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<u>1</u>	<u>ZUSAMMENFASSUNG</u>	<u>5</u>
<u>1</u>	<u>SUMMARY</u>	<u>7</u>
<u>2</u>	<u>INTRODUCTION</u>	<u>9</u>
2.1	General issues	9
2.1.1	Parasitic nematodes	9
2.1.2	Tropomyosin	10
2.2	Part I – RNA interference in <i>Heligmosomoides polygyrus bakeri</i>	13
2.2.1	Genetic manipulation of parasitic nematodes	13
2.3	Part II – Influence of rAv-TMY on a murine model of allergic airway inflammation	16
2.3.1	Immune modulation and evasion by helminths	16
2.3.2	Asthma	18
2.3.2.1	Immunological reactions leading to airway hyper-reactivity	18
2.3.3	Worms and allergy	20
2.4	Aims	23
<u>3</u>	<u>RESULTS</u>	<u>24</u>
3.1	Part I – RNA interference in <i>Heligmosomoides polygyrus bakeri</i>	24
3.1.1	Target gene and sequence fragments	24
3.1.2	Feeding of <i>Hp-tmy</i> dsRNA does not induce a phenotype in <i>H. p. bakeri</i> , but in <i>C. elegans</i>	24
3.1.3	<i>H. p. bakeri</i> larvae ingest fewer bacteria compared to <i>C. elegans</i> larvae	26
3.1.4	Electroporation with siRNA does not induce a phenotype in adult <i>H. p. bakeri</i>	27
3.1.5	Soaking with <i>Hp-tmy</i> dsRNA does not induce a phenotype in <i>H. p. bakeri</i>	28
3.1.6	BLAST analysis to identify RNAi-related orthologous in helminths	30
3.2	Part II – Influence of rAv-TMY on a murine model of allergic airway inflammation	33
3.2.1	rAv-TMY modulates airway hyperresponsiveness	33

3.2.2	rAv-TMY affects migration and TLR-2 expression of macrophages	41
4	<u>DISCUSSION</u>	43
4.1	Part I – RNA interference in <i>Heligmosomoides polygyrus bakeri</i>	43
4.2	Part II – Influence of rAv-TMY on a murine model of allergic airway inflammation	48
4.2.1	Tropomyosin as an allergen	48
4.2.2	rAv-TMY alleviate OVA induced airway hyperresponsiveness	51
4.2.2.1	<i>The role of IgG in rAv-TMY mediated immune modulation</i>	52
4.2.2.2	<i>Influence of rAv-TMY on cytokine expression</i>	52
4.2.2.3	<i>rAv-TMY alters eosinophil migration by repressing chemokine expression</i>	57
4.2.2.4	<i>rAv-TMY immune modulation does not involve regulatory T cells</i>	60
4.2.2.5	<i>rAv-TMY influence IgA expression</i>	60
4.2.3	Model of rAv-TMY immune modulation	62
4.2.4	rAv-TMY promotes TLR-2 expression on macrophages and their efflux from the peritoneum	66
5	<u>SUMMARY / OUTLOOK</u>	68
6	<u>MATERIALS AND METHODS:</u>	69
6.1	Animal models	69
6.1.1	Animals used for experiments	69
6.1.2	... in RNAi experiments	69
6.1.2.1	<i>Feeding of <i>C. elegans</i></i>	69
6.1.3	... in Asthma experiments	69
6.1.3.1	<i>Model of murine airway inflammation</i>	69
6.1.3.2	<i>Bronchoalveolar lavage (BAL)</i>	70
6.1.3.3	<i>Histological analysis of lung</i>	70
6.2	Molecular biology and biochemistry	71
6.2.1	... in RNAi experiments	71
6.2.1.1	<i>Feeding - Plasmid preparation and transformation</i>	71

6.2.1.2	<i>Soaking - Target design, plasmid preparation and in vitro transcription</i>	71
6.2.1.3	<i>RNA extraction from <i>H. p. bakeri</i></i>	71
6.2.1.4	<i>cDNA synthesis</i>	72
6.2.1.5	<i>qPCR TaqMan® relative gene expression assay for RNAi experiments</i>	72
6.2.2	<i>... in Asthma experiments</i>	73
6.2.2.1	<i>Plasmid preparation and transformation</i>	73
6.2.2.2	<i>RNA extraction from lung tissue</i>	73
6.2.2.3	<i>cDNA synthesis</i>	74
6.2.2.4	<i>SYBR green relative gene expression assay for asthma experiments</i>	74
6.2.2.5	<i>Purification of recombinant Av-TMY</i>	76
6.2.2.6	<i>Endotoxin removal by EndoTrap</i>	76
6.2.2.7	<i>Limulus amoebocyte test (endotoxin measurement)</i>	77
6.2.2.8	<i>Quantification of protein</i>	77
6.2.2.9	<i>SDS-PAGE (sodium-dodecyl-sulfate polyacrylamid gel electrophoresis) and coomassie staining</i>	77
6.3	Parasitological methods	77
6.3.1	<i>Parasite maintenance</i>	77
6.3.1.1	<i>Life cycle of <i>H. p. bakeri</i></i>	77
6.3.1.2	<i>Life cycle of <i>A. viteae</i></i>	78
6.3.2	<i>... in RNAi experiments</i>	78
6.3.2.1	<i><i>E. coli</i> HT115 as food source</i>	78
6.3.2.2	<i>Preparation of eggs and feeding of <i>H. p. bakeri</i></i>	78
6.3.2.3	<i>Feeding on bacteria expressing the green fluorescent protein (gfp)</i>	79
6.3.2.4	<i>Electroporation of adult worms and larvae</i>	79
6.3.2.5	<i>Soaking of <i>H. p. bakeri</i> adult worms</i>	79
6.4	Immunological methods	80
6.4.1	<i>Enzyme-linked immunosorbent assay (ELISA)</i>	80
6.4.1.1	<i>Serum levels of IgE (total and OVA-IgE), OVA-IgG1, OVA-IgG2a</i>	80
6.4.1.2	<i>Serum levels of IgG subclasses specific for OVA or rAv-TMY</i>	80
6.4.1.3	<i>Serum / BALF levels of total IgA</i>	81
6.4.1.4	<i>Cytokine detection</i>	81

6.4.2	Cytometric Bead Array	81
6.4.3	Flow cytometric analysis	81
6.5	Cell culture techniques	82
6.5.1	Preparation of splenocytes and peribronchial lymph node cells (PBLNC)	82
6.5.2	Peritoneal lavage and preparation of peritoneal exudate cells (PEC)	82
6.6	Statistics	82
6.7	Database analysis	83
6.8	Material	83
6.8.1	Laboratory equipment	84
6.8.2	Buffers and media	84
6.8.3	Protein purification buffers	85
6.8.4	Chemicals and biologicals	85
6.8.5	Commercial Kits	86
6.8.6	Software	86
6.8.7	Antibodies and secondary reagents	86
6.8.7.1	<i>FACS reagents</i>	86
6.8.7.2	<i>Antibody detection</i>	87
<u>7</u>	<u>ABBREVIATIONS</u>	<u>88</u>
<u>8</u>	<u>REFERENCES</u>	<u>92</u>
<u>7</u>	<u>PUBLICATIONS AND PRESENTATIONS</u>	<u>113</u>

1 Zusammenfassung

Parasitische Würmer gehören mit über 3,5 Milliarden Betroffenen zu den weltweit verbreitetsten Infektionskrankheiten. Der Erfolg dieser Parasiten beruht auf ihren ausgefeilten Mechanismen mit denen sie das Immunsystem ihrer Wirte manipulieren. Interessanter Weise gehen Wurminfektionen mit einer geringeren Wahrscheinlichkeit an Allergien zu erkranken einher. Parasiten manipulieren ihre Wirte u. a. durch die Sekretion verschiedener immunmodulatorischer Moleküle. Wie genau diese Moleküle wirken und welche Mechanismen evtl. noch eine Rolle spielen ist weitgehend unbekannt.

Diese Arbeit verfolgte zwei Ziele. Zum einen sollte RNA interference (RNAi), eine Methode zur genetischen Manipulation, anhand des Modellmoleküls Tropomyosin etabliert werden. Zum anderen wurden die Auswirkungen von rekombinantem Tropomyosin der Filarie *Acanthocheilonema viteae* (rAv-TMY) auf die Entstehung entzündlicher Atemwegserkrankungen im Mausmodell untersucht.

RNAi basiert auf dem Einbringen doppelsträngiger RNA in den Zielorganismus, was zum Abbau der homologen mRNA und damit zu einem „knock down“ des Zielgens führt. RNAi wird in unterschiedlichen Organismen wie z.B. dem freilebenden Nematoden *Caenorhabditis elegans* eingesetzt. In dieser Arbeit konnte am Beispiel des essentiellen Muskelproteins Tropomyosin gezeigt werden, dass der Strongylide *Heligmosomoides polygyrus bakeri* im Gegensatz zu *C. elegans* unempfindlich für RNAi ist. Dies war unabhängig davon ob eine invasive Transfektionsmethode zur Einbringung von dsRNA wie Elektroporation oder eine nicht invasive wie „Feeding“ oder „Soaking“ genutzt wurde. Eine Analyse verschiedener EST-Datenbanken ergab das Strongyliden und anderen parasitischen Nematoden verschiedene Gene der RNAi-Maschinerie fehlen. Unter anderem auch die sog. SID Gene, die in *C. elegans* für die exogene Aufnahme und den internen Zell zu Zell Transport von dsRNA verantwortlich sind.

Desweiteren wurde das rekombinante Muskelprotein rAv-TMY in einem OVA induziertem Asthmamodell eingesetzt. Tropomyosin ist u.a. als Lebensmittelallergen aus Schalentieren bekannt, scheint aber, trotz seiner IgE induzierenden Wirkung, keine allergischen Reaktionen in Wurminfektionen hervorzurufen. Tiere wurden während der Sensibilisierungsphase mit

rAv-TMY behandelt, so dass das Molekül bereits während der Initiation der Immunreaktion wirken konnte. Eine viermalige Behandlung mit rAv-TMY in einem Zeitraum von vier Wochen führte zu verringerten entzündlichen Reaktionen in den Atemwegen. Die Analyse immunologischer Parameter ergab, dass rAv-TMY signifikant den Einstrom von Entzündungszellen in die Atemwege, allen voran Eosinophile, reduziert. Dies lässt sich durch die verringerte Ausschüttung an IL-5, Eotaxin und MCP-5 zurückführen. Zudem wurde die Bildung von antigenspezifischen IgE verringert während sich die Produktion blockierender IgG1 Antikörper erhöhte.

Zusammenfassend belegen die vorliegenden Daten, dass sich RNAi als Manipulationsmethode für Nematoden nicht oder nur in geringem Maße eignet.

Weiterhin konnte mit dieser Arbeit gezeigt werden, dass rAv-TMY anti-allergische Eigenschaften hat. Dieser Umstand ist umso interessanter, als Tropomyosin aus Schalentieren als Allergen bekannt ist. Somit scheinen es Würmer geschafft zu haben ihre „Allergene“ so zu modifizieren, dass diese trotz ihrer teilweise allergenen Eigenschaften (IgE, IL-4) kaum, oder sogar antiallergisch wirken. Damit stellt rAv-TMY ein interessantes Kandidatenmolekül zur Behandlung allergischer Reaktionen dar. Desweiteren kann der Vergleich von allergenem, nicht allergenem und modulatorischem Tropomyosin wichtige Informationen über die allgemeinen Eigenschaften von Allergenen und ihrer molekularen Struktur geben.

Parasiten, Nematoden, parasitische Nematoden, Filarien, Hakenwürmer, Heligmosomoides polygyrus, Acanthocheilonema viteae, Asthma, Allergie, RNAi, Tropomyosin, Immunmodulation

1 Summary

Parasitic worms are among the world's most prevalent infectious diseases with more than 3.5 billion. The success of these parasites is based on their sophisticated ways to manipulate the immune system of their hosts. Interestingly, worm infections abate the risk to develop allergic disorders. Parasites manipulate their hosts amongst others by the secretion of various immunomodulatory molecules. How exactly these molecules operate and what mechanisms may play a role is so far largely unknown.

This work had two goals. First, to establish RNA interference (RNAi), a method of genetic manipulation, using tropomyosin as target gene. Second, to examine the effects of recombinant tropomyosin of the filariae *Acanthocheilonema vitae* (rAv-TMY) on the formation of an inflammatory airway disease in mice.

RNAi is based on the introduction of double-stranded RNA into a target organism, leading to the degradation of homologous mRNA and thus resulting in a "knock down" of the target gene. RNAi is used in various organisms such as the free-living nematode *Caenorhabditis elegans*. Using tropomyosin as example, this study demonstrates that the strongylid *Heligmosomoides polygyrus bakeri*, in contrast to *C. elegans*, is insensitive to RNAi. This was regardless of whether an invasive transfection method was used, such as electroporation or a non-invasive, such as "Feeding" or "soaking". An analysis of various EST databases revealed that strongylids and other parasitic nematodes lack different genes of the RNAi machinery. Among others, the so-called SID genes, which are responsible for uptake and spreading of dsRNA in *C. elegans*.

Furthermore, the recombinant muscle protein rAv-TMY was applied in an OVA-induced asthma model. Tropomyosin is a known food allergen of shellfish but despite inducing an IgE response helminth tropomyosin seems not to cause allergic responses during infections. Animals were treated during the sensitization phase but prior to airway provocation with rAv-TMY, so that the molecule could interfere with the initiation of the immune response. A four-time treatment with rAv-TMY over a period of four weeks resulted in decreased inflammatory responses in the airways. The analysis of immunological parameters showed that rAv-TMY significantly reduces the influx of inflammatory cells into the airways, especially eosino-

phils. This can be explained by the decreased secretion of IL-5, eotaxin and MCP-5. In addition, the formation of antigen-specific IgE was impaired whereas the production of the blocking antibody IgG1 was increased.

In summary, the present data show that RNAi is not or only to a small extent useful as method to genetically manipulate nematodes.

Furthermore, this work demonstrates the anti-allergic properties of rAv-TMY. This fact becomes even more intriguing when considering that tropomyosin is a known allergen of shellfish. Thus, helminths seem to have modified their "allergens" in such a way that they do not exacerbate allergy, but in fact can even abrogate allergic responses. For this reason rAv-TMY becomes an interesting model molecule for the treatment of allergic diseases. Furthermore, the comparison of allergenic, non-allergenic and modulatory tropomyosin might put some light on the nature of allergens and their molecular patterns.

Parasites, nematodes, parasitic nematodes, filariae, hookworms, Heligmosomoides polygyrus, Acanthocheilonema viteae, asthma, allergy, RNAi, tropomyosin, immunomodulation

2 Introduction

2.1 General issues

2.1.1 Parasitic nematodes

Nematode infections are one of the most widespread infectious diseases with over 3.5 billion infected people worldwide [1,2]. The evolutionary success of these parasites is based on their sophisticated ability to regulate the host immune system. The modulation of the immune response was acquired during the long lasting “evolutionary arms race” between parasite and host. Since this is a reciprocal process the host immune system became adapted to the parasites, an evolutionary process which becomes obvious when the worms are eliminated. The evaluation of anti-helminth treatments revealed an increased risk to develop allergic disorders upon clearance of the worm infection [1]. This phenomenon is also seen in westernized countries where improved sanitation and hygiene standards go along with increased prevalence for allergic disorders. Understanding the modulatory mechanisms might enable us to utilize the modulatory function of helminth derived molecules.

This study investigates the use of RNAi as a method of reverse genetics in parasitic nematodes. For this purpose an intestinal hookworm of rodents, *Heligmosomoides polygyrus bakeri bakeri*, was used. *H. p. bakeri* is kept in BALB/c mice, easy to maintain under laboratory conditions and has a simple life cycle (Fig. 1a). *H. p. bakeri* belongs to the genus strongylida and is widely used as a model for human hookworm infections.

In the second part the ability of *Acanthocheilonema viteae* derived tropomyosin to interfere with allergic reaction was studied. *A. viteae* is a parasite of gerbils that dwells in the subcutaneous tissues. It belongs to the Filarioidea and is used as a model organism for filarial diseases in man such as onchocerciasis. Details of the life cycles can be found in Fig. 1b.

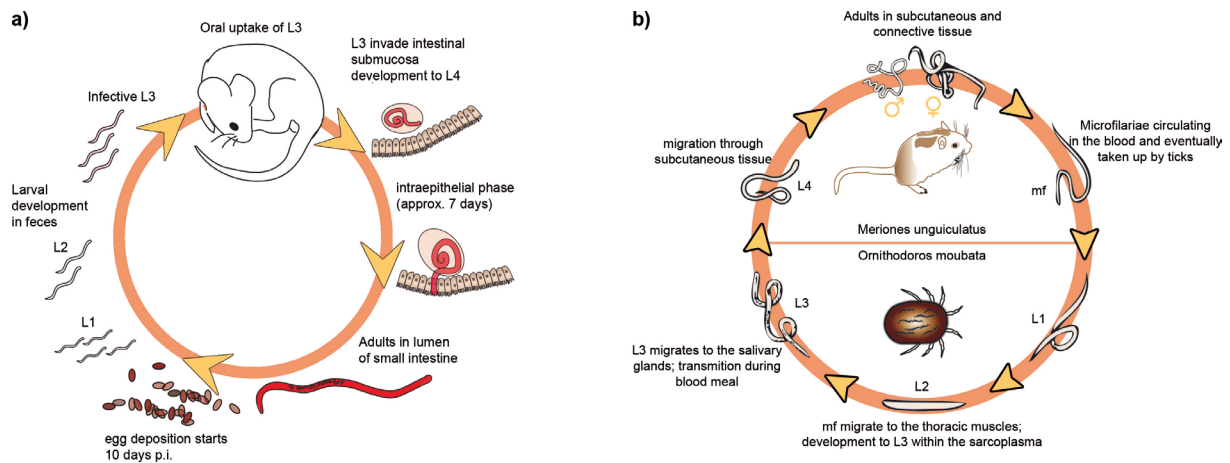


Fig. 1 a) Life cycle of *H. p. bakeri*. Mice are infected by oral uptake of infectious larvae (L3) which invade the gastric and duodenal submucosa within hours. Cyst formation occurs during the histotrophic phase of larval development from L3 to L4 stage. The L4 reenters the gut lumen and develops to adult worms which start to release eggs by day 10 p.i. Hatching of eggs and development to the L3 stage takes place within the feces. L3 actively migrate away from the feces to be ingested. Modified from Rausch S., 2008; L: larvae **b) Life cycle of *A. viteae*.** *A. viteae* is a tissue dwelling filariae of rodents. The host becomes infected during the blood meal of the arthropod host (tick - *Ornithodoros moubata*). After a migration phase, the developing L4 settles in the connective or subcutaneous tissues. The development to adults is completed 6 weeks p.i. and the females start to release microfilariae (mf) which circulate in the blood stream and are taken up by ticks during the blood meal. Mf leave the tick stomach and migrate to the thoracic muscles where they develop to L3 within the sarcoplasm. Developed L3 immigrate into the salivary glands and wait for the next blood meal. L: larvae, mf: microfilariae

2.1.2 Tropomyosin

Parasitic nematodes constantly excrete or secrete potent allergens or release them when dying. Among these is tropomyosin a muscle protein abundant in most species.

Tropomyosin is present in all eukaryotic cells and found in multiple isoforms depending on the type of tissue [1]. It is expressed as long filaments that form dimers of ~ 20 Å in diameter and ~ 400 Å in length in a coiled-coil manner [2]. The coiled-coil structure results from heptad repeats (abcdefg) where 'a' and 'd' are hydrophobic amino acid residues and the other positions represent polar or ionic amino acids. The 'a' and 'd' positions interact with the same positions of the other chain stabilizing the coiled-coil structure. The residues 'b', 'c' and 'f' are located in the outer part and can interact with other proteins such as actin. The N- and C-termini of each filament have overlapping residues that lead to a head-to-tail association [1]. These repeated filaments are associated with actin chains where they regulate myosin binding together with the troponin complex in a Ca^{2+} dependent reaction [3]. Therefore

tropomyosin is essential for the proper function of the movement apparatus but also believed to be involved in the intracellular transportation system of non-muscle cells [4].

