose uptake, type 1 diabetes (T1D) is caused by the destruction of the insulin-producing ß-islet cells of the pancreas. T1D accounts for 5-10% of all diabetes cases and especially in industrial countries the incidence is dramatically increasing (169, 170). The subsequent loss of insulin production leads to increased blood glucose levels that led before the discovery of insulin by Banting and Best in 1922 ultimately to death (171). As genetic factors alone cannot explain the recent increase in T1D incidences, many studies support that environmental factors have to be taken into consideration. This "hygiene hypothesis" suggests that improved hygiene in developed countries changes the surrounding environment of the inhabitants of developed countries with decreasing bacterial, viral, fungal and parasitic infections. The human immune system evolved in the presence of parasitic helminths (172-174), but due to improved hygiene and living conditions in developed and newly industrialized countries; humans are barely exposed to helminths any longer. This lack of helminth infections prevents immunomodulatory responses triggered by the worm, consequently the immune system becomes hyper-responsive and more susceptible to autoimmune diseases. As reviewed in Berbudi, Ajendra 2015, studies using several species of helminths and/or their derived products demonstrated that helminths have the ability to prevent or delay T1D onset (166).

Immunological mechanisms leading to this helminth-mediated protection include a shift to a type 2 immune response, as shown for *Heligmosomoides polygyrus* (175), *Strongyloides venezuelensis* (176), *Trichinella spiralis* (177) or *Schistosoma mansoni* (178). Hübner et al. demonstrated that protection from T1D onset using *L. sigmodontis* is independent of a type 2

immune shift, but requires TGF-ß (179, 180). At the same time the regulatory cytokine IL-10 was not required. As infections with living worms have several disadvantages as a therapeutic approach, most recent studies are focusing on helminth-derived products like worm-antigen or eggs. Administration of *F. hepatica* (181) or *S. mansoni* products reduced the incidence of T1D in the NOD mouse model (182) and treatment with a crude *L. sigmodontis* extract delayed T1D onset in NOD mice and lowered diabetes incidence (179). Common for all published studies is the fact, that protection was only achieved when administration was started in mice younger than 6 weeks of age. This is due to the physiology of the mice used, the non-obese diabetic (NOD) mice. The NOD mouse model is a widely used tool in diabetic research. It was generated in Japan (183) and particularly female NOD mice are prone to develop T1D with around ~80% probability. The infiltration of autoreactive T cells, which leads to the destruction of ß-islet cells, starts by the age of 6 weeks. The older the mice, the more infiltration and insulitis can be observed, and mice cannot be protected anymore with the aforementioned infections or helminth product administration.

1.12 Objectives of this thesis

A – Combination therapy of LsAg and proinsulin to protect NOD mice from T1D onset

As infections with living helminths as well as administrations with helminth products only protect from T1D before the onset of insulitis, i.e. in NOD mice 6 weeks of age and younger, new treatment regimens have to be tested. A way to enhance the effect of helminth antigen treatment is to combine the helminth antigen induced non-specific regulatory immune responses with the induction of protective diabetes-specific responses. Bresson et al. demonstrated that combination of anti-CD3 and proinsulin treatments are able to reverse recent diabetes onset in NOD mice (184). The first aim of this thesis was to investigate

whether combined administration of intraperitoneal LsAg with intranasal proinsulin has a synergistical effect and protects NOD mice from T1D onset after the development of insulitis. In complimentary project A it was investigated which immune responses are induced with repeated injections of LsAg in BALB/c mice.

B – The role of the NOD2-receptor during L. sigmodontis infection

A possible impact of the NOD2 receptor on the outcome of filarial infection is particularly interesting as many filarial nematodes harbour the endosymbiont *Wolbachia*, which possesses a potential ligand for NOD2 activation. Furthermore, reporter cell lines demonstrated that NOD2 gets activated by *Wolbachia* containing crude worm extracts. For this thesis, NOD2 deficient mice were to be naturally infected with *L. sigmodontis* and parasite burdens as well as immunological responses at different time points post infection were to be analysed. Obtained results should further support our understanding of the interactions between the parasitic filariae and the host's immune system. The proposed role of the NOD2 receptor during filarial infections would increase our knowledge for protective mechanisms and may be also relevant for infections with the causative agents for LF and onchocerciasis. Further complimentary projects were performed to investigate additional components of the protective immune response regarding their role during infection with *L. sigmodontis*. This included the role of the IL-33 receptor ST2 (complimentary project B), the role of the cytokine interleukin-6 (complimentary project C) as well as the impact of *L. sigmodontis* infection in mice lacking T cells, B cells and NK cells, the Rag2IL-2R$\gamma^{-/-}$ mice (complimentary project D).

2. Materials & Methods

2.1 Mice

All mice (*Mus musculus*) used were maintained and bred under specific pathogen-free conditions according to animal welfare guidelines in the animal facilities of the Institute for Medical Microbiology, Immunology and Parasitology (IMMIP). Animals were kept at 22°C and a 12h day-night-rhythm. Water and food pellets were provided ad libitum.

C57BL/6 NOD2$^{-/-}$ mice were obtained from Prof. Dr. Thomas Kufer / Dr. Michael Schramm (Institute of Medical Microbiology, Immunology and Hygiene, University Hospital Cologne, Germany). C57BL/6 mice obtained from Janvier Labs (Le Genest Saint Isle, France) were used as corresponding WT controls. For T1D experiments, non-obese diabetic NOD mice were used, which were maintained at the House of Experimental Therapy (HET, University Hospital Bonn). For experiments sex- and age-matched groups were used; only female mice were used for diabetes experiments.

2.2 Life cycle of *L. sigmodontis*

The life cycle of the parasite *L. sigmodontis* is kept in a secluded area of the animal facilities of the IMMIP. As an obligate intermediate host, the tropical rat mite *Ornithonyssus bacoti* is used. Mites were bred in small basins, covered in fine litter. The mite containing basins were kept in incubators at 28°C and 80% humidity. Mites were fed with fresh mice blood thrice a week. To maintain the life cycle of *L. sigmodontis*, chronically *L. sigmodontis*-infected cotton rats (*Sigmodon hispidus*) were exposed to mites. The mites subsequently take up L1 larvae during their blood meal and can then be used to infect further animals. Cotton rats were examined for blood microfilariae load. For this, a small amount of blood was taken and MF were counted using a microscope (see section 2.6) and cotton rats harbouring ~1000 MF/µl blood were used for the infection of mites. For infection of mites, a cotton rat was placed in a

cage with the contents of up to two mite basins. With their blood meal, mites are infected with MF. After the blood meal, mites fall back to the bedding in the basin. The bedding was collected in an Erlenmeyer-flask and closed with a polyamid sieve-texture (Gaze). The flasks were kept in incubators in basins filled with soap water. After infection, the cotton rat was placed over night above soap water, so that remaining mites fell into the water. The next day, cotton rats were placed in new cages until next infection. Within the mites, the microfilariae develop to infectious L3 larvae within 10-12 days. To control the parasitemia, a few mites were dissected regularly and L3 larvae were counted.

2.3. Infection with *L. sigmodontis*

For experiments performed, mice were infected either naturally, or via intradermal or subcutaneous injection.

For natural infection mice were placed in bedding with mites, which were infected for 10-12 days, overnight. For this, mite-containing bedding was placed in basins and mice were placed into the basin in cages. All used mice groups were infected with the same batch of mites to ensure a similar infection rate. Mites transmit infectious L3 larvae during their blood meal to the mice and mites fall back to the bedding of the basins afterwards. Next day, the bedding was collected and discarded. Mice were placed in cages above soap water to let the rest of the mites fall into the water. The day after, mice are placed in new cages with new bedding and food. For quarantine, cages were changed daily for five days, after the fifth day, mice were brought to a different part of the animal facilities and remained there until the required infection time point.

For subcutaneous injection of *L. sigmodontis*, mites were dissected under the binocular and motile infectious L3 larvae were collected. 40 L3 larvae were taken up in 1ml of PBS and then injected subcutaneously into mice. Control mice were injected with PBS only. For intradermal injection, 20 L3 larvae in 100µl PBS were injected per mice in the shaved

area of the upper hind leg region. Two days prior to injection, mice were unhaired using commercially available depilatory cream. Control mice were injected with 100µl PBS.

2.4 Euthanasia of mice

Mice were euthanized using an overdose of Isoflurane (1-cloro-2, 2, 2-trifluoroethyldifluoro-methylether; Forene®, Abbolt, Wiesbaden). For this, mice were placed into a sealed plastic box and were indirectly exposed to an overdose of Isoflurane until they died.

Experiments using the $NOD2^{-/-}$ mice were performed 4, 5, 7, 10, and 15 days post infection (dpi). NOD mice for T1D experiments were euthanized 24h, two weeks and four weeks after combination therapy.

2.5. Determination of worm load

To determine possible differences in worm burden between $NOD2^{-/-}$ mice and WT controls, all worms were isolated after euthanasia of mice and counted. To ensure that all filariae were counted, pleural lavage was filtered using sterile 41µm gaze filter, and the filter was afterwards placed in a Petri dish. Additionally, the peritoneal cavity was checked for worms. L3 and L4 larvae were counted using a binocular. Adult worms were counted and differentiated into male and female worms without magnification.

2.6. Determination of microfilarial load

To determine the microfilarial load of mice, blood was taken from the facial vein. 30-50µl blood was taken and resuspended in 1ml of 1x red blood lyses buffer (10x RBC lyses buffer, eBioscience, San Diego) and kept at room temperature (RT) until counting. Before counting, blood was centrifuged at 400g for 5min at RT (Eppendorf Centrifuge 5810R). The supernatant was discarded and the pellet resuspended, completely transferred to an object slide and covered with a cover slip. The whole slide was counted with the aid of a microscope using the 10x objective.

2.7 Plasma isolation

For plasma collection, blood was collected in heparin tubes. Blood samples were centrifuged at 7000g at RT for 5 min. Plasma was taken subsequently and kept for further use at -20°C.

2.8. Isolation of thoracic cavity cells

Euthanized mice were disinfected with 70 % ethanol and fixed on a Styrofoam board dorsally. Using sterile surgical instruments, the mouse was opened ventrally starting at the abdomen in a V-shape up to the thorax. With a little cut below the last costal arch, the thoracic cavity was opened. With a sterile Pasteur pipette, the thoracic cavity was lavaged using 1ml of cell culture medium (RPMI 1640, PAA) or PBS (PAA laboratories, Pasching) to isolate cells. Hereby, the first ml of medium was collected separately in an Eppendorf tube, centrifuged for 5min at 400g and the supernatant und kept for further use at -20°C. The pellet was combined with the further lavage of the thoracic cavity using another 4 ml, which was added to a 15 ml falcon and placed on ice for further use. With the thoracic cavity lavage worms were also isolated. For this, the lavage was filtered using gaze-filter paper.

2.9. Isolation of lymphocytes from the pancreas

To remove the pancreas from mice, the peritoneal cavity was opened and the pancreas was isolated and placed into a tube containing 1ml of harvest buffer (PBS, 5% FCS, 1M glucose, 1 complete mini-EDTA free protease inhibitor cocktail tablet (Sigma-Aldrich, Taufkirchen, Germany)). Using scissors, pancreas was minced into small pieces and washed twice for 5min at 400g using ice-cold PBS. Washed pancreatic fragments were resuspended in 1ml pre-warmed Digest Buffer (PBS with Ca^{2+} and Mg^{2+}, 5% FCS, 2,5mg/ml Liberase, 5mg/ml DNAse (Roche, Basel, Switzerland)) and incubated for 10min in a water bath at 37°C. Digestion was stopped by topping up with cold 10mM EDTA in PBS and washed at 400g for 5min. Supernatant was removed and remaining pancreas fragments were forced through a 70µm cell strainer into a Petri dish using a 2ml syringe plunger. Strainer and Petri dish were

rinsed with cold Iscove's modified Dulbecco's medium IMDM (PAA, Pasching, Austria), cell suspensions were transferred to a 15ml falcon and washed twice with cold medium, and centrifuged for 5min at 400g. Following the second wash, the pellet was resuspended in 3ml of 33% Percoll gradient and centrifuged for 25min at 800g without brake at RT. Following centrifugation, the pellet was kept and the supernatant was discarded. The pellet was washed in cold medium again and then counted prior to culture, restimulation or FACS staining.

2.10 Isolation of cells from pancreatic lymph nodes

Pancreatic lymph nodes (pLN) were removed carefully and placed in cold medium. To isolate cells, pLNs were forced through a 70µm cell strainer into a small Petri dish using a 2ml syringe plunger. Strainer and Petri dish were rinsed regularly to elute as many cells as possible. In the next step, cells were centrifuged at 400g for 7min at 4°C, the supernatant was discarded and the pellet was resuspended in 1ml of medium. Cells were then counted and kept on ice for further use in FACS analysis.

2.11 Preparation of cells from the thoracic cavity

The first ml of cells, which were isolated from the thoracic cavity of the mice, was centrifuged at 400g for 5min. The supernatant was put into new Eppendorf tubes and kept at -20°C for further analysis per ELISA. The pellet was combined with the 4ml of thoracic cavity wash; cells were counted and adjusted according to the experiment's protocol. A part of the cells were restimulated ex vivo for ELISA. Cells were adjusted to 2Mio. cells /ml and either unstimulated or stimulated with 50µg/ml LsAg or 2,5ng/ml ConA. Cells were then incubated for 72h at 37°C, 5% CO_2 in an incubator. Following incubation, supernatants of culture were taken and centrifuged at 400g for 5min for further use as samples for ELISA analysis.

2.12 Spleen cell isolation

After the isolation of the thoracic cavity cells, the spleen was excised and kept on ice for further use. To isolate cells from spleen, each spleen was forced through a 70µm cell strainer

into a small Petri dish using a 2ml syringe plunger. Strainer and Petri dish were rinsed regularly to elute as many cells as possible. In the next step, cells were washed at 400g for 7min at 4°C, the supernatant was discarded and subsequently a red cell lyses was performed. For this, the pellet was resuspended in 1ml of red blood lyses buffer and incubated for 5min. Reaction was stopped by topping up with 10ml RPMI medium. After another washing step (7min, 400g), the pellet was resuspended in 10ml of RPMI cell culture medium, cells were counted and adjusted for further use in FACS analysis or were restimulated similar to thoracic cavity cells for ELISA.

2.13 Determination of cell number

To determine the numbers of isolated thoracic cavity cells as well as pancreas or spleen cells, the Casy® Cell Counter + Analyser System Model TT (Roche) was used. First, a Casy® tube was filled with 10ml of Casyton® buffer and 10µl of the sample was added. After vortexing, the Casy® tube was placed into the measuring capillary and the software determined the cell count.

2.14 Enzyme-linked immunosorbent assay (ELISA)

Cytokines from supernatants of restimulated and unstimulated spleen cells, thoracic cavity cells and others were quantitatively determined using the Sandwich-ELISA method. All ELISAs were performed according to manufacturer's protocols (BD Pharmingen, eBioscience). ELISA plates (Greiner Bio-One) were coated with primary antibodies, diluted in either PBS (IL-5, IL-6) or coating buffer (other cytokines; Na_2HPO_4 0.1M [Merck], pH9.0). Plates were incubated over night at 4°C. The next day, plates were washed once with washing buffer (PBS/0,05% Tween20 (Sigma-Aldrich)) and then blocked using either PBS/1% BSA (PAA, Linz, Austria) or eBioscience Assay diluent for 2h at RT on a plate shaker. After another washing step, samples were added in duplicates or triplicates for a minimum of 2h at RT or overnight at 4°C. For blank controls, PBS was added. Serial

dilutions of standard samples were also added as a reference. After washing five times, the second antibody, the detection antibody, was added for another 2h. Following incubation, plates were washed for five times and the enzyme, horseradish peroxidise (HRP) streptavidin was added and incubated for 1h at RT. The substrate tetramethylene benzidine (TMB) was then added after another washing step to the samples. After a change in colour was detected, the reaction was stopped by adding of 1M H_2SO_4 (Merck). The optical density (OD) of the reaction was then measured at 450nm using SpectraMAX 340 Pc plate reader (Molecular Devices, Ismaning, Germany).

For measurement of *L. sigmodontis*-specific antibodies like IgG1, IgG2a or IgE, plates were coated with 10µg/ml of *L. sigmodontis* antigen extract in PBS overnight. The next steps were performed similar to the cytokine ELISA as describe above.

2.15 Fluorescence activated cell sorting (FACS)

To determine the cell populations within the pleural cavity, spleen, pancreatic lymph nodes or pancreas, cells were analysed via flow cytometry.

Within the flow cytometer, cells flow through a capillary and pass different lasers. The cells reflect a part of the light, with reflection correlating with size and granularity of the cells. These two parameters are displayed on the Forward Scatter (FSC), the dimension for the volume and size of the cell, as well as on the Sideward Scatter (SSC), the dimension for the granularity.

Prior to the analysis, cells were washed with PBS/1% BSA and the subsequent pellet was incubated with Fc receptor block (Sigma) in PBS/1% BSA for at least 1h. After another washing step, cells were ready to be stained according to protocol. 100.000 to 500.000 cells in 100µl PBS/1% BSA were transferred into FACS tubes and then stained using fluorescent-marked monoclonal antibodies. As controls, Fluorescence minus one (FMOs) were used,

samples which lack one of the used antibodies. These control samples were used to set the gates for the specific cell populations. To set the voltages for each channel, BD compensation beads were used. All measurements were performed at a BD Canto 1 (BD Biosciences, Heidelberg, Germany). The flow speed was adjusted to ≤ 8.000 events per second. The data were recorded at logarithmic scale, integrating the forward and sideward scatter (FSC, SSC) width and height and plotted bi-exponential. For intracellular staining of Foxp3, RELMα or intracellular cytokines, cells were fixed and permeabilized with 300µl of fix/perm buffer o/n at 4°C. After cells were washed and incubated for 30min with Fc-receptor block. Following cells were washed twice with permeabilization buffer for 10min at 400g, before cells were then stained in the permeabilization buffer as described above.

2.16 Histology

To determine the quantity of pancreatic ß-islet cells from diabetic mice, histology was performed on pancreata from NOD mice. Pancreata were isolated from mice and directly placed in a 15ml falcon and completely covered in 4% formalin to fix the organ. The preparation of the organs for the histology was performed by employees of the Institute for Pathology of the University Hospital Bonn. Briefly, organs were dehydrated using different concentrations of ethanol and in the next step cleared form all alcohol. The following day pancreata were placed in paraffin blocks and cut in thin slides of 5µm with the aid of a microtome. Cut slides were immediately placed on an object slide and dried o/n at 35°C. The slides were then dehydrated using decreasing concentrations of alcohol, starting with 3x xylol, 100% ethanol, 96% ethanol, 80% ethanol, 70% ethanol, 50% ethanol and finally distilled water. Slides were dipped into each concentration for around 2mins. After the dehydration, slides were placed for 10mins into Mayersches' Hämalaun and then watered for 30min in the sink with tap water. After washing with distilled water, slides were counter-stained with 0.1% eosin. In the next step the slides were dipped again into alcohol like describe above, starting

with distilled water and using increasing concentrations of ethanol. Following staining, a cover slip was added on the slide and optical glue was used to protect the slide. Pancreatic islet cells could now be counted using a microscope.

2.17 Neutrophil depletion

In vivo depletion of neutrophils was achieved by injecting 500µg/ml anti-Ly6G antibody intraperitoneally 18h prior to infection with *L. sigmodontis*. Controls were injected at the same time point with PBS. Successful depletion was confirmed via flow cytometry.

2.18 Macrophage depletion

To deplete macrophages in vivo, 100µl of clodronate containing liposomes and PBS containing liposomes as negative controls were i.p. injected 24h prior to infection as well as 7 and 11 days post infection with *L. sigmodontis*. Successful depletion of macrophages was confirmed by flow cytometry.

2.19 IL-5 depletion

In vivo depletion of IL-5 was achieved via i.p. injection of 10µg/mouse 24h prior to infection and 3, 7, 10, and 13 days post infection with *L. sigmodontis*. Isotype controls were used as negative controls. Successful depletion of macrophages was confirmed by flow cytometry staining of eosinophils and IL-5 ELISA.

2.20 In vitro analyses of neutrophils

To enrich neutrophils, mice were injected with 1ml of Casein solution. To obtain the Casein solution, casein was dissolved in 1M NaOH in PBS and heated to 70°C for 2h. Afterwards, 10mM $CaCl_2$ and 5M $MgCl_2$ were added and the solution was autoclaved for 1h at 125°C. The casein solution was i.p. injected 18h and 3h prior to necropsy of mice. Cells were harvested via peritoneal lavage and centrifuged for 10min at 800g. The pellet was resuspended in PBS and cells were counted. Cells were then further purified using autoMACS

anti-PE staining for Gr1 positive neutrophils (autoMACS Pro Separator, Miltenyi, Köln). An additional adherence step was performed for 2h to get rid of remaining macrophages. Neutrophils were then plated at 1Mio cells/well and stimulated for 18h using 200ng/ml LPS, 500ng/ml MDP, 500ng/ml Pam_3Cys and 25µg/ml LsAg. After incubation at 37°C, supernatants were harvested and stored at -20°C for further analysis per ELISA.

2.21 Gene expression analysis

For a detailed analysis of the molecular process within the skin after *L. sigmodontis* infection, gene expression analysis was performed using skin tissue. Three hours after injection of motile L3 larvae or LsAg, skin was isolated and immediately frozen in liquid nitrogen. Skin was then further prepared for RNA isolation using a homogenizer for 2x20s (PreCellys, Bertin Technologies, Montigny-le-Bretonneux, France). RNA was isolated from homogenized skin using Trizol extraction (Ambion, Austin, USA) and the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). RNA was quantified by NanoVue (GE Lifescience, Chalfont St Giles, Great Britain) and quality was assessed using the Experion gel electrophoresis system (BioRad, Hercules, USA). cDNA was synthesized with the RT2 first strand kit (Qiagen). A customized RT2 PCR array (Qiagen) was performed on cDNA using a Rotor Gene Q (Qiagen). The complete list of genes included in this array is reported in table S1. Samples were performed in triplicates. Data was analysed using the online RT2 Profiler PCR Array Data analysis 3.5 software at the sabiociences.com website (Qiagen). Gene expression was normalized to 3 housekeeping genes (Actb, B2m, Gapdh). From the array several genes were chosen and single-PCR reactions were performed using Bio-Rad PrimePCR protocol (Bio-Rad).

2.22 Reporter assays

Human embryonic kidney cells transfected with either murine NOD1 gene or murine NOD2 gene were obtained from Invivogen (San Diego, USA) and were maintained according to the

manufacturer's protocol. Cells were kept in culture with DMEM growth medium containing 4.5g/l glucose, 10% FCS, 50U/ml penicillin and 50µg/ml streptomycin (all from PAA, Austria). Cells were passaged after reaching a confluency of 70-80%. For the reporter assay, cells were harvested using PBS, counted with a Neubauer-chamber and then a cell suspension was prepared using HEK-blue detection medium. Cells were then added to an ELISA-plate (40000 cells per well) and 10µg/ml LsAg, LsAg –W., MDP, DAP and water control were added additionally. Plate was incubated at 37°C with 5% CO2 for 10 hours and secreted embryonic alkaline phosphatase (SEAP) reporter protein was measured using a spectrometer at 650nm. To lower background signalling, L-homoarginine (Alfa Aesar, Heysham, UK) as a phosphatase-inhibitor was added additionally to the worm antigens.

2.23 Statistics

Statistical analyses were performed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, California, USA), using the Mann Whitney u test for nonparametric comparison of two groups. For comparison of more groups One-Way ANOVA followed by Dunn's post hoc test was used to assess statistical significance. All data, if not mentioned otherwise, are shown as median. P values ≤ 0.05 were considered as significant.

3. Results

3.1. Type 1 Diabetes

Helminth infections and helminth antigens can prevent or delay the onset of autoimmune diseases including T1D (166). The success of these therapies in regard to T1D is however limited as protection is only given, when NOD mice are infected with helminths or treated with helminth antigens before the onset of insulitis, i.e. before 6 weeks of age. With the experimental setup used in this thesis we aimed to improve the already known protective effect of LsAg (179) in combining it with proinsulin. In complimentary project A it was demonstrated that LsAg injections induces immune responses similar to infections with *L. sigmodontis*.

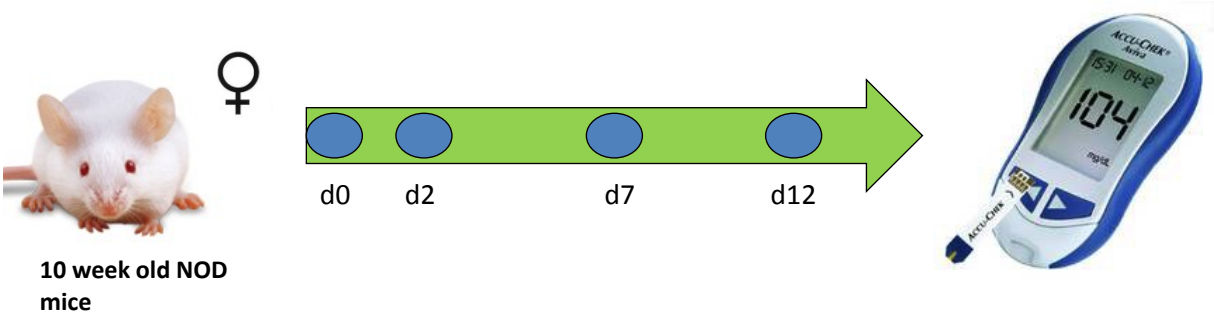


Figure 5: Experimental setup of the combination therapy. 10 week old female NOD mice were treated at indicated days with LsAg i.p., proinsulin intranasal or a combination of both, control animals were treated with PBS

3.1.1 Combination therapy prevents T1D in 10 week old NOD mice

In order to investigate if a combined therapy using LsAg and proinsulin prevents T1D onset in 10-week old female NOD mice, mice were either treated with LsAg, proinsulin or a combination of both (CT). Control animals were treated with PBS. Starting two weeks after treatment, blood glucose levels were measured every other week until 40 weeks of age. Diabetes onset was defined as a blood glucose level of >230mg/dl, mice showing an increased blood glucose level for two measurements in a row, were euthanized. As shown in Figure 6,

only two out of fifteen CT-treated mice (13.3%) developed T1D, whereas eight out of thirteen proinsulin-only treated (61.5%) and eight out of twelve LsAg treated mice developed diabetes (66.7%). Mice treated with PBS as negative controls developed in seven out of thirteen cases T1D (53.8%). Using the Mantel-Cox-test a statistical significant decrease in T1D development was observed in CT-treated mice when compared to mice treated with the single components. This result demonstrates that each component of the CT-therapy by itself does not prevent T1D at this late time point, the combination however was successful. T1D onset occurred in PBS- and LsAg-treated mice starting at 14-16 weeks of age and at 20 weeks of age in proinsulin treated mice (Fig.6).

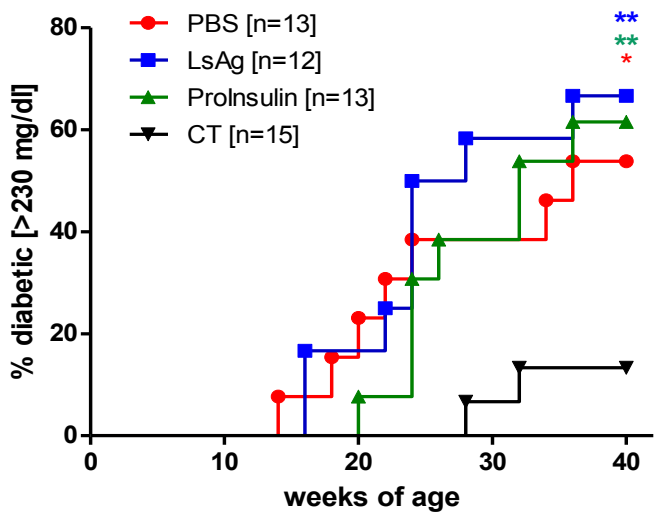


Figure 6. Combined treatment of LsAg and Proinsulin protects 10 week-old NOD mice from T1D onset. Frequency of diabetic NOD mice after treatment with a combination of LsAg and proinsulin (CT), treatments with LsAg or proinsulin alone, and PBS as control. Pooled data from four independent experiments. Survival curve was analyzed for statistical significance using Log-rank (Mantel-Cox) test. **p<0.01 *p<0.05.

3.1.2 Combination therapy does not prevent T1D in 12-week old NOD mice

Since the combination therapy proved to be successful using it in 10-week old female NOD mice, it was next investigated if CT can also protect 12-week old female NOD mice - a time point, where insulitis is further progressed. As illustrated in Figure 7, CT in 12-week old mice

was not successful anymore. While all negative control mice treated with PBS developed T1D by 22 weeks of age, half of the CT treated mice were diabetic by 30 weeks of age. Mice treated with either LsAg or Proinsulin also developed T1D and no positive protective effect was apparent (LsAg 100% T1D onset at week 30; Proinsulin 75% T1D onset at week 30).

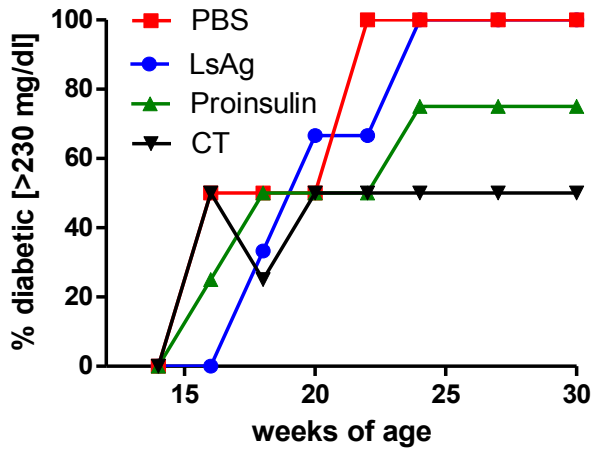


Figure 7. Combination therapy does not protect 12 week-old NOD mice from diabetes onset. Frequency of diabetic NOD mice after treatment with a combination of LsAg and proinsulin (CT), treatments with LsAg or proinsulin alone, and PBS as control. Four mice per treatment group.

3.1.3 Less infiltration of autoreactive T cells in pancreata of 10-week old NOD mice after combination therapy

One hallmark of T1D development is the inflammation of the pancreas and the subsequent loss of pancreatic ß-islet cells. To analyse the effect of the CT on the pancreatic ß-islet cells when administered in 10 week-old NOD mice, pancreata of CT and PBS treated mice were histologically assessed 4 weeks after treatment. As shown in Figure 8, pancreata from CT-treated animals displayed significantly less inflammation than pancreata from PBS controls. Although pancreata of NOD mice with combination therapy also presented intra insulitis (Fig. 8 C) and peri-insulitis, the frequency of non-infiltrated, healthy islets (as shown in Fig. 8B) was significantly higher with 48.1 % in CT-treated animals vs. 10.0 % in PBS controls. Accordingly, the frequency of islets showing signs of cellular infiltration was increased in

PBS controls, with 65.8 % of islets having intra insulitis compared to 29.4 % in CT-treated NOD mice.

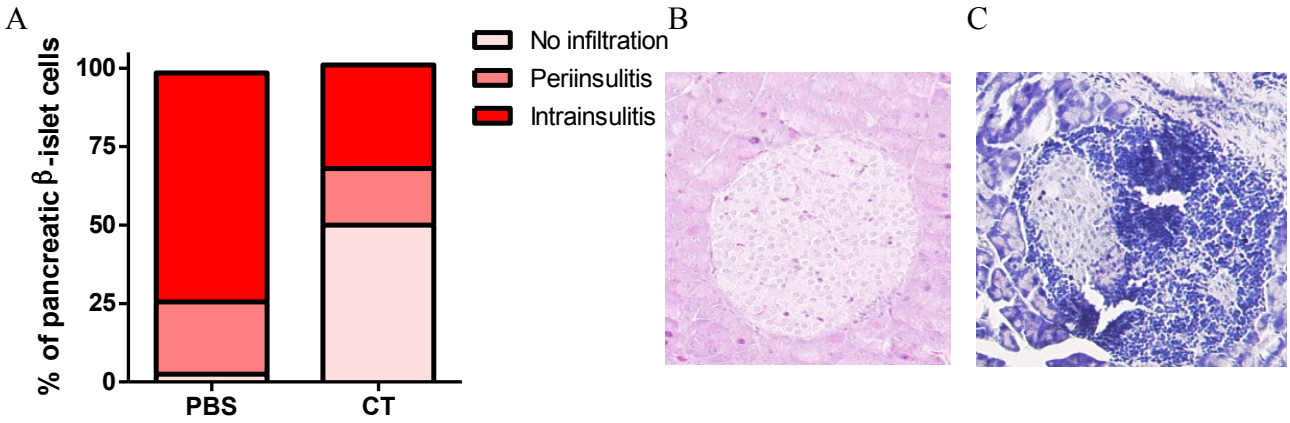


Figure 8. **Pancreatic ß-islet cells of NOD mice 4 weeks after CT treatment exhibit less insulitis**. A) Bars show histologically determined frequencies of ß-islet cells with no cellular infiltration, peri-insulitis and intra-insulitis of CT-treated NOD mice and PBS controls. Pooled data from three independent experiments with 4-5 mice per group per experiment. Data was analyzed for statistical significance by Mann-Whitney U-test, with differences between CT- and PBS-treated animals for intra-insulitis being $p<0.001$ and differences for no infiltration being $p<0.001$. B) Histological example of healthy islet cells without infiltration, C) example of intra-insulitis during T1D in NOD mice with destruction of islet cells.

3.1.4 Splenic T cell frequencies do not differ between CT-treated NOD mice and PBS controls

To analyse whether the combination therapy has a systemic effect, important cell types of the spleen known to be involved in T1D development were investigated via FACS analysis.

24h after the last treatment, no differences were observed for CD4+ and CD8+ T cells as well as Foxp3+ regulatory T cells between CT-treated NOD mice and corresponding PBS controls (Fig. 9A-C). Median values for CD4+ T cells were 24.6% in mice after CT treatment and 23.6% for PBS controls. Frequency of CD8+ T cells were lower in both tested groups, with median being 13.6% for CT animals and 13.2% for PBS animals. The median value for the frequency of regulatory T cells was at 1.6% in CT animals and 1.1% in the corresponding PBS controls.

Analysis of the activation marker CD69 however displayed significant differences, as the MFI of CD69 on CD4+ T cells and on CD8+ T cells was significantly increased after CT treatment compared to PBS controls (Fig. 9D, E). CD62L expression did not differ in both tested

groups. Other tested cell types like macrophages or dendritic cells did not show any consistent differences at investigated time points.

A separate experiment analysing the immune response 4 weeks after treatment, did not differ from the 24h time point, as no differences were observed between CT treated mice and PBS controls (data not shown).

To analyse whether the CT therapy has a systemic effect, ELISA using serum of NOD mice treated with CT and PBS were analysed. However, measured cytokines IL-10, IFNγ and IL-6 were below the detection limit of the ELISA (data not shown).

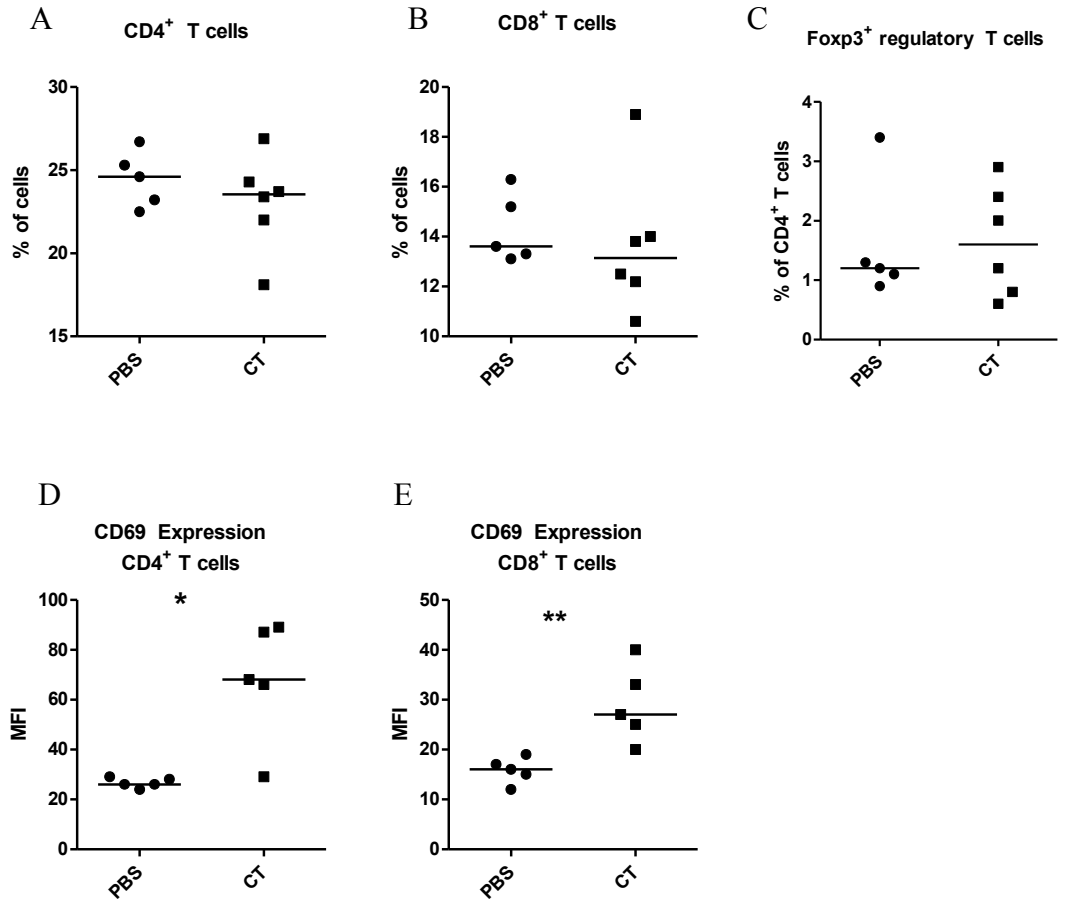


Figure 9. Combination therapy does not alter splenic CD4+ and CD8+ T cell frequencies. A) Frequency of CD4+ T cells B) CD8+ T cells as well as C) regulatory T cells in spleens of NOD mice treated with combination therapy and PBS controls. Treatment started at 10 weeks of age and experiment was performed 24h after the last treatment. Mean fluorescence intensity (MFI) of CD69 expression on CD4+ T cells (D) and CD8+ T cells (E) in spleen of NOD mice. Data representative for two independent experiments with at least 5 animals per group. Both groups were tested for significances using non-parametric Mann-Whitney U-test (**p<0.01, *p<0.05).

3.1.5. Increased frequency of CD4+ T cells in pancreata of CT treated mice

The pancreas is the most affected organ during the development of T1D. To investigate whether the CT therapy has an effect on the pancreas, pancreata from mice treated with CT and PBS as control were isolated, digested and homogenized to isolate cells for FACS analysis. Four weeks after the last treatment, no differences were observed for frequencies of CD4+ and CD8+ T cells in both tested groups (Fig. 10A, B). Frequencies of Foxp3+ CD4+ T cells were increased after CT treatment, but this difference did not reach statistical significance (Fig. 10C, p=0.54). Other tested cell types like DCs or macrophages were either not detectable or did not show any differences in frequency.

24h after last treatment significantly increased frequencies of both, CD4+ and CD8+ T cells were observed after CT therapy compared to PBS controls. While at the 24h time point 6.7% CD4+ T cells were detected after CT therapy and 2.8% CD8+ T cells, 4 weeks after treatment the frequency of CD4+ T cells increased to 24.4%, while the CD8+ T cells just increased marginally to 5.5% (Fig. 10D, E; p=0.0028 for CD4+ T cells, p=0.0079 for CD8+ T cells). Expression of the regulatory T cell subset marker Foxp3 was measured for the 24h time point, but no differences were detected between both tested groups. Further differences within the pancreas were observed regarding the F4/80+ macrophage population. The frequency of macrophages was significantly increased in CT treated mice compared to PBS controls (22% in CT animals compared to 5.5% in controls; Fig.10G, p=0.0043). The T cell populations were also tested for their expression of the activation marker CD69. As shown in Figure 10 H and I, T cells in pancreata of NOD mice treated with CT had a higher activation rate compared to T cells from PBS controls. The difference was statistical significant for the CD4+ T cell population with p=0.008, the difference for CD8+ T cells did not reach statistical significance.

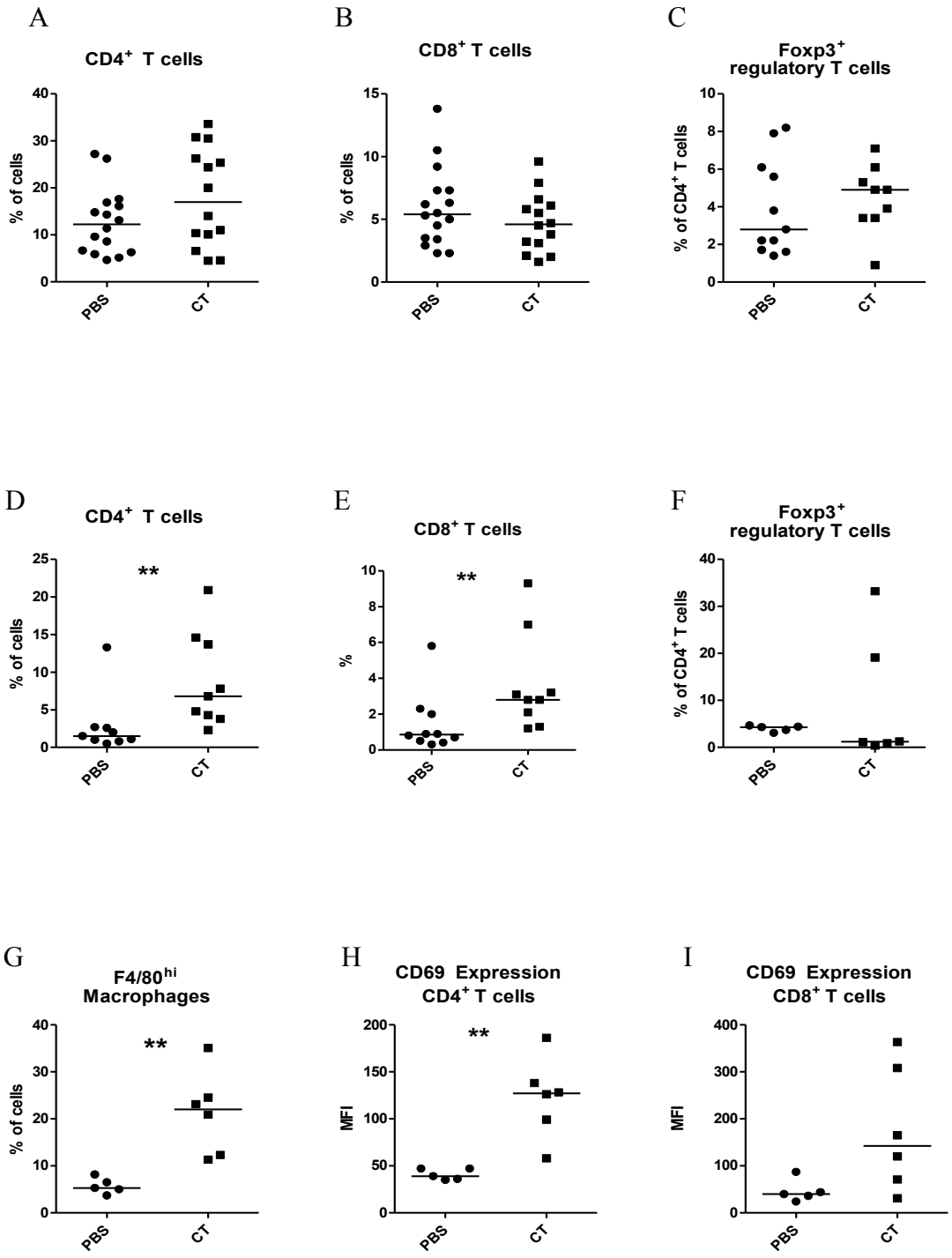


Figure 10. Significantly altered cell composition in the pancreas of CT animals 24h after treatment. A) Frequency of CD4+ T cells, B) CD8+ T cells, and C) regulatory T cells in pancreas of NOD mice treated with combination therapy (CT) and PBS controls 4 weeks after last treatment. D) Frequency of CD4+ T cells E), CD8+ T cells, F) regulatory T cells, and G) macrophages in pancreas of NOD mice treated with CT and PBS controls 24 hours after last treatment. Mean fluorescence intensity (MFI) of CD69 on CD4+ and CD8+ T cells in pancreas (H, I). Pooled data of two independent experiments with at least 5 animals per group (A-E), single experiment (F-I). Both groups were tested for statistical significances using non-parametric Mann-Whitney U-test (**$p<0.01$, *$p<0.05$).

3.1.6. Combination therapy leads to a significant increase in regulatory T cell frequencies in the pancreatic lymph nodes

Pancreatic lymph nodes (pLN) are important for priming immune responses during T1D and therefore cells from pLNs of CT treated mice and PBS controls were isolated to determine the cellular composition via FACS analysis.

At both tested time points pLNs were significantly increased after CT treatment compared to PBS controls (Fig.11A). While pLNs of the PBS controls had around 500.000 cells, CT treated animals showed a significant increase in cell numbers with a median value of 3.1 Mio cells 24h after treatment and 1.94 Mio cells four weeks after treatment (Fig.11A). Macrophages are known to be implicated in the process of T1D development. Frequencies of F4/80+ macrophages were obtained via FACS analysis for time points 4 weeks after treatment and 24h after treatment. At the 4 week time points only a very low frequency of macrophages was detected within the pLNs and no differences were found between both tested groups (Fig.11C). However, 24h after last treatment, CT treated animals had a significantly decreased frequency of macrophages compared to PBS controls as is shown in Figure 11B. As the alternatively activated phenotype of macrophages may be important for the protective effect by reducing autoreactive inflammatory responses, macrophages were stained for RELMα expression. However, at both tested time points, no differences were detected in the MFI of RELMα between CT treated mice and PBS controls (Fig.11D), suggesting that most of the macrophages in the PBS controls are representing an inflammatory, classical phenotype. Probably the most important cell types in the development of T1D are the T cells. Similar to the analysis of pancreas and spleen, cells of the pLN were investigated for CD4+ and CD8+ T cells at time points 4 weeks and 24h post treatment. Frequency of CD4+ and CD8+ T cells were comparable between CT treated mice and PBS controls at both tested time points. Frequency of CD4+ T cells were 44.8% 24h after treatment in CT treated mice compared to 39.6% in controls; at the 4 week time point the frequencies were 52.4% in CT mice and 44%

in PBS controls. The population of CD8+ T cells was in general lower than the CD4+ T cell population. At the 4 week time point 16.8% of the pLN cells in CT treated mice were CD8+ T cells compared to 19.8% in PBS controls; at the 24h time point the values were 20.2% and 18%, respectively (Fig.11E, F). However, as significantly bigger pancreatic lymph nodes were observed in the CT treated mice, absolute numbers of CD4+ and CD8+ T cells were significantly increased in mice treated with the CT (data not shown). The most interesting finding of this study referred to the frequency of the Foxp3+ CD4+ T cells. As these cells are known for their regulatory role during helminth infections and known to be induced by pro-insulin therapies, the pLN cells were assessed for Foxp3 expression. 24h post treatment CT treated mice displayed a significantly increased frequency of CD4+ Foxp3+ regulatory T cells with the median being at 8.3% compared to 2.9 % in PBS controls (Fig.12A, p= 0.004). At the 4 week time point no differences between CT animals and the PBS controls were observed with the frequency being 5.1% for the CT group and 8.6% for the PBS group (Fig.12B). Figure 12 C and D are showing representative dot plots for the regulatory T cell subset in the pLN of the CT treated group and PBS control.