Interestingly tropomyosin is the major food allergen of shellfish and considered a pan allergen due to its wide distribution. Shellfish tropomyosin has several B-cell epitopes that feature high cross-reactivity with invertebrate tropomyosins of different species (Fig. 2). Mapping the IgE epitopes for shrimp tropomyosin showed that the epitopes cluster in five regions of the protein that correspond to the binding regions of the two tropomyosin helices. These regions are in contrast to the actin binding regions less conserved between invertebrate and vertebrate tropomyosin providing an explanation for the different immunogenicity [5]. However, invertebrate tropomyosin does not only induce B-cells but has also the ability to induce a strong T-cell response [6]. The induced antibody responses are highly cross-reactive between invertebrate species [7]. The fact that parasitic worms constantly confront their hosts with large numbers and amounts of antigens would suggest a strong allergic response to these antigens and cross-reactivity to the respective antigens of other species. This becomes even more evident if one considers the example of patients infected with the filariae *Onchocerca volvulus* that can harbor up to 12 million microfilariae which are subjected to constant turnover. Furthermore, tropomyosin was found to be expressed not only in the muscle layers but also in the cuticle of *O. volvulus* and *Trichinella spiralis* exposing the antigen directly to the immune system [8,9]. In regard to natural worm infections it seems, however, that the parasites induce no or only mild allergic reactions. The question of clinically relevant cross-reactivity between invertebrate tropomyosins has been addressed only in a few studies of which none dealt with parasitic tropomyosin [10,11,12]. In contrast, vertebrate tropomyosin has been reported to be non-allergenic in humans [13] probably due to the high homology among vertebrate tropomyosins.

A. viteae tropomyosin (Av-TMY) used in this study was characterized as an important antigen in vaccination studies. Animals immunized with irradiated L3 become resistant to challenge infection. Sera of these mice strongly react with a 41 kDa band in a Western Blot using whole worm extract [14]. Intriguingly, using Av-TMY in a receptor study revealed TLR-2 dependent signaling. HEC cells transfected with a TLR-2 luciferase reporter plasmid produced a strong signal upon stimulation with rAv-TMY that was not seen using a TLR-4 reporter construct.

Similar, RAW cells (a macrophage cell line) started to expression of IL-4 and IL-10 in response to rAv-TMY. The expression of IL-4 and IL-10 was abrogated when macrophages were treated with anti-TLR-2 mAb but not when an anti-TLR-4 mAb was used. Furthermore, macrophages isolated *ex vivo* from MyD88 knockout mice showed an impaired IL-10 expression upon Av-TMY stimulation indicating Av-TMY signaling is MyD88 dependent (Eckert J., unpublished data). The fact that filarial tropomyosin protects mice against challenge infections [15] but at the same time is able to induce a regulatory cytokine seems to be a conflicting capacity. On the other hand a modulatory function of this molecule might explain why tropomyosin of filariae seems not to induce drastic allergic reactions in a natural infection. The above mentioned findings lead to the speculation that Av-TMY might be less allergenic at least in regard to cross reactivity and might possess modulatory capacities that can have spillover effects on allergic reactions induced by unrelated allergens.

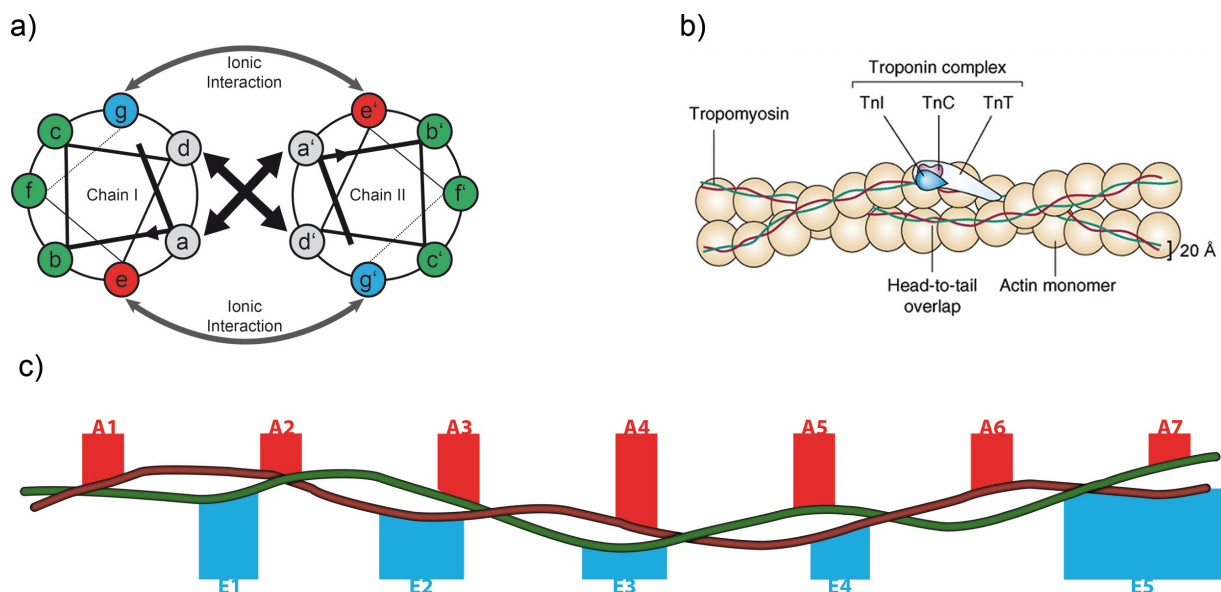


Fig. 2 Structure and epitopes of tropomyosin. **a)** Tropomyosin consists of two 400 Å α -helices that show a sequence of seven repeating amino acids where the positions 'a' and 'd' are hydrophobic amino acid residues and the remaining positions are taken by polar or ionic amino acids. The 'a' and 'd' positions of each chain interact with each other resulting in a coiled-coil filament that is stabilized by the interaction of 'e' and 'g'. The positions 'c', 'b' and 'f' are located in the outer part of the filament enabling them to interact with actin. The N- and C-terminus have overlapping residues which create long filaments by a head-to-tail overlap (modified from Stewart, 2001 and Perry, 2001). **b)** Tropomyosin filaments run along actin filaments where they are complexed with Troponin. After Ca^{2+} influx, this complex is shifted by 35° and enables myosin to bind to actin for movement (modified from Gordon, 2000). **c)** Schematic representation of actin binding sites A1-A7 of mammalian tropomyosins and the IgE binding regions of *P. aztecus* Tropomyosin described by Ayuso, 2002. The IgE regions show partial overlap with the actin binding sites, indicating that actin binding sites are not excluded from being immunogenic. (extracted from Perry, 2001 and Sereda, 2008)

2.2 Part I – RNA interference in *Heligmosomoides polygyrus bakeri*

2.2.1 Genetic manipulation of parasitic nematodes

To date genetic manipulation of parasitic nematodes has proven largely unsuccessful. The various reasons include the complex lifecycles, poor accessibility and the small amount of genome information available. The technique of RNA interference (RNAi) gave rise for hope to have a method that prevents the need to (stably) integrate the gene of interest into the genome and thereby knock down genes in parasitic nematodes. To establish RNAi, *H. p. bakeri*, a well-established murine model for intestinal nematode infections was used. *H. p. bakeri* exhibits the characteristic features of a nematode infection [16,17], is easy to maintain in the laboratory and therefore suitable to study RNA interference (RNAi). RNAi is a highly conserved mechanism whose function is to protect the cells from double-stranded RNA (dsRNA) derived from virus infections or to regulate transposable elements and gene transcription [18,19]. RNAi operates via degradation of messenger RNA (mRNA) that shows homology to the dsRNA (see Fig. 3). RNAi was first described in the free-living nematode *Caenorhabditis elegans* [20] but is in the meantime established for a range of organisms including plants [21], insects [22] and vertebrates [23,24]. However, only nematodes and planarians [25,26,27,28] as well as plants [29] develop systemic RNAi. In *C. elegans* it was shown that RNAi is dependent on several proteins that enable uptake, spreading and processing of dsRNA [28,30,31,32,33]. Furthermore, it was shown that RNAi is heritable for more than three generations in *C. elegans* [20,34]. Although RNAi is a conserved mechanism, the way of dsRNA uptake differs between organisms. In *C. elegans* the systemically expressed proteins SID-1 and SID-2 facilitate the passive uptake of dsRNA as well as the systemic spread within the worm, whereby long sequences of dsRNA are favored (>100bp) and DNA is completely discriminated. Structure, kinetic and ATP independency makes it likely that SID-1 forms channels in the membrane for dsRNA diffusion [28,31]. SID-2 is mainly located in the intestine and is only involved in the uptake of exogenous dsRNA into the cells [35]. This uptake machinery makes *C. elegans* susceptible to a variety of application methods like soaking and feeding.

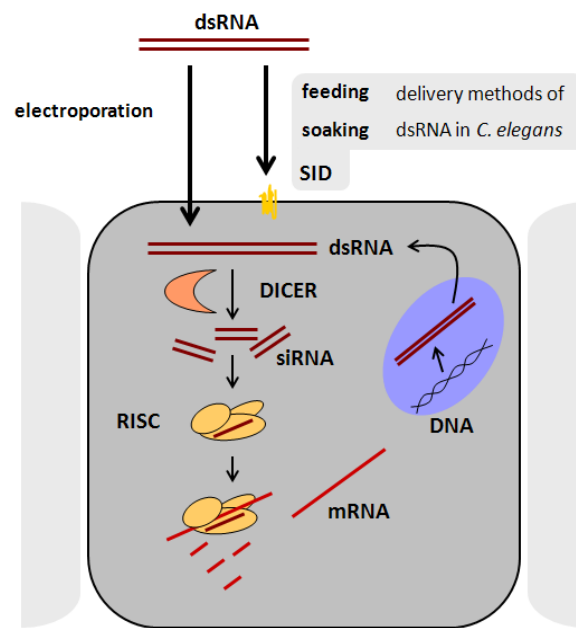


Fig. 3 Principles and function of RNAi. RNAi was first discovered in *C. elegans*. This free-living nematode possesses trans-membrane proteins (SID-1/SID-2) that allow an ATP independent uptake of dsRNA into the cells. In experimental settings this can be utilized to deliver dsRNA by simply feeding dsRNA expressing bacteria to the worms or by soaking them in dsRNA containing medium. Alternatively dsRNA can be introduced by electroporation. The natural function of RNAi is to protect the cell from double-stranded RNA viruses or for post-transcriptional gene silencing (PSTG). The artificially introduced or cell produced dsRNA is recognized by DICER, an enzyme that cleaves the dsRNA strand into ~21 bp small interfering RNAs (siRNA). A single strand of siRNA is then incorporated into the RNA induced silencing complex (RISC) that degrades homologues mRNA and thereby silencing the respective gene. dsRNA: double stranded RNA, SID: systemic RNAi deficient, siRNA: small interfering RNA, RISC: RNA induced silencing complex

Soaking is an easily applicable method by which the worm is simply soaked in dsRNA containing media. In *C. elegans* soaking was used for high-throughput analyses and also the first RNAi experiments with parasitic helminths used soaking [36,37,38,39,40].

Feeding is based on dsRNA-producing bacteria fed to *C. elegans*. The dsRNA is delivered via the intestinal epithelium of the worm. Meanwhile feeding is the standard method in *C. elegans* for functional genome screenings [41]. Additionally, feeding of dsRNA has also been used to manipulate parasitic nematodes such as the gastrointestinal nematode *Trichostrongylus colubriformis* [37] or planarians such as *Schmidtea mediterranea* [25].

The third method used in helminths, electroporation, is based on the formation of small pores within the cell membrane upon an electric pulse [37,42,43,44]. For this method 21 bp long small interfering RNAs are designed and used to transfect the organism.

In this work all three delivery methods were used to transfect different life stages of *H. p. bakeri*.

2.3 Part II – Influence of rAv-TMY on a murine model of allergic airway inflammation

2.3.1 Immune modulation and evasion by helminths

By definition parasitism is a close relationship in which one organism, the parasite, live at the expense of the host. Endoparasitic worms live within their respective host and are therefore directly exposed to the host immune system for a long period. Hosts have a variety of mechanisms to expel parasites, which in turn have developed intriguing ways to circumvent this leading to a well-balanced host-parasite equilibrium.

The interaction between the host immune system and the worm might start when a larvae enters the host or hatches from an egg. The invader is immediately recognized as foreign by epithelial cells, innate immune cells and antigen presenting cells (APC) through their released or membrane bound antigens. The antigens are taken up by the APCs, such as dendritic cells (DC), macrophages (MØ), B-cells, processed and presented to CD4⁺ cells via MHC class II upon migration to the draining lymph nodes and the spleen. Extracellular pathogens usually favor the development of a Th2 response that leads to the production of the typical Th2 cytokines IL-4, IL-5, IL-9 and IL-13. In this environment B-cells start to produce high amounts of antibodies that can opsonize the parasite thereby attracting eosinophils, basophils and macrophages to attack the parasite. The recruitment of cells is in addition supported by mast cells that release histamines, prostaglandins and chemokines upon FcεR bound IgE cross-linking. The recruitment of further immune cells leads to a strongly Th2 biased environment around the parasite. Subsequently the parasite can either be directly killed or encapsulated or intestinal worms can be expelled by mucus hyper production and muscle hyper contractility induced by IL-13 and IL-9 [45].

However, some worms can live up to 15 years in their respective hosts despite being confronted to the immune system [46]. Parasites have evolved different strategies to evade the constant attack of the host immune system. The evasion strategies include the shedding of opsonising antibodies either mechanically through movement/molting or chemically by proteases preventing cell based killing. Some worms also escape inflammation by constantly moving or hiding in nodules. But the probably most elaborate survival strategy is an active

interference with and modulation of the host immune system. This not only prolongs parasite survival but also prevents immune reactions detrimental for the host. Surprisingly helminths are known to skew the host immune system towards Th2 which seems to be contradictory with the long survival of these parasites. On the other hand helminth infections are often found to induce regulatory T cells [47] and are associated with elevated levels of the regulatory cytokine IL-10 [48,49]. They are also accompanied by increased IgG4 antibody titers in humans a subclass that is unable to activate complement and less efficient in opsonising parasites to phagocytic cells. The modulation of the preferentially induced Th2 immune response is presumably achieved by the release of modulating molecules. A few have been isolated and characterized. One example are the homologues of human cystatin family released by different nematodes [50,51]. The cysteine proteinase inhibitor Bm-CPI-2 released by *Brugia malayi* targets cathepsin B, L, S and an endopeptidase within the MHC class II pathway thereby inhibiting the antigen presentation [52]. Bm-CPI-2 shows homologies to human cystatin C which is expressed at high levels in immature DCs and down regulated during maturation [53]. Nematode cystatin could therefore keep dendritic cells in an immature status leading to T cell anergy [54]. Av17, a cystatin released by *A. viteae*, operates in a similar way. In addition Av17 triggers macrophages to produce the regulatory cytokine IL-10. IL-10 abates the expression of MHC class II and CD80/CD86 on DCs and subsequently the antigen presentation [55]. IL-10 also skews the IgE/IgG4 ratio towards the blocking IgG4 subclass [56]. Modulation and communication of cells is strongly dependent on mediators such as cytokines and chemokines. It comes as no surprise that parasites also utilize these pathways for their purposes. The best investigated examples are the TGF- β homologue (TGH) of *B. malayi* [57,58] and the macrophage-migration inhibition factor (MIF) produced by various parasitic nematodes [59,60]. Bm-TGH2 binds to mammalian TGF- β receptors and can thereby modulate the immune response similar to TGF- β . Mammalian MIF is a pro-inflammatory cytokine that induces TNF- α expression in macrophages. The *B. malayi* released MIF acts in the same way on human monocytes *in vitro* what is somehow contradictory to the anti-inflammatory aim of the parasite. However, the authors claim that under *in vivo* conditions the permanent release of Bm-MIF could lead to an anti-inflammatory response [59]. Besides these “intended” immune modulators helminths release a whole assortment of “unintended” antigens. This especially holds true for filarial nematodes which produce high numbers of micro-

filaria (Mf) circulating in the bloodstream or migrating in the tissue. Upon death or killing by macrophages the antigens are released rising the risk of an unregulated immune response. Interestingly parasites appear to have shaped their most immunogenic antigens to be less immunogenic or even to be modulatory. One example is tropomyosin (TM). The immunogenicity of TM seems to be dampened in filarial infections where filarial TM is released in high amounts but without inducing strong allergic responses nor enforcing the response to TM of different species.

2.3.2 Asthma

Allergic disorders such as asthma, atopic dermatitis and hay fever are on the rise in western countries. The hallmark of allergic disorders is an inappropriate immune response to otherwise harmless antigens. With regard to asthma about 40% of the western population is atopic but only 7% develop asthma [61]. Atopic individuals show a remarkable IgE response to common environmental antigens whereas asthmatic individuals in addition develop a severe inflammation followed by structural changes of the airways.

2.3.2.1 Immunological reactions leading to airway hyper-reactivity

The onset of asthma is a highly complex mechanism that can be divided into three phases: induction, early asthmatic reaction (EAR) and late asthmatic reaction (LAR).

A fundamental process in the establishment of asthma is the sensitization to an allergen when it is first inhaled. The antigen is taken up by intraepithelial dendritic cells, a process enhanced by allergen specific surface bound IgE. The engulfed antigen is processed leading to its presentation on MHC class II molecules, expression of co-stimulatory molecules (CD80, CD86) and the migration of DCs to the mediastinal lymph nodes. Sensitization of T-cells occurs only in contact with mature antigen presenting DCs expressing CD80/CD86 otherwise leading to T-cell anergy [61]. The decision if DCs mature depends on the co-stimuli during antigen uptake. It has been shown that immunogenic antigens often bear either an enzymatic activity or contaminated with LPS stimulating epithelial cells via protease activated receptors (PAR) or Toll-like receptors (TLR) to produce a variety of mediators like TSLP, IL-6, GM-CSF and CCL17. This not only attracts lymphocytes but also provides a maturation signal for DCs. After priming naïve CD4⁺ cells to become Th2 T-cells DCs migrate back to the airways

to become potent producers of mainly Th2 cytokines [54]. The following cascade leads to a massive influx and activation of different cell types.

Among them mast cells to some extent play a role in linking innate and adaptive immunity by creating certain cytokine environment and by presenting antigens. As cells of the first line defense mast cells produce a myriad of different molecules which are released upon an appropriate stimulus. Cross linking of IgE bound to the high affinity receptor Fc ϵ leads to degranulation [61]. The preformed granules contain effector molecule like histamine, proteases, prostaglandins and cytokines. Together they increase smooth muscle contractility, microvascular and tissue permeability and are also thought to contribute to airway remodeling [62]. Among the secreted cytokines are IL-4 and IL-5 leading to eosinophil maturation and recruitment. Mast cells are recruited by stem-cell factor (SCF) released by epithelial cells that binds to the KIT receptor on the surface [62]. Attracted mast cells localize in the airway connective-tissue, mucosa and in association with smooth muscle cells [61,63,64]. In the LAR mast cells are highly sensitized with IgE due to the massive upregulation of the Fc ϵ R. At that stage of the disease they are the main link between IgE and airway hyperreactivity (AHR).