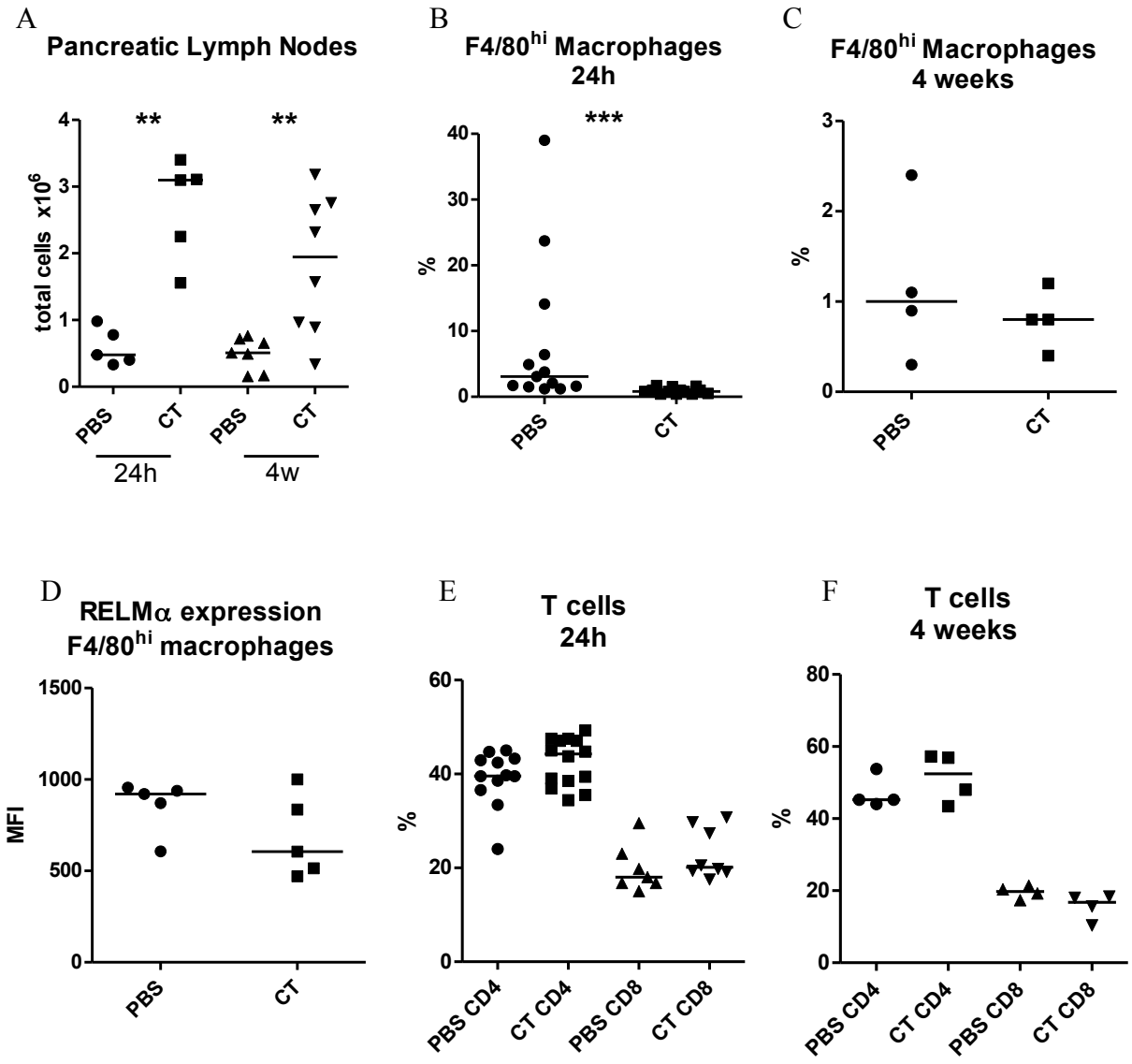


Figure 11. Increased pLN cell numbers, but reduced macrophage frequencies after CT treatment. A) Number of pancreatic lymph node cells 24h and 4 weeks after combination therapy (CT). Frequency of F4/80+ macrophages B) 24h and C) 4 weeks after last treatment in both tested groups. D) Mean fluorescence intensity (MFI) of RELMα expression of macrophages of CT treated animals and PBS controls. Frequency of CD4+ and CD8+ T cells in pLN 24h after last treatment (E) and 4 weeks after treatment (F). Pooled data of two independent experiments with at least 5 animals per group (A, B, E). Both groups were tested for statistical significances using non-parametric Mann-Whitney U-test (**$p<0.01$, *$p<0.05$).

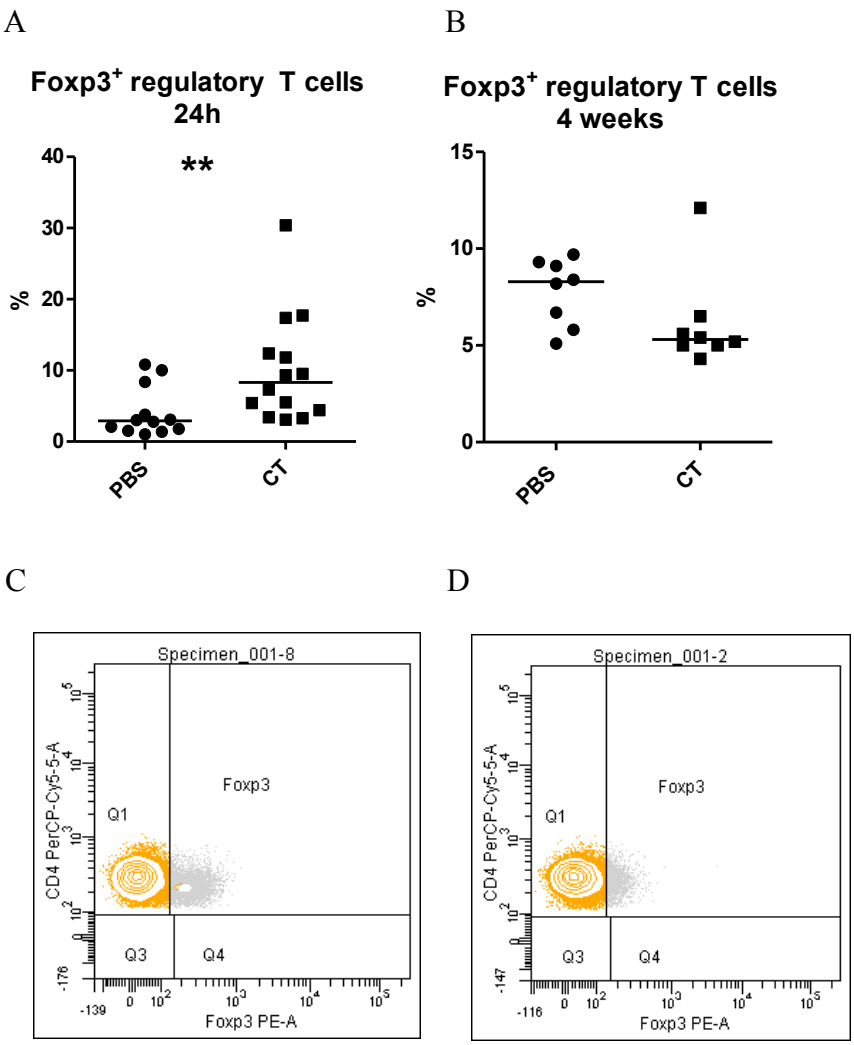


Figure 12. Increased frequency of regulatory T cells after combination therapy. A) Frequency of regulatory T cells in pLNs 24h after last treatment with combination therapy (CT) or PBS. Pooled data from three independent experiments. B) Frequency of regulatory T cells in pLNs 4 weeks after last treatment. Pooled data from two independent experiments. Data were analysed for A) and B) using the Mann-Whitney U-test, (**$p<0.01$). C) Representing dot plot of the Foxp3 expressing population in the FACS analysis of a combination therapy treated NOD mouse, D) representing dot plot for PBS control.

These experiments demonstrate that a combined therapy of crude worm extract and intranasal proinsulin can prevent T1D in up to 10-week old NOD mice. This protection was associated with a reduced inflammation of the pancreatic islets and an increased frequency of regulatory T cells within the pancreatic lymph nodes. Additionally, frequencies of inflammatory macrophages were significantly decreased after CT-treatment.

3.2. The role of the NOD2-receptor during *L. sigmodontis* infection

3.2.1 NOD2 receptor is activated by *Wolbachia*-containing worm extract

With NF-κB-reporter assays it was investigated, whether NOD1- and NOD2-transfected HEK cells gets activated by LsAg. The optimal concentration of LsAg and LsAg-W was determined performing the reporter assay with different concentrations. Using the concentration of 10µg/ml for both LsAg and LsAg -Wolb, we could demonstrate that complete crude worm extract (LsAg) lead to a significantly increased NFκB-activation via the NOD1 and NOD2 receptor compared to corresponding samples without *Wolbac*hia. The used positive controls MDP for NOD2 and DAP for NOD1 were activated specifically to the corresponding receptor. Cells stimulated with inactive MDP as well as H_20 did not show any activation. To dampen background-signalling, both crude worm extracts were stimulated in presence of 250mM L-homoarginine, a protease-inhibitor which does not affect the activity of SEAP. These in vitro experiments demonstrate that LsAg activates the NLRs NOD1 and NOD2 in a *Wolbachia* dependent manner.

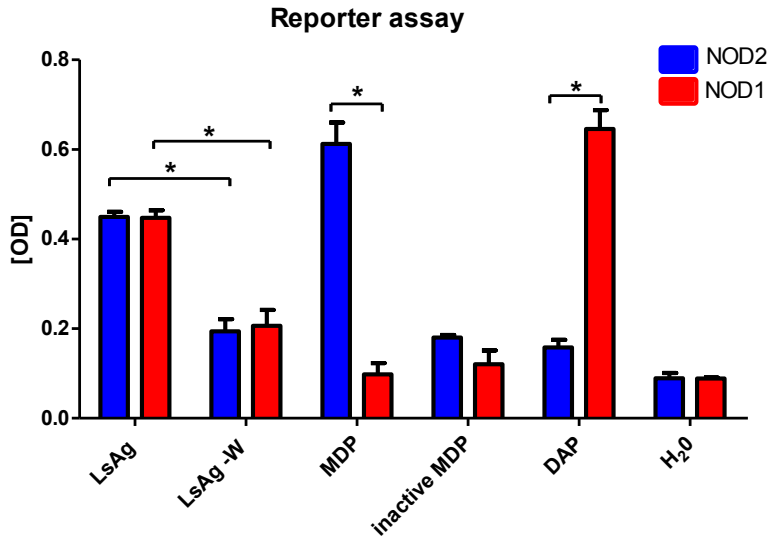


Figure 13. NOD1 and NOD2 receptor are activated by *Wolbachia* containing crude worm extract. NF-κB activation of HEK cells transfected with NOD1 (red) or NOD2 (blue) after stimulation with 10µg/ml of complete *L. sigmodontis* antigen (LsAg),or *L. sigmodontis* antigen depleted from *Wolbachia* (LsAg -Wolb.), MDP, Tri-DAP, inactive MDP and water for 10h. Additionally, 250mM L-homoarginine was added as a phosphatase-inhibitor to LsAg and LsAg-W. Representative data of six independent experiments. Data was tested for statistical significances using Mann-Whitney-U-test (*$p<0.05$).

3.2.2 NOD2$^{-/-}$ mice are more susceptible to *L. sigmodontis* infection than WT controls

To investigate whether the NOD2 receptor is implicated during parasite infections, NOD2$^{-/-}$ mice were naturally infected with the rodent filarial nematode *L. sigmodontis* and worm burden was assessed at different time points after infection.

As shown in Figure 14A, 60dpi no differences were observed between NOD2$^{-/-}$ mice and WT controls, both groups showed only a few remaining adult worms in the pleural cavity and therefore were able to successfully eliminate the infection. Neither NOD2$^{-/-}$ nor C57BL/6 WT controls did develop a patent infection, as no microfilariae were found in the peripheral blood. However, experiments 35dpi and 15dpi showed a significantly increased worm burden in NOD2$^{-/-}$ mice compared to WT controls (Fig.14B, C). While at 35dpi median values for worm burden were 35.7 in NOD2$^{-/-}$ compared to 7.6 worms in WT controls, the experiment at 15dpi resulted in lower median values with 2.5 in WT controls and 6 in NOD2$^{-/-}$ mice. This increased worm burden in NOD2$^{-/-}$ mice was also observed at early time points of infection when testing 10dpi and 5dpi, shortly after L3 larvae reach the thoracic cavity (median 10dpi NOD2$^{-/-}$ 31, WT 5; 5dpi NOD2$^{-/-}$ 27, WT 17.5 worms; Fig.14E, F). An experiment performed 15 days post infection using ASC$^{-/-}$ and IL1ßR$^{-/-}$ besides NOD2$^{-/-}$ mice and WT controls demonstrated that only NOD2$^{-/-}$ displayed a significantly increased worm burden compared to the other three groups (Fig.14G). The median worm burden was 1.5 worms/mouse for ASC$^{-/-}$, 1 worm/mouse in IL1ßR$^{-/-}$ and 2.5 worms/mouse in WT controls and therefore comparable. The NOD2$^{-/-}$ however had significantly more *L. sigmodontis* worms in the thoracic cavity with a median value of 6 (range 3-12 worms/mouse). However, this experiment was performed once and required a repeat, especially as the worm burden was in general low.

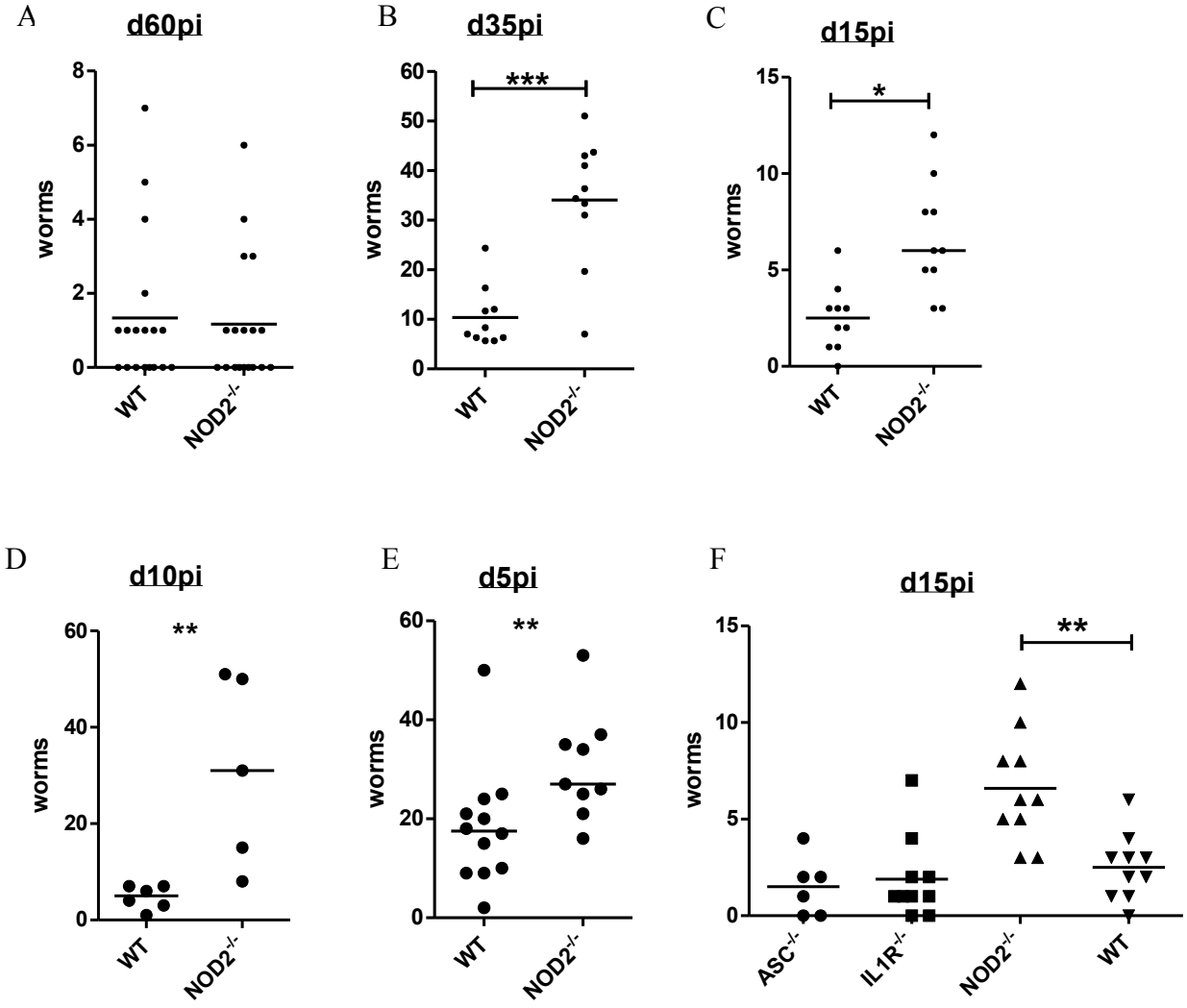


Figure 14. Increased worm burden in NOD2$^{-/-}$ mice at early time points of infection. Worm burden in the thoracic cavity of WT and NOD2$^{-/-}$ mice 60 (A), 35 (B), 15 (C), 10 (D) and 5 (E) dpi. Data was tested for statistical significance using the Mann-Whitney U-test. Representative data of three or more experiments per time point with at least 5 mice per group. F) Worm burden of NOD2$^{-/-}$ and WT mice as well as ASC$^{-/-}$ and IL1R$^{-/-}$ mice 15dpi. Data was tested for significance using the Kruskal-Walli-Test followed by Dunn's post-test. (***p<0.001, **p<0.01, *p<0.05).

3.2.3 No differences in the composition of major cell types and cytokine concentrations in the pleural cavity of infected NOD2$^{-/-}$ compared to WT controls during early time points of infection

To investigate whether the immune response against *L. sigmodontis* is impaired or altered in NOD2$^{-/-}$ mice compared to WT controls and therefore could be associated with the increased worm burden, FACS analysis to analyse the cellular composition of the pleural cavity and ELISA to analyse the cytokine profiles 15 and 5 dpi was performed.

Important cell types were analysed 15dpi and no differences were observed for the absolute numbers of macrophages, CD4+ T cells, B cells, neutrophils, and eosinophils. As shown in Figure 15A, frequencies of those cell populations were comparable between NOD2$^{-/-}$ mice and WT controls. Similarly, ELISA data tested of the first ml of the pleural lavage showed comparable values for NOD2$^{-/-}$ and WT controls as shown in Figure 15B-E. Concentrations of IL-4, IL-5, IL-6, and IL-10 did not show any differences between NOD2$^{-/-}$ mice and WT controls. Similarly, measurements of LsAg-specific immunoglobulins like IgG1, IgG2a/b, IgE, and IgM in serum did not reveal any differences between NOD2$^{-/-}$ mice and WT controls (data not shown).

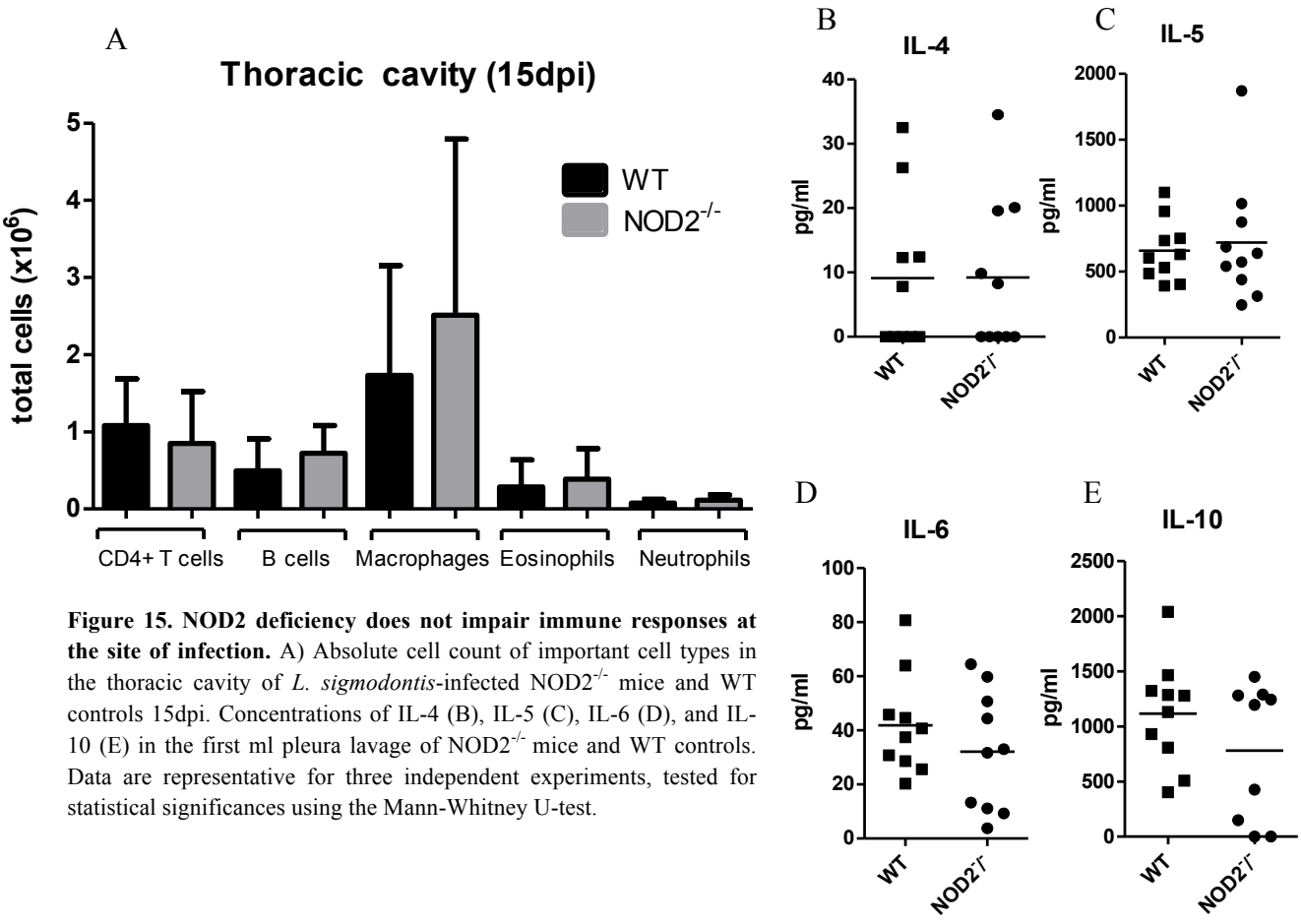


Figure 15. NOD2 deficiency does not impair immune responses at the site of infection. A) Absolute cell count of important cell types in the thoracic cavity of *L. sigmodontis*-infected NOD2$^{-/-}$ mice and WT controls 15dpi. Concentrations of IL-4 (B), IL-5 (C), IL-6 (D), and IL-10 (E) in the first ml pleura lavage of NOD2$^{-/-}$ mice and WT controls. Data are representative for three independent experiments, tested for statistical significances using the Mann-Whitney U-test.

Accordingly, experiments were performed for the time point 5dpi. No differences were observed for this early time point and the absolute numbers of important cell populations remained low and were similar between NOD2$^{-/-}$ mice and WT controls (Fig.16A). ELISAs were performed, but did also not reveal differences in the concentrations of IL-4, IL-6 and IL-10 (Fig.16B, C, E). However, IL-5 was significantly increased in NOD2$^{-/-}$ mice compared to WT controls (Fig.16C, p=0.0025), with median values being 169 pg/ml in NOD2$^{-/-}$ mice compared to undetectable values in WT controls.

Similar to the pleural cavity cells, cells isolated from the spleens were analysed. Data from FACS analysis and ELISA did not reveal any constant differences (data not shown).