Eosinophils are thought to be the main effector cell type in the airways and also found in sputum and bronchoalveolar lavage fluid. Eosinophils derivate from CD34⁺ bone marrow precursors under the influence of IL-3, IL-5, GM-CSF and eotaxin 1-3 [65]. Upon activation with IL-5 they start to mature and express several receptors such as CCR3, CCR5, L-selectin, β 2 integrin [66]. These receptors are used to attract eosinophils along a gradient of different chemoattractants (PGD₂, CCL5, CCL7, CCL11, CCL24, RANTES) to and within the airways. Reaching their destination eosinophils become activated by stimuli such as secretory IgA, IgG, IL-5, GM-CSF and IL-3 [67]. Next they release cytotoxic molecules (MBP, ECP), lipid mediators (PGE₂, leukotrienes), growth factors (TGF- β , VEGF) and cytokines (IL-1, IL-2, IL-6, IL-4, IL-5, IL-13, TNF- α) [65] after cross linking of Fc ϵ RI/Fc α RI or stimulation of the complement receptors [67,68].

Macrophages play an important role in induction and maintenance of asthma. As effector cells they are a source of reactive oxygen products, lysosomal enzymes and leukotrienes. As conductors they help to orchestrate the ongoing inflammation by attracting neutrophils, monocytes and T-cells but also limiting the cytokine response by releasing proteases such as

MMP9 and regulatory cytokines like IL-10 and TGF- β [61,64,69]. However, the precise role of macrophages in asthma is largely unknown [61].

Finally B cells are a relevant source of antigen-specific IgE under Th2 conditions which contributes to airway inflammation. B cells primed by mucosa derived DCs turn into IgA secreting plasma cells that migrate to the submucosa releasing high amounts of sIgA that can activate eosinophils. Under regulatory conditions B cells can also produce the blocking antibody IgG4 that counteracts the effects of IgE [70]. In addition B cells also serve as antigen presenting cells and some subsets can have regulatory functions by producing IL-10 [71].

In summary the induction of asthma is coupled to many different cell types and mediators which are precisely regulated. The Factors such as genetic background, nutrition and hygiene influencing this network are hardly understood.

2.3.3 Worms and allergy

The observation that a westernized lifestyle goes along with an increase of allergic disorders led to the formulation of the hygiene hypotheses by Strachan [72]. He postulated that a reduced exposure to Th1 driving infections during childhood as a result of improved sanitation and vaccination shifts the immune response to a Th2 response. This again escalates the risk to develop allergic disorders. The hygiene hypotheses has been refined and extended over the last 20 years mainly by including parasitic infections. To narrow down the factors influencing the hygiene hypotheses such as bacterial and viral stimuli turned out to be difficult. This is of no surprise as allergies are usually multi-factorial diseases depending on several factors like the genetic background, environmental pollution, nutrition and site of inflammation. However, one trait that turned out to be quite consistent is the inverse correlation of helminth infections and the prevalence for allergic disorders although exceptions occur here as well. On the first glance it seems to be paradox that Th2 skewing infections should protect from Th2 based allergies. A lot of research has been invested resulting in the hypothesis of counter-regulation. It is hypothesized that the affords of the parasite to dampen the immune response against itself has bystander effects dampening the reaction to an allergen as well (see Fig. 4) [73,74,75,76]. IL-10 plays a central role in this hypothesis as a regulatory cytokine. IL-10 is produced by a wide variety of cells such as monocytes, macrophages, dendritic cells,

B cells, T cells and NKT cells following many different stimuli including stimulation by bacteria and parasites [73]. Interestingly, there is evidence that asthmatic patients have general lowered IL-10 expression [77,78,79] and therefore might tend to establish polarized immune reactions. Helminths have evolved sophisticated mechanisms to use the homeostatic features of IL-10 to counterbalance the Th2 bias induced by their antigens [53].

There are accumulating reports on the positive influence of helminths on the course of allergic disorders. They include reports on the negative correlation between helminth infections and allergies [80,81,82,83,84,85,86,87,88,89], studies linking the effect of anti-helminth treatment with worsening of allergies [90,91] and studies on the underlying immunological parameters [92,93]. Nevertheless there are also reports on detrimental effects of worm infections on allergic reactions [90,94,95,96]. It was also shown that helminth infections can have a different impact on different kinds of allergic disorders. For hookworm infections it was found that they can improve asthma but exacerbate dermatitis [97]. This was confirmed by meta-analysis of recent publications showing that *Ascaris* infections are likely to increase incidence of asthma whereas hookworms reduce incidence indicating that species and localization of the parasite might also be important factors [98]. The above mentioned studies clearly show the ability of helminths to suppress allergic responses as consequence of released molecules that are able to mediate this modulation. A few immune modulators of helminths have been isolated and tested in a murine system for their suppressive capacities. Probably the best characterized molecule is ES-62 of the rodent filariae *A. viteae*. ES-62 forms complexes with Toll-like receptor 4 (TLR-4) and thereby inhibits the FcεRI-induced degranulation of human mast-cells. It protects mice from arthritis and mast-cell hypersensitivity in the lung and skin [99,100,101]. A second molecule derived from *A. viteae* is the cystatin Av17. Av17 is able to suppress inflammatory responses both in a Th2 model of asthma and a Th1 colitis model. The effects are dependent on macrophages and IL-10 [102]. Another helminth molecule is PAS-1 of the roundworm *Ascaris suum*. PAS-1 can reduce the airway hyperreactivity against the allergenic protein of *A. suum* (APAS-3) in a IL-10/IFN-γ dependent fashion [103,104,105].

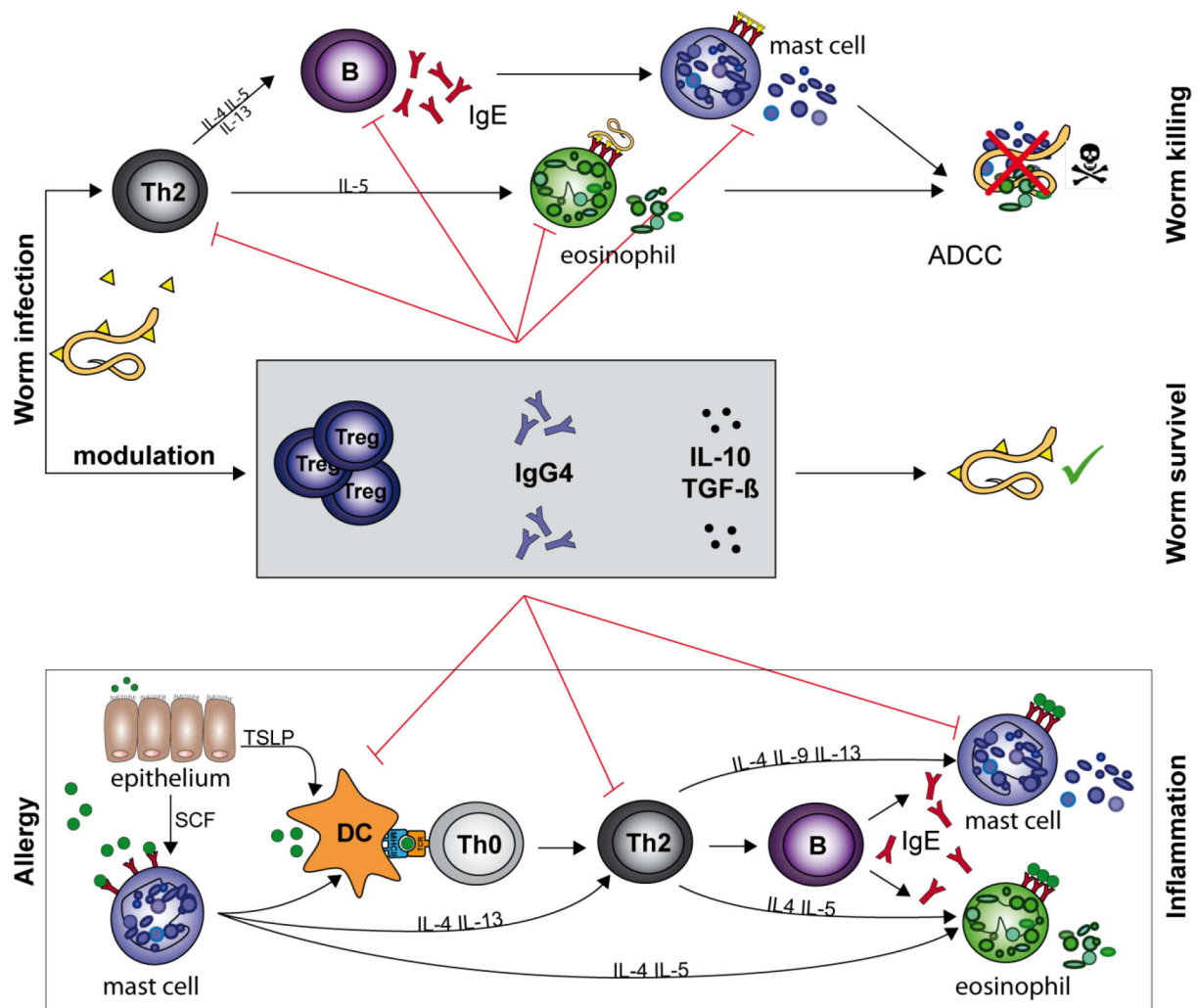


Fig. 4 Immune modulation by helminths. Worm infections commonly induce a Th2 immune response with high IgE levels, eosinophilia and mastocytosis. The release of cytotoxic substances by mast cells and eosinophils as well as antibody dependent cellular cytotoxicity can lead to killing of the parasite (upper panel). To prevent killing, helminths use different mechanisms to modulate the immune system for their purposes. Common traits are the induction of T regulatory cells, blocking IgG4 (mouse IgG1) antibodies and the expression of the regulatory cytokines IL-10 and TGF-β (central panel). A bystander effect of helminth induced immune modulation is the interference with allergen induced allergic inflammation. Allergic inflammations are characterized by provoking a similar, Th2 based, immune response as seen in helminth infections. A crucial step in the initiation of allergy is the allergen-specific activation of DCs. Epithelial cells play an important role in directly priming the DC via TSLP to become a Th2 promoting cell. The recruitment and activation of mast cells by release of SCF also supports a Th2 bias. Modulating effects of helminths can interfere with the induction and maintenance of allergy at different levels. IL-10 and IgG4 interfere with antigen presentation by DCs and thereby the induction of Th2 T cells. DCs modulated by IL-10 fail to mature and release less inflammatory cytokines. T regulatory cells prevent the production of pro-inflammatory cytokines by releasing IL-10 and TGF-β or a direct cell-cell interaction. Moreover, blocking antibodies of the IgG4 subclass can prevent IgE based activation and degranulation of mast cells and eosinophils. Adapted from Schnöller, 2008 and Barnes, 2008 B: B cell, Th: T helper cell, DC: dendritic cell, Treg: T regulatory cell, Th2: T-helper cell 2, MHC class II: major histocompatibility complex II, Ig: immunoglobulin, IL: interleukin, TSLP: thymic stromal derived lymphopoietin, SCF: stem cell factor; TCR: T cell receptor; ADCC: antibody dependent cellular cytotoxicity

2.4 Aims

Part I

The aim of this part was to establish RNAi in *H. p. bakeri* using a protein with an easily detectable phenotype. Tropomyosin was a promising candidate as it produces an easily detectable phenotype. Tropomyosin has also been used by others to establish RNAi and is reported to produce a strong phenotype in *C. elegans* [106,107] and in *T. colubriformis* [37].

Part II

The second part investigates the possible role of *A. viteae* tropomyosin in the modulation of the immune response to an unrelated allergen (Ovalbumin) in a rodent model of allergic airway inflammation

3 Results

3.1 Part I – RNA interference in *Heligmosomoides polygyrus bakeri*

3.1.1 Target gene and sequence fragments

H. p. bakeri-tropomyosin (Hp-tmy) was chosen as a model target gene, because tropomyosins have been successfully knocked down in several other organisms. To attempt RNAi in *H. p. bakeri* the complete sequence of Hp-tmy (*cHp-tmy*) with a length of 855bp including start and stop codon as well as a 165bp fragment (*Hp-tmy-1*) covering the 5' end and a 273bp fragment (*Hp-tmy-2*) covering the 3'end was used (Fig. 5). dsRNA (Ec-malE) derived from maltose-binding protein of *E. coli* (*Ec-MBP*) and dsRNA of the *Ce-unc-22* gene were used for control treatments. The *C. elegans unc-22* gene encodes for twitchin, a protein involved in muscle regulation, and was used as positive control for *C. elegans* and negative control for *H. p. bakeri*, respectively.

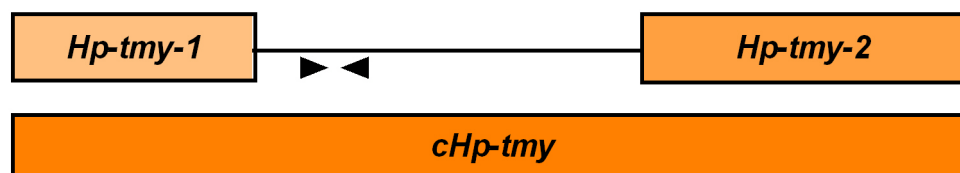


Fig. 5 Schematic illustration of *H. p. bakeri* tropomyosin (*Hp-tmy*) fragments used for RNAi. *Hp-tmy-1* and *cHp-tmy* were used for feeding, *Hp-tmy-1* and *Hp-tmy-2* for soaking experiments; location of the Real-Time PCR primers is marked by opposing arrowheads. *cHp-tmy*: complete *H. p. bakeri* tropomyosin; *Hp-tmy-1* & *Hp-tmy-2*: fragments of *H. p. bakeri* tropomyosin.

3.1.2 Feeding of *Hp-tmy* dsRNA does not induce a phenotype in *H. p. bakeri*, but in *C. elegans*

Initial tests revealed that *E. coli* HT115 (the strain producing recombinant dsRNA) supports the development of *H. p. bakeri*. The hatching rate and the proportion of larvae reaching the L3 stage were similar after feeding on lawns of *E. coli* HT115 as compared to lawns of *E. coli* OP50, the standard strain used for maintenance of *H. p. bakeri* larvae.

E. coli HT115 expressing *cHp-tmy* or *Hp-tmy-1* dsRNA were fed to *H. p. bakeri* larvae. As controls, *H. p. bakeri* were kept on lawns of *E. coli* HT115 expressing *Ec-malE*, *Ce-unc-22* or no dsRNA. After seven days of feeding on recombinant bacteria the number of *H. p. bakeri* reaching the L3 stage did not significantly differ between the groups (Fig. 6a). Microscopic examination of vital L3 did not show differences in the morphology between the groups. Thus, keeping of *H. p. bakeri* larvae on lawns of *E. coli* HT115 expressing dsRNA of *Hp-tmy* failed to induce a phenotype.

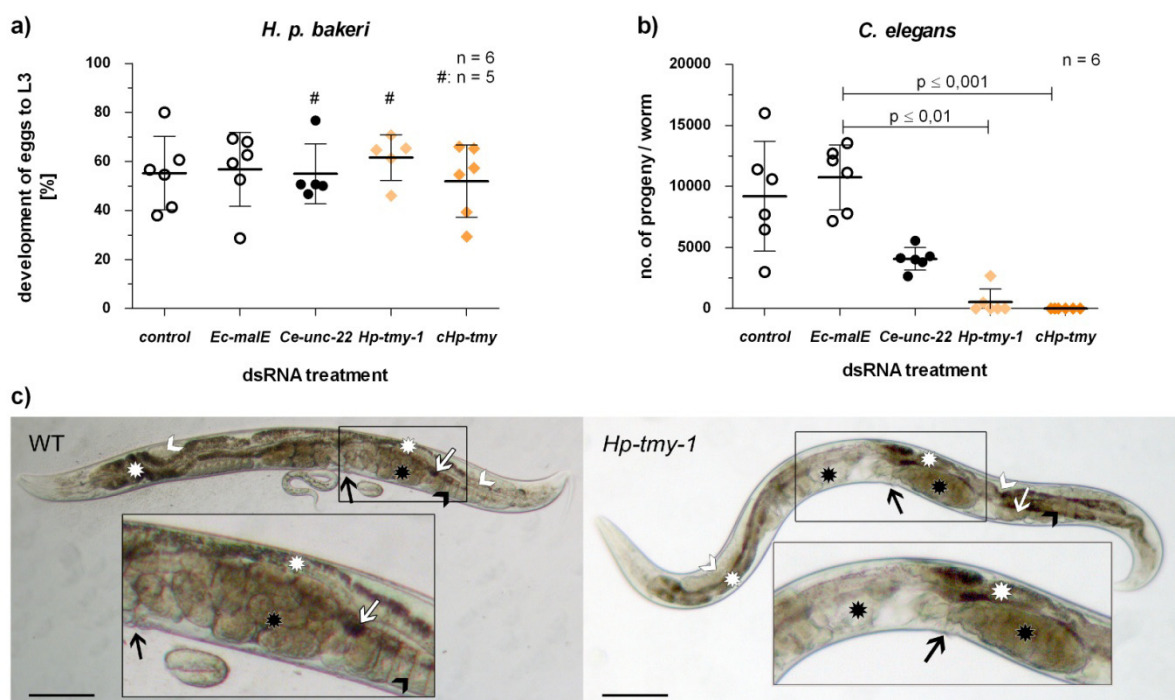


Fig. 6 Feeding of *Hp-tmy* dsRNA does not induce a phenotype in *Heligmosomoides polygyrus bakeri* but in *Caenorhabditis elegans*. (a) Shown is the development of *H. p. bakeri* eggs to L3. *H. p. bakeri* larvae were fed on bacteria expressing *cHp-tmy* or *Hp-tmy-1* dsRNA, irrelevant dsRNA (*Ec-malE*, *Ce-unc*) or bacteria containing an empty L4440 plasmid (control). (b) Shown is the number of worms produced by a single *C. elegans* hermaphrodite upon treatment. *C. elegans* worms were fed on recombinant bacteria expressing dsRNA of *cHp-tmy*, *Hp-tmy-1* or *Ce-unc-22*, irrelevant dsRNA (*Ec-malE*) or bacteria containing an empty plasmid (control). Error bars indicate the standard deviation of the mean of six independent experiments, (ANOVA, df=4, p<0,0001; Dunn's post-hoc test, significant differences are denoted in the graph). (c) *C. elegans* developed body morphology defects (bmd-phenotype) after feeding for three days on bacteria expressing *Hp-tmy-1* dsRNA. Wild Type (WT): untreated hermaphrodite with complete organs. The reproductive tract shows the didelphic ovaries with highly structured oocytes and a uterus containing developing eggs. The intestine is filled with bacterial debris. *Hp-tmy-1*: a hermaphrodite treated with *Hp-tmy-1* dsRNA. The ovaries contain only rudimentary structures of oocytes. The uterus is empty or contains degraded eggs. The intestine is partly atrophied. white arrow: spermatheca; black arrow: vulva; white arrowheads: ovaries; black arrowhead: oocytes; black asterisk: developing eggs within the uterus; white asterisk: intestine; bar: 130 μm.

In contrast, feeding of the same recombinant *E. coli* HT115 expressing dsRNA of *cHp-tmy* or *Hp-tmy-1* to *C. elegans* caused a significant reduction of progeny compared to the control groups (Fig. 6b). Moreover, morphological changes of *C. elegans* after feeding with *cHp-tmy* and *Hp-tmy-1* dsRNA resembled the well described phenotypes seen in knock down mutants of *C. elegans* obtained by feeding on dsRNA of *C. elegans* tropomyosin (*Ce-tmy*), namely body morphological defects, sterility, embryonic arrest and abnormal egg laying (Fig. 6c). Feeding of the *cHp-tmy* dsRNA to *C. elegans* had the strongest impact. *cHp-tmy* dsRNA inhibited the release of eggs and was finally lethal to the adults. *Hp-tmy-1* dsRNA also showed significant effects on *C. elegans* but single hermaphrodites were still able to release low amounts of eggs resulting in a small number of offspring. Feeding of bacteria expressing *Ce-unc-22* dsRNA (positive control) to *C. elegans* also led to a reduced number of offspring (Fig. 6b & 6c) and caused a “twitching” phenotype.