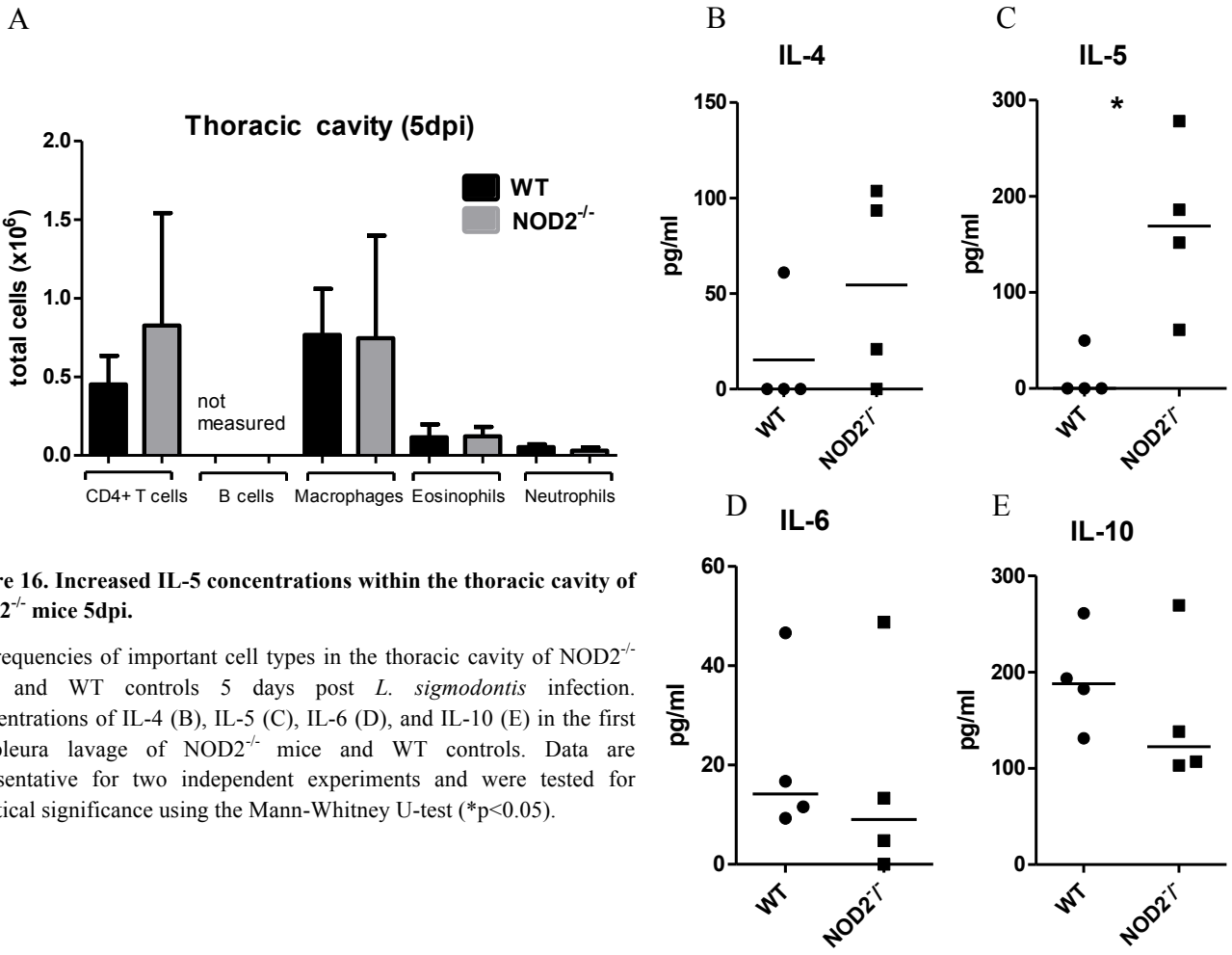


Figure 16. Increased IL-5 concentrations within the thoracic cavity of NOD2$^{-/-}$ mice 5dpi.

A) Frequencies of important cell types in the thoracic cavity of NOD2$^{-/-}$ mice and WT controls 5 days post *L. sigmodontis* infection. Concentrations of IL-4 (B), IL-5 (C), IL-6 (D), and IL-10 (E) in the first ml pleura lavage of NOD2$^{-/-}$ mice and WT controls. Data are representative for two independent experiments and were tested for statistical significance using the Mann-Whitney U-test (*p<0.05).

3.2.4 Macrophage depletion does not reverse the phenotype of infected NOD2$^{-/-}$ mice

Macrophages are one of the major immune cells expressing the NOD2-receptor. To investigate the importance of macrophages during *L. sigmodontis* infection, macrophages were depleted using clodronate-containing liposomes on 7 and 10dpi and worm burden was assessed at 15dpi.

As shown in Figure 17 A, clodronate treatment did not reverse the observed phenotype in NOD2$^{-/-}$ mice. While NOD2$^{-/-}$ mice still had a significantly increased worm burden in the pleural cavity compared to WT mice, clodronate treatment did not further increase the worm burden, resulting in similar levels as in control liposome treated NOD2$^{-/-}$ mice and WT controls. This suggests that at the time worms are present in the thoracic cavity, the NOD2 receptor expressed in macrophages does not play an essential role. Successful depletion was confirmed via FACS analysis (Fig.17B).

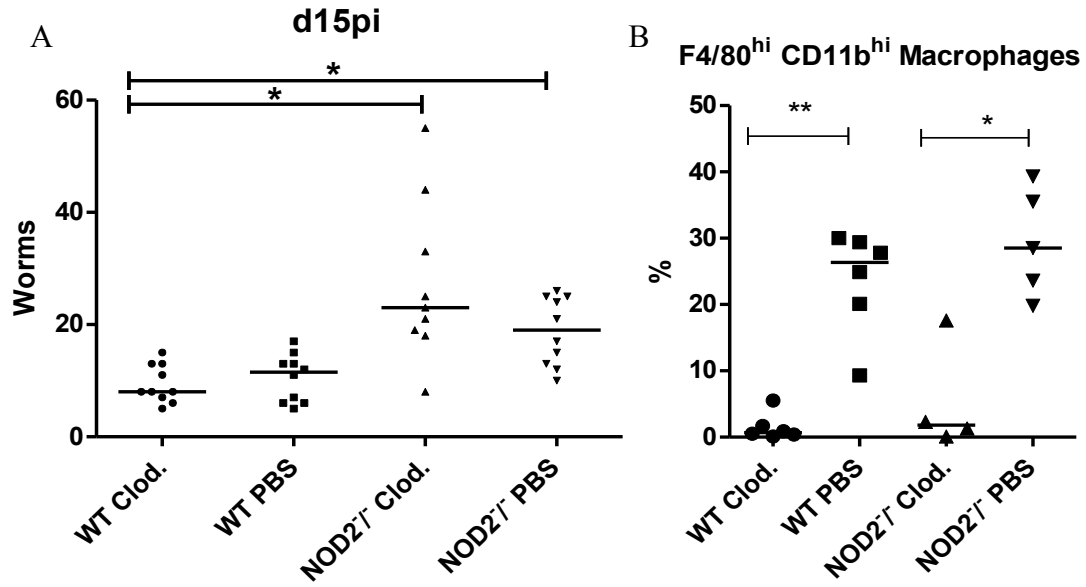


Figure 17. Depletion of macrophages does not reverse the phenotype in NOD2$^{-/-}$ mice. A) Worm burden 15dpi in WT mice and NOD2$^{-/-}$ mice after depletion of macrophages using clodronate (clod.) liposomes and PBS liposomes as control. Pooled data from two experiments with at least 4 mice per group B) Frequency of macrophages in peritoneum, confirming the successful depletion of F4/80hiCD11bhi macrophages. Data were tested for statistical significance using Kruskal-Wallis test followed by Dunn's post-test (**p<0.01, *p<0.05).

3.2.5. IL-5 depletion does not reverse the phenotype of NOD2$^{-/-}$ mice

One difference observed in the infection experiments using NOD2$^{-/-}$ mice was the increased IL-5 levels in the thoracic cavity lavage 5dpi as well as increased levels of IL-5 from ex vivo stimulated splenocytes (185). This may suggest a supporting role for IL-5 on increased worm burden at early time points of infection in the NOD2$^{-/-}$ mice. To investigate the role of this cytokine, IL-5 was depleted using an anti-IL-5 antibody 24h before infection as well as on 3, 7, 10, and 13 dpi. Worm burden were analysed at 15dpi. Successful depletion of IL-5 was confirmed by staining for pleural eosinophils via flow cytometry as is shown in Figure 18B.

Data from this experiment proved that IL-5 did not have a beneficial role for worms in NOD2$^{-/-}$ mice as NOD2$^{-/-}$ mice treated with depleting antibodies had a similar worm burden at the site of infection as NOD2$^{-/-}$ mice treated with isotype control (Fig.18A). Both groups had a significantly increased worm burden compared to WT controls treated with the isotype controls. This experiment proves that IL-5 is not the driving force behind the increased worm burden in NOD2$^{-/-}$ mice.

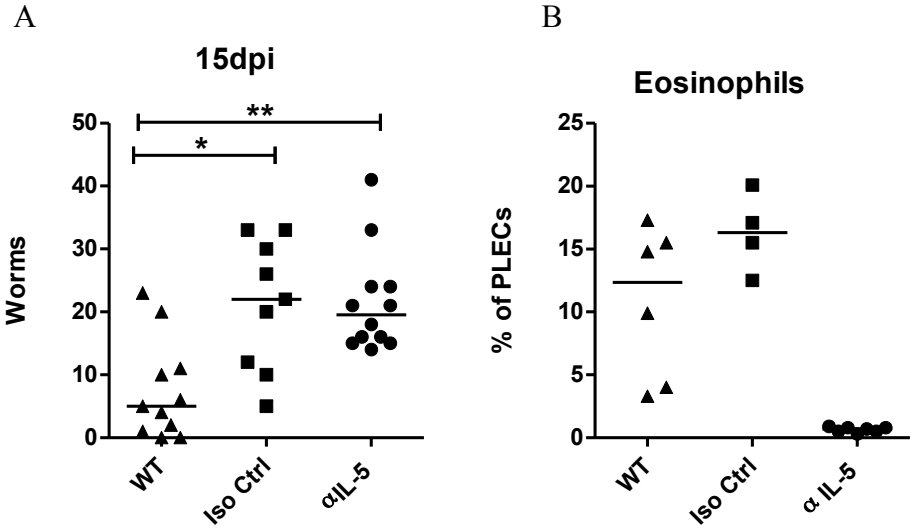


Figure 18. Depletion of IL-5 does not reverse the phenotype of NOD2$^{-/-}$ mice. A) *L. sigmodontis* worm burden 15dpi in WT mice and NOD2$^{-/-}$ mice treated with isotype control as well as anti-IL5. B) Frequency of eosinophils in the thoracic cavity after depletion of IL-5 (αIL-5) as well as isotype control treatment (Iso Ctrl) in NOD2$^{-/-}$ mice. One experiment. Data was analyzed for significance using the Kruskal-Wallis-test followed by Dunn's post-test (**p<0.01, *p<0.05).

3.2.6. No impaired production of IgM in NOD2$^{-/-}$ mice during early *L. sigmodontis* infection

As IgM is the earliest produced immunoglobulin and involved in the activation of the complement system as well as in protective immune response against helminths (Mohanty 2002), antibody titers of IgM were tested in the first days after *L. sigmodontis* infection. As shown in figure 19, no differences were found in *L. sigmodontis*-specific IgM antibody levels in the serum of NOD2$^{-/-}$ mice and WT controls on 4dpi (A), 7dpi (B) as well as 10dpi (C). This leads to the conclusion that IgM is not mediating the increased worm burden in NOD2$^{-/-}$ mice.

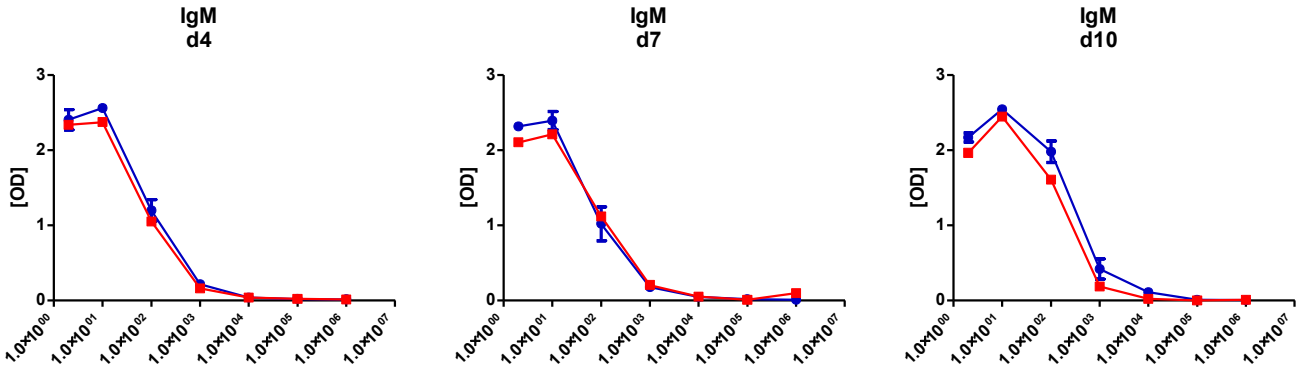


Figure 19. LsAg-specific IgM antibody titers of NOD2$^{-/-}$ mice and WT controls are similar. Graphs are showing the optical density [OD] of LsAg-specific IgM on 4 (A), 7 (B) and 10 (C) dpi. Blue lines indicate IgM levels in serum of NOD2$^{-/-}$ mice, red lines of WT controls. One experiment per time point with at least 4 mice per group was performed.

3.2.7 NOD2$^{-/-}$ do not have an increased worm burden after subcutaneous injection of L3 larvae

Since experiments during the early time points of infection did not reveal any differences in the cellular composition or cytokine milieu which could account for the increased worm burden in NOD2$^{-/-}$ mice, it was assumed that the mechanisms leading to the increased worm burden must occur before the L3 larvae reach the thoracic cavity. The skin represents the first major obstacle for invading L3 larvae. Using subcutaneous injection of L3 larvae the skin barrier was surpassed and 15dpi the worm burden in the thoracic cavity was analysed. Subcutaneous injection of 40 L3 larvae led to no difference in worm burden between NOD2$^{-/-}$

mice and WT controls 15dpi at the site of infection (Fig.20). While the majority of the injected L3 larvae did not migrate to the thoracic cavity, numbers of retrieved worms were comparable in both tested groups. These results proved that the decisive NOD2 depending mechanism must occur within the skin.

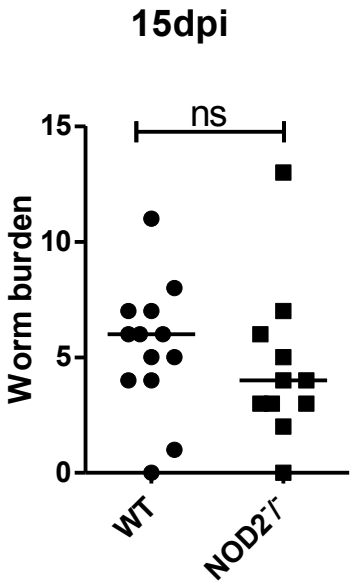


Figure 20: Subcutaneous injection of L3 larvae does not lead to an increased worm burden in NOD2$^{-/-}$ mice. Worm burden at day 15pi after subcutaneous injection of 40 L3 per mouse. Data are shown as median and were tested for statistical significance using Mann-Whitney U-test. Pooled data from two independent experiments using 4-6 mice per group.

3.2.8 Impaired neutrophil influx to the skin of NOD2$^{-/-}$ mice

To obtain a detailed picture of the immune responses within the skin after infection with *L. sigmodontis,* a crude worm extract was intradermally injected and skin tissue was isolated 3h post injection for FACS analysis. Analysis revealed an impaired immune response within the skin of NOD2$^{-/-}$ mice compared to WT controls. As shown in Figure 21A, frequency of Gr1^{+}CD11b^{+} neutrophils in the skin were significantly upregulated in WT mice after LsAg injection with a frequency of 5% in the WT LsAg injected group compared to 1% of neutrophils after PBS injection. This difference was less prominent in NOD2$^{-/-}$ mice after LsAg injection, with neutrophil frequencies of 2.5% in LsAg injected mice compared to 0.8%

after PBS injection ($p<0.05$). When comparing LsAg treated WT mice with NOD2$^{-/-}$ mice after LsAg injection, the frequency of neutrophils was significantly decreased in the NOD2$^{-/-}$ mice (Fig.21A). No effect was observed for the frequency of F4/80^{+} CD11b^{+} macrophages. While both LsAg-treated groups showed a slight increase in macrophage frequency, these differences were not statistically significant (Fig.21B). Another cell type implemented in the early immune response to LsAg are mast cells, which were identified as cKit^{+} F4/80^{-}. While an increased mast cell frequency in WT mice injected with LsAg compared to PBS controls was observed, this difference did not reach statistical significance (Fig.21C). No differences were seen in NOD2$^{-/-}$ mice groups in regard to mast cell frequencies (Fig.21C). Cells were also stained for eosinophils with Siglec F, but no eosinophils were detected within the skin following LsAg injection, confirming other studies describing the time point 3h post injection to be too early for eosinophils.

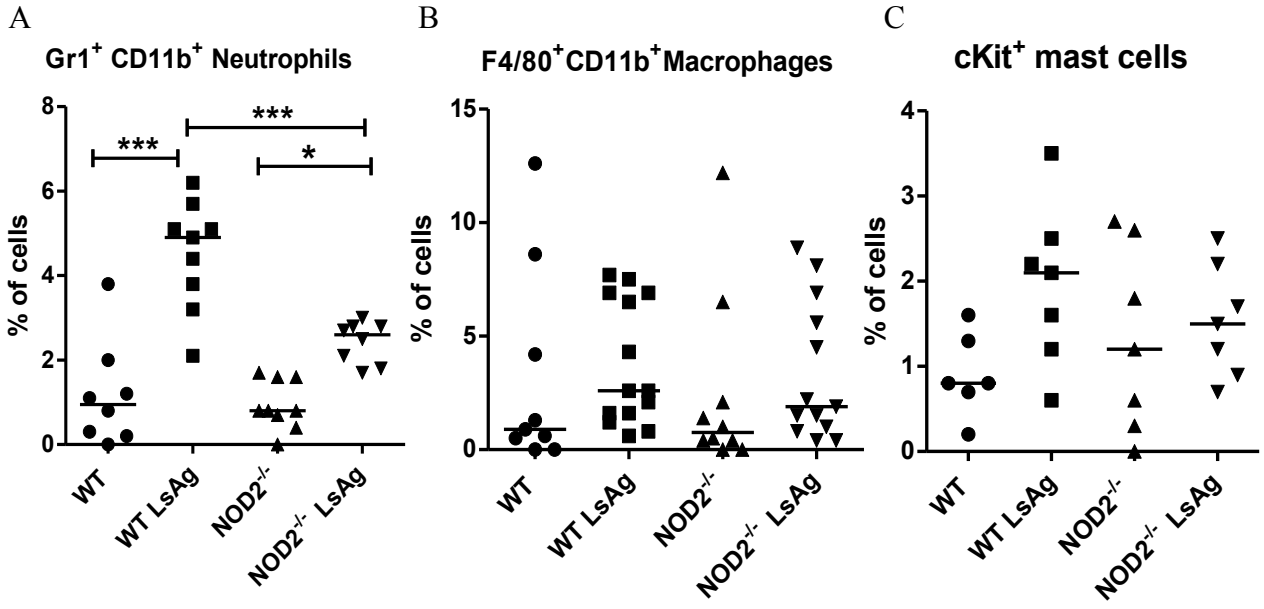


Figure 21: Impaired neutrophil influx to LsAg within the skin of NOD2$^{-/-}$ mice. Frequency of neutrophils (A), macrophages (B) and mast cells (C) in the skin three hours after intradermal LsAg injection in NOD2$^{-/-}$ mice and WT controls. Controls were treated with PBS. Pooled data from three independent experiments (A, B), one experiment (C). Data was analyzed for statistical significances using the Kruskal-Wallis-test followed by Dunn's post-test (***$p<0.001$, *$p<0.05$).

3.2.8 Blood neutrophil levels are lower in NOD2$^{-/-}$ mice

Further evidence that the NOD2 receptor is important for establishing a proper neutrophil recruitment to the site of infection was observed in the blood. While naïve NOD2$^{-/-}$ mice and WT controls both displayed a similar blood neutrophil frequency between 1.3% and 0.6%, 24h post infection NOD2$^{-/-}$ mice had a significantly lower frequency of neutrophils compared to WT controls. While WT mice had a median of 7.5% neutrophils in the blood, the level in NOD2$^{-/-}$ mice was significantly lower with 3.5% (Fig.22A, B; Mann-Whitney U-test p=0.02). However, at later time points, i.e. 5dpi, blood neutrophil frequencies were significantly increased in NOD2$^{-/-}$ mice (Fig.22C). From day 5 onwards, when L3 larvae are found within the thoracic cavity, no consistent differences were found in the pleural cavity neutrophil population (Fig.16A).

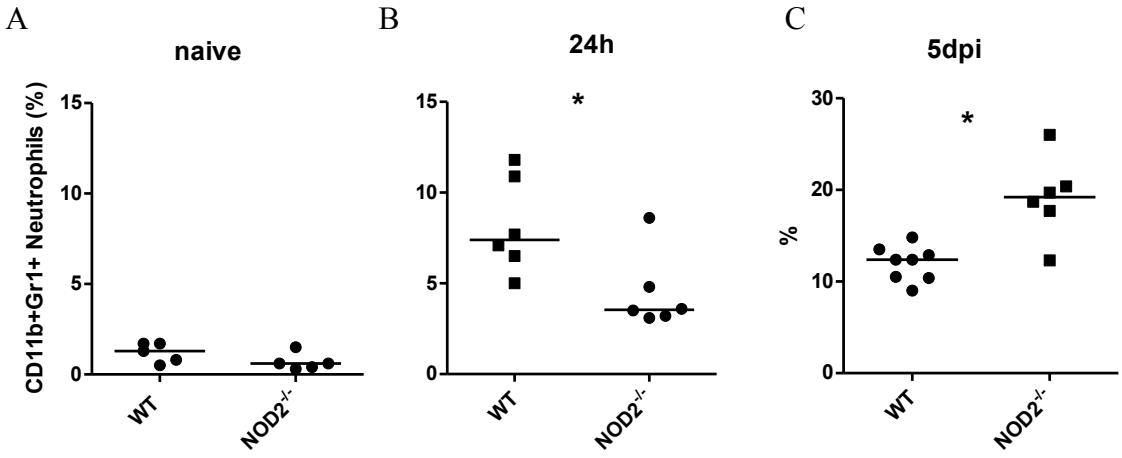


Figure 23: Impaired neutrophil recruitment in NOD2$^{-/-}$ mice upon *L. sigmodontis* infection. Frequency of blood CD11b+Gr1+ neutrophils in naïve mice (A) and 24h (B) as well as 5 days (C) after infection with *L. sigmodontis*. Representative data of two independent experiments. Data was analyzed for statistical significances using the Mann-Whitney U-test (*p<0.05).

3.2.10 Depletion of neutrophils leads to an increased worm burden in WT mice

To directly assess the role of neutrophils within the first days of infection, neutrophils were depleted using an anti-Ly6G antibody and worm burden was subsequently analysed 15dpi.

Neutrophils were depleted 1h prior to infection by intraperitoneal injection of 500µg anti-Ly6G, control groups were injected with the same amount of the corresponding isotype control. In further experiments, the antibody was injected 3d as well as 24h prior to infection as well as 3dpi; in a third experiment the antibody was solely injected 24h prior to infection. Independent of the depletion scheme chosen, comparable results were obtained. Worm burden of WT mice with depleted neutrophils was increased compared to isotype-treated WT controls. However, NOD2$^{-/-}$ mice with depleted neutrophils displayed a similar worm burden as their corresponding isotype controls (Fig.23). The significantly increased worm burden in isotype treated NOD2$^{-/-}$ mice compared to isotype-treated WT mice was not given any more when comparing neutrophil depleted WT mice and isotype-treated NOD2$^{-/-}$ mice (Fig.23). This further highlights the major role of neutrophils within the first days of infection and that the neutrophils are acting in a NOD2-dependent manner.