An alignment of *cHp-tmy* with tropomyosin of *C. elegans* revealed 85% identity to the *Ce-tmy-I* splicing form. Hence, feeding of *C. elegans* with *cHp-tmy* dsRNA resulted in a phenotype expected for knock down of tropomyosin, although the sequence identity between *cHp-tmy* and *Ce-tmy-1* is below 90%. The successful RNAi in *C. elegans* suggests that the technique works, and implies that the lack of a phenotype in *H. p. bakeri* must be owing to either an impeded uptake of RNA and/or a deficiency of elements of the RNAi machinery in the parasitic nematode.

3.1.3 *H. p. bakeri* larvae ingest fewer bacteria compared to *C. elegans* larvae

To determine the amount of bacteria taken up by *H. p. bakeri* as compared to *C. elegans*, GFP-expressing *E. coli* were fed to the respective larvae and the uptake was followed microscopically. These experiments showed that *C. elegans* L1 larvae ingest substantially more bacteria than *H. p. bakeri* L1 (Fig. 7). Whereas *C. elegans* larvae continuously ingest bacteria, so that the pharynx is constantly filled with a green fluorescing mass, *H. p. bakeri* larvae only pick up single bacteria from time to time. These pass the oesophagus as single spots, before the fluorescence extinguishes when the bacteria reach the intestine.

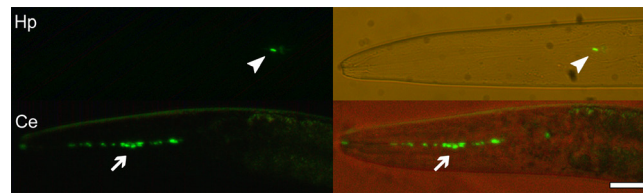


Fig. 7 *Heligmosomoides polygyrus bakeri* larvae ingest fewer bacteria than *Caenorhabditis elegans* larvae. Shown are examples of a single L1 of each species fed on *E. coli* expressing the green fluorescent protein. The *H. p. bakeri* L1 takes up single bacteria (Hp, arrowhead), whereas the *C. elegans* L1 continuously takes up large amounts of bacteria (Ce, arrow); bar: 10 μ m.

3.1.4 Electroporation with siRNA does not induce a phenotype in adult *H. p. bakeri*

To test whether electroporation is the method of choice larvae (L1) as well as adult worms were electroporated with available siRNAs labeled with the fluorescent dye Cy3 suspended in trehalose buffer previously used by Issa et al. [37] or commercial electroporation buffer (Ambion, Austin, USA). Electroporation of larvae resulted in massive death of 70-90 % of larvae irrespective of the conditions for buffer, voltage and time (see p. 79 Tab. 7). Electroporation with Cy3-labeled siRNA did not result in a detectable fluorescent signal within the larvae. Conversely, electroporation of adults with a single pulse of 20 ms @ 500V led to single spots of Cy3-siRNA between cuticle and hypodermis. However, these penetration events occurred rarely in relation to the worm size (Fig. 8).

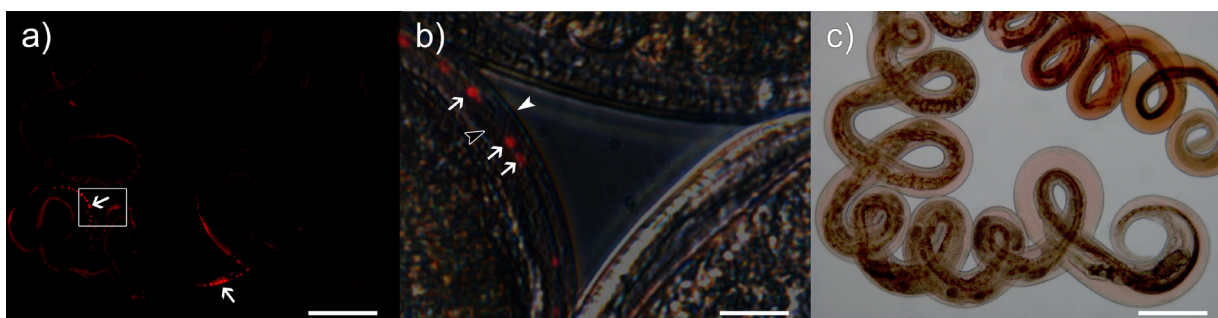


Fig. 8 Cy3-siRNA penetrates the cuticle of adult *Heligmosomoides polygyrus bakeri* upon electroporation. (a) Electroporation of adult *H. p. bakeri* with dye-labeled siRNA resulted in local uptake, but no spreading. (b) Higher magnification shows the localization of the siRNAs between cuticle and hypodermis. (c) bright field of the *H. p. bakeri* worm shown in a). arrow: single fluorescent spot; white arrowhead: cuticle; black arrowhead: hypodermis. a) 550 nm; bar: 500 μ m b) 550 nm; bar: 40 μ m c) bright field; bar: 500 μ m.

3.1.5 Soaking with *Hp-tmy* dsRNA does not induce a phenotype in *H. p. bakeri*

Larvae of *H. p. bakeri* do not lend themselves to soaking as L1/L2 exhibit a reduced lifespan in liquid media and L3 are physiologically inactive. Therefore adult worms were used for soaking in dsRNA. To test whether RNA is taken up by soaking, adult *H. p. bakeri* worms were exposed to *Hp-tmy-1* dsRNA labeled with the dye Cy3. After 18 h of soaking the dsRNA accumulated in the pharynx and to a minor extent in the intestine of adult worms (Fig. 9a). Fluorescence was visible in the lumen of the pharynx and also in the surrounding tissue, indicating that dsRNA was taken up by cells. However, a systemic spread of dsRNA was not detectable. Worms kept in RPMI without dsRNA did not show any fluorescence (Fig. 9b upper worm).

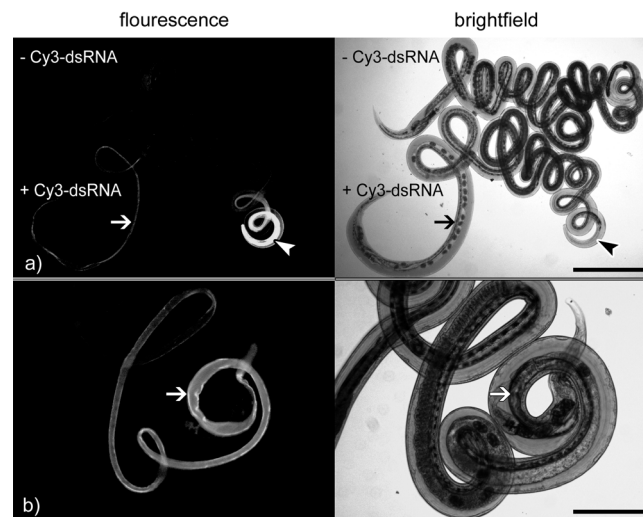


Fig. 9 Soaking of *Heligmosomoides polygyrus bakeri* adults results in uptake of dsRNA into pharynx and gut. (a) Shown are two adult worms soaked in RPMI with and without Cy3-siRNA. No fluorescence was found in the control group (upper worm of a: -Cy3-dsRNA) whereas worms exposed to Cy3-labeled dsRNA showed strong fluorescence in the pharynx and weak fluorescence in the intestine (lower worm of a: +Cy3-dsRNA) highlighting the major side of uptake; bar: 1,7 mm. (b) The intestine also served as side of dsRNA-uptake visible at higher magnification. Panel b) also depicts the invasion of the dsRNA into the epithelial layer seen by the stronger fluorescence of the tissue compared to the lumen of the gut; bar: 0,7 mm, arrowhead: pharynx; arrow: intestine.

Next, adult worms were soaked in 2 μ M dsRNA of *Hp-tmy-1* and *Hp-tmy-2*, respectively, for different time periods and analyzed for potential morphological alterations. A soaking period of 24 h did not result in morphological changes compared to the control groups. Extended soaking for six days resulted in 37% of altered worms ($p < 0.05$ as compared to the control group) (Fig. 10a) that showed particular morphological changes before dying. In female

worms, the ovaries and the gut were disintegrated and in some worms egg fragments were floating in the pseudocoelomic cavity (Fig. 10 b & c). These lesions did, however, not necessarily stop the worms from moving. The proportion of damaged and subsequently dead worms was lower (16%) after soaking in *Hp-tmy-2* dsRNA. However, also some worms

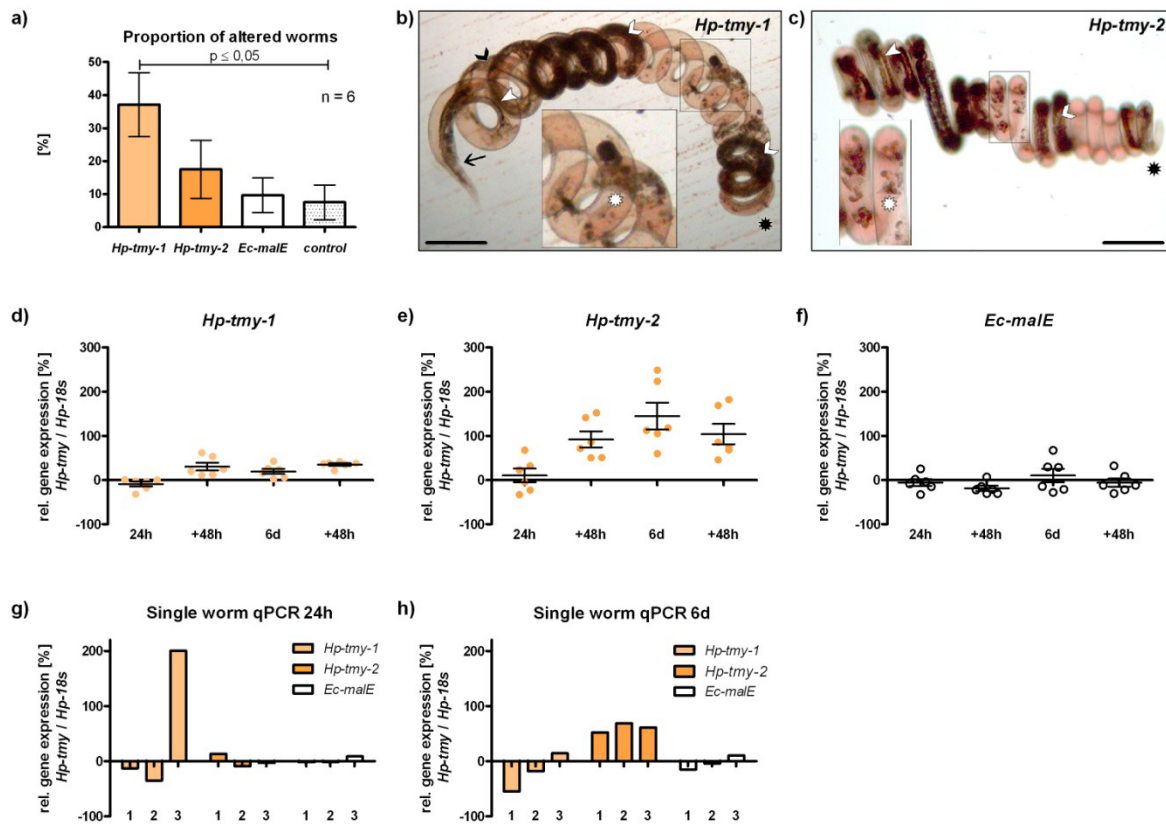


Fig. 10 Soaking failed to induce RNAi in *Heligmosomoides polygyrus bakeri* adults. (a) Soaking of adult worms for 6 days in *Hp-tmy* dsRNA resulted in increased morbidity and increased ageing after treatment with *Hp-tmy-1* in comparison to untreated (control) and *Ec-malE*-treated worms. (b) and (c) Treatment with *Hp-tmy-1* and *Hp-tmy-2* leads to a disintegrated gut and disrupted ovaries; bar: 2 mm. (d) Treatment with *Hp-tmy-1* dsRNA did not induce significant changes in the expression of *Hp-tmy* compared to the *Ec-malE* control group analyzed by qPCR. (e) Treatment with *Hp-tmy-2* led to an upregulation of the tropomyosin expression. (f) Treatment with *Ec-malE* control dsRNA did not change the expression level of tropomyosin. Single worm qPCR revealed different expression levels between individuals upon treatment with dsRNA for 24 hours (g) or 6 days (h). Error bars indicate the standard error of mean of six independent experiments, (ANOVA, $df=4$, $p<0.05$; Dunn's post-hoc test, significant differences are denoted in the graph). Black arrow: vulva; black arrowhead: Uterus; white arrowheads: ovaries; white triangle: intestine; white asterisk: disrupted ovary; black asterisk: anterior end.

treated with *Ec-malE* (10%) dsRNA and even a few control worms (7%) showed these alterations. To correlate these morphological alterations with possible RNAi effects, the levels of tropomyosin mRNA were analyzed by qPCR of worm pools cultured together in one well (Fig. 10 d-f). Treatment with *Hp-tmy-1* dsRNA did not significantly change the tropomyosin mRNA level after soaking for 24 h or 6 days, while treatment with *Hp-tmy-2* dsRNA led to increased

mRNA levels after 6 days. The tropomyosin expression seen after soaking for 24 h or 6 d did not change substantially when worms were cultured for further 48 h in dsRNA free medium. Worms kept in *Ec-malE* dsRNA did not show significant alterations of tropomyosin mRNA levels.

To analyze possible individual differences between worms, RNA of single females soaked either 24 h (no morphological alterations) or 6 days (all with morphological alterations) was used to determine the expression levels of tropomyosin. These experiments revealed substantial individual differences between worms, particularly after soaking in *Hp-tmy-1* (Fig. 10 g & h). However, the levels of mRNA were not correlated with the degree of morphological alterations.

3.1.6 BLAST analysis to identify RNAi-related orthologous in helminths

As feeding, electroporation and soaking of *H. p. bakeri* did not yield a clearly visible phenotype, the question stand to reason whether this failure could be due to the absence of genes required for uptake and/or processing of dsRNA. To address this hypothesis the EST database of the closely related ruminant nematode *Haemonchus contortus*, the genome database of the filarial nematode *B. malayi* as well as the EST database of the trematode *Schistosoma mansoni* was searched for sequences homologous to 18 RNAi related proteins of *C. elegans* (Tab. 1). Out of 18 *C. elegans* sequences only six showed significant homologies to genes in the *H. contortus* database, ten to *B. malayi* sequences and seven to sequences in the *S. mansoni* genome database.

RNAi proteins can be divided into different subclasses for uptake, processing, mRNA regulation, amplification, and degradation of dsRNA (see Tab. 1). For *H. contortus*, no matches were obtained for proteins involved in RNA-uptake (SID-1, SID-2) and dsRNA-regulation (ERI-1). Best matches for *H. contortus* proteins were obtained for proteins belonging to dsRNA-processing (DICER, DRH-1) and dsRNA-amplification (RRF-1, EGO-1, RDE-3) and mRNA regulation (TSN-1) class. For *B. malayi* and *S. mansoni* similar results were obtained with some additional sequences (see Tab. 1) including one homologous sequence encoding for a protein involved in RNA-spreading (RSD-3).

In summary none of the used transfection methods for RNAi - feeding, electroporation and soaking - yield a clear phenotype. Only soaking resulted in a locally confined alteration of adult worms. The local restriction might be explained by the finding that the Genome of *H. contortus*, a close relative of *H. p. bakeri*, does not contain certain components of the RNAi machinery necessary of an effective and systemic knock down.

Tab. 1 Analysis of core proteins of RNAi in three different genomes; The EST database of *H. contortus* (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus), the WGS database of *B. malayi* (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and the GAV3 database of *S. mansoni* (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni) were searched for orthologous proteins of the RNAi pathway of *C. elegans* by tBLASTn. Sequences with a p-value below e^{-5} were designated as probably homologue. Homologues sequences were subsequently used to search for conserved domains in the conserved domain database available on NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). EST, expressed sequence tag; WGS, whole genome shotgun; GAV3, genome assembly version 3; CDD, conserved domain database; RdRP, RNA dependent RNA polymerase

<i>C. elegans</i>			<i>H. contortus</i>		<i>B. malayi</i>		<i>S. mansoni</i>		
	Gene	Function / Phenotype	Ref.	EST	CDD	WGS	CDD	GAV3	CDD
				P-value	Homologues Domain	P-value	Homologues Domain	P-value	Homologues Domain
Uptake	<i>sid-1</i>	Uptake of dsRNA into cells and spread between cells	[28,30,31,108]	0,9997	----	0,0005	----	0,0067	----
	<i>sid-2</i>	Uptake of dsRNA from the intestine	[35]	----	----	0,9997	----	0,2	----
	<i>rsd-2</i>	Silencing defect in germline cells	[27]	----	----	0,9		0,13	----
	<i>rsd-3</i>	Silencing defect in germline cells	[27]	0.085	----	5.0e-18	ENTH_epsin	3e-16	ENTH_epsin
	<i>rsd-4</i>	Silencing defect in somatic and germline cells				No sequence data available			
	<i>rsd-6</i>	Silencing defect in germline cells	[27]	0.55	----	----	----	0.0026	----
Processing	<i>dcr-1</i>	Ribonuclease	[109,110]	1.9e-42	HELICc/PAZ/DSRM	8.2e-45	RIBOc, PAZ_dicer_like, DEXHc	4.9e-34	RNase III, DSRM, PAZ, HELICc, DEXHc
	<i>drh-1</i>	Helicase; required for somatic and germline RNAi	[110]	1.3e-15	DEXHc/HELICc	3.1e-18	HELICc, Type III restriction enzyme	1.1e-08	DEXHc
	<i>rde-1</i>	Accumulation of dsRNA	[34]	0,66	Piwi/ago	1.6e-10	DEXDc, Piwi_ago-like	1.0e-17	PIWI, PIWI-ago
	<i>rde-4</i>	dsRNA recognition and binding protein	[34]	----	----	----	----	----	----
mRNA Regulation	<i>tsn-1</i>	Nuclease	[111]	2.7e-66	Tudor	6.2e-46	Tudor	4.0e-108	Tudor
	<i>vig-1</i>	predicted RNA-binding protein	[111]	0.9994		3.2e-20	mRNA binding family	----	----
	<i>rde-2</i>	functions downstream of rde-1	[112,113]	----	----	0.78	----	----	----
	<i>mut-7</i>	RnaseD; required for RdRP and	[114]			0.0035	----	----	----
Amplification	<i>rff-1</i>	for somatic RNAi	[115]	6,6e-16	----	2.0e-42	RdRP	----	----
	<i>ego-1</i>	RdRP	[116]	8,7e-20	----	1.2e-43	RdRP	0,9993	----
	<i>rde-3</i>	Nucleotidyltransferase; function in RNAi unknown	[117]	9.1e-15	----	7.8e-09	----	7.0e-05	poly(A) polymerase
	<i>eri-1</i>	Exonuclease; degrades siRNA	[118]	0.992	----	2.6e-20	Exonuc_X-T, EXOIII	1.4e-30	exonuclease

3.2 Part II – Influence of rAv-TMY on a murine model of allergic airway inflammation

In order to investigate possible functions of filarial derived tropomyosin in modulating inflammatory immune responses, rAv-TMY was applied in an *in vivo* model of OVA induced airway inflammation. To provoke an allergic response the mice were sensitized two times with OVA/Alum intraperitoneally and subsequently challenged by administration of OVA into the airways. A preventative model was used in which the treatment can possibly interfere with the sensitization phase. Mice were treated four times with 20 µg *E. coli* expressed Av-TMY or the mouse derived control protein dihydrofolate reductase (DHFR) over a period of three weeks, challenged twice with OVA at day 28 / 29 and scarified at day 32 (Fig. 11).

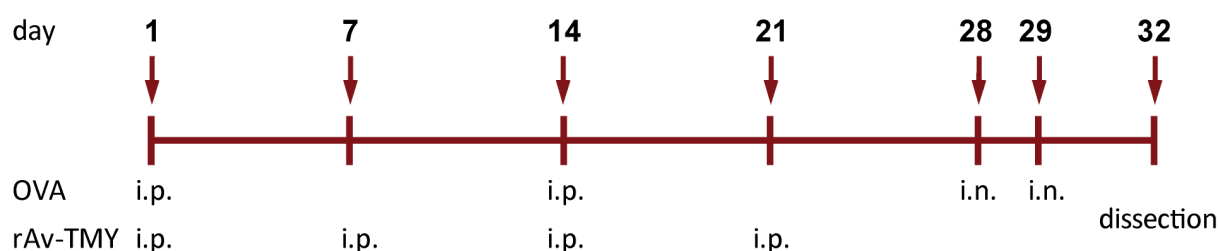


Fig. 11 Model of a murine allergic airway inflammation. The scheme presents a preventative model allowing the interference of rAv-TMY with the onset of the allergic inflammation. Animals were sensitized two times with 20 µg OVA-Alum and in parallel treated four times with 20 µg rAv-TMY. 28 days after the first sensitization, airway inflammation was induced by two intranasal applications of 50 µg OVA. Two days later animals were dissected. i.p. intraperitoneal, i.n. intranasal

3.2.1 rAv-TMY modulates airway hyperresponsiveness

Sensitization and challenge of mice with OVA induced a strong Th2 biased immune response. This was reflected by mucus hypersecretion, a massive cell influx into the airways, a strong OVA specific IgE response and a Th2 cytokine environment. Application of rAv-TMY but not rDHFR induced changes in a way that the immune reaction in the lung was less responsive to provocation by OVA. Several parameters were analyzed.

First, the cell composition in the bronchoalveolar lavage fluid (BALF) was analyzed mainly in regard to eosinophils, a main effector cell in allergic airway diseases. rAv-TMY abated the cell influx into the lung after challenge with OVA. Treated animals showed significantly reduced

total cell numbers ($p = 0,007$), significant less lymphocytes ($p = 0,0048$) and significantly reduced numbers of eosinophils ($p = 0,01$) in the BALF (Fig. 12).

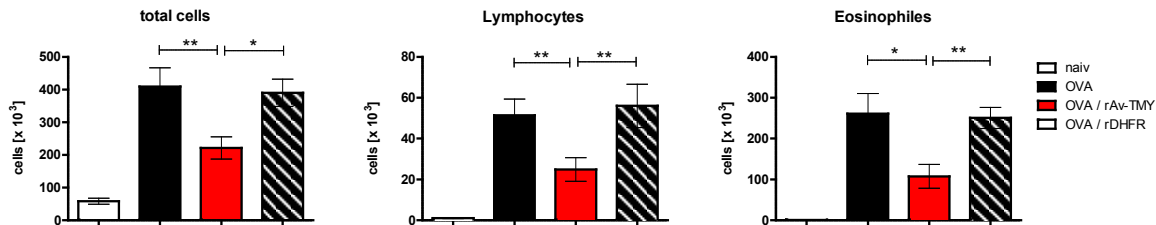


Fig. 12 rAv-TMY abate cell influx into the airway lumen. rAv-TMY treatment resulted in a significant reduction of total cells, lymphocytes and eosinophils within the BALF. Mice were sensitized with OVA-Alum or PBS and treated with PBS, rAv-TMY or rDHFR respectively. 32 days post first treatment animals were scarified, the lungs lavaged with PBS and the cells in the BALF differentiated in a cytospin preparation. Error bars indicate standard error of mean of two independent experiments with 6-7 animals per group (ANOVA $p \leq 0,05$, Mann Whitney test, * $p \leq 0,05$, ** $p \leq 0,01$); BALF bronchoalveolar lavage fluid

The reduction of eosinophils was also reflected in the airway interstitium seen by histological sections (Fig. 13). Both sensitized only and rAv-TMY treated animals had large cell infiltrations around the vessels and bronchioles compared to PBS treated. However, rAv-TMY treated animals showed lower no. of eosinophils within these cell infiltrations.

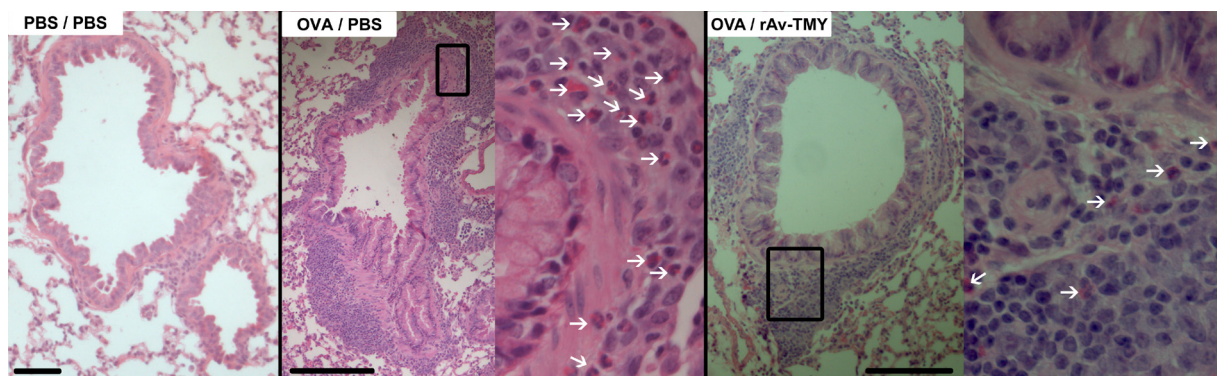


Fig. 13 Eosinophil migration to the airway interstitium is reduced by rAv-TMY treatment. HE staining of lung sections showed large cell infiltration around the vessels and bronchioles in only sensitized and treated animals but with a smaller proportion of eosinophils in treated vs. only sensitized animals seen at higher magnification (white arrows). 32 days post first treatment animals were sacrificed, lungs flushed with PBS to remove excessive blood from the vessels and conserved in 3,5% formalin. Paraffin embedded slices were HE stained. bar: 100 μ m, HE: hematoxylin and eosin

Second, the influence of rAv-TMY on the IgE response as a central maker for allergic responses was investigated. Total IgE levels were lowered in treated animals without reaching significance. In contrast OVA specific IgE production was significantly inhibited by rAv-TMY

treatment compared to animals sensitized only with OVA ($p < 0.0001$) or treated with rDHFR ($p = 0.0075$) (Fig. 14).

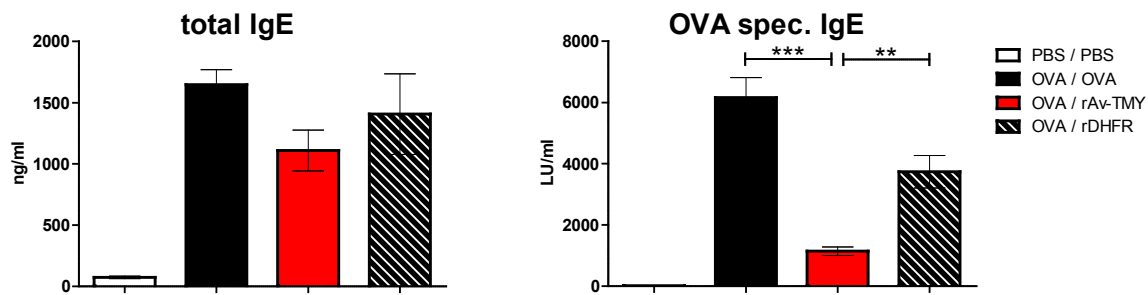


Fig. 14 OVA specific IgE production is inhibited by rAv-TMY treatment. rAv-TMY diminished total IgE production compared to OVA or rDHFR treated animals without reaching significance (a). OVA specific IgE levels were significantly reduced compared to the OVA or rDHFR group (b). In the PBS group hardly any IgE was detectable (a & b). IgE content was measured in a sandwich ELISA. OVA spec. IgE was analyzed using biotinylated OVA as secondary reagent and values are expressed as lab units (LU). Error bars represent standard error of mean of two independent experiments with groups of 6-7 animals (ANOVA $p \leq 0.05$, Mann Whitney test, $**p \leq 0.01$, $***p < 0.001$)

Third, mucus production as a clinical relevant parameter was altered by rAv-TMY. Analysis of histological sections showed reduced mucus production in the large airways between rAv-TMY treated and control animals as seen by PAS staining for mucin (Fig. 15).

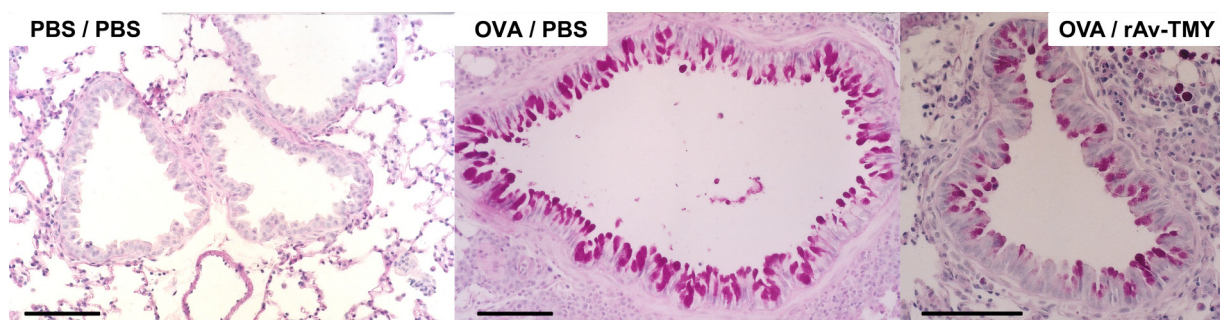


Fig. 15 Mucus production is reduced by rAv-TMY treatment. Staining of lung sections with PAS revealed a reduced mucin production (purple color) by goblet cells in bronchi and bronchioles of rAv-TMY treated compared to sensitized only animals. PBS treated animals did not produce any mucin (a). bar: 100 μ m, PAS: periodic acid-Schiff

Both specific immunotherapy (SIT) as well as helminth infections are shown to alter the pattern of immunoglobulin G subclasses. To address the question if rAv-TMY is able to modulate IgG responses antigen specific subclass ELISA's were performed. rAv-TMY treated animals

develop a strong rAv-TMY specific IgG1 (human IgG4) response (Fig. 16a). In contrast OVA sensitized and challenged animals showed a modest induction of OVA specific IgG1 that was not altered by rAv-TMY treatment (Fig. 16b). Similar effects were observed concerning the other immunoglobulin subclasses IgG2a (human IgG1) and IgG2b (human IgG3) (Fig. 16 c & d). The sera levels of both subclasses responding to rAv-TMY were elevated in rAv-TMY treated animals but almost no IgG2a or IgG2b antibodies against OVA (data not shown) could be detected. In summary rAv-TMY induced a strong specific IgG response accompanied by an abated allergen specific IgE response.

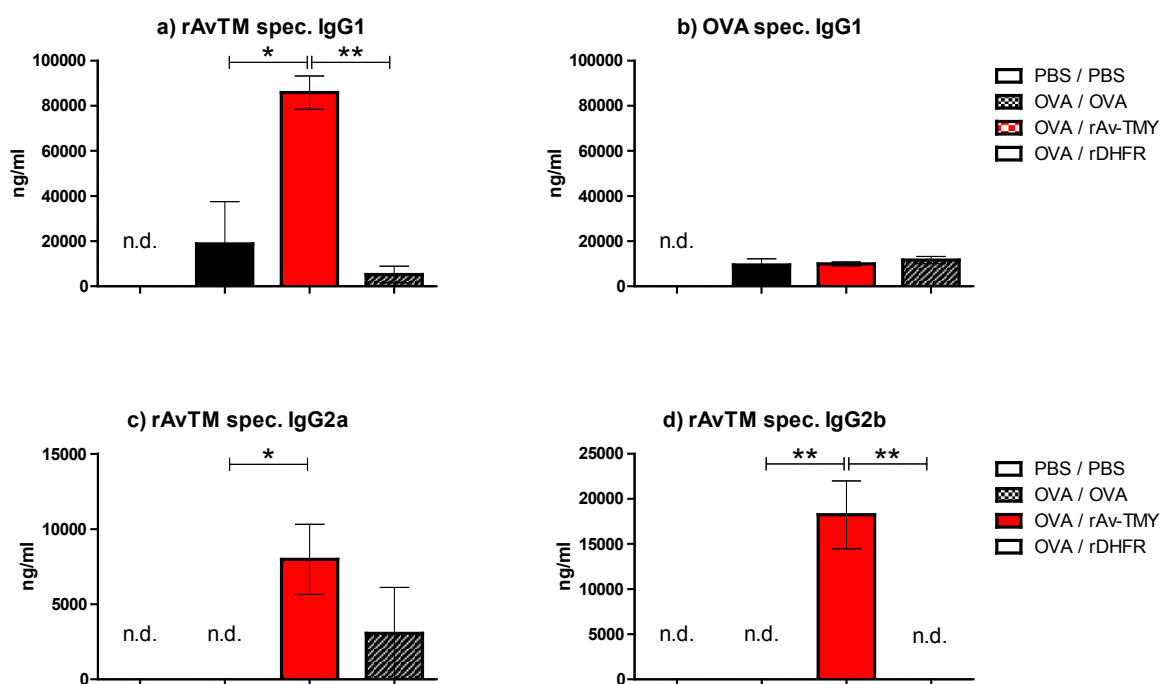


Fig. 16 Changes in the composition of IgG subclasses in sera induced by rAv-TMY. The graphs depict three different IgG subclasses found in sera of mice. A strong IgG1 response specific for rAv-TMY was observed in the rAv-TMY group (a) whereas OVA did not induce specific IgG1 antibodies (b). A similar effect was found for IgG2a (c) and IgG2b (d). Antibodies were detected by ELISA using subclass specific detection antibodies. Error bars indicate standard error of mean for a representative experiment with 7 animals per group (ANOVA $p \leq 0,05$, Mann Whitney test, * $p < 0,05$, ** $p \leq 0,01$). n.d.: not detectable

Next it was of interest if the local and systemic cytokine pattern were affected by rAv-TMY. Cytokines are of importance for the local orchestration of allergic airway inflammation. The Cytokine composition of BALF was analyzed using a cytometric bead array (CBA). The Th2 cytokines IL-5 and to lesser extent IL-13 were found in lower concentrations in rAv-TMY treated animals whereas the IL-4 levels showed no differences compared to the control

groups. With regard to IL-13 the control protein rDHFR had also an inhibiting effect. The regulatory cytokine IL-10 remained the same expression in all groups (Fig. 17d) and the Th1 cytokine IFN- γ was below the detection limits (data not shown).

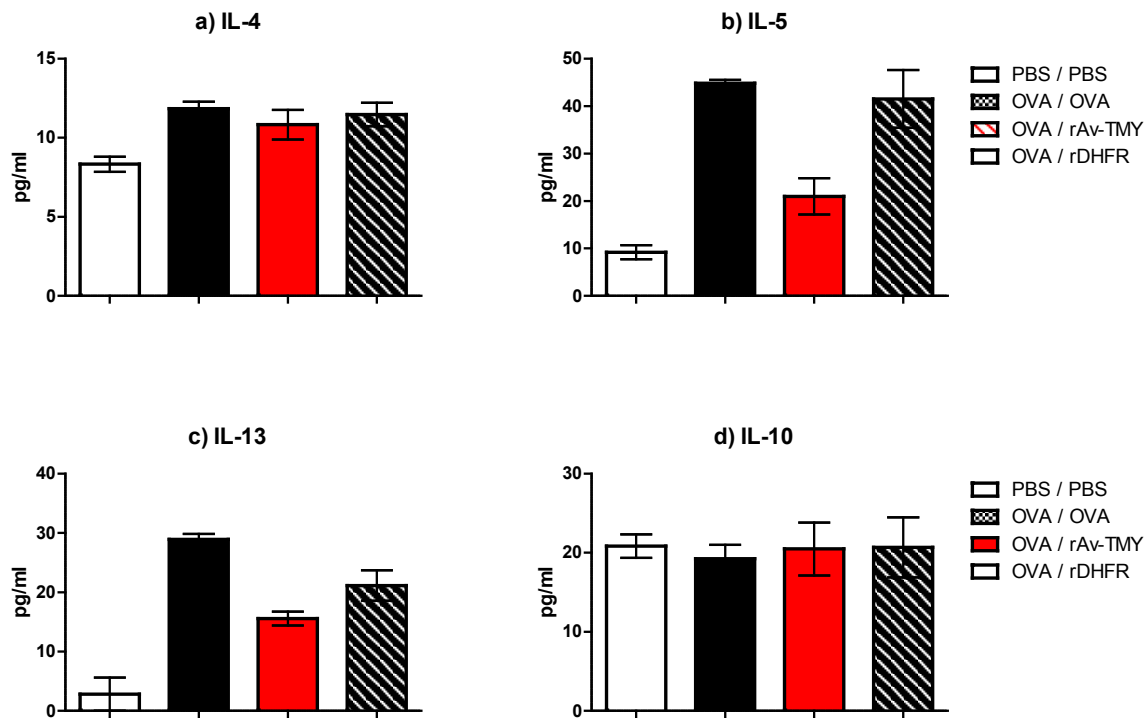


Fig. 17 Cytokine pattern in the BALF changes in response to rAv-TMY. Cytokines in the BALF were measured by CBA. IL-5 and IL-13 secretion was prominently induced in OVA only sensitized animals. This effect was reduced by half in rAv-TMY treated animals. No differences between the groups could be detected for IL-4 and IL-10. Error bars indicate standard error of mean of 3 animals per group; BALF bronchoalveolar lavage fluid

Since allergic reactions are mostly dominated by Th2 cytokines the question arises whether this cytokines are altered by a rAv-TMY treatment also in a systemic manner. In addition the expression of the regulatory cytokine IL-10 and the Th1 cytokine IFN- γ was measured. Regarding the Th2 cytokines both OVA and rAv-TMY induced IL-4, IL-5 and IL-13 as well as the regulatory cytokine IL-10 in the spleen. In contrast IFN- γ was only expressed by spleen cells of rAv-TMY treated animals in response to the same antigen (Fig. 18).