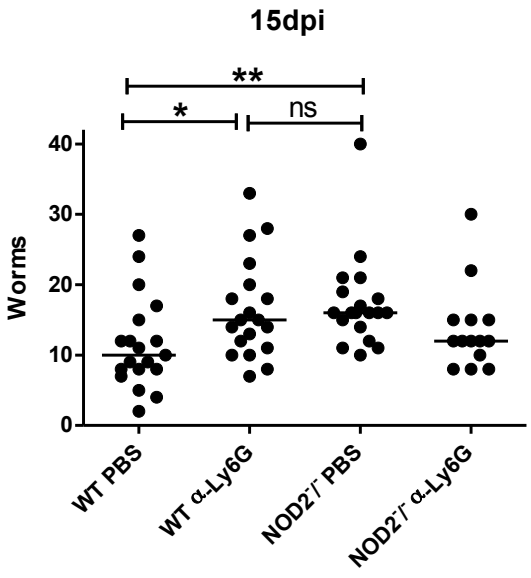


Figure 23: Depletion of neutrophils leads to an increased worm burden in WT mice, but not in NOD2$^{-/-}$ mice. Worm count on day 15 after *L. sigmodontis* infection, with mice either treated with α-Ly6G to deplete neutrophils or isotype as controls. Pooled data from three independent experiments with at least 4 mice per group. Data was analyzed for statistical significances using the Kruskal-Wallis-test followed by Dunn's post-test for chosen groups (**p<0. 01, *p<0.05).

3.2.11 Neutrophils of NOD2$^{-/-}$ mice are functionally not impaired

While the previous data suggests that neutrophil recruitment in NOD2$^{-/-}$ mice is impaired, in vitro studies were performed to investigate whether neutrophils of NOD2$^{-/-}$ mice are functionally impaired compared to WT neutrophils.

To collect neutrophils, NOD2$^{-/-}$ mice and C57BL/6 WT controls were injected twice with casein solution (18h and 3h prior to necropsy) and neutrophils were harvested via peritoneal lavage. Collected cells were further treated as described in the material and methods section 2.20, plated, stimulated overnight and supernatants were analysed by ELISA for pro-inflammatory cytokines. After isolating neutrophils, a purity of around 80% for both groups was determined via flow cytometry. As shown in Figure 24A-D, supernatants of unstimulated neutrophil cultures had the lowest production for all tested cytokines, namely MIP2, KC, IFNγ and TNF. Further, no differences were observed in MIP2, KC, IFNγ, and TNF concentrations between neutrophils of NOD2$^{-/-}$ and WT mice when cells were stimulated with LPS as TLR4 ligand, TLR2 ligand Pam3Cys (P3C), LsAg or LsAg depleted of *Wolbachia*. While IFNγ concentrations remained at low baseline levels for all tested stimuli, MIP2 and KC were barely detected after MDP stimulation of neutrophils of both tested groups. This data suggests that there is no functional impairment in pro-inflammatory cytokine and chemokine production of neutrophils of NOD2$^{-/-}$ mice compared to WT controls. Furthermore, this observation shows that in vitro TLR stimulants are more potent inducers of MIP2 and KC from neutrophils than MDP.

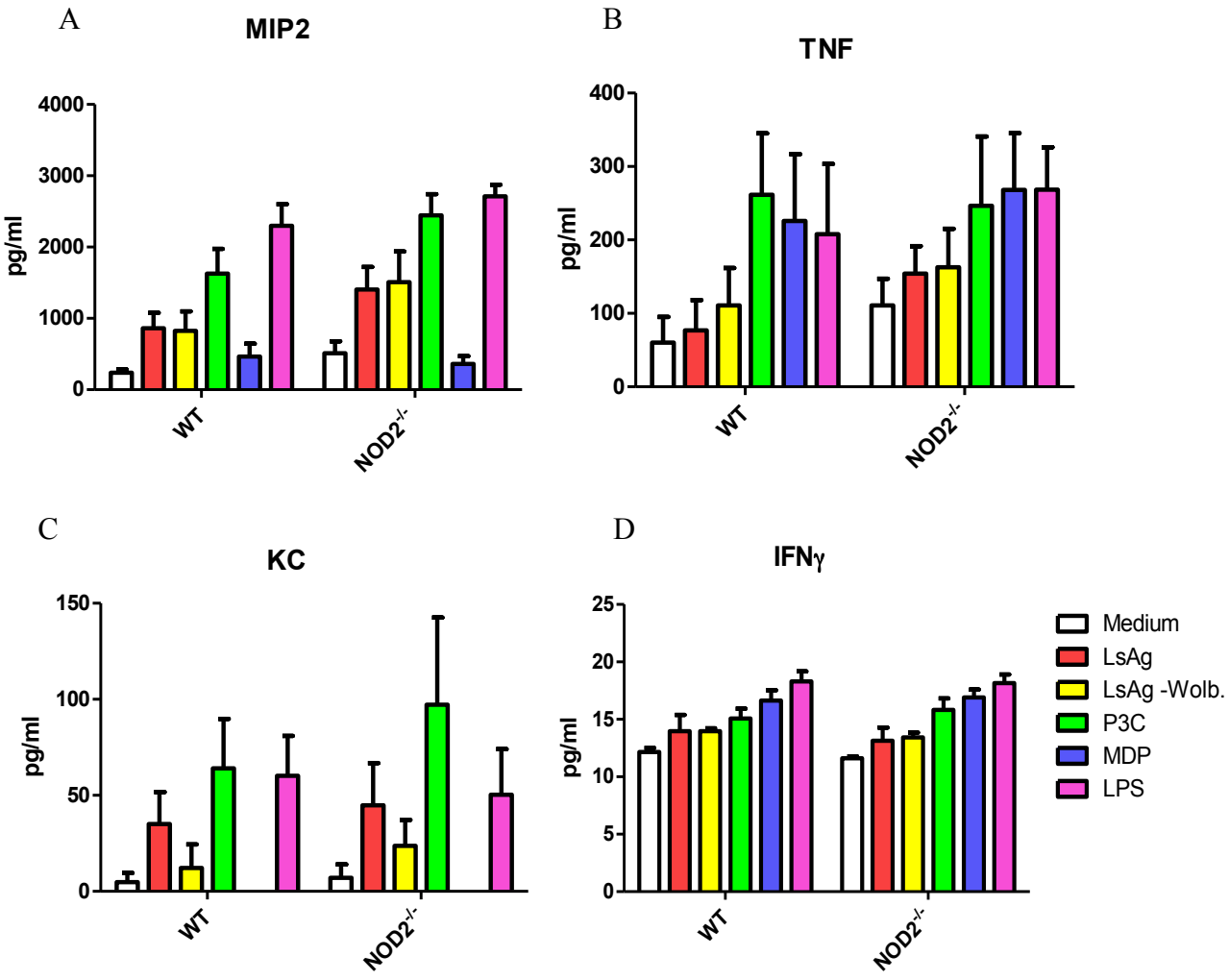


Figure 24: Pro-inflammatory cytokine and chemokine production is not impaired in neutrophils of NOD2$^{-/-}$ mice. Concentrations of TNF (A), MIP2 (B), KC (C), and IFNγ (D) of neutrophils from WT and NOD2$^{-/-}$ mice after 18h in vitro stimulation with crude worm extract (LsAg), crude worm extract depleted from Wolbachia (LsAg -Wolb.), Pam-3-cys (P3C), muramly-dipeptide (MDP) and lipopolysaccaride (LPS).

3.2.12 Genes associated with neutrophils are significantly less expressed in NOD2$^{-/-}$ mice upon L3 injection

For a more detailed analysis of the molecular processes within the skin upon *L. sigmodontis* exposure, NOD2$^{-/-}$ mice and WT controls were intradermally injected with 40 L3 larvae and skin was isolated three hours post injection for gene expression analysis via PCR array. In the PCR array a broad range of genes which are known to be involved in immune responses against helminths and filarial infections, were tested. An overview of all tested genes is shown in supplement table 1. As expected, NOD2$^{-/-}$ mice and WT controls share a broad number of

genes, which are upregulated after L3 injection when compared to PBS injected WT controls (Fig.25). However, data from PCR arrays also identified several genes which show a stronger fold change in L3 injected NOD2$^{-/-}$ mice compared to PBS treated WT controls, while none of the tested genes proved to be downregulated when comparing WT after L3 injection to NOD2$^{-/-}$ with L3 injection. Most importantly, when comparing gene expression of L3 injected NOD2$^{-/-}$ mice with L3 injected WT controls, a generally reduced expression of inflammatory genes was detected in NOD2$^{-/-}$ mice (Fig.26).

As the experiments described above focused on neutrophils, a main focus was also put on genes associated with neutrophils in the PCR arrays. According to the previous findings on impaired neutrophil recruitment to the site of LsAg injection in the skin, L3 injections in NOD2$^{-/-}$ mice resulted in a less prominent expression of ELANE ($p=0.013$), the gene encoding for neutrophil expressed elastase as well as NGP (neutrophilic granule protein; $p=0.046$) and NOS2 (inducible nitrite oxide synthase II; $p=0.021$), which is also expressed in neutrophils, compared to WT controls. This difference in gene expression of these neutrophil-associated genes further confirms the hypothesis that neutrophils are essentially implemented in the NOD2 depending mechanisms causing the increased worm burden. Furthermore, genes associated with eosinophils were analyzed. The array revealed that genes encoding for eosinophil peroxidase (EPX; $p=0.007$) and Siglec F ($p=0.029$) were downregulated in NOD2$^{-/-}$ mice after L3 exposure compared to WT controls. Interestingly, also mast cell-related genes were less strongly expressed in the skin of L3 injected NOD2$^{-/-}$ mice. This included the genes for the histamine receptors H4 ($p=0.014$) and H3 ($p=0.047$). Since the gene expression of several cytokines like IL-5 ($p=0.0002$), IL-9 ($p=0.015$), and IL-21($p=0.003$), as well as IL-6, IL-10, IL-12a, IL-17a, and IL-25 were less strongly induced in NOD2$^{-/-}$ mice, it can be assumed that the inflammatory responses in the skin of NOD2$^{-/-}$ mice upon L3 injection was much weaker than in the corresponding WT controls (Table 1).

Other relevant genes downregulated in NOD2$^{-/-}$ mice included Relm ß (p=0.004) and granzyme B (p=0.009; see Fig.25, full list of genes supplement table1). The PCR array also revealed several genes which were upregulated in L3-injected NOD2$^{-/-}$ mice compared to L3-injected WT controls. Genes expressing for the chemokines CCL3 and CCL4, also known as MIP1α and MIP1β, CXCL2 (MIP-2), and CD14 were stronger expressed in NOD2$^{-/-}$ mice after L3 injection than in WT controls. Another upregulated gene was MMP9 (matrix metallopeptidase 9), an enzyme involved in the degradation of the extracellular matrix. In conclusion, NOD2$^{-/-}$ mice had a reduced inflammatory gene expression in the skin upon invading L3 larvae when compared to WT controls. PCR array data were further analysed by R (www.r-project.org) and packages from the Bioconductor project (www.bioconductor.org). P-values were then calculated using the limma package (Bioconductor.org) and corrected for multiple testing using Benjamini-Hochberg procedure. p-values of <0.05 were considered statistically significant. Figure 26 presents the genes which were significantly downregulated in NOD2$^{-/-}$ mice compared to WT controls, both upon L3 injection.

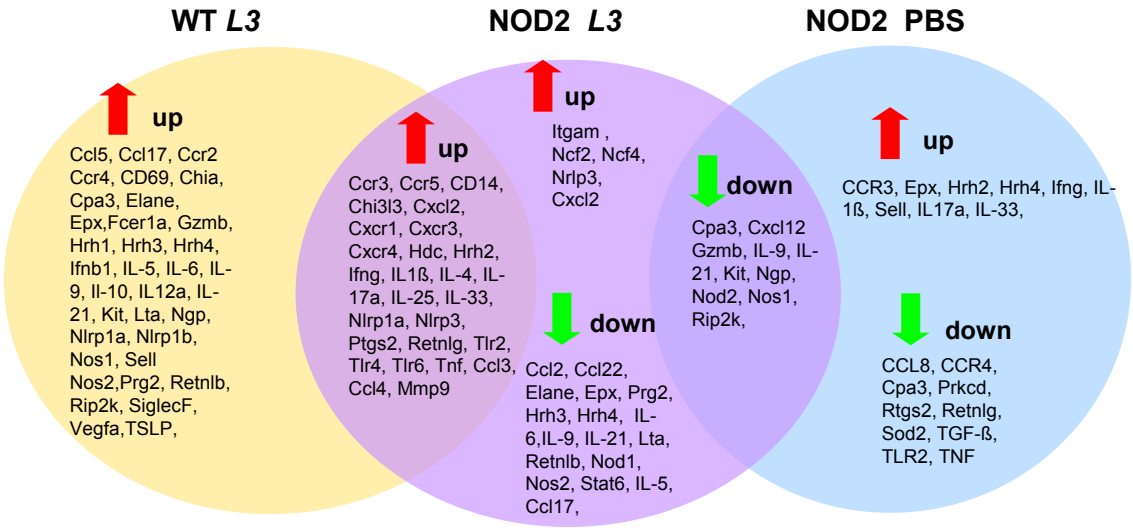


Figure 25. Decreased expression of inflammatory genes in NOD2-deficient mice. Gene expression profile of skin tissue from NOD2$^{-/-}$ mice and WT controls 3h post L3 injection, controls were injected with PBS. Diagram shows investigated genes with decreased or increased expression of all tested groups compared to naive C57BL/6 WT mice. Fold change cut-off for the genes set at >2 with three mice per group.

Table 1. Decreased expression of inflammatory genes in NOD2-deficient mice. Genes in skin tissue with significantly decreased expression in NOD2$^{-/-}$ mice after intradermal L3 injection compared to WT mice after L3 injection. All nominal significant genes are listed and raw p-values are given. Fold change cut-off for the genes set at >2 with three mice per group.

Gene	p-value*	
IL5	0.0002	Interleukin-5
Il21	0.003	Interleukin-21
Retnlb	0.004	RELM beta
Nos1	0.005	Nitric oxide synthase 1
Epx	0.007	Eosinophil peroxidase
Ifng	0.008	Interferon gamma
Gzmb	0.009	Granzyme B
Elane	0.013	Neutrophil elastase
Hrh4	0.014	Histamine receptor H4
Prg2	0.015	Proteoglycan 2
Il9	0.015	Interleukin-9
Nos2	0.021	Inducible nitric oxide synthase 2
SiglecF	0.029	Siglec F
Ngp	0.046	Neutrophilic granule protein
Hrh3	0.047	Histamine receptor H3

4. Discussion

4.1. The protective effect of worm extract administration on T1D

Due to improved hygiene and life style changes in industrialized countries parasite/helminth infections have become rare. Coincidently this decrease in parasite infections correlates with the rising incidences of autoimmune diseases. The human immune system evolved in the presence of helminth infections (186, 187) and the eradication of the infection may lead to an imbalanced, hyper-responsive immune system. An increasing number of studies have demonstrated the extent to which helminth infections regulate the immune system. Therefore, studies proving the immunomodulatory capacity of helminth infections and antigens are becoming more prominent. Hübner et al. demonstrated the protective effect of *L .sigmodontis* infection on T1D (179). While the protection was clearly proven, only mice up to 6 weeks of age were protected from T1D onset. The first goal of this thesis was to improve the therapy.

4.1.1. Combination therapy protects up to 10-week old NOD mice

T1D development was significantly decreased in CT-treated mice compared to proinsulin and LsAg-single treated or PBS-treated controls, demonstrating that each component of the therapy by itself does not prevent T1D, but a combination of both treatments was successful when treatment was started at 10 weeks of age. This suggests that combining insulin-specific therapies with helminth antigen treatment improves protective immune responses and allows prevention of diabetes onset even after insulitis occurs. Efficacy of an experimental antigen-specific therapy with intranasal proinsulin was previously enhanced by depletion of T cells via anti-CD3 treatment (184). The proinsulin thereby is required for the induction of insulin-specific regulatory T cells. While this approach was able to reverse recent diabetes onset in NOD mice, depletion of T cells may impair the immune system and increase the risk of opportunistic infections (188, 189). While helminths induce regulatory immune responses in their hosts and were shown in the past to prevent or delay diabetes onset in NOD mice,

several studies suggested that they do not impair protective immune responses (156, 157, 190). Similar to the treatment with LsAg, other helminth-derived antigens like *S. mansoni* egg antigen were only shown to prevent or delay diabetes onset in NOD mice at 4-6 weeks of age. The same accounts for treatment with *Dirofilaria immitis* antigen, which completely prevented insulitis and diabetes onset only when given to 6-week-old female NOD mice (191) as well as for excretory-secretory products from *Fasciola hepatica*, which only protected NOD mice from diabetes onset when administered at 4 weeks of age (181). It can be assumed that at later time points, helminth antigen therapy would have no effect, as inflammation of the pancreas would progress to a state where the immunomodulatory effects of helminth antigen treatment alone would not be sufficient to prevent further loss of pancreatic islet cells. In contrast, combination therapy prevented T1D in up to 10-week old mice, a time point where inflammation in the pancreas is progressed. However, this combination therapy was not able to protect pre-diabetic NOD mice at 12 weeks of age. Several options can improve our tested combination therapy which could lead to protection of mice older than 10 weeks. As the main goal is the induction of insulin-specific regulatory T cells, an approach similar to Grinberg-Bleyer et al. might be promising. In their study, a treatment with IL-2 induced pancreatic regulatory T cells which reversed T1D onset in NOD mice (192).Therefore, adding IL-2 to our combination therapy would be a promising way to improve the therapy. Another option for a triple therapy approach would be to include anti-CD3 antibodies to proinsulin and LsAg. The temporary depletion of T cells in combination with proinsulin proved to be successful in the study of Bresson et al., so a one-time depletion of T cells before starting the combination therapy could increase the efficacy that was already achieved with our approach (184). Further studies however should focus on the improvement of our treatment to make it suitable for mice older than 10 weeks of age. Identification of the active component within the LsAg would not only be beneficial for this combination therapy, but would also aid other

models where LsAg proves to be a suited therapy. Also, administration time points and injection routes can be adjusted. Instead of an intraperitoneal injection of LsAg, subcutaneous injections may lead to a depot effect which allows a prolonged exposure to the parasite antigens and improve efficacy. In mice older than 10 weeks, four injections may be not sufficient to prevent the development of autoimmune responses long-term, meaning additional treatment time points have to be evaluated.

4.1.2. Protection is associated with increased regulatory T cells in the pLN

As inflammation of the pancreas occurs during the development of T1D, pancreatic ß-islet cells of NOD mice with CT treatment and PBS controls were histologically assessed. Pancreata from CT-treated animals had less inflammation than pancreata from PBS controls. Although pancreata of NOD mice with combination therapy also presented intrainsulitis and peri-insulitis, the frequency of non-infiltrated islets was significantly higher in CT-treated animals compared to PBS controls. Accordingly, the frequency of islets showing signs of cellular infiltration was increased in PBS controls. Some level of insulitis was expected in CT-treated NOD mice as the treatment was initiated at a time point after insulitis onset. Nevertheless, less pronounced insulitis in CT-treated animals was evident and indicates that LsAg in addition to proinsulin treatment delays further destruction of pancreatic islets. A reduced cellular infiltration into the pancreatic islets and a greater frequency of non-infiltrated, healthy islet cells was previously observed in LsAg-treated mice when treatment was started at 6 weeks of age (179). Similarly, mice treated with anti-CD3 and proinsulin showed predominately islets with mild peri-insulitis while islets of diabetic mice were heavily infiltrated and destroyed (184). A therapy which would lead to an efficient regeneration of pancreatic ß-islets cells would be a desirable therapeutic approach. However, this has not been realized yet and so far the only available curative therapy for T1D is the replacement of the lost ß-islet cells by islet transplantations from donors (193). In vitro generation of ß-islet cells

from stem cells would deliver an unlimited source for transplantation, but until now generation of mature ß-cells from stem cells has not been achieved yet (194). In mouse-models the development and possible ability of ß-islet cells to regenerate are still being discussed. A study by Cano et al. however presented a transgenic mouse, in which the pancreas has the ability to fully recover from almost complete ablation of all existing β-islet cells (195).

Several studies have proposed a broad range of mechanisms which may account for the protective effect seen by helminth antigen treatment (as reviewed in (166)). To get a better understanding of the immunological effects CT treatment induces, pancreatic lymph nodes (pLN) were analysed one day as well as four weeks after CT treatment and were compared to PBS treated mice. As islet-reactive T cells are primed in the pLN before they infiltrate the pancreas and damage ß-islet cells (196), immune cells within the pLNs were analyzed. Statistical significant differences were detected 24 hours after treatment in the pLNs of CT-treated mice compared to PBS controls. CT treatment led to a significantly increased total number of cells in the pancreatic lymph nodes, which was still present 4 weeks after treatment. For T cells, which are the major cause of insulitis, no differences were found in regard to CD4+ and CD8+ T cell frequencies. More detailed analysis of the T cell subsets would clarify the function of these cells, as investigations of cytokine production or activation of these cells could be subject of further experiments. Autoreactive T cells are hereby an important factor since these are the cell types which lead to destruction of the pancreatic islet cells. Currently, it is not possible to investigate these T cell subsets in detail, due to the lack of suitable methods to isolate or even identify them in the NOD mouse model. Another possible experiment could focus on antigen-specific immune responses. While both CD4+ and CD8+ T cells are known to be involved in the destruction of β-islet cells, in particular CD4+ T cells are also known to have potential regulatory features which can suppress the functions of

autoreactive T cells (197). Supporting this hypothesis, a significant increase in CD4+Foxp3+ regulatory T cell frequencies was observed after CT treatment in the pLNs. Several experimental therapeutic approaches for T1D are trying to increase regulatory T cell numbers and their functional capacity (198). Salomon et al. showed that the failure to generate regulatory T cells leads to accelerated diabetes progression in NOD mice (199) and Green et al. proved that pLN derived regulatory T cells are extremely potent and crucial for prevention of ß-islet cell destruction (197). Another study by Nti et al. demonstrated in accordance with our study, that recovery of ß-islet cells strongly correlates with the regulatory T cell population in the pLNs (200). Furthermore, Bresson et al. state in their study, that proinsulin administration alone would not prime a sufficient number of regulatory T cells (184). A further significant difference after CT treatment was observed in the frequency of macrophages, which were significantly reduced in pLNs of CT-treated mice. The expression of RELMα, a marker for alternatively activated macrophages, was similar between both tested groups, suggesting that the protective effect by CT therapy is not mediated via the induction of AAM. However, the observed reduction of macrophage frequency can be linked to the protective effect seen in CT treated mice. Classically activated macrophages are known to contribute to ß-islet destruction by inducing inflammation producing IL-1ß, TNF or nitric oxide (201) and our CT treatment leads to a downregulation of these macrophages. Immunological analyses of the spleen did not reveal any differences within the cellular composition suggesting that the CT treatment has only a limited systemic impact.

4.1.3 Outlook T1D

This thesis describes a therapy to protect 10-week old NOD mice from diabetes onset. The next step would be to achieve remission from recent diabetes onset similar to the study by Bresson et al. or the total protection of NOD mice from T1D onset regardless of age. A potential therapeutic approach would be the administration of a triple therapy using anti-CD3,

proinsulin and LsAg. A similar approach would be also a suitable therapy for human T1D cases. T1D is a disease which is associated with a high number of genetic factors (202). Early identification of these genes especially in children and obese persons would allow labeling individuals at risk, who could then be treated with a combination therapy. It is also known that children, whose parents suffer from T1D, have a higher risk of getting this disease, too. Thus, targeting individuals at risk before the onset of T1D is a valid approach. The induction of regulatory T cells to protect from T1D is already a widely-used approach in clinical studies, especially for children at risk (203). A more difficult task would be the identification of the active component within the LsAg. That component could not only be used during T1D, but also for other autoimmune diseases as well as situations, when a regulation of the immune system is necessary.