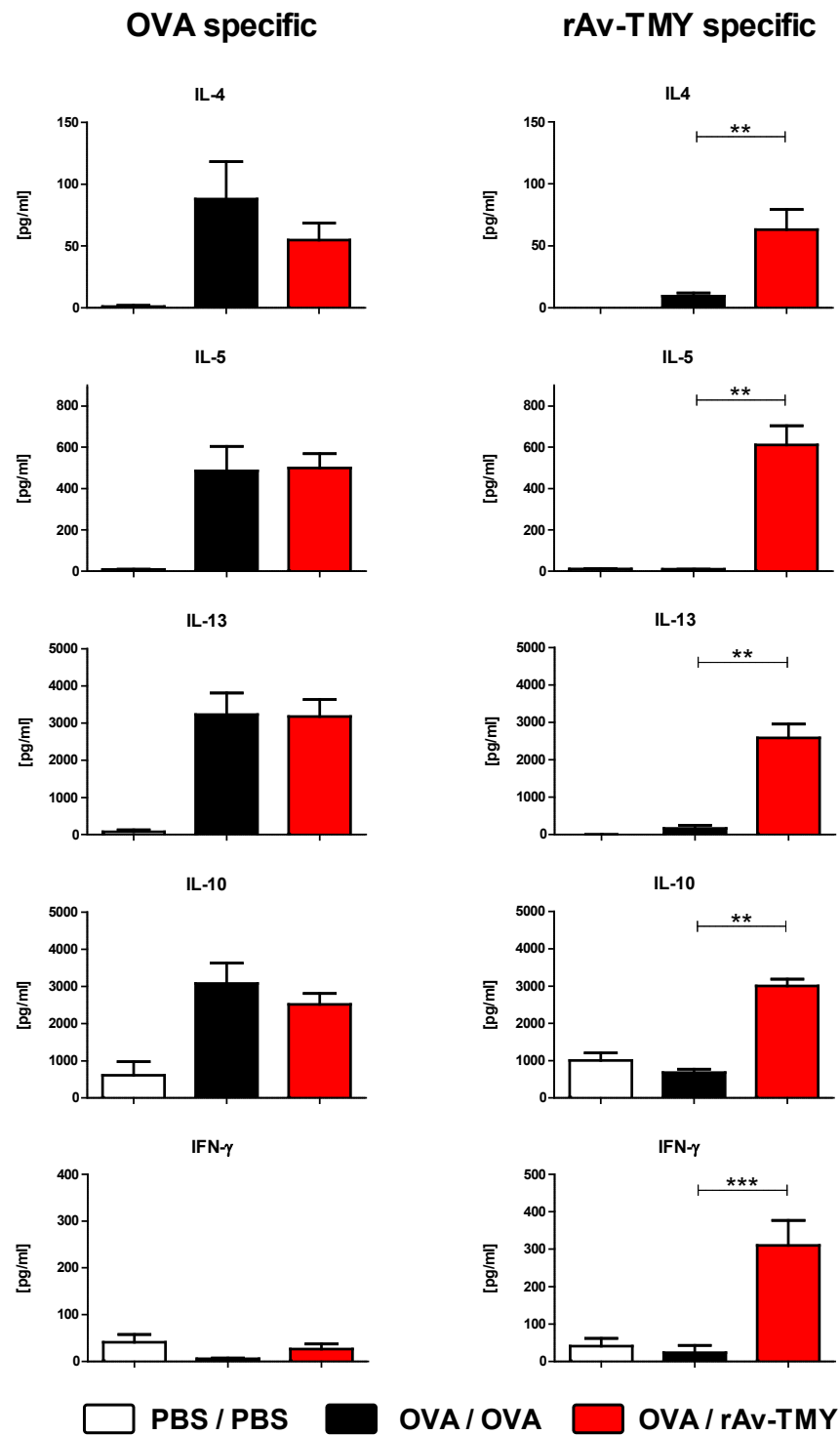


Fig. 18 rAv-TMY and OVA induced cytokine responses by restimulated splenocytes. Splenocytes were restimulated with either OVA (OVA specific) or rAv-TMY (rAv-TMY specific) and cytokine content in the supernatant was measured by ELISA. Error bars indicate standard error of mean of a representative experiment with 6 animals per group. (ANOVA $p \leq 0,05$, Mann Whitney test, ** $p \leq 0,01$, *** $p < 0,001$)

Similar to cytokines, chemokines play an important role in the cell recruitment, cell activation and particularly in the micro environmental allocation of cells in the airways. To unravel the chemokine expression lung tissue was analyzed by quantitative real-time PCR. Expression of CCL11, CCL12 and CCL24 - three main chemokines attracting and guiding eosinophils - were drastically reduced in treated animals vs. non-treated (Fig. 19). CCL12 is also a chemoattractant for monocytes. Other chemokines and receptors known to play a role in eosinophil recruitment and tethering such as VCAM-1, CCR3, CCL2 and CCL5 were not influenced by rAv-TMY treatment (data not shown).

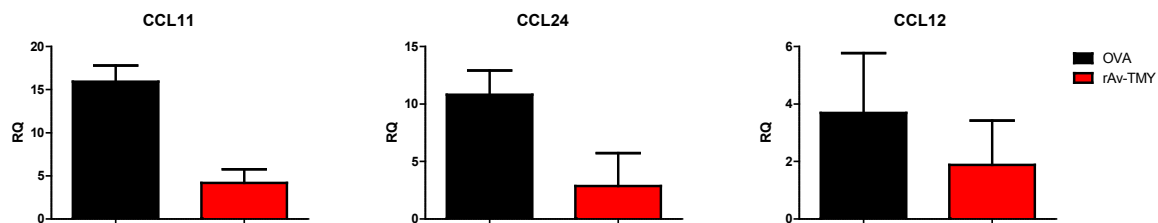


Fig. 19 rAv-TMY abrogates expression of chemokines in the airways induced by OVA . The graph illustrates the relative gene expression of the indicated chemokines compared to PBS treated animals. CCL11 (Eotaxin-1), CCL24 (Eotaxin-2) and CCL12 (MCP-5) are responsible for the recruitment and distribution of eosinophils. All three chemokines were upregulated in OVA sensitized animals, an effect that was abrogated in rAv-TMY treated animals. Ct values were normalized against endogenous control and expressed as fold change to PBS treated group (analyzed with StatMiner). Error Bars indicate standard deviation of a group with six animals. RQ: relative quantification, CCL: chemokine (CC-motif) ligand, MCP: monocyte chemotactic protein

Several parasites and consequently their released molecules have been shown to modulate the host immune system by inducing regulatory T cells. Therefore it was of interest if rAv-TMY also promotes the development of regulatory T cells and their accumulation in the lung draining lymph nodes. To investigate this question the numbers of CD4⁺CD25⁺CD103⁺ cells in the draining lymph nodes was measured by FACS. Most CD4⁺ T cells express the IL-2 receptor CD25 on their surface. Activated regulatory T cells which preferentially migrate to sides of inflammation additionally express the activation marker CD103. Only a minor difference between naïve animals versus OVA or rAv-TMY treated animals but no difference between the OVA versus rAv-TMY treated animals was observed. These findings suggest that the ameliorating effects of rAv-TMY are not due to activated Tregs within the PBLNs (Fig. 20).

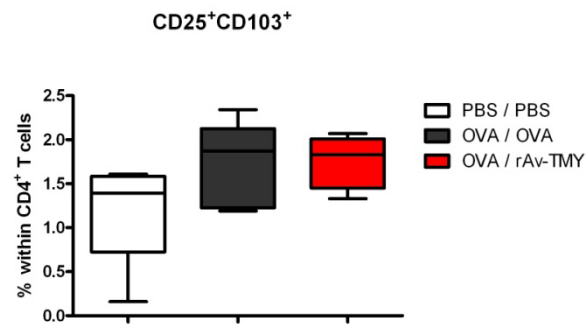


Fig. 20 rAv-TMY does not change the frequency of activated Treg cells in then PBLNs. Shown is the proportion of CD4⁺ T cells that express the IL-2 receptor CD25 and the activation marker CD103 in the lung draining lymph nodes (PBLNs). Neither sensitization alone nor the combination with rAv-TMY treatment changed the proportion of CD25⁺CD103⁺ cells in the CD4⁺ compartment compared to PBS treated animals. Graph represents a group with six animals. Boxes indicate interquartile range and median. Whiskers represent min and max values.

Immunoglobulin A is the main mucosal antibody and shown to be involved in the activation of eosinophils. IgA is described to have activating and inhibitory functions in humans but the definite role in asthma is unclear. Nevertheless, it was of interest if rAv-TMY would change the IgA content of BALF and serum. rAv-TMY treated animals secreted significant less total IgA into the BALF compared to OVA sensitized only animals ($p = 0,039$) (Fig. 21) whereas sera IgA was unchanged in all groups (data not shown).

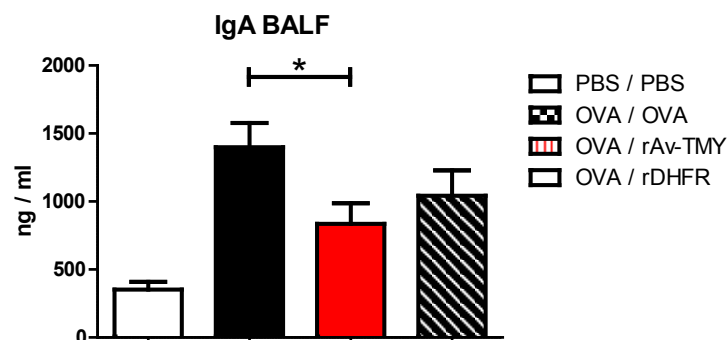


Fig. 21 rAv-TMY inhibits secretion of IgA in bronchoalveolar fluid. The graph depicts the concentration of secreted IgA in the BALF. Mice that received rAv-TMY secreted significantly less IgA into the airway lumen then mice only sensitized. A reduction in IgA levels was also seen in rDHFR treated animals although it did not reach significance. Total IgA was measured by ELISA. Error bars indicate standard error of mean of two independent experiments with 6-7 animals per group (ANOVA $p \leq 0,05$, Mann Whitney test, * $p \leq 0,05$); BALF bronchoalveolar lavage fluid

3.2.2 rAv-TMY affects migration and TLR-2 expression of macrophages

The intraperitoneal application of rAv-TMY has to influence somehow the immune system to create the effects seen in the asthma model. Preliminary *in vitro* studies showed a TLR-2 / MyD88 dependent induction of IL-10 by rAv-TMY in macrophages. Moreover, there are studies correlating TLR-2 expression and decreased susceptibility to allergies and reports on helminth derived molecules acting via TLRs. This led to the speculation that the immune modulation seen by rAv-TMY treatment might be mediated by macrophages in a TLR-2 dependent mechanism. To address these question mice were injected with tropomyosin and rDHFR as control molecule. 18 h afterwards the PECs were analyzed in regard to the cell composition. Macrophages (F4/80^{high}) were additionally analyzed in respect to TLR-2 and MHC class II expression. As presented in Fig. 22 treatment with rAv-TMY lead to a nearly complete loss of F4/80^{high}Gr-1⁻ expressing cells (macrophages) in the peritoneum in comparison to naive animals. Treatment with rDHFR also reduced this cell population, however, not as drastic as rAv-TMY. Analyzes of the TLR-2 expression on F4/80^{high}Gr-1⁻ cells revealed a significant increase of TLR-2 expressing macrophages in the rAv-TMY group compared to the naive or rDHFR treated group. Macrophages of rAv-TMY treated animals expressed high levels of MHC class II compared to PBS or rDHFR treated mice showing a higher activation status (data not shown). The results of this study clearly indicate that macrophages are induced by rAv-TMY to express high amounts of TLR-2 and that these cells emigrate from the peritoneum into different tissues.

In summary this work demonstrates that rAv-TMY induced a systemic Th2 response in an inflammation model indicated by the expression of IL-4, IL-5 and IL-13 but most importantly induced also regulatory IL-10 and the Th1 cytokine IFN- γ . Strikingly, rAv-TMY abates mucus hypersecretion and cell influx in the airways and leads to a modified Th2 response in the local airways indicated by reduced IL-5 but increased IL-4 and unchanged IL-10 expression. Application of rAv-TMY strongly induced TLR-2 expression on macrophages which started to emigrate from peritoneum. TLR-2 signaling on macrophages is therefore a likely mechanism by which rAv-TMY induce immune modulation.

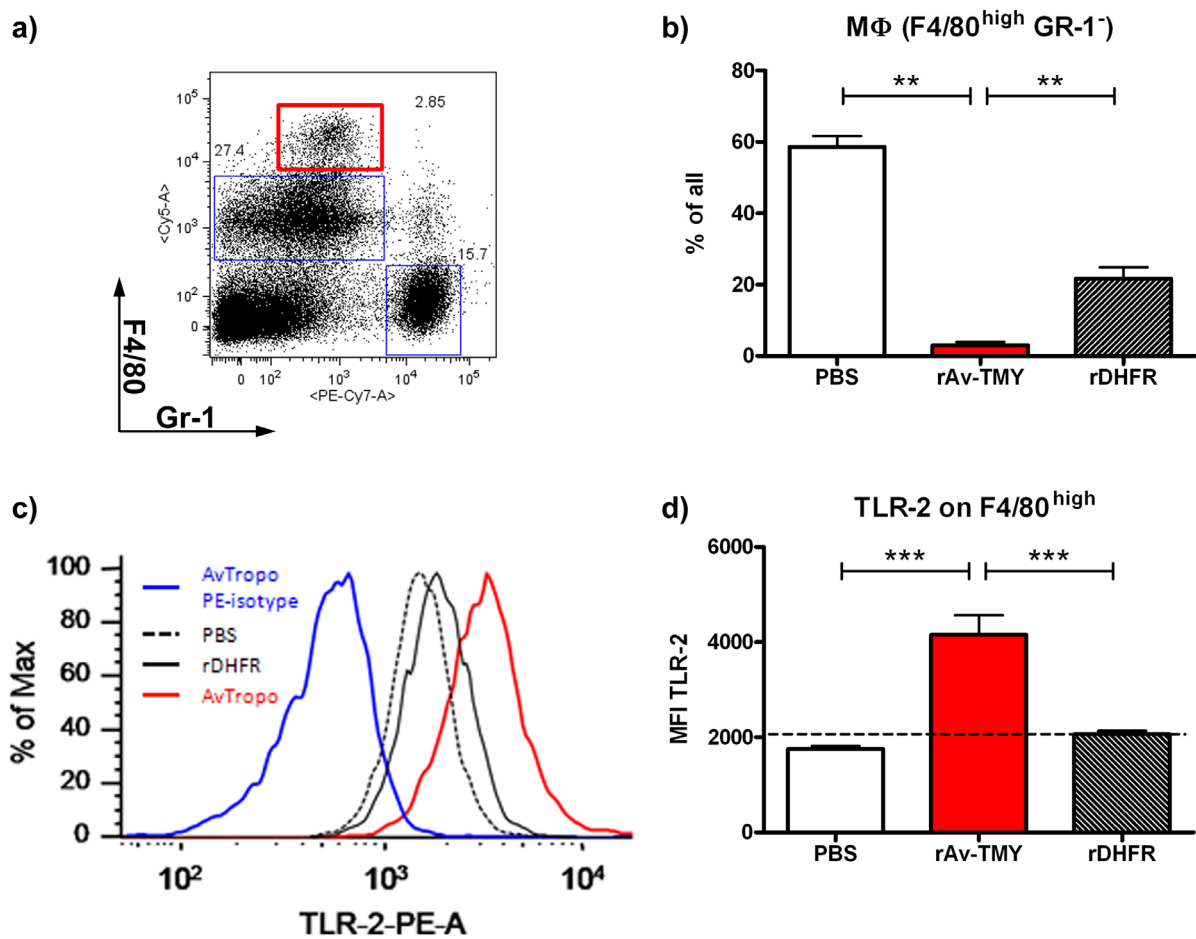


Fig. 22 rAv-TMY induces TLR-2 expression on macrophages and efflux of these cells from the peritoneum. PBS, rAv-TMY or rDHFR were injected into the peritoneum of mice and the peritoneal exudate cells were analyzed by FACS for expression of F4/80 and TLR-2 after 18h. a) Dot plot of PECs of one representative animal treated with rAv-TMY. b) Proportion of $F4/80^{high} Gr-1^{-}$ (macrophages) of all PECs. c) Shown is a histogram of TLR-2-PE labeled $F4/80^{high}$ cells and a PE-isotype (irrelevant antibody) control. A shift to the right indicates a higher expression of TLR-2, leading to an increased fluorescence intensity of labeled cells. d) Mean fluorescent intensity of anti-TLR-2 labeled macrophages. Error bars indicate standard error of mean of 5-6 animals per group. (ANOVA $p \leq 0,05$, Mann Whitney test, ** $p \leq 0,01$, *** $p < 0,001$)

4 Discussion

Worms are important parasites because of their ability to modulate the host immune response and in succession also diseases. Tools to manipulate helminths will help to dissect the mechanisms of helminth immune modulation. First, this work used tropomyosin to investigate the potential to use RNA interference as a tool to knock down genes in the murine strongylid *H. p. bakeri*. Second, filarial tropomyosin was used to reveal its potential immunomodulatory capacities of this molecule in a murine model of allergic airway diseases.

The data presented in the first part of this work disclose RNAi to be a rather inapplicable method for reverse genetics in strongylids and probably also other parasitic nematodes of mammals. The fact that parasitic nematodes obviously missing certain proteins of the RNAi machinery has wide implications discussed later on.

Second, the data on treatment with rAv-TMY in a murine asthma model revealed an immunomodulatory function of this molecule which will be discussed in the second part.

4.1 Part I – RNA interference in *Heligmosomoides polygyrus bakeri*

This work aimed to establish a method to genetically manipulate parasitic nematodes in order to have a tool which allows to study the immunomodulatory functions of single helminth derived molecules *in vitro* and *in vivo*. The potential use of the main delivery methods for RNAi – feeding, electroporation and soaking was explored. None of them yield a clear phenotype nor reduced levels of tropomyosin mRNA, indicating an insufficient uptake of dsRNA and/or a malfunctioning RNAi machinery.

The feeding method, which is well-established for *C. elegans*, was of particular interest as it allows *H. p. bakeri* to develop under nearly natural conditions on a bacterial lawn. However, feeding of recombinant bacteria did not induce RNAi in *H. p. bakeri* although both *Hp-tmy* dsRNAs used exhibited a strong impact on development, morphology and morbidity of *C. elegans*. Sequence alignment of the fragment *Hp-tmy-1* with the isoform CeTM1 of *C. elegans* revealed 88% identity, while *Hp-tmy-2* had 85% identity to CeTM1. Mismatches between the *H. p. bakeri* and *C. elegans* tropomyosin sequence were distributed over the complete sequence with a bias towards the 3' end. According to the dsRNA restriction by DICER it

is very likely that the resulting siRNAs contained one or more mismatches to the respective *C. elegans* mRNA. Therefore, our results support that induction of RNAi in *C. elegans* is not dependent on 100% sequence identity between siRNA and target mRNA as suggested by Jackson et al. [119]. Induction of a strong phenotype in *C. elegans*, but not in the homologous system, could have several reasons: (i) the RNA was not efficiently delivered to the gut of the worms, (ii) the RNA delivered to the gut was not taken up into the tissues, and/or (iii) later steps of RNAi did not work in *H. p. bakeri*. Another factor to be considered is the fast turnover of proteins within the short life-cycle of *C. elegans*, making the free living nematode possibly more susceptible to the consequences of RNAi as compared to *H. p. bakeri*. For *C. elegans* it was found that the genes responsible for dsRNA uptake are mainly expressed in the intestine [28,31,35] and, that the concentration of dsRNA in the environment is important for effective RNAi [30,31,35]. The fact that *H. p. bakeri* L1 take up only a fraction of recombinant bacteria ingested by *C. elegans* L1 leads to a much lower exposure of *H. p. bakeri* L1. In addition, *C. elegans* feeds constantly on bacteria throughout all life stages, whereas *H. p. bakeri* takes up bacteria only in certain phases of the life cycle, namely in the L1 and L2 stage. However, the reduced exposure to bacteria would not completely exclude uptake of dsRNA through the intestinal wall. Therefore, additional factors like deficiencies of specific RNA transporters or other downstream elements of the RNAi machinery could contribute to the failure of RNAi by feeding in *H. p. bakeri*. Our results are in accordance with the report of Geldhof et al. [44] who did not obtain a phenotype by feeding of dsRNA of different genes including paramyosin to *H. contortus* larvae. In contrast, Issa et al. reported on a developmental retardation of *T. colubriformis* larvae after feeding of tropomyosin dsRNA [37]. It is possible that this nematode takes up more bacteria as compared to *H. p. bakeri*, thus allowing penetration of dsRNA into the gut epithelium in quantities sufficient to cause RNAi.

As a second method electroporation was used to apply RNA. The structure mostly exposed to electroporation is the multi layered nematode cuticle, an acellular product shed by hypodermal cells. As transport of low molecular substances through the cuticle has been described in *Brugia pahangi*, *Dirofilaria immitis* and *A. suum* [120,121,122] we hypothesized that passage of siRNAs through the cuticle might deliver the molecules to the hypodermis. Electroporation was lethal to a large proportion of larval stages although a variety of conditions were used and no penetration of dye labeled RNA was observed. These results are in

contrast to Issa et al. who showed a developmental retardation of *T. colubriformis* L3 upon electroporation with siRNA [37]. Along the same lines, Geldhof et al. showed a reduction of the transcript level by qPCR for the two genes *Hc-ben-1* (β -tubulin) and *Hc-sod-1* (superoxide dismutase) after electroporation of *H. contortus* L1/L2 stages, but did not describe phenotypic effects due to the high proportion of dead larvae produced by this method [44]. It is possible that *H. p. bakeri* larvae have a particularly strong cuticle that does not allow the penetration of dsRNA. However, our study shows that electroporation of adult *H. p. bakeri* with labeled siRNA resulted in locally restricted signals within the cuticle. This indicates that electroporation has the capacity to penetrate a cuticle but the efficiency is probably too low for systemic RNAi. In contrast, the pre-adult stage of the trematode *S. mansoni*, the schistosomulum, which possess a tegument instead of a cuticle shows a robust phenotype after electroporation and is therefore more susceptible to RNAi via electroporation [123,124,125].