4.2. The role of the NOD2- receptor during infection with *L. sigmodontis*

One major target of the WHO is the eradication of helminth diseases like lymphatic filariasis and onchocerciasis as a public health problem by the year 2020 and 2025, respectively. To achieve this, a much broader understanding of the immune response to filariae with all its components is crucial. With the use of the laboratory mouse model *L. sigmodontis*, our understanding of the complex mechanisms in the host-parasite interaction has vastly improved. With the second part of this thesis, a protective role of the intracellular receptor NOD2 during helminth infections was demonstrated. The NOD2 receptor is an intracellular receptor binding the bacterial cell wall component MDP. MDP itself has not been clearly identified in *Wolbachia* yet, but the *Wolbachia* do synthesize lipid II (144, 204), from which MDP is derived. Additionally, NF-κB-reporter assays of this thesis using NOD2 transfected HEK-cells demonstrated that crude worm extract (LsAg) containing *Wolbachia* lead to NF-κB activation, while *Wolbachia*-depleted worm extract (LsAg –W.) exhibited a significantly lower activation rate. Interestingly, not only the NOD2 receptor, but also the NOD1 receptor

gets activated by LsAg in a *Wolbachia*-dependent manner. Both receptors are widely expressed in various cell types and are an important part of the recognition of bacterial infections with the ligand for NOD2 being MDP and the NOD1 receptor recognizing bacterial molecules containing D-glutamyl-meso-diaminopimelic acid (DAP) moiety (205). They both signal through the RIP2/TAK1 pathway and activate a proinflammatory immune response (206). Our reporter assays demonstrated that the *Wolbachia* endosymbiont of *L. sigmodontis* serves as a potential ligand for the NOD2 receptor and therefore using NOD2$^{-/-}$ mice, a possible role of this receptor during *L. sigmodontis* infection was investigated.

4.2.1. NOD2$^{-/-}$ mice are more susceptible to infections with *L. sigmodontis*, but have no impaired immune responses within the thoracic cavity

NOD2$^{-/-}$ mice had an increased parasite burden within the thoracic cavity from the earliest time point L3 larvae reached the thoracic cavity at 5dpi until 35dpi. Around 60dpi, NOD2$^{-/-}$ mice eliminated the infection similar to their corresponding C57BL/6 WT controls. Accordingly, no microfilaremia occurred, suggesting that embryogenesis was inhibited in the female worms of NOD2$^{-/-}$ and WT mice. Due to their genetic background and their immune response, C57BL/6 mice are semi-susceptible to infection with *L. sigmodontis*. BALB/c mice on the other hand are fully permissive to infection and the parasite can undergo the full life cycle within these mice (33). However, under certain circumstances, even C57BL/6 mice develop microfilaremia. For instance, C57BL/6 mice with a Rag2IL-2Rγ knock-out - lacking T, B, and NK cells - develop a high microfilariae burden and therefore become fully permissive as was shown as a complimentary project of my thesis (76). The lack of NOD2 however did not lead to patent infections, leading to the assumption, that this receptor may not play a decisive role during later stages of filarial infections. Fitting to this finding, investigated immune responses at the site of adult *L. sigmodontis* residence were not impaired in NOD2 deficient mice. Tested cell types were comparable in their frequency to WT controls and no differences in cytokine concentrations were observed beside IL-5. The increased IL-5

production 5dpi can be associated with the increased worm burden at this early time point and may be produced by ILC2 cells. These innate immune cells are one of the first responders to helminth infections and produce high amounts of IL-5 (and IL-4, IL-13) after stimulation by the alarmins IL-33 and IL-25 as well as TSLP – cytokines which are induced probably by epithelial cells of the lung (207) and during infection with L. sigmodontis (96). Own first results identified ILC2 within the thoracic cavity and the lungs during the early stage of *L. sigmodontis* infection. These ILC2 could be the early source of important type 2 cytokines during the infection. Increased IL-5 concentrations in NOD2$^{-/-}$ mice correlated with a retarded development of *L. sigmodontis*. Thus, L4 larvae at 15dpi were significantly shorter and by 35dpi most of the larvae have not undergone a successful molting into adult worms in NOD2$^{-/-}$ mice (185). Additionally, L4 larvae which managed to molt were significantly shorter adults compared to adult worms in the C57BL/6 WT controls. It can be speculated that parasites need an intact immune system within the host for proper growth and development as the selective pressure induced by the immune response triggers mechanisms – on cellular and molecular level – leading to molting and growing as well as sexual maturity. As the group of Babayan et al. showed, helminths can benefit from immunological pressure and worms develop faster, if this pressure is given. One cytokine which was given a role in this context is IL-5 (208). Migrating larvae will also be exposed to inflammatory responses on their way to the pleural cavity and this "priming" might lead to their molting and growth. Since we observed lower inflammation in the NOD2$^{-/-}$ mice within the first days of infection, the L3 larvae meet less immunological pressure on their way to the site of infection, which may delay the subsequent molting. It can also be assumed that due to the increased worm burden, living conditions and space in the thoracic cavity are more limited, forcing the worms to grow smaller in size.

4.2.2. More L3 larvae reach the thoracic cavity of NOD2$^{-/-}$ mice compared to WT controls

A number of studies have reported that NOD2 deficiency is associated with an increased bacterial burden. Examples for this phenomenon are infections with *Chlamydophila pneumonia* and *Legionella pneumophila* (135, 137). In the latter case, mice deficient for NOD2 failed to recruit neutrophils to the lung and exhibited an increased bacterial load (137). In infections with *C. pneumonia*, NOD2$^{-/-}$ mice proved to have a delayed bacterial clearance (135). A study by Deshmukh et al. demonstrated a role for NOD2 on *Staphylococcus aureus* infection, as NOD2$^{-/-}$ mice were more susceptible to *S. aureus* and exhibited a higher bacterial burden due to defective neutrophil functions, but no impaired neutrophil recruitment (209). While bacterial infections induce a different immune profile than helminth infections, a common pattern is evident for NOD2 deficiency. In accordance to the bacterial studies, experiments of this thesis demonstrate increased burdens of the infectious agent, i.e. *L. sigmodontis* worms. As mentioned before, studies dealing with the NOD2-receptor in context with parasite infections are rare. The study by Shaw et al. using *Toxoplasma gondii* infections also demonstrated an increased parasite load as a result of decreased clearance, associated with lower production of IFNγ and a T cell-intrinsic effect in IL-2 production. While this study was later proven to be rebuttable, the characteristic feature of increased parasite load was also observed here (140). The only study so far dealing with helminth infections and NOD2 deficient mice is by Bowcutt et al. using the gastrointestinal nematode *Trichuris muris*. Similar to our observations Bowcutt and colleagues observed an increased parasite burden. They linked this increased worm burden to an impaired recruitment of CD103+ dendritic cells, which are known to majorly contribute to resistance against *T. muris* infections (210). All these studies demonstrate that the NOD2 receptor is indeed important for the control and initiation of infectious diseases, regardless of the site of infection. The finding that already 5dpi a significantly increased worm burden in the NOD2$^{-/-}$ mice was present during *L.*

sigmodontis infection suggests that the NOD2-dependent mechanisms must occur before this event. According to the life cycle of *L. sigmodontis* L3 larvae enter the vertebrate host with the blood meal of the arthropod vector and can be found within the skin for up to 48h before migrating through the lymphatics to the thoracic cavity (64, 211). As already more L3 larvae reached the thoracic cavity, it can be assumed that the NOD2 depending mechanism occurred within the skin or during the following migration phase. Subcutaneous injections of L3 larvae in NOD2$^{-/-}$ mice led to the same worm burden in the pleural cavity when compared to C57BL/6 WT mice. Thus, bypassing the immune response within the skin resulted in a comparable worm burden, confirming that the NOD2 dependent mechanisms must occur within the skin.

4.2.3. Protective immune responses within the skin are impaired in NOD2$^{-/-}$ mice

Although all filarial nematodes have to invade the skin and pass this immune barrier successfully to reach their final site of residence, the skin stage is not fully understood so far. Using intradermal injections of crude worm extract, recruited cells to the skin were analyzed. Three hours after injection the immune response was dominated by neutrophils and macrophages, as seen in the results of the flow cytometry measurement. Resident cells of the skin like keratinocytes or Langerhans cells are most probably implemented, too. Even though they were not measured via FACS, the PCR array contained several genes associated with these cell types. One such gene is the gene encoding for langerin, a protein expressed by the Langerhans cells of the skin, which was lower expressed in NOD2$^{-/-}$ mice. Similarly, several chemokines and cytokines produced by keratinocytes and Langerhans cells were found to be expressed at reduced levels in NOD2$^{-/-}$ mice compared to WT controls, including CCR4, CCL22 and CCL17. While the flow cytometry measurement revealed that cells like macrophages and mast cells were at the same frequency in WT and NOD2$^{-/-}$ mice, neutrophil frequencies were significantly lower in NOD2$^{-/-}$ mice. An influx of neutrophils was also

observed in the study by Porthouse et al. 3h after intradermal injection of *Brugia pahangi* L3 larvae. The exact mechanisms leading to the recruitment and activation of neutrophils were not defined. Results from this thesis suggest that *Wolbachia* are essentially involved in the recruitment of neutrophils to the site of larval entrance. Larval death in response to the very early host response may lead to *Wolbachia* release and subsequent neutrophil recruitment. Another possibility is the release of *Wolbachia* by intact L3 larvae, similar to adult worms. It was shown for *O. volvulus*, that intact adult worms are surrounded by neutrophils within the nodules, an observation which was gone when patients were treated with antibiotics (212). Neutrophils were already described to be important in the host immune response in mouse models and human infections of filariasis. *Wolbachia* bacteria induce neutrophil recruitment in murine and human filarial infections (88, 213, 214) and activated neutrophils can ingest *Wolbachia* following their release as was shown in *O. volvulus* infections (214). Porthouse et al. also showed in their study a loss of almost 80% of invading L3 larvae within the skin, as only 22% of the larvae were recovered 3h post injection. This further proves the importance of the skin barrier and that only the strongest larvae are able to pass the skin and reach the final site of residence. In this thesis a decreased frequency of neutrophils was observed in the skin three hours after crude filarial extract injection. After natural infection, lower frequencies of neutrophils within the peripheral blood were identified in $NOD2^{-/-}$ mice. Taken together, this demonstrates a delayed or impaired recruitment of these cells. Eosinophils were previously shown not to be involved in the early skin stage of primary infections (64) and increased numbers of eosinophils were also not detected on a cellular level within the skin at the 3h time point and in the blood 24h after infection. The NOD2 receptor has been shown to be expressed in various cell types present in the skin, which could mediate the neutrophil recruitment. Langerhans cells and dermal dendritic cells (DDC) are highly effective antigen-presenting cells, which are probably the cells that encounter the invading parasites first and

are possible inducers of neutrophil activation and recruitment. However, a study by Cotton et al. demonstrated that *B. malayi* L3 larvae failed to activate these cells (215). L3 larvae were able to avoid APC detection within the skin and did not induce cytokine production, which would have further triggered the immune response (215). However, it has to be noted that this study was mainly done with in vitro generated cells and may not adequately represent the interactions as they occur in vivo within the skin as a complex immune organ. The most abundant cell type within the skin is keratinocytes and the NOD2 receptor was also shown to be expressed within these cells and the NOD2 ligand MDP proved to be the most potent inducer of cytokine production (133, 216). Furthermore, Leire at al demonstrated that keratinocytes produce VEGF, IL-6, and IL-8, which not only support vascular permeability, wound healing and skin cell proliferation, but also contribute to neutrophil migration and activation (217). Interestingly, $NOD2^{-/-}$ mice were also associated with defects in wound healing processes as illustrated by Campbell et al. $NOD2^{-/-}$ mice showed in this study an initial delay in neutrophil recruitment and worsened damage of cutaneous wounds (113). Taken these studies together, it can be hypothesized that *Wolbachia* released by incoming L3 larvae are activating an immune response within the skin. The cellular inducer of the immune response remains unknown, but possible candidates include keratinocytes, but also resident APCs - cell types known to express the NOD2 receptor and being inducers of neutrophil recruitment. Neutrophils are essentially implemented cell types in the very early immune response against *L. sigmodontis* and significantly lower frequencies of neutrophils recruited to the entrance site of infectious larvae within the skin of $NOD2^{-/-}$ mice were observed. Because of this impaired recruitment, immune responses within the skin were weaker and allowed more L3 larvae to pass the skin barrier and to migrate on to the thoracic cavity, causing the increased worm burden observed from 5dpi onwards.

4.2.4 NOD2-dependent neutrophil recruitment leads to the increased worm burden

Apart from the investigations on the cellular level, the skin was tested for gene expression after L3 injection. This provided a more detailed picture of immunological processes on a molecular level. As expected, more genes associated with inflammatory responses had an increased expression in WT mice after injection with L3 larvae. In contrast, L3 injected NOD2$^{-/-}$ mice, consistent with the findings on the cellular level, had a lower expression of many genes involved in inflammatory responses compared to WT controls. While observations on molecular levels often do not directly translate into observations on the cellular level, similarities especially with genes associated with neutrophils were found. Fitting to the decreased frequency of neutrophils in the skin 3h post filarial antigen injection, genes like ngp (neutrophilic granule protein), elane (neutrophil-expressed elastase) and neutrophil activation protein prg2 were less expressed in NOD2$^{-/-}$ mice following L3 injection. The gene expressing for IL-6 was also found to be lower expressed in NOD2$^{-/-}$ mice. While IL-6 has a broad range of functions, it is especially important for neutrophil activation and trafficking (218-220). Genes associated with mast cells were also found to be lower expressed in NOD2$^{-/-}$ mice after L3 injection when compared to WT controls. This included the mast cell surface marker cKit (CD117) as well as the histamine receptors 1 to 4. Histamine receptor 2 and 4 were thereby already described to have functions in the recruitment of neutrophils (221-223). Interestingly, eosinophil associated genes like siglec f, epx and eotaxin were also lower expressed in NOD2$^{-/-}$ mice. On the cellular level, eosinophils were not detected, but in combination with the decreased IL-5 expression in the skin, it can be assumed that eosinophil functions and activation can be regulated by the NOD2 receptor, especially during the early phase of infection. Since eosinophils were previously described to only get involved in the immune response against invading larvae from 7dpi onwards (64), eosinophils are probably not the main reason for the increased worm burden in NOD2$^{-/-}$ mice.

Jiao et al. described in their study a NOD2 dependent activation of eosinophils during atopic dermatitis-like skin inflammation (224). Wong et al. also demonstrated a NOD2-mediated eosinophil activation in bronchial epithelial cells (225). The PCR array results of this thesis are in accordance with these observations as a lower expression of eosinophil-associated genes was reported when the NOD2 receptor is lacking.

Summarizing the cellular and molecular observations, neutrophils are the crucial cell type involved in the NOD2-depending immune response against invading L3 larvae within the skin. This role was further highlighted by neutrophil depletion experiments. Depletion of neutrophils within the first days of infection led to a significantly increased worm burden in C57BL/6 WT mice compared to isotype-treated WT controls. This increase in worm burden was however not observed in $NOD2^{-/-}$ mice. It can be assumed that due to the vastly reduced recruitment of neutrophils in $NOD2^{-/-}$ mice, neutrophil depletion did not result in a further increased worm burden as seen in the WT controls. A delayed or impaired neutrophil recruitment in $NOD2^{-/-}$ mice was already described by several groups in the context of bacterial infections or wound healing processes (113, 126, 135, 137). All these studies are in accordance with observations of this study. A functional impairment of neutrophils within $NOD2^{-/-}$ mice as observed in the study by Desmukh et al. (209) was not observed in this thesis.

4.2.5. Outlook

This thesis demonstrates the first evidence for a NOD2-mediated immune response against invading L3 larvae. While a direct binding of MDP to the NOD2 receptor has been just proven recently (109), the direct interaction of the NOD2 receptor to *Wolbachia* proteins is still subject of ongoing studies. This data would complete the observations of this study and the signaling pathway. However, molecular evidence for MDP in the cell wall-less *Wolbachia* genus is not reported yet, although there are several indications. Also the endocytic uptake and internalization of MDP to bind to the NOD2 receptor is still unknown. A study from the Michigan Medical School is hereby providing first evidence for a clathrin- and dynamin-dependent endocytosis pathway that mediates MDP uptake and subsequent NOD2 activation (226). A new study in the context of the skin defect Blau-syndrome even reports the increased propensity for NOD2 to relocate to the plasma membrane (112). This new feature of the NOD2-receptor would open up more possibilities in the ligand-binding mechanisms. Aside from all the molecular functions which still have to be investigated, the role of the NOD2-receptor during infections with other parasitic worms, especially *Wolbachia* lacking filariae, would be interesting. NOD2 is majorly associated with gastrointestinal and bowel diseases (e.g. Crohn's disease), so its functions during infections with gastrointestinal helminths would be intriguing and should be a target for further studies. Furthermore, it has to be evaluated in which regard the NOD2 receptor can be used as a therapeutic target, especially during the skin stage of human helminth infections. Since our reporter assays also proved that NOD1 gets activated by filarial antigens, experiments using NOD1$^{-/-}$ mice should determine the role of the NOD1 receptor during *L. sigmodontis* infection.

Complimentary project A – In vivo LsAg-stimulation

Infection with *L. sigmodontis* leads to a typical Type 2 immune response with increased production of the type 2 cytokines IL-4, IL-5, IL-10, and IL-13, high amounts of parasite specific IgE and IgG1 and increased numbers of eosinophils. At the same time helminth infections induce a regulatory milieu within the host, mainly driven by regulatory T cells, IL-10 and TGFß. This not only allows the parasite itself to persist for a long time within the host, it also dampens pathology of Th1-driven diseases by reducing pathology caused by a type 1 immune response, e.g. autoimmune diseases.

In this study we demonstrate that a repeated injection with crude worm extracts in BALB/c mice leads to similar type 2 immune responses as a natural infection with *L. sigmodontis*. Understanding this mechanism is of essential importance in regard to identify the best possible treatment regimens for experimental autoimmune disease models.

Materials & Methods:

BALB/c mice were repeatedly injected intraperitoneally with 100µg/ml *L. sigmodontis* antigen (LsAg, d0, d7, d14, and d21) and control mice were injected with PBS. On d28 mice were euthanized, opened and the immune response was investigated. To analyze the short-term effect of LsAg, another group obtained a single injection one day before the necropsy.

Blood was taken for LsAg-specific ELISA, cells from the peritoneum as well as the spleens were isolated for FACS analysis, and supernatants from these cells were used for ELISA after 72h stimulation with LsAg and ConA.

Results:

Repeated LsAg injection leads to significantly increased numbers of peritoneal cells and eosinophils.

Repeatedly injected mice had a significantly increased number of peritoneal cells (median: 1.03Mio; min: 0.65Mio; max: 3.07Mio) compared to one time injected mice (median: 0.48Mio; min: 0.32Mio; max: 0.61) or PBS controls (median: 0.59Mio; min: 0.36Mio; max:

0.96Mio; Fig.26A), significant differences were especially observed in the frequencies of peritoneal granulocyte populations.

Frequencies of peritoneal eosinophils were significantly increased after repeated LsAg injection (median: 7.2 %) compared to one time injection (4.75%) or PBS controls (2.55%) (p= 0.0017; Fig.26C). While repeated injection with LsAg leads to higher numbers of eosinophils, one time LsAg injection led to a high recruitment of neutrophils (p=0.009; Fig. 26B). The frequency of neutrophils was significantly increased in the peritoneum after one time injection (median: 8.4%) compared to repeated injections (0.3%) or the PBS controls (0.45%). While the CD4+ T cell population was the same in the repeated LsAg injected mice and the PBS controls (2.6% vs. 2.55%), the mice injected one time with LsAg showed a significantly higher frequency of CD4+ T cells (4.45%, Fig. 26E).

In contrast, spleen cells did not show any differences in the cellular composition with the different treatment regimens (data not shown).

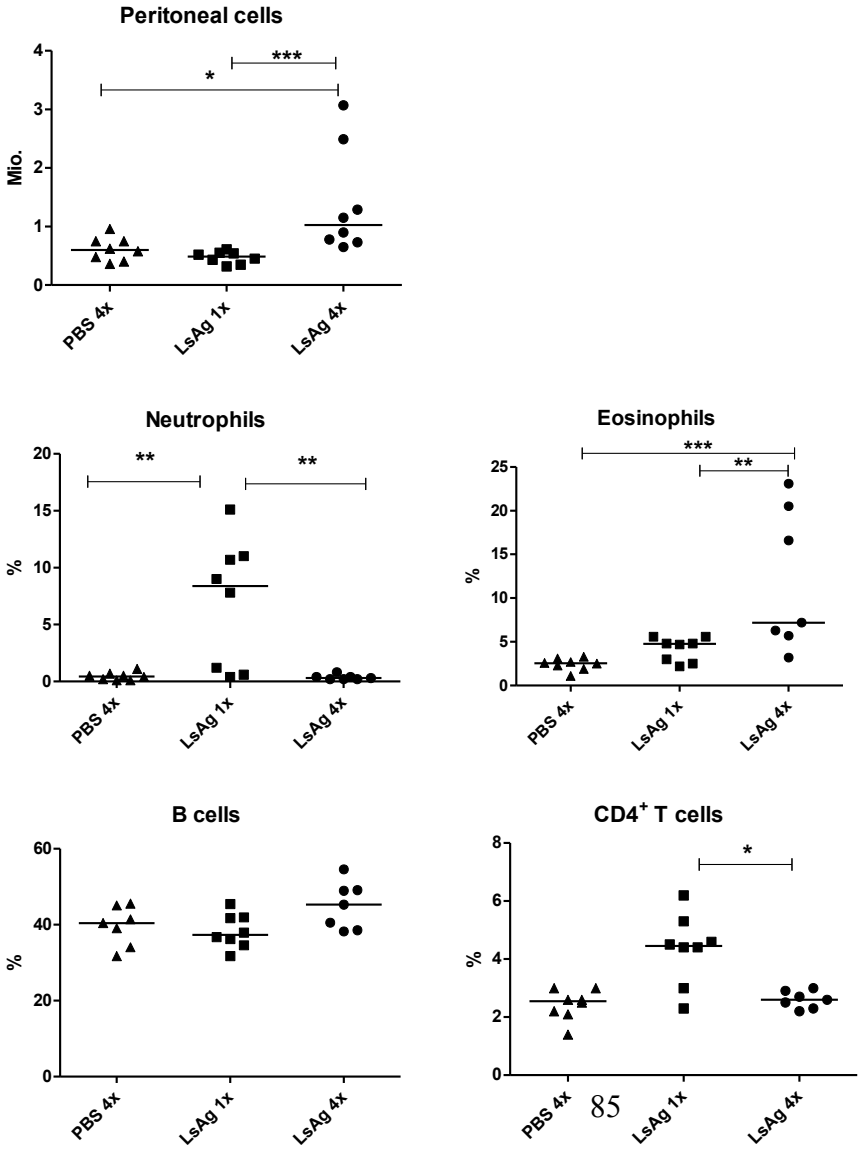


Figure 26: Repeated LsAg injection leads to an increased number of peritoneal cells. Total peritoneal cell number and frequencies of neutrophils, eosinophils, B cells and CD4+ T cells in mice treated with 4x LsAg, 1x LsAg and PBS. Data is representative for three independent experiments with at least 7 mice per group. Data was tested for statistical significance using the Kruskal-Wallis test followed by Dunn's post-test.

Chronic LsAg injection leads to an increased induction of alternatively activated macrophages

Frequencies of F4/80^{hi} peritoneal macrophages did not reveal any differences in all three tested groups (medians: 4xLsAg 17.5%; 1xLsAg 17.9%; 4xPBS: 16.5%, Fig.27A). Since a helminth-induced type 2 immune response is characterized by the induction of alternatively activated macrophages (AAMO), F4/80hi macrophages were tested for their RELMα expression via FACS analysis. Mice repeatedly injected with LsAg had a median value of 96.7% of AAMO and thus showed a significantly increased frequency than the mice injected with one time LsAg (18.8%) or PBS (25.1%, Fig.27B).