As a third approach soaking, a method that has recently been used for RNAi with the strongylids *T. colubriformis* and *H. contortus* was used [37,44]. Soaking of dsRNA is only applicable to parasite life stages that can be cultured in liquid media. L1 and L2 stages of *H. p. bakeri* develop best on solid media ([44], own unpublished results). The L3 is a kind of “dauer-stage” that has a less active metabolism and we considered it to be an inappropriate target for RNAi after disappointing preliminary experiments with sheathed and exsheathed L3 (data not shown). Soaking of adult worms showed that the pharyngeal region, and to a minor extent the intestine, was filled with dye labeled material. However, a spreading into other organs or the pseudocoelomic cavity was not detectable, making a systemic RNAi unlikely. Nonetheless, soaking of adults in dsRNA for at least 6 days resulted in increased numbers of dead and damaged worms. The damaged worms resembled very much *C. elegans* after treatment with *Hp-tmy-1* or *Hp-tmy-2* dsRNA or treatment with *Ce-tmy* dsRNA described in literature [106,107]. However, as these morphological changes only occurred after 6 days of *in vitro* culture, and were then also seen in the control groups (although to a much lesser extent), it was suspected that the alterations could also be age-related. Indeed, ageing *C. elegans* showed a similar phenotype (data not shown). Furthermore, a disintegrated gut and disintegrated ovaries of still living female nematodes were also reported for old *Onchocerca volvulus* worms [126]. This view was supported by the fact that the occurrence of morphological alterations was not correlated with reduced levels of tropomyosin

RNA after soaking. In addition, tropomyosin mRNA levels of single worms soaked with *Hp-tmy-1* or *Hp-tmy-2* and showing morphological changes (day 6) varied widely. Therefore, no correlation between altered morphology and mRNA levels could be established on the levels of individual worms. Our observations are compatible with a relatively inefficient and local uptake of dsRNA that would result in local damage of the gut by RNAi. As a consequence treated worms might have suffered from more stress than the control groups, which might have led to increased ageing, but not to a specific phenotype. The reasons for an increase of transcript levels in *Hp-tmy-2* treated worms are unclear. A similar effect was also reported by Geldhof when *H. contortus* L3 were electroporated or soaked in *Hc-vha-10* dsRNA targeting the vacuolar ATPase [44]. One possible explanation is a feedback regulation that senses the decrease of the transcript level of a certain gene and reacts with enhanced transcription. However, other groups reported a clear down regulation of the transcript level in parasitic nematodes upon dsRNA treatment [39,127].

Successful RNAi depends on a complex protein machinery. The failure to produce a phenotype by RNAi in *H. p. bakeri* might be due to the absence of certain of these elements. This question was addressed by a database search for proteins that are part of the RNAi machinery. Since no genome information for *H. p. bakeri* is available the expressed sequence tag (EST) database of the closely related strongylid *H. contortus* for orthologs of *C. elegans* RNAi related proteins was searched. The most prominent proteins found belong to protein complexes involved in dsRNA-processing (DICER), mRNA-degradation (RISC) and dsRNA-amplification (RdRP) [32].

However, the database did not contain orthologs for SID-1 (uptake and spreading), SID-2 (uptake) or RSD-3 (spreading) [27,31,32,35]. Hence, the presence of the RNAi core proteins and the absence of dsRNA uptake proteins suggest that RNAi could occur in strongylid nematodes if the problem of uptake could be overcome. Without a system for spreading of dsRNA, the effects of RNAi could, however, be local and confined to accessible and sensible organs like the gut. The lack of uptake and/or spreading system would explain the paucity of reports on successful RNAi in parasitic strongylids. Hussein et al. could knock down two different acetylcholinesterases (AChEs) in *Nippostrongylus brasiliensis* detected by a decrease in enzymatic activity [128]. *T. colubriformis* was reported to be most susceptible to RNAi when larval

stages were electroporated with siRNAs of ubiquitin [37]. This finding supports our hypothesis that parts of the machinery for RNAi are present in parasitic nematodes and can act if the missing uptake proteins are replaced by an invasive delivery method like electroporation. This is also supported by an extensive study performed by Geldhof et al. [44]. Out of 11 genes of *H. contortus* larvae only two genes, encoding for β -tubulin and superoxide dismutase could be knocked down, when larvae were electroporated or soaked, whereas feeding failed completely. Furthermore, a previous report showed a clear down regulation of two tubulin mRNAs of *H. contortus* upon soaking of L3, L4 and adults with dsRNA fragments [127].

Our data support the notion that dsRNA is not efficiently taken up, but can be processed by *H. p. bakeri*, which is in line with the results presented by others [44,129,130]. Obviously, uptake and spreading of dsRNA is important, but factors such as sequence specificity, high or low gene expression, stage specific expression, stability of the target protein or strength and structure of the cuticle are likely to influence whether RNAi induces a phenotype or not in nematodes. Filarial nematodes for example are known to take up low molecular weight nutrients via their cuticle [120,121,122] and might be more capable to acquire high amounts of dsRNA although they do not possess the specific uptake proteins SID-1 and SID-2 as indicated by our results (see p. 32 Tab. 1) and others [131]. The few publications on filarial nematodes suggest a functioning RNAi machinery. Treatment of adult *B. malayi* with dsRNA via soaking of two housekeeping genes led to the reduction of the respective mRNA level and was lethal, and treatment with dsRNA of a microfilarial sheath protein inhibited proper sheath formation [36]. Furthermore, Pfarr et al. demonstrated RNAi in adult *Litomosoides sigmodontis* and proved off target effects using high concentration (>3.5M) of dsRNA via soaking experiments [39]. A reduced lifespan and inhibition of molting was observed via soaking of *A. viteae* in dsRNA targeting chitinase [132] and also L3 of *O. volvulus* failed to molt after soaking in cathepsin-L and -Z dsRNA [38]. The latter authors propose a transfer of dsRNA through the cuticle, since some of the L3 showed a signal in the hypodermal region after exposure to labeled dsRNA. In this respect it is interesting that our database analysis revealed that *B. malayi* exhibits a gene with similarities to the *rsd-3* of *C. elegans* [32]. RSD-3 comprises an epsin N-terminal homology domain (ENTH) that belongs to a family of endocytic adaptor proteins, which are thought to be involved in vesicle trafficking. In *C. elegans* RSD-3 is specifically involved in systemic RNAi of exogenous dsRNA [27,29,32]. Thus, it is possible that RSD-3 plays

the same role in filarial nematodes and makes them more amenable to RNAi. This might also be true for trematodes such as *S. mansoni*, in which the *rsd-3* gene is present (see Tab. 1). *S. mansoni* was reported to produce a long lasting and reproducible phenotype by electroporation with dsRNA of cathepsin B and alkaline phosphatase [123,124,133].

4.2 Part II – Influence of rAv-TMY on a murine model of allergic airway inflammation

Generally helminth infections induce a Th2 response to which the parasites have become adapted in a way that they are able to modulate this otherwise harmful immune response [53,134,135,136]. The modulating properties of helminths include the modulation of the antibody response, induction of IL-10 and TGF- β production and the expansion and differentiation of regulatory T cells [137,137,138,139]. One evolutionary trait that developed to interfere with the host immune response is the release of modulating molecules. A few of the responsible products released by different helminth parasites have been characterized [50,60,99,102]. However, the detailed mechanisms how helminths modulate the host immune reaction remains barely understood. This study aims to reveal the potentially immunomodulatory functions of *A. viteae* tropomyosin a molecule otherwise shown to be an allergen [140,141]. The fact that filarial nematodes persist in their respective hosts for a long time in general without inducing signs of severe allergic reactions led to the speculation that filarial tropomyosin might have lost the immunogenic capacity seen in invertebrates of other phyla. Furthermore preliminary *in vitro* studies revealed IL-10 inducing capacity of *A. viteae* tropomyosin. This indicates a potential regulatory function of Av-TMY. In this work the capacity of rAv-TMY to modulate an allergic immune response to an unrelated antigen was investigated. rAv-TMY was tested to interfere with the onset of an OVA induced airway inflammation. The results clearly indicate that rAv-TMY is able to ameliorate the symptoms of allergic airway inflammation.

4.2.1 Tropomyosin as an allergen

As initially described tropomyosin is a major food and aeroallergen of invertebrates [141,142]. The gut mucosal immune system is usually confronted with a myriad of bacterial and food derived antigens. In healthy individuals the immune system is primed to be tolerant

against harmless antigens but under certain conditions the induction of oral tolerance fails. Several factors leading to intolerance have been discussed. The route of uptake for instance seems to play an important role. There is incidence that the aberrant uptake of allergens via the skin or by inhalation (e.g. house dust mite tropomyosin) - bypassing the gut-mucosal barrier - can lead to sensitization. Sensitized individuals subsequently react allergic to orally ingested antigens [143,144]. Other factors like genetic determinants, commensal gut flora, digestion of antigens during uptake and age are playing important roles in establishing atopic allergies. However, invertebrate tropomyosin exhibits strong IgE cross reactivity making a cross-sensitization likely. It is shown that sera of patients with allergy to indoor arthropods react also to shrimp tropomyosin indicating that a inhalation of tropomyosin of mites or cockroaches can lead to food allergy against shrimp tropomyosin [7,145,146]. Helminths also induce a strong IgE response among which tropomyosin is also recognized. The induction of reactive IgE by nematode tropomyosin [8,15,147] and their cross-reactivity to shrimp and house dust mite [8,148,149] has been demonstrated. The broad cross reactivity to tropomyosin gives rise to the question if helminth tropomyosin could induce allergic reactions to itself and/or tropomyosin of different organisms. Considering the fact that filarial nematodes express tropomyosin on their surface [8,9] and that the continuing turnover of microfilariae confronts the host with relevant amounts of tropomyosin makes this question even more appropriate. So far there are no studies linking helminth infections with the induction or enhancement to the clinical outcome of food allergies. However, for allergic airway disorders some correlations have been found. For *Ascaris* a correlation between the infection and an increased risk to develop asthma was demonstrated in cross-sectional study of children in China [95]. In contrast Wördemann et al. reported *A. lumbricoides* infections to be protective for atopic dermatitis but *Enterobius vermicularis* and hookworm infections to be risk factors to develop atopic dermatitis and/or allergic rhinoconjunctivitis [89]. On the other hand there is accumulating data showing an inverse correlation between helminth infections and the risk for allergic disorders. Van den Biggelaar and coworkers found a positive correlation between helminth infections and a negative skin prick test for house dust mite allergen in Gabonese school children. Interestingly a negative skin prick test was not correlated with lower total or specific IgE levels indicating that reduced IgE or induction of polyclonal IgE is not the main reason for reduced skin reactivity [80]. A further study of van den Biggelaar revealed a posi-

tive correlation between helminth infections, increased IL-10 levels and lower prevalence of a positive skin reaction to house-dust mite [92]. These studies suggest that helminth infections do not prevent atopy but reduce the risk to develop an allergic reaction. This notion is supported by a study where allergic and non-allergic subjects of different socio-economic levels were compared. Individuals of high (HSEL) and medium socio-economic levels (MSEL) showed higher skin reactivity to common environmental allergens than their counterparts of low socio-economic levels (LESL). Lower skin reactivity correlated with high total IgE levels and a much higher worm burden [85]. Most studies were done with adults or older children leaving out an important time frame in which individuals first encounter environmental antigens and also helminths. Dagoye and colleagues addressed this question in a nested case-control study supporting a negative correlation between helminth infections and wheezing in children between one and four years old [81]. However, there are further reports providing evidence for the beneficial capacities of helminth infections in regard to allergic inflammation [83,86,91,92,93]. Surveys regarding whole helminth infections of course do not exclude the allergenicity of a single parasite molecule. Helminths are shown to release several immunomodulators inducing a regulatory phenotype that can counterbalance the allergenic features of single molecules.

Nevertheless, there are reasons arguing against a strong allergenicity of Av-TMY. First, Asturias and colleagues showed that tropomyosin is probably not a major allergen as sera of *Anisakis simplex* patients were found to be non-reactive with native or recombinant *A. simplex* tropomyosin [147]. Second, the prevalence of helminth infections in developing countries is very high whereas the incidents of food allergies is very low compared to western countries [143]. This negative correlation again argues against a strong allergenic or cross-reactive property of helminth derived allergens, in that case tropomyosin. Third, the recognition of tropomyosin by IgE does not necessarily mean that tropomyosin is also allergic. As mentioned atopic individuals often found to be non-allergic. Furthermore for peanut allergens it was shown that IgE antibodies of atopic patients with severe reactions recognize more peanut epitopes than those with mild or no symptoms [150,151] and stronger binding of antigen could account for an enhanced immune response. Hence, tropomyosin of worms might induce a more polyclonal IgE response which results in a reduced allergenicity. The

above discussed points show that the allergenicity of an allergen is dependent on many factors and that an IgE response is not necessarily followed by an allergic reaction.

4.2.2 rAv-TMY alleviate OVA induced airway hyperresponsiveness

To test the hypothesis that rAv-TMY exhibit immunomodulating capacities to bystander allergens rAv-TMY was applied in preventative model of OVA induced airway inflammation. Animals were sensitized to OVA and during sensitization treated with rAv-TMY or the control protein rDHFR respectively. Airway inflammation was provoked by intranasal application of OVA. Changes in the inflammatory response between treated and untreated (sensitized only) animals were evaluated by measuring the cell influx into the airway lumen, antibody response and mucus production.

A hallmark of asthma is the increase in CD4⁺ T cells of the Th2 subtype combined with massive influx of eosinophils into the airways whereas in healthy individuals Th1 cells dominate [63]. In this study rAv-TMY drastically reduced the numbers of immigrating immune cells into the BALF. The reduction in lymphocytes goes along with a reduction in T helper cells thereby limiting the release of pro-inflammatory cytokines and the promotion of a Th2 bias (reviewed in [63,64,69]). The importance of mainly CD4⁺ T cells was demonstrated by Gavett and colleagues who showed that the depletion of CD4⁺ T cells in a murine *in vivo* model of asthma abrogated antigen induced AHR and airway eosinophilia [152]. It is of importance that eosinophils, the main effector cells in allergic airway inflammation, were strongly reduced in the BAL [65,67]. As an important source of mediator and effector molecules such as IL-13, histamines, leukotrienes (LT) and platelet-activating factor (PAF) reduced eosinophil numbers might lead to diminished mucus production [65]. Reduced eosinophil numbers were also seen in the lung interstitium by histological sections. Notably the size of cell infiltrations around the vessels and bronchioles did not differ between treated and untreated animals but the proportion of eosinophils compared to total cells within these cell conglomerates was substantially decreased.

Another feature of asthma is a remarkable increase in allergen specific IgE antibodies [61]. Antigens bound to IgE effectively cross-link FcεRs on mast cell resulting in degranulation and tissue damage. As first line effector cells mast cells have an important role in induction and

maintenance of inflammation upon allergen exposure [153]. rAv-TMY significantly diminished the production of allergen specific IgE thereby limiting the activation of mast cells.

As an outcome of reduced cell influx and lowered IgE levels symptoms of the allergic airway inflammation measured by mucus hyper secretion were reduced. The mucus production was, however, not completely absent. This indicates that rAv-TMY does not completely block the effector functions but rather mitigate symptoms.

4.2.2.1 *The role of IgG in rAv-TMY mediated immune modulation*

Helminth infections are known to induce a shift in the IgE/IgG4 ratio towards IgG4 in humans [49,139]. It was therefore of interest if rAv-TMY is able to induce such a shift. Indeed rAv-TMY induced a dramatic rise in rAv-TMY specific IgG1 (human IgG4) antibodies whereas OVA did not induce an IgG1 response at all. Increased IgG1 levels have at least two implications. First, IgG1 can compete for IgE epitopes on the antigen. This prevents cross-linking of FcεRs on mast cells resulting in a reduced degranulation frequency [70,154]. Second, IgG1 can cross-link the FcγRIIb with FcεRI/FcγRIIb on mast cells what cause an inhibitory signal [70,155]. rAv-TMY was also potent in stimulating the production of IgG2a and IgG2b two subclasses generally considered to occur under Th1 conditions [155]. If and to what extent this antibodies might contribute to the ameliorating effects of rAv-TMY stays elusive. They might somehow have a “diluting” effect by competing for receptor binding sites and epitopes. In humans it is well established that specific immune therapy leads to high antigen specific IgG1 (mouse IgG2a) titers but the role in desensitization are still unknown [70,156,157]. Wilson and co-workers found an increase in allergen specific IgG2a antibodies in *H. p. bakeri* infected mice sensitized to OVA although not reaching statistical significance, arguing for a role of IgG2a in the abatement of allergy. [137].

4.2.2.2 *Influence of rAv-TMY on cytokine expression*

To address the question about possible mechanisms and the involvement of mediators the local and systemic cytokine pattern was analyzed. Allergies are commonly associated with a Th2 dominated cytokine environment [63]. Analysis of the BALF revealed similar IL-4 and IL-10 levels in all groups and a substantial reduced IL-5 expression in rAv-TMY treated vs. OVA only sensitized animals. Both, OVA and rAv-TMY were able to stimulate spleen cells to pro-

duce the before mentioned cytokines with rAv-TMY being a little bit less effective to induce IL-4 expression. This constellation suggests a modified Th2 response which is characterized by an increased expression of IL-10 and IL-4 but reduced amounts of IL-5 [93,158]. The expression of IL-4 and the induction of IgE indicate that rAv-TMY features some characteristics of an allergen. On the other hand the induction of IL-10 adds a regulatory element. Intriguingly rAv-TMY was also able to induce IFN- γ production by spleen cells and reduce the effector cytokine IL-13 in the BALF. The possible roles of this cytokines will be discussed in the following part.

IL-4 is a key cytokine in the induction of a Th2 response and is associated with allergies and helminth infections. IL-4 can be initially induced by antigen presentation to TCR in a MHC class II context. The activation of GATA-3 then leads to expression of IL-4 and IL-2. IL-4 itself acts as a positive feedback cytokine for Th2 differentiation. This process is counter regulated by IFN- γ [159]. It might be speculated that the induction of IFN- γ by rAv-TMY account for the diminished IL-4 production by spleen cells. However, spleen cells responded with production of IL-4 and IL-13 upon stimulation with OVA or rAv-TMY. Consistently, a class switch to IgE, reflected by high amounts of total IgE was found. In the airways rAv-TMY reduced the IL-13 but not the IL-4 production compared to sensitized only animals. Importantly, IL-4 is also responsible for the down regulation of the high affinity IgE receptor Fc ϵ RI on mast cells [68,160]. Taken together this might imply that IL-4 on the one hand induces IgE on the systemic level but on the other hand down regulates the respective receptor in the local environment. Low expression levels of Fc ϵ RI would imply a desensitization of mast cells despite high levels of IgE. Consistent with this study protective effects against OVA induced airway inflammation were also reported for whole helminth infections. Protection was found even though a Th2 response against the parasite was mounted seen by unchanged or even increased IL-4 levels in the BAL of infected animals [137,161]. Furthermore IL-4 recruits eosinophils from the blood stream by inducing the expression of endothelial adhesion molecules, most prominently VCAM-1 [65,66]. Congruent with the unaltered IL-4 levels in the BALF of treated animals VCAM-1 was not changed in the airways seen by qPCR (data not shown). This indicates that rAv-TMY does not alter the trans-endothelial migration of eosinophils.