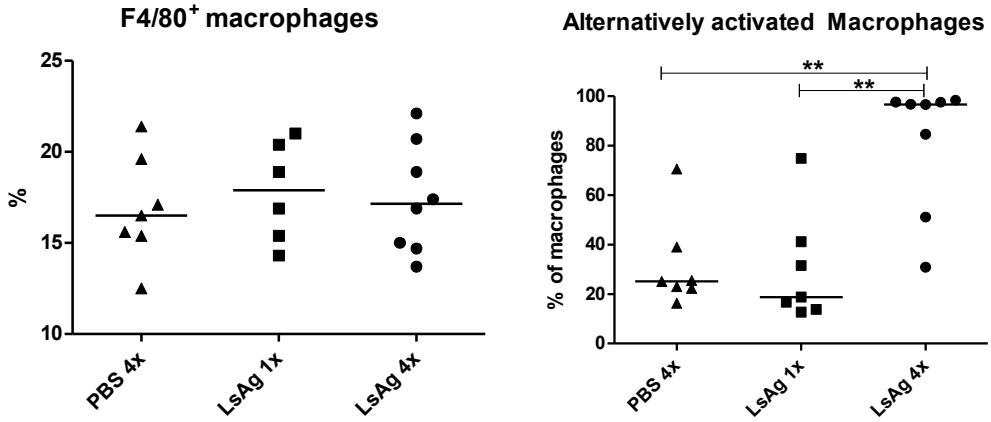


Figure 27: Increased induction of alternatively activated macrophages after repeated LsAg injection. Frequency of macrophages in the peritoneum after four times LsAg injection, one time LsAg injection and in PBS controls as well as frequency of RELMα+ macrophages. Representative data of three independent experiments with at least 7 mice per group. Data was tested for statistical significance using the Kruskal-Wallis test followed by Dunn's post-test.

Ex vivo stimulated peritoneal cells from repeated LsAg-treated mice produce significantly higher amounts of type 2 cytokines compared to PBS controls

Ex vivo stimulation with LsAg and ConA led to significantly higher production of the type 2 cytokines IL-4, IL-5, IL-10, and IL-13 (Fig.28A-D). Fitting to that result, concentrations of

the pro-inflammatory cytokines IFNγ (Fig.28E) and IL-6 (Fig.28F) were significantly downregulated in the repeated LsAg-treated mice compared to PBS controls.

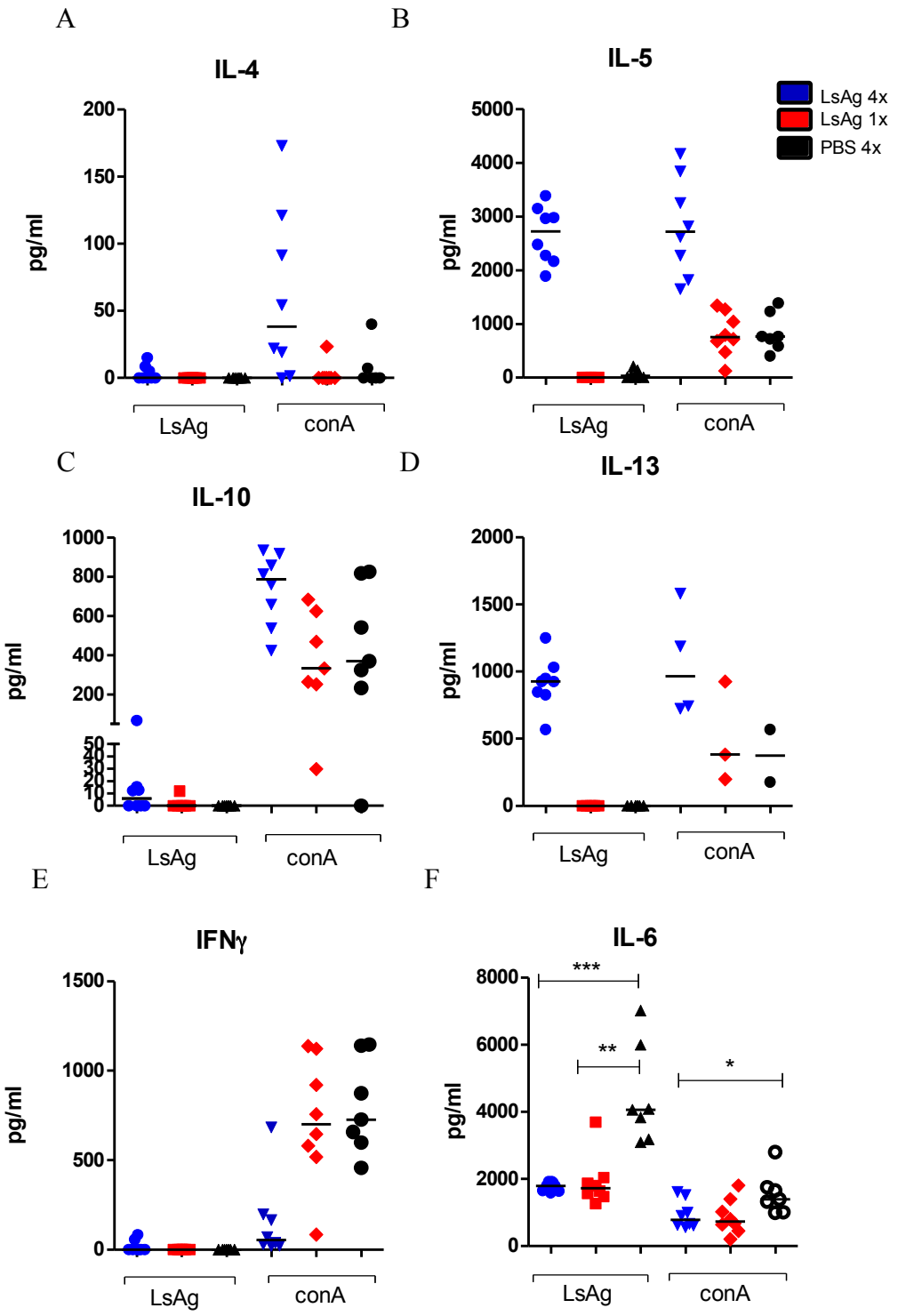


Figure 28: Increased production of type 2 cytokines after repeated LsAg stimulation. Concentrations of IL-4 (A), IL-5 (B), IL-10 (C), IL-13 (D), IFNγ (E), and IL-6 (F) after ex vivo stimulation of peritoneal cells with LsAg and ConA. Mice were treated 4xLsAg (blue), 1x LsAg (red) and PBS (black). Representative data of three

independent experiments with at least 8 mice per group. Data was tested for statistical significance using the Kruskal-Wallis test followed by Dunn's post-test.

Repeated LsAg treatment leads to increased LsAg-specific antibodies

As shown in Figure 29, the concentration of total IgE and IgG1 was significantly increased in repeated LsAg treated mice compared to PBS controls and one time treated mice, whose numbers were similar. Similarly, the OD-values of LsAg-specific titers for IgE and IgG1 were elevated in the repeated LsAg treated mice. While this was also the case for LsAg-specific IgG2a/b, total IgG2a levels did not reveal any differences among the tested groups.

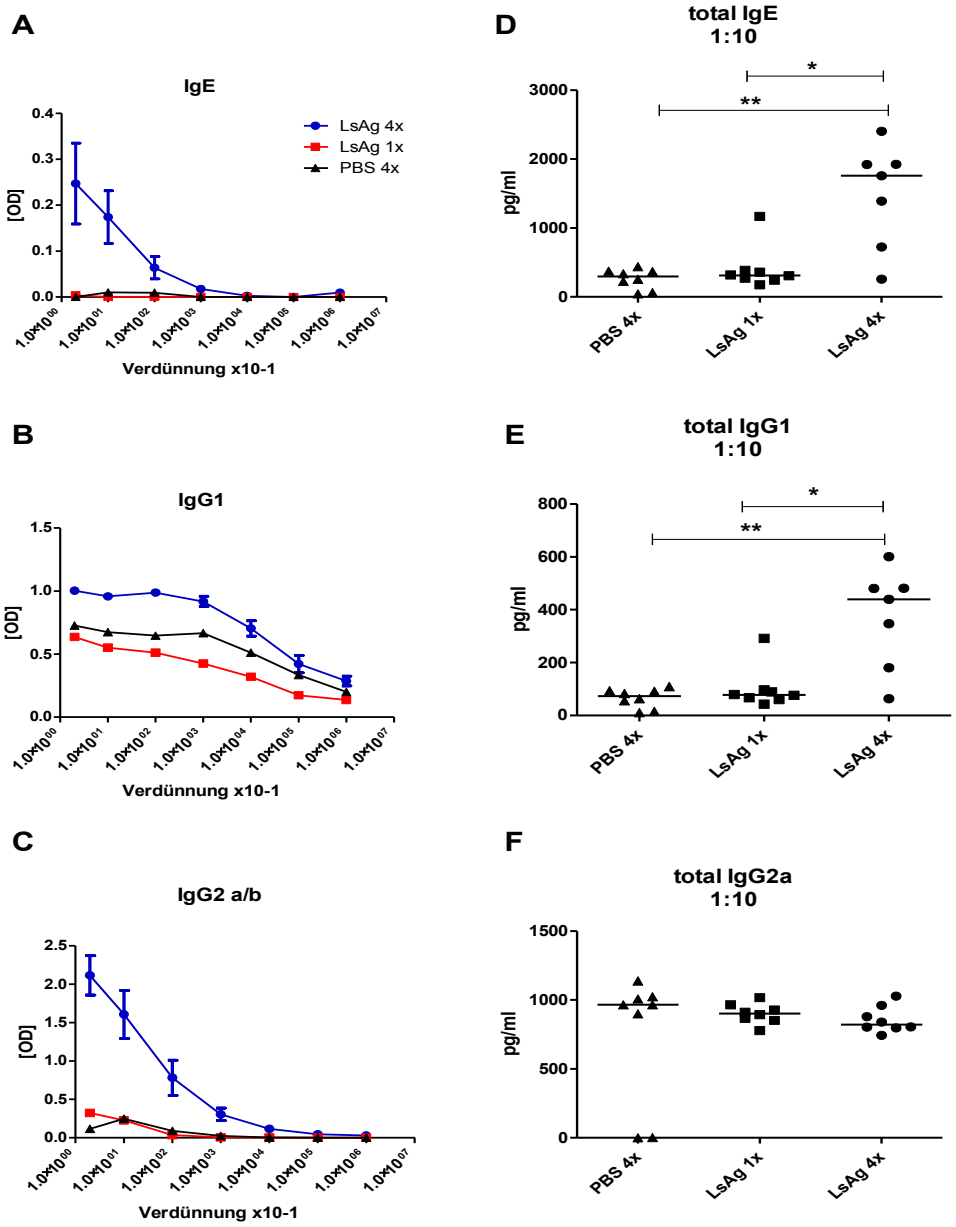


Figure 29: Increased antibody production after repeated LsAg stimulation. Optical density [OD] of LsAg-specific IgE (A), IgG1 (B) and IgG2a/b (C) as well as concentrations of total serum IgE (D), IgG1 (E) and

IgG2/b (F) in mice treated four times with LsAg, one time LsAg as well as in PBS controls. Representative data of three independent experiments with at least 7 mice per group. Data was tested for statistical significance using the Kruskal-Wallis test followed by Dunn's post-test.

Discussion and Outlook:

The data from these experiments clearly demonstrate that repeated injections of LsAg induces to a certain extent an immune response similar to a natural infection with *L. sigmodontis*. Immunological features, which are found in natural *L. sigmodontis* infection and after repeated LsAg injections are an increased eosinophil number in the pleural cavity, induction of AAMs and increased production of type 2 cytokines like IL-4, IL-5, IL-10, and IL-13. The initial source of IL-4, IL-5 and IL-13 may be ILC2 (227) and the increased production of IL-5 leads then to the increased eosinophil population; IL-4 and IL-13 are responsible for the switch of macrophages to the alternatively activated phenotype (228). The increased production of LsAg-specific and total IgE and IgG1 is also fitting to the installment of a type 2 immune response after repeated LsAg injections. Even though certain aspects of an infection, like the movement and tissue damage caused by the filariae as well as released excretory/secretory products, are missing when using the crude worm extract, LsAg treatment surrogates several aspects of a natural infection and is therefore of interest for experimental models of allergy and autoimmune diseases. The one-time injection of LsAg was tested to evaluate, whether one administration of worm extract is sufficient to induce an immune modulating effect. However, the one-time injection of LsAg led to an increased influx of neutrophils into the peritoneum, suggesting the injection leads to an initial inflammation. This could be due to the *Wolbachia*, which are still present in the crude worm extract. To really access the role of the *Wolbachia* during the initiating of the inflammation, additional experiments should be performed using crude worm extract depleted from *Wolbachia*.

In the next steps (229), treatment with LsAg was optimized. Several experiments were performed to identify the minimum amount of antigen required to induce the observed type 2

immune response. Furthermore, different preparations of antigens were tested, e.g. antigen produced from male and female adult worms as well as worm antigen from worms depleted from *Wolbachia*. Treatments of the LsAg with proteinase K, deglycosylation of the antigen as well as treatment with heat was done and suggested that the active component of the LsAg is a glycogen similar to ES-62.

A successful optimization of the LsAg administration and purification of the active component is important for future experiments, as it could have the potential to replace the complexity of natural infection and crude antigen mixtures.

Complimentary project B – The role of the ST2 receptor during *L. sigmodontis* infection

Interactions of the recently discovered Th2 cytokine IL-33 with its receptor ST2 leads to amplified type 2 immune responses (230). As type 2 immune responses are known to mediate protection against helminth infections, it was hypothesized that the lack of ST2 would lead to an increased susceptibility to filarial infections.

Materials & Methods:

ST2 deficient and immunocompetent BALB/c mice were infected with the filarial nematode *L. sigmodontis*. At different time points after infection (35, 60, 90 dpi) mice were analyzed for their worm burden and immune responses were examined at the site of infection, the thoracic cavity, and systemically using cells derived from the spleen.

Results

As demonstrated in my diploma thesis, *L. sigmodontis* infected ST2-ko mice had significantly reduced total numbers of thoracic cavity cells and splenocytes compared to infected immunocompetent controls. At early time points restimulated thoracic cavity cells of ST2-deficient mice produced lower amounts of type 2 cytokines, although this difference was not apparent after the onset of microfilaremia, the release of filarial progeny. Microfilaria levels were significantly increased in ST2-ko mice (Fig.30A, B), whereas the adult worm burden was not affected by the absence of ST2 (data not shown).

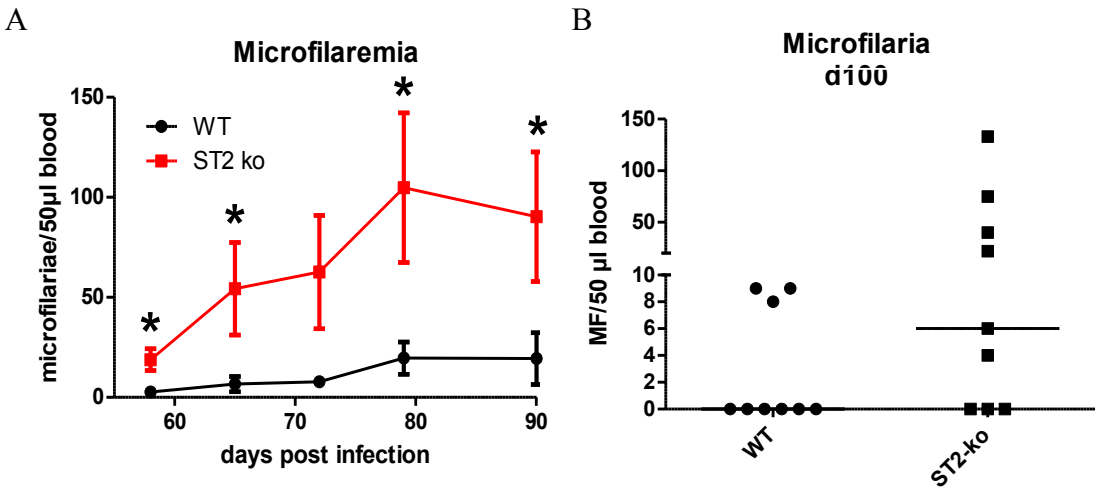


Figure 30: Deficiency of ST2 leads to a pronounced microfilaremia. (A) Microfilariae count in 50µl of peripheral blood (B) microfilaria burden on d100 post infection in ST2-ko mice and WT controls (A) Pooled data from three independent experiments. (B) Representative data of two independent experiments. Differences were tested for statistical significance by Mann-Whitney-U-test, *p<0.05.

In further experiments performed during my PhD thesis, microfilariae were injected into naïve WT and ST2-ko mice to investigate whether the lack of ST2 impairs the clearance rate of peripheral microfilariae.

As shown in Figure 30A, ST2-ko mice had a delayed clearance of injected microfilariae compared to WT controls. As early as 1h after microfilariae inoculation, ST2-ko mice retained significantly more microfilariae compared to WT controls (WT: average of 13 microfilariae/ 50µl blood, ST2-ko: 33 microfilariae/50µl blood). Significantly increased numbers of microfilariae in the peripheral blood of ST2-ko mice was maintained until 13 days post inoculation. Indeed, whereas all ST2-ko mice were still microfilariae positive 13 days post injection, 50% of the WT controls had no detectable peripheral blood microfilariae at this time point. On day 23 post inoculation no microfilariae were detectable in WT animals but 60% of the ST2-ko mice still had viable circulating microfilariae. On day 42 post inoculation all ST2-ko mice had successfully cleared all peripheral microfilariae.

As microfilariae can be found in the spleen after inoculation, it was investigated whether the impaired clearance of injected microfilariae in ST2 deficient mice was mediated by the spleen. Thus, naïve mice were splenectomized prior to inoculation with microfilariae. One hour after microfilariae injection, splenectomized WT mice had microfilariae levels that were comparable with splenectomized ST2-ko animals (both groups median 35 microfilariae/50µl blood) and were significantly higher than the levels seen in WT controls, which had undergone sham surgery (median 18 microfilariae/50µl blood; Fig.31B). Sham treated ST2 deficient mice had microfilariae levels (median 27 microfilariae/50µl blood) that were significantly increased compared to sham treated WT controls, but lower microfilariae loads than splenectomized groups (Fig.31A). This demonstrates that the spleen is essential in the clearance of peripheral blood form microfilaria.

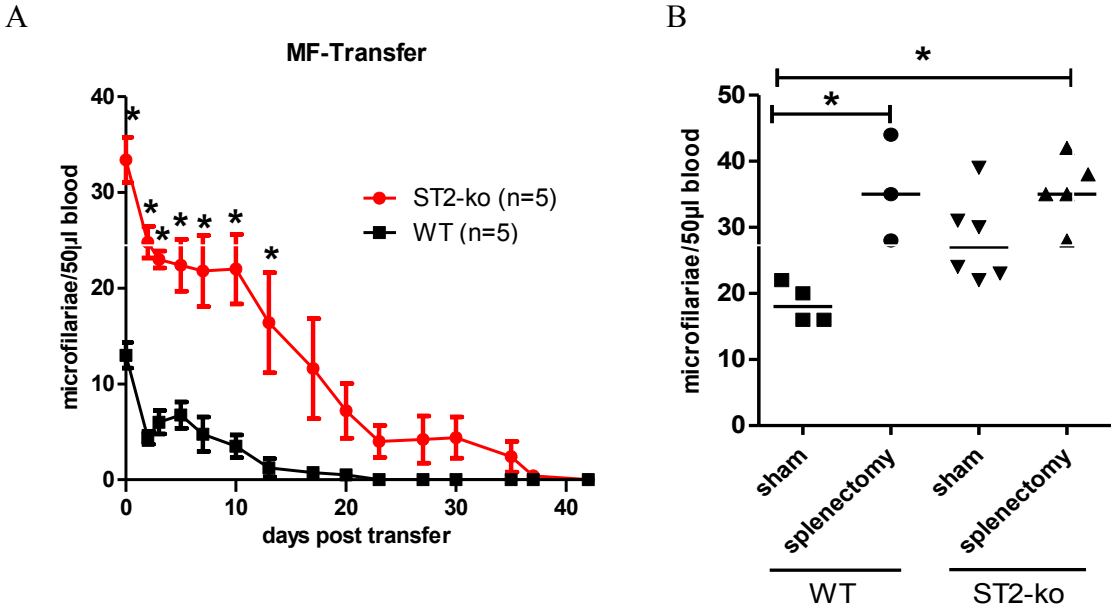


Figure 31: ST2-ko mice have a delayed splenic clearance of transferred microfilariae. Kinetics of blood microfilariae numbers in ST2-ko mice and wild type (WT) controls after i.v. inoculation of 50.000 microfilariae (A). Blood microfilariae counts in splenectomized and sham-treated ST2-ko mice and WT controls one hour after inoculation with 50.000 microfilariae per mouse (B). Differences were tested for statistical significance by Mann-Whitney-U-test, *p<0.05. Representative data shown in (A) is from two independent experiments with at least 6 mice per group.

Discussion and Outlook:

In conclusion, our study investigated for the first time the impact of ST2 on the development of a non-enteric helminth using ST2-deficient mice and infections with the filarial nematode *L. sigmodontis*. The absence of the ST2 receptor had no effect on protective immune responses against the adult stage of *L. sigmodontis*, but led to a significantly higher microfilarial burden. Embryogenesis was not altered in the ST2 deficient mice. Furthermore, the increased microfilarial burden in ST2-ko mice did not correlate with an impaired Th2 cytokine response after the onset of microfilaremia, but was shown to be due to an impaired splenic clearance of microfilariae.

This study was published in 2014 in PLOS One with the title "**ST2 deficiency does not impair type 2 immune responses during chronic filarial infections but leads to an increased microfilaraemia due to an impaired splenic microfilarial clearance**" (96).

Complimentary project C – The role of Interleukin-6 during *L. sigmodontis* infection

IL-6 is a cytokine with a broad range of functions. In this study, the role of IL-6 during infection with the filarial nematode *L. sigmodontis* was investigated. Previous experiments using IL-6 deficient mice demonstrated that IL6$^{-/-}$ mice had a significantly increased worm burden after natural infection with *L. sigmodontis* compared to WT controls at early time points of infection (185). This increased worm burden was no longer observed after the molt to adult worms and during chronic infection. Further experiments proved that worm burden in IL-6$^{-/-}$ mice were already increased at the time point the infectious L3 larvae reach the pleural cavity. Immune responses were analyzed in the pleural cavity, but did not reveal significant differences accounting for the increased worm burden. Blocking mast cell degranulation reduced the worm burden in IL-6$^{-/-}$ mice partially, suggesting that release of mast cell derived mediators improve larval migration to a certain degree. Circumventing the skin barrier by s.c. injection resulted in a similar worm load in IL-6$^{-/-}$ and WT mice, a phenomenon also observed in the experiments using NOD2$^{-/-}$ mice. To get a detailed picture of the immune response within the skin, L3 larvae were injected intradermally and skin was isolated three hours later for RNA isolation. PCR arrays were performed of the skin to reveal differences in the immune responses on molecular levels between IL-6$^{-/-}$ mice and PBS controls.

Material & Methods:

IL-6 deficient mice and WT controls were intradermally injected with LsAg or PBS as controls and skin cells were isolated three hours post injection for FACS analysis. For PCR arrays, mice were injected intradermally with 40 L3 larvae and skin was isolated three hours after injection. RNA isolation was performed like described in section 2.21., using the

RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Statistical analysis of array data was done like described in section 2.22.

Results:

While WT mice exhibited a significantly increased frequency of neutrophils after LsAg injection compared to WT PBS controls, neutrophil frequencies of IL6$^{-/-}$ mice treated with LsAg and PBS were similar and did not show significant differences (Fig.32A), indicating an impaired immune response within the skin when IL-6 is missing. Similarly, macrophage frequencies in the skin of WT mice treated with LsAg increased compared to WT PBS controls, while LsAg injection in IL6$^{-/-}$ mice did not lead to such an increased macrophage frequency (Fig.32).The PCR array data revealed that IL-6$^{-/-}$ mice injected with LsAg showed significantly lower expression of several genes important for immunity when compared to LsAg-injected WT controls. These genes included *Cxcr1* ($p<0.05$), a receptor mainly expressed on neutrophils and macrophages and *Elane* ($p>0.05$), the neutrophil-derived elastase. This observation is in accordance with our FACS data. Furthermore, *Tslp* (thymic stromal lymphopoietin, $p<0.05$), *Hrh4* (histamine receptor 4, $p>0.05$), and *IL6* were downregulated in LsAg-treated IL-6$^{-/-}$ mice compared to WT controls (Table 2).

Genes increased in IL-6$^{-/-}$ mice upon LsAg injection included *kit* ($p<0.05$), a marker for mast cells, the mast cell Chymase 1 (*cma1*, $p>0.05$) as well as genes for the histamine receptors 1 and 2 (*hrh1/hrh2*, both $p>0.05$) and the gene for eosinophil major basis protein ($p<0.05$).

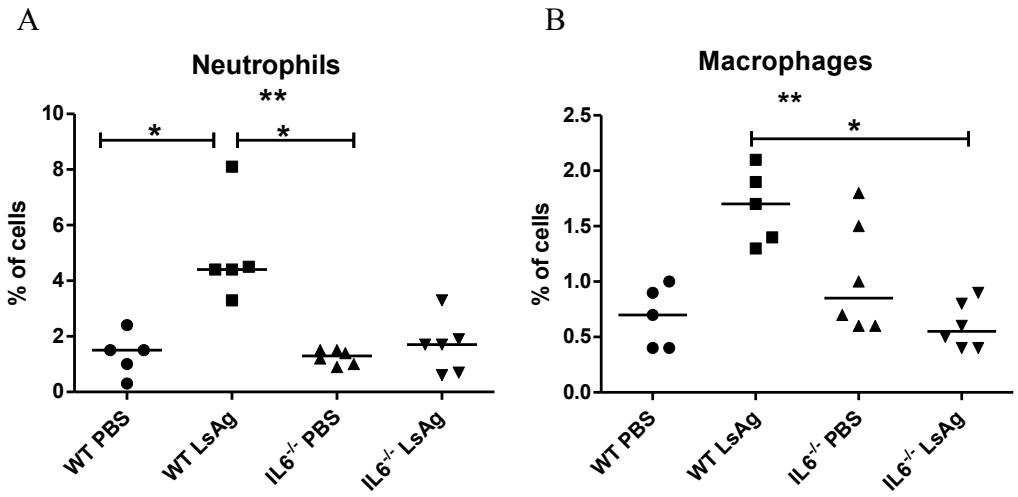


Figure 32. Impaired recruitment of neutrophils and macrophages in response to filarial antigen within the skin of IL-6$^{-/-}$ mice. Frequency of (A) neutrophils and (B) macrophages of IL-6$^{-/-}$ and wild type (WT) animals 3h after intradermal injection with LsAg or PBS. Differences were tested for statistical significance by Mann-Whitney-U-test (Data is shown as median*p<0.05; **p<0.01).

Table 2: Gene expression in skin of IL-6$^{-/-}$ and WT animals three hours after LsAg injection. The table shows genes up- and downregulated in wild type BALB/c mice compared to IL-6$^{-/-}$ mice 3h upon LsAg injection. Bold numbers indicate significantly up- or downregulated gene expression. Three mice per group, data is shown as mean.

BALB/c LsAg vs. IL6ko LsAg					
Gene Symbol	**Fold Regulation**	**p-value**	**Gene Symbol**	**Fold Regulation**	**p-value**
Tslp	2,3107	**0,01607**	**Kit**	-5,6373	**0,012635**
Cxcr1	2,8448	**0,037499**	**Prg2**	-4,5263	**0,046351**
Chia	2,3812	0,08559	**Cma1**	-2,2013	0,054101
Hrh4	3,6935	0,102471	**Il4**	-4,7185	0,102993
Chi3l3	2,8317	0,191815	**Nos1**	-2,7992	0,223604
Elane	3,014	0,331063	**Hrh2**	-2,3922	0,307639
Il6	38,0107	0,350403	**CCL3**	-2,0586	0,345303
Tlr6	4,0512	0,657386	**Hrh1**	-3,9222	0,373112

Discussion:

Our data suggest that neutrophils are less abundant in response to *L. sigmodontis* exposure and facilitate larval survival and migration to the pleural cavity, leading to increased worm numbers at early time points of infection. This study demonstrates that the increased worm

burden in IL-6$^{-/-}$ mice was neither due to impaired protective immune responses within the pleural cavity nor due to an increased vascular permeability that facilitates larval migration to the pleural cavity. Moreover, we demonstrate a key role for the cytokine IL-6 during early phases of *L. sigmodontis* infection. IL-6 is a key mediator for immunity against invading L3 larvae orchestrating recruitment of neutrophils and macrophages, which entrap incoming infectious larvae. Surprisingly, this mechanism is very similar to the investigated mechanism in the NOD2-project. Using the fully permissive BALB/c strain as well as the semi-permissive C57BL/6 mouse strain, we could demonstrate the key role of neutrophils during the early immune response within the skin. The lack of neutrophils lead in both, NOD2$^{-/-}$ and IL-6$^{-/-}$ mice to significantly increased worm burden at site of infection during early time points, proving their essential role in the skin stage of filarial infection. In the next steps, the focus should be on the neutrophils and their exact function during the protective effect. Neutrophils can act in various ways and it should be investigated how the protection is mediated. Cytokine and chemokine production by neutrophils was partly investigated in the NOD2-project as shown in section 3.2.11, furthermore, characterization of neutrophilic extracellular traps (NETs) using in vivo-imaging, as well as identification of granule proteins would provide more insight about the detailed mechanism.

Complimentary project D – Infection of Rag2IL-2Rγ$^{-/-}$ mice with *L. sigmodontis*

While the BALB/c mouse is fully permissive for infections with *L. sigmodontis*, C57/BL6 mice are only semi-susceptible to this infection. In C57BL/6 mice, *L. sigmodontis* never produce microfilariae and most adult worms are killed shortly after the molt into adult worms. In this study, C57BL/6 mice with a Rag2IL-2Rγ$^{-/-}$ were examined for worm development and patency. Rag2IL-2Rγ$^{-/-}$ mice are deficient for T cells, B cells and NK cells and therefore impaired in potential immune responses, which could lead to the killing of adult worms in WT C57BL/6 mice.

Material & Methods:

Rag2IL-2Rγ$^{-/-}$ mice and corresponding WT controls were naturally infected with *L. sigmodontis* and worm burden, cellular composition via flow cytometry as well as cytokine milieu via ELISA were analyzed 30 and 72dpi.

Results:

The worm burden was comparable between both tested groups 30dpi, however, by 72dpi, parasites were still present in Rag2IL-2Rγ$^{-/-}$ while the WT controls eliminated the infection as expected. Adult worms were not only present and motile, but also produced a high number of microfilariae, which were found in both, the thoracic cavity and the peripheral blood (Fig.33A-E).

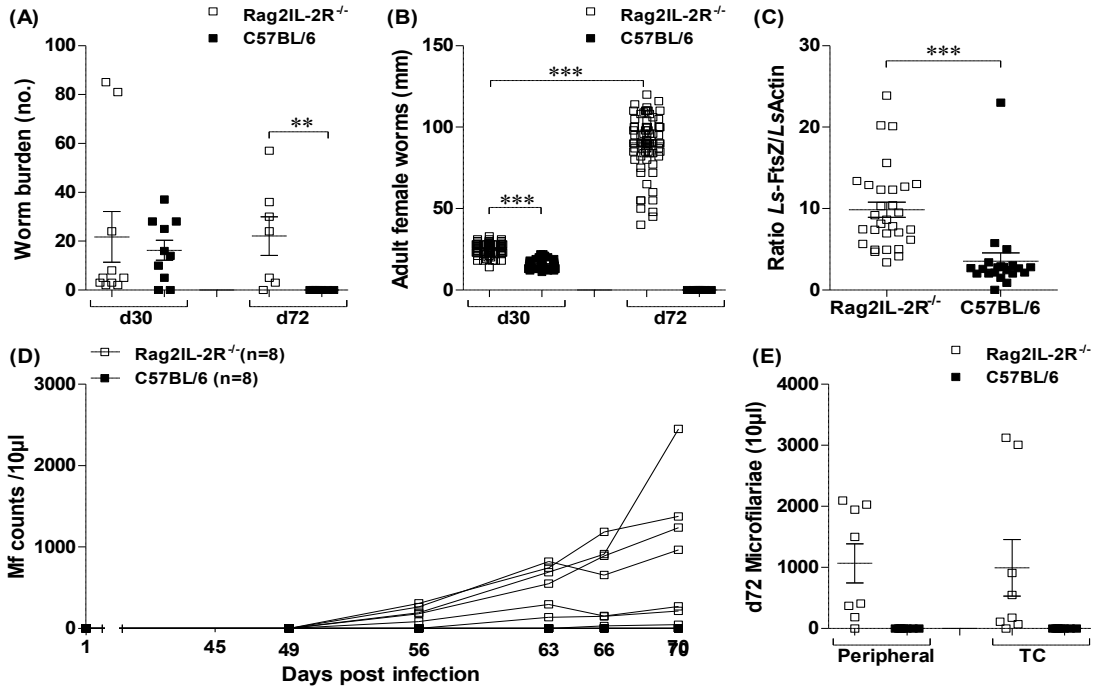


Figure 33: *L. sigmodontis* infection in RAG2IL-2Rγ$^{-/-}$ mice leads to patency. (A) Worm burden of both tested groups of mice 30dpi and 72dpi. (B) Adult female worm length. (C) Levels of *Wolbachia* DNA were determined via a duplex PCR in individual adult female worms.(D) Microfilariae levels in the peripheral blood from 49dpi until 70 dpi and microfilariae load within thoracic cavity (TC) fluid and blood 72dpi (E). Graphs show data from one infection experiment with n=10 WT and n=10 Rag2IL-2Rγ$^{-/-}$ on d30 and n=8 WT and n=8 Rag2IL-2Rγ$^{-/-}$ until d72. Differences were tested for statistical significance by Mann-Whitney-U-test (**p<0.05, ***p<0.001).

Tested cytokines like IL-4, IL-6, TNF as well as chemokines like MIP2, RANTES and eotaxin were all significantly lower in the thoracic cavity of the knock-out mice compared to the WT controls 30dpi. (Fig.34). 72dpi this difference was still observed for TNF and MIP-2.

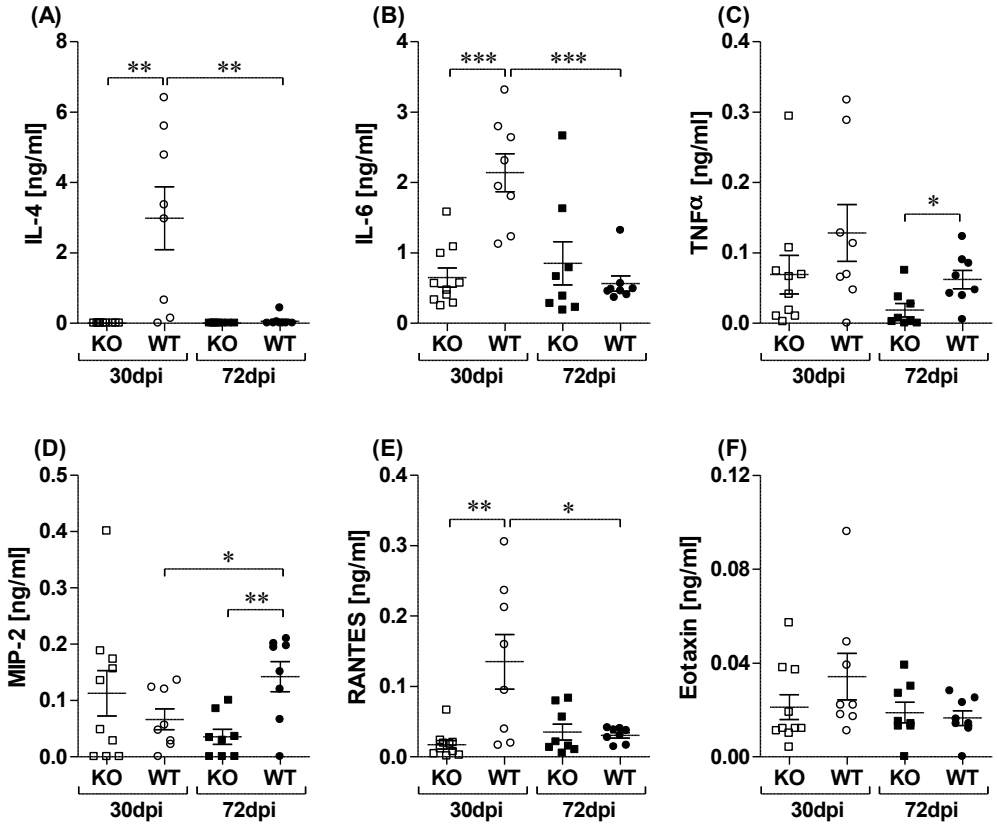


Figure 34: Low cytokine and chemokine production in the thoracic cavity of *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ mice. Levels of IL-4(A), IL-6 (B), TNF (C), MIP-2 (D), RANTES (E) and eotaxin (F) were measured in 30 and 72dpi by ELISA. Graphs show data from one infection experiment with n=10 WT and n=10 KO-mice on d30 and n=8 WT and n=8 KO-mice on d72. Differences were tested for statistical significance by Mann-Whitney-U-test (**p<0.05, ***p<0.001).

Important cell types were tested in the thoracic cavity, as shown in figure 35. While neutrophil frequencies were significantly increased in Rag2IL-2Rγ$^{-/-}$ mice, eosinophil and macrophage populations were significantly lower in Rag2IL-2Rγ$^{-/-}$ mice compared to WT controls. In accordance, no AAMs were found in Rag2IL-2Rγ$^{-/-}$ mice (Fig.35F).

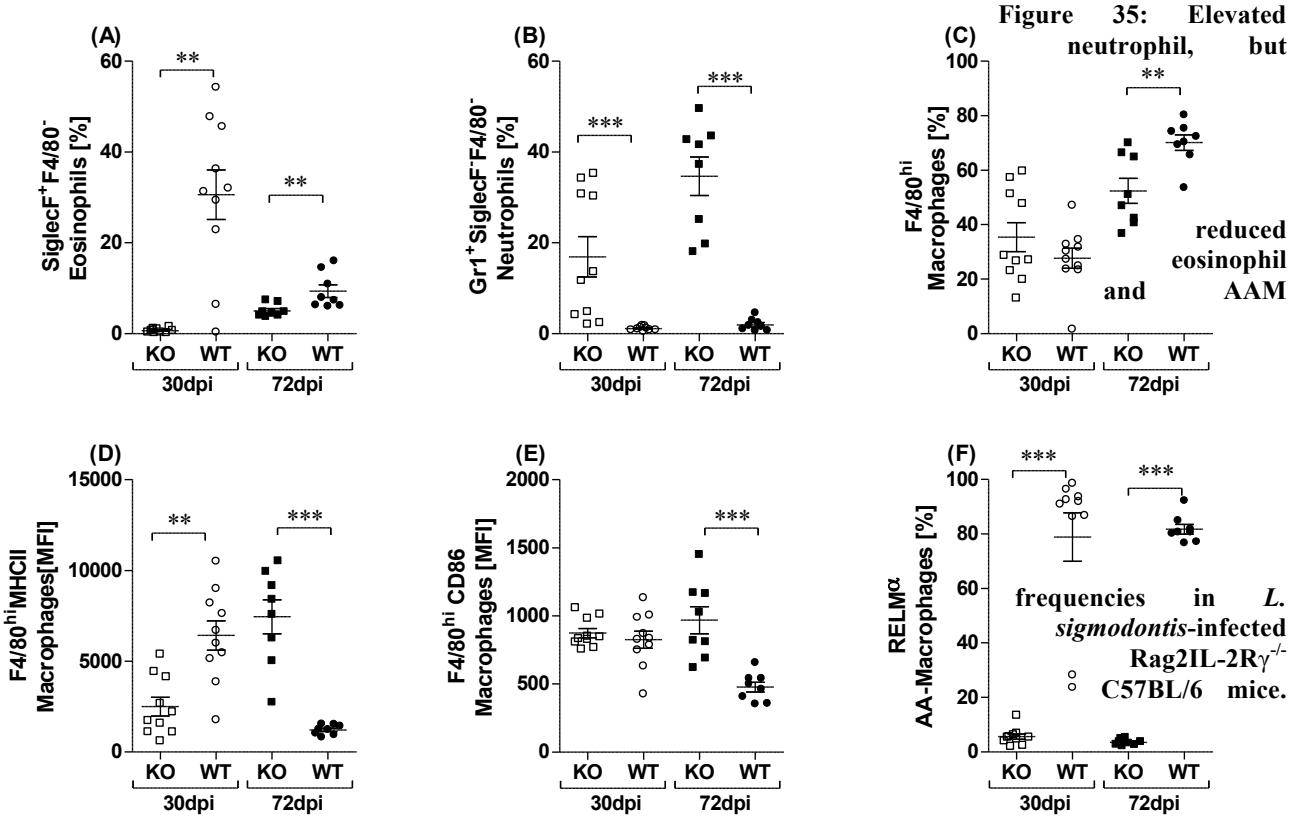


Figure 35: Elevated neutrophil, but reduced eosinophil and AAM frequencies in *L. sigmodontis*-infected Rag2IL-2Rγ$^{-/-}$ C57BL/6 mice.

Thoracic cavity cells, isolated from individual mice on d30 or 72p.i. were assessed for the frequency of (A) eosinophils, (B) neutrophils and (C) macrophages. MFI for (D) F4/80hi MHCII+, (E) F4/80+CD86+ and (F) RELMα+ on macrophages is shown. Pooled data for two independent experiments per time point. Differences were tested for statistical significance by Mann-Whitney-U-test (**p<0.05, ***p<0.001).

Discussion & Outlook:

The data of these experiments demonstrate that adaptive immune responses and or NK cells are responsible for the prevention of development of patent *L. sigmodontis* infections in semi-susceptible C57BL/6 mice. The combined lack of T, B and NK cells facilitate the development of patency leading to 100% prevalence. Previous experiments using B cell deficient µMT C57BL/6 mice did not lead to patent infections, demonstrating that the lack of B cells alone is not influencing the patency (79). However, B1 deficiency (Xid) in BALB/c mice leads to an increased worm burden and microfilaremia, which was similarly observed in CD4+ T cell depleted mice (77, 208). T and B cells are driving the immune response via the production of cytokines, and as observed in our ELISA data, cytokine levels in Rag2IL-2Rγ$^{-/-}$

mice were significantly lower at both tested time points. This impaired cytokine production also influenced the immune response at the site of infection. Eosinophil and AAM frequencies were significantly lower in Rag2IL-2Rγ$^{-/-}$ mice, demonstrating that these cells need T cell derived cytokines for their development and expansion.

Further studies have to analyze the impact of specific cell populations including B and T cells with their subsets as well as NK cells. Rag2IL-2Rγ$^{-/-}$ mice reconstituted with different cell populations could give more insight to their role in the development of patency. This would give a detailed look of the reconstituted cell type as well as their secreted chemokines and cytokines and their specific role during infection with filarial nematodes.

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List of abbreviations

AAM	alternatively activated macrophages
APC	allophycocyanine
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
CCL / CCR	chemokine (c-c motif) ligand / receptor

CD	cluster of differentiation
cfu	colony forming units
ConA	concanavalin A
CO_2	carbon dioxide
CXCL	chemokine C-X-C motif ligand
DAMP	damage-associated molecular pattern
DEC	diethylcarbamazine
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpi	days post infection
E/S	excretory/secretory
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
FACS	Fluorescence activated cell sorting
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	fluorescence minus one
FoxP3	forkhead box protein P3
FSC	forward scatter
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
HEK	human embryonic kidney cells
hi	high expressing
HRP	horseradish peroxidase
IFNγ	interferon gamma
Ig	Immunoglobulin
IL	Interleukin
i.p.	intra-peritoneal
i.v.	intra-venous
ko	knock-out
L1	first larval stage
L3	third larval stage
L4	fourth larval stage

LF	lymphatic filariasis
lo	low expressing
LPS	lipopolysaccharide
LsAg	*Litomosoides sigmodontis* antigen
Ls-Wolb.	Antigen from tetracycline-treated *L. sigmodontis* adult worms
MACS	magnet activated cell sorting
M	molarity (mol/L)
med.	medium (RPMI1640)
min	minute(s)
MIP	macrophage inflammatory protein
mf/MF	microfilariae
MFI	mean fluorescence intensity
MHC	mayor histocompatibility complex
ml	milliliter
MyD88	myeloid differentiation primary response protein 88
NLR	Nod-like receptors
NO	nitric oxide
NOD	non-obese diabetic mice
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOS	nitric oxide synthase
OD	optical density
o/n	over night
Pam3Cys	P3C Pam3Cys-Ser-(Lys)4, Trihydrochloride
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	para-formaldehyde
PI	propidium iodide
PRR	pattern recognition receptor
RAG	recombination activating genes

RBC	red blood cell
RELM	resistin-like molecule
Ripk2	Receptor-interacting serine/threonine-protein kinase 2
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT-PCR	realtime PCR
s.c.	subcutaneous
SEM	standard error of mean
SIRS	systemic inflammatory response syndrom
SSC	side scatter
Treg	regulatory T cell
TGFβ	transforming growth factor beta
Th	T helper cell
TLR	toll-like receptor
TMB	tetramethybenzidine
T_{reg}	regulatory T cells
UV	ultraviolet
TNF	tumor necrosis factor
WHO	World Health Organization
WT	wild type
+/-	positive/negative

Publications in peer-reviewed journals

ST2 deficiency does not impair type 2 immune responses during chronic filarial infection but leads to an increased microfilaremia due to an impaired splenic microfilarial clearance.

Ajendra J, Specht S, Neumann AL, Gondorf F, Schmidt D, Gentil K, Hoffmann WH, Taylor MJ, Hoerauf A, Hübner MP.

PLoS One. 2014 Mar 24;9(3):e93072. doi: 10.1371/journal.pone.0093072. eCollection 2014.

Chronic filarial infection provides protection against bacterial sepsis by functionally reprogramming macrophages.

Gondorf F, Berbudi A, Buerfent BC, **Ajendra J**, Bloemker D, Specht S, Schmidt D, Neumann AL, Layland LE, Hoerauf A, Hübner MP.

PLoS Pathog. 2015 Jan 22;11(1):e1004616. doi: 10.1371/journal.ppat.1004616. eCollection 2015 Jan.

Parasitic helminths and their beneficial impact on type 1 and type 2 diabetes.

Berbudi A, **Ajendra J**, Wardani AP, Hoerauf A, Hübner MP.

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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.

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Combination of worm antigen and proinsulin prevents type 1 diabetes in NOD mice after the onset of insulitis.

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Conferences and schools

Participation and oral presentation at "Short Course for Young Parasitologists" in Bernhard-Nocht-Institute Hamburg, 8th-11th March, 2013 (DGP); **2nd prize "Best Talk**" (Talk title: "*Litomosoides sigmodontis*: Effects of crude worm extract and its immune modulating effects during type 1 diabetes")

Participation and oral presentation at 18th Symposium „ Infection and Immune-defense" at Burg Rothenfels 12th-14th March, 2014 (DGHM, DGfI); (Talk title: NOD2 mediates protective immune responses against invading infectious *L. sigmodontis* larvae)

Participation and oral presentation at "Paratrop - Joint Meeting of Parasitology and Tropical Medicine" in Zürich, 16th- 19th July, 2014 (DGP); (Talk title: NOD2 deficiency impairs early protective, neutrophil-dependent immune response and leads to an increased filarial worm burden)

Participation and poster presentation at 44th Annual Meeting of the German Society for Immunology (DGfI) Bonn, 17th-20th September, 2014; (Poster title: Combination therapy using filarial antigen and intranasal pro-insulin protects against Type 1 Diabetes in NOD mice after insulitis onset)

Participation and oral presentation at "International Filariasis Meeting 2014" in Natural History Museum, Paris, France; 26th -27th September, 2014, (Talk title: NOD2 dependent neutrophil recruitment is required for early protective immune responses against invading infectious L3 larvae)

Participation and poster presentation at "11th Spring School of Immunology" Ettal (DGfI) 08th-13th March, 2015, (Poster title: Participation and poster presentation at "9th Spring School of Immunology")

Participation and oral and poster presentation at "Science Day 2015" of the "Immunosensation excellence cluster", Bonn, 2th-3th November 2015; **Prize "Best Poster"** (Abstract title: $NOD2^{-/-}$ mice are more susceptible to infection with *Litomosoides sigmodontis* due to an impaired early protective immune response")

Participation and oral presentation at the 27th Annual Meeting of the German Society for Parasitology (DGP), Göttingen, 9th-12th March 2016; (Talk title: A new role for the NOD2-receptor: NOD2 deficiency leads to an impaired early immune response during the skin stage of *Litomosoides sigmodontis* infection)

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