The recruitment and maturation of eosinophils is thought to be mainly managed by IL-5. Consistently with reduced IL-5 levels in the BALF and Sera (data not shown) the influx of eosinophils was significantly reduced in treated animals. Eosinophils derive from CD34⁺ lymphohemopoietic stem cells. They start to mature in response to IL-3 and GM-CSF but IL-5 is most important for proliferation, release from bone marrow, maturation and terminal differentiation [67,162]. In addition IL-5 induces degranulation of eosinophils [67]. So far IL-5 is the main player in recruiting eosinophils but redundant mechanisms must exist. Flood-Page and colleagues found that treatment with the anti-IL-5 antibody mepolizumab only reduced airway eosinophil numbers by 50% and had no effects on the airway hyperreactivity. Interestingly, staining for major basic protein revealed no differences between treated and placebo group [163]. This indicates that 50% of eosinophils can account for the same amount of released granule proteins as in untreated individuals. A stronger degranulation of the remaining eosinophils might be due to a different/enhanced degranulation signal that compensates the lacking eosinophils. The absolute eosinophils numbers are therefore not the only factor contributing to inflammation. However, an impact of helminthes or their products on the local and systemic IL-5 production in allergic disease models have been described. Kitagaki and co-workers showed that *H. p. bakeri* infection reduced IL-5 secretion in the BALF and caused an intermediate response of splenocytes upon antigen or mitogen stimulation in an OVA induce asthma model [164]. The impact of an *H. p. bakeri* infection on the IL-5 concentration in the BALF was confirmed by Wilson et al [137]. Reduced IL-5 levels in the BALF were also reported for treatment with the MIF homologue of *A. simplex* [60]. Moreover egg antigen of *Schistosoma japonicum* potently inhibited IL-5 production measured in the BALF and sera of OVA sensitized mice [165]. In contrast, Wang and colleagues found a profound increase in the IL-5 production by CD4⁺ T cells and a secretion into the BAL in mice infected with *Strongyloides stercoralis* [166]. In general helminth infections are associated with induction of IL-5 expression [167]. The differential expression of IL-5 in spleen and airways found in this study and by others [158,164] points towards a compartmentalization of the immune response induced by helminthes or their products. This might lead to a Th2 response on the systemic level but a modified Th2 response in the local tissue. The alteration of IL-5 and IL-4 expression might also have an indirect impact on the IL-13 production, probably one of the main effector cytokines regulating mucus hyperplasia and AHR. IL-13 is mainly released by

Th2 T cells but also by mast cells, basophils and eosinophils. IL-13 induced STAT6 signaling can trigger the expression of a variety of mediators such as chemokines (eotaxin, MCP) and goblet cell inducing mediators (LT, TGF- α et al.) [168]. IL-13 was reduced by half in the BALF of rAv-TMY treated animals compared to sensitized only animals. This effect might be explained by the decreased influx of cells into the airways and the reduced activation status. Nonetheless, rDHFR had a similar effect indicating that this protein is not completely inert in this system. Reduced IL-13 secretion to the BALF together with ameliorated airway inflammation was also reported for treatments with the *A. simplex* MIF homologue and the *A. suum* PAS-1 protein [60,103]. In contrast, infections with male *S. mansoni* (precluding eggs) were reported to increase IL-13 secretion in the BALF and to induce an antigen specific IL-13 expression by spleen cells despite protecting animals from OVA induced airway inflammation [161]. In the present study spleen cells of rAv-TMY treated animals responded to OVA and rAv-TMY by expressing IL-13. Taken together rAv-TMY seems to differentially alter the IL-13 expression locally and systemically. The observation that rAv-TMY changes the IgE / IgG1 ratio and suppresses OVA specific IgE despite a systemic IL-4 and IL-13 production raises the questions which signal is responsible for this outcome. One candidate is IL-10. IL-10 is shown to potently trigger B cells to express IgG4 and suppress IgE [56,169,170,171]. This is in line with several findings showing that helminth infections induce IL-10 production together with mouse IgG1 [172] or human IgG4 [173] respectively. Moreover it was shown that specific immunotherapy has similar effects [157,169,174]. However, the suppressive function of IL-10 is meanwhile extended to interference with eosinophil survival and cytokine release, the deactivation of mast cells and hampering antigen presentation by DCs (see also Fig. 23; reviewed in [55,175]). Arock and colleagues showed a reduced eosinophil survival by an IL-10 dependent suppression of GM-CSF released by bone marrow-derived mast cells [176]. These findings were extended by Takanashi and co-workers showing a suppression of LPS induced autogenous production of GM-CSF leading to reduced survival of eosinophils *in vitro* [177]. IL-5 expression in T cells, however, seems to be also suppressed by IL-10 [178,179]. IL-5 and GM-CSF induce the expression of the anti-apoptotic gene Bcl-x_L in eosinophil [180]. A suppression of IL-5 and/or GM-CSF by IL-10 might therefore lead to increased apoptosis of eosinophils. The anti-inflammatory effects of IL-10 *in vitro* are meanwhile confirmed by *in vivo* inflammation models. Tournay et al. demonstrated suppressive effects of endogenous IL-10

on allergen induced airway inflammation. IL-10^{-/-} mice developed stronger eosinophilia and had a higher baseline level of AHR compared to wild type mice [181]. Moreover Fu and co-workers achieved reduced allergen responsiveness by intra-tracheal application of IL-10 expressing plasmids [182]. It is therefore of no surprise that helminth induced modulation is also mediated by IL-10. Kitagaki and colleagues showed that suppression of OVA induced airway inflammation by a *H. p. bakeri* infection was abrogated in IL-10 knock out mice [164]. In contrast Wilson and co-workers reported IL-10 independent mechanism in the same model. Applying an anti-IL-10 mAb did not influence the protective capacities of a *H. p. bakeri* infection and protection could be transferred by CD4⁺ cells from IL-10^{-/-} mice [137]. Nevertheless, an important role of IL-10 mediating tolerance in OVA induce airway inflammation was also reported for infections with *N. brasiliensis* and male *S. mansoni* [161,183]. Furthermore IL-10 induced by *Hymenolepis diminuta* could ameliorate symptoms in a Th1 based colitis model [184]. In the same line Schnöller et al. showed the suppressive effects of single nematode derived molecule in a model of allergic airway inflammation and colitis. This process was found to alter the antigen presentation by APCs rather than inducing regulatory T cells [102]. The multi suppressive effects of IL-10 suggest this cytokine to be a main player in the rAv-TMY induced modulation. From this study it remains unclear whether IL-10 might be only important as an initial trigger to induce the antibody shift and/or to interfere with the onset of inflammation or if it is needed on a constant level.

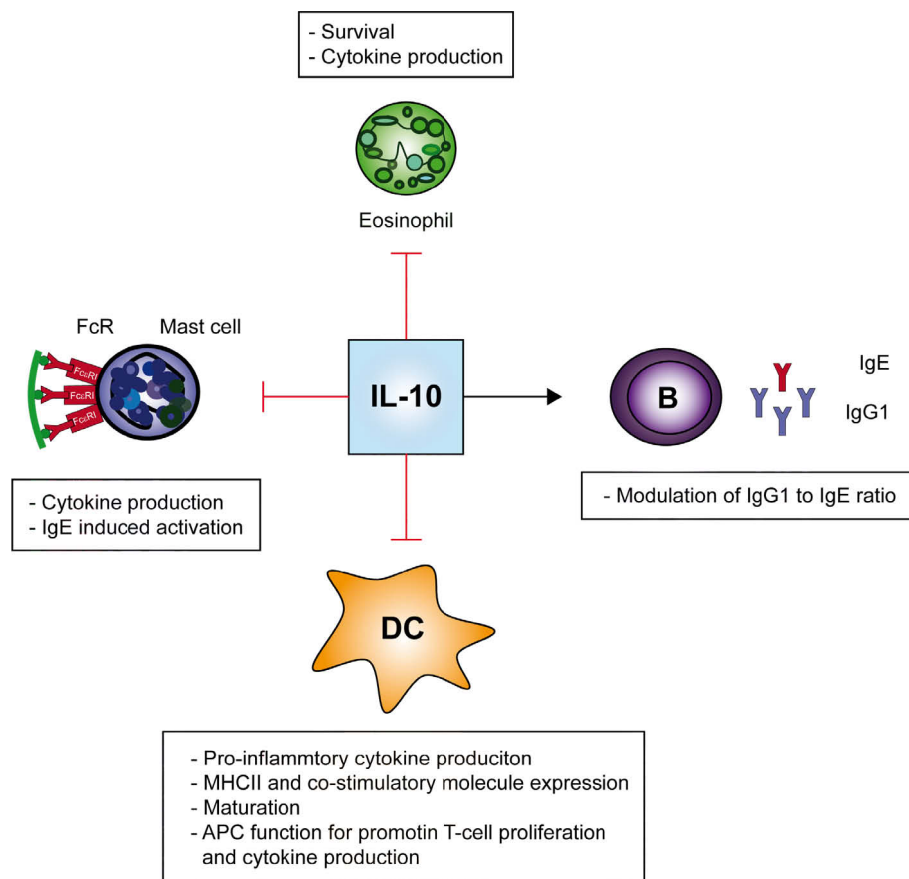


Fig. 23 Potential role of IL-10 in orchestrating a modified Th2 immune response. The early induction of IL-10 by rAv-TMY might lead to a modified Th2 response that interferes with the onset and severity of airway inflammation. IL-10 might suppress the onset of inflammation due to reduced IgE mediated degranulation and cytokine release by mast cells in the airways. IL-10 thereby creates a local anti-inflammatory environment that feeds back also on dendritic cells and other APCs. The production of pro-inflammatory cytokines by DCs is inhibited by IL-10. By reducing the expression of MHC class II and co-stimulatory molecules on DCs IL-10 prevents or abates the subsequent induction of Th2 T cells in the draining lymph nodes. This results in a reduced inflammation in the late asthmatic reaction (LAR) upon allergen restimulation. In LAR the effector function of mast cells can be inhibited by a shift in the IgE / IgG1 ratio towards blocking IgG1 antibodies. In addition the survival, cytokine production and degranulation of eosinophils in the airways can be diminished. Overall these effects can lead to an attenuated inflammatory response and reduced symptoms. Modified from Havrylowicz & O'Garra, 2005. DC: dendritic cell, B: B cell, APC: antigen presenting cell, MHC class II: major histocompatibility complex class II, FcR: fragment crystallizable (region) receptor

4.2.2.3 rAv-TMY alters eosinophil migration by repressing chemokine expression

As stated above IL-5 might not be the only chemoattractant and activation signal for eosinophils. Furthermore, IL-13 is shown to potently induce chemokines such as eotaxin and monocyte chemoattractant protein (MCP) and reduced IL-13 levels might alter the chemokine expression. To clarify a possible impact of rAv-TMY treatment in the regulation of the chemokine production the expression of several cytokines in the airways was analyzed. As

mentioned before the VCAM-1 expression was not altered leaving the trans-endothelial migration of eosinophils intact. However, the allocation of eosinophils within the interstitium was different in treated animal, suggesting an altered homing signal. One putative candidate is CCL12 (MCP-5) that showed lowered mRNA levels in the lung tissue. CCL12 is described to rather alter the trafficking of eosinophils through the lung interstitium than to recruit eosinophils from the blood stream [185]. MCP-5 is expressed at early time points of the allergic response what might explain the minor difference between OVA and rAv-TMY groups in this study [186].

Two other chemokines - eotaxin-1 (CCL11) and eotaxin-2 (CCL24) - showed a decrease expression in the lung of rAv-TMY treated animals. Both target CCR3 and are able to exclusively guide eosinophils along the gradient to its destination [67,187]. Analysis of the CCR3 expression in the airways conducted conflicting results in either being up- or down regulated upon rAv-TMY treatment (data not shown). This might be due to the fact that only little parts of the lung were used for RNA isolation which might contain unequal numbers of eosinophils. Little is known about the regulatory pathways concerning eotaxin. It was demonstrated that IL-4, IL-13, TNF- α and histamine induce eotaxin expression alone or in a synergistical way in human fibroblasts or airway smooth muscle cells [188,189,190,191]. However, eotaxin inducing effects of IL-4, IL-13 and TNF- α were not seen in endothelial or epithelial cells [192] conflicting with the notion that epithelial cells are one of the major sources of eotaxin beside mast cells. Therefore the way how rAv-TMY could trigger the down regulation of eotaxin stays elusive. Nevertheless, the suppression of eotaxin seems to be a common feature of helminth infections resulting in reduced eosinophilia in the respective inflammation models. Infections with *H. p. bakeri* were shown to reduce eotaxin levels and eosinophilia in the BALF of BALB/c mice sensitized to OVA and in C57BL/6 mice sensitized to the house dust mite antigen Der p 1 [137]. Similar to *H. p. bakeri* *N. brasiliensis* abates eotaxin expression and eosinophilia in the lung. Interestingly, in natural infections without sensitization to OVA *N. brasiliensis* induced strong eosinophilia in the lung. The cell influx followed a kinetic with eosinophilia peaking at two weeks post infection and returned to baseline levels as soon as eight weeks post infection. This kinetic was also seen in OVA sensitized animals showing that the immune response modulated by a *N. brasiliensis* infection seems to override the response to the allergen OVA [193]. However, single helminth derived molecules were also re-

ported to impact eotaxin expression. The protein PAS-1 of *A. suum* was shown to reduce eotaxin expression and eosinophilia in the BAL of OVA sensitized mice. Intriguingly this effect was abrogated in IFN- γ and IL-10 but not in IL-12 knockout mice indicating an important role of these mediators in the regulation of eotaxin [103]. It might be that the induction of IL-10 and IFN- γ expression by rAv-TMY in spleen cells is also mediating the suppression of eotaxin. Conversely, treatment with excretory/secretory products of *N. brasiliensis* (NES) protected mice from OVA induced airway inflammation in an IL-10 and IFN- γ independent fashion [183]. A possible explanation might be the difference between a single molecule and a mixture of several potentially modulating proteins. The latter one might influence the immune response through different and possibly IL-10/IFN- γ independent ways. Furthermore it was shown that metalloproteases released by the hookworm *N. americanus* cleaves eotaxin *in vitro* and *in vivo* [194]. The matrix metalloproteases (MMP) 9 and 2 are described to be important in the regulation of cell recruitment probably by a degrading chemokines. However, the effects of matrix metalloproteases are conversely discussed in being beneficial or detrimental in the course of allergic airway inflammation. MMP9 knockout mice showed increased cell recruitment into the lungs and increased levels of CCL11 and CCL22 in an OVA induced airway inflammation. This indicates a critical role of MMP9 in the regulation of eosinophil migration [195]. Similar results were obtained in an *Aspergillus fumigatus* allergen induced model of airway inflammation. Using MMP9^{-/-} and MMP9/MMP2 double knockout mice the authors proposed independent and overlapping contributions of this two proteases to cell egression from the lung by regulating the transepithelial chemokine gradient [196]. However, opposite results were obtained in a different study using also MMP9 knockout mice. Upon OVA challenge significantly less mononuclear cells infiltrated the lung and AHR was reduced compared to wild type mice [197]. In mycobacteria infections the expression of MMP9 in macrophages is induced in a TLR-2 dependent manner [198]. As rAv-TMY induces the expression of TLR-2 on macrophages it is reasonable that rAv-TMY might induce metalloproteases in a TLR-2 associated way thereby influencing the chemokine mediated cell influx and/or egression.

Intriguingly, eotaxin-2 has been shown to cooperate with IL-5 in the lung to orchestrate eosinophilia, mucus hyperproduction and AHR in an OVA-induced model of airway inflammation. Yang and colleagues also demonstrated that the local presence of both IL-5 and eotaxin-2 in

the airways is crucial to induce pulmonary eosinophilia and AHR [199]. This model fits well to the findings that rAv-TMY treatment reduce IL-5 production in the BALF and sera (data not shown), eotaxin-2 expression in the lung tissue and subsequently the IL-13 secretion into the BALF. However, reduced expression of eotaxin can have further implications. In the last years a phenomenon called “piecemeal degranulation” (PMD) got into the focus of research [200]. Basophils, mast cells and eosinophils are able to differentially secrete their products. Eotaxin was shown to induce the differential secretion of IL-4 by eosinophils [201]. If this process can for example also trigger the exclusive secretion of IL-13 by either mast cells or eosinophils is unknown but this mechanism adds a further explanation for micro environmental regulation of inflammation.

4.2.2.4 rAv-TMY immune modulation does not involve regulatory T cells

Helminths have been shown to induce regulatory T cells as an important cell type in helminth mediated immune modulation and in regulation of allergic airway diseases [137,165,202,203]. Furthermore, rAv-TMY induced IL-10 expression what might suggest the involvement of regulatory T cells. Treg cells are known to impair the ability of DCs to activate effector T cells by cell-cell interaction, IL-10 and TGF- β production and the expression of CTLA4 soon after migration to the lymph nodes. The re-migrating DCs then lack the ability to activate T cells in the airways [47]. Therefore the proportion of CD4⁺ cells expressing the Treg marker CD25 and the activation marker CD103 in the PBLNs was investigated. No differences in the proportion of Treg cells within the PBLNs primed to migrate to the airways were found in all groups compared to the naïve animals suggesting that Treg cells do not play an important role in rAv-TMY induced immune modulation. The slight increase in Treg cells in sensitized and treated animals might be a response to the ongoing inflammation in both groups. However, as CD25 can be also upregulated on activated conventional CD4⁺ T cells and PBLN cells were not analyzed for Foxp3 expression, an impact of rAv-TMY on the induction of Treg cells cannot be completely excluded.

4.2.2.5 rAv-TMY influence IgA expression

In allergic airway diseases the antigen is first encountered by Immunoglobulin A at the mucosal surface. IgA was long time thought to have only a “neutralizing” function by excluding the

multitude of dietary, environmental and microbial antigens that bombard the mucosal surface. Meanwhile it has become clear that IgA and its receptor Fc α RI regulate various responses such as degranulation, phagocytosis and cytokine release by granulocytes, monocytes and macrophages in humans [204]. It was therefore of interest to analyze if rAv-TMY would alter the IgA expression and secretion into the BALF respectively. Total IgA secretion into the BALF was significantly reduced in rAv-TMY treated animals compared to OVA alone treated whereas the sera levels of IgA were the same in all groups (data not shown). The role of serum IgA antibodies is mostly unknown but it was proposed that the role of circulating IgA is to keep the immune system under tight steady state control [205]. Therefore, one would expect serum IgA expression to be unchanged in mucosal associated diseases such as asthma. Concerning secretory IgA (sIgA) there exist conflicting data. sIgA is the most abundant immunoglobulin in mucosal secretions with a variety of five receptors expressed for example on eosinophils [204]. Schwarze and colleagues found an improvement of allergic symptoms (reduced AHR, eosinophil infiltration, IL-4 & IL-5 and restored IFN- γ) in a ragweed model of allergic airway disease when they administered specific ragweed (Amb a I) IgA in the airways i.e. high levels of sIgA correlated with abatement of symptoms [206]. In contrast it was reported that in an OVA induced asthma model AHR, eosinophilic infiltration and Th2 cytokine production was accompanied with a strong increase in total IgA in the BALF as well as in the supernatant of *ex vivo* mononuclear cells of the lung [207]. Along the same line a more recent report showed that IgA induce degranulation of eosinophils more potently than IgE indicating an underestimated role of IgA antibodies in eosinophil activation [208]. Notably IgA was also found to have regulatory functions. Woerly et al. could induce IL-10 secretion from human blood eosinophils when sIgA immune complexes were present and IL-2/IFN- γ secretion by targeting CD28 on eosinophils. Combining the IgA and CD28 stimulus abrogated the secretion of all three cytokines [209]. Furthermore it was shown that monomeric targeting of the Fc α RI ameliorated allergic response in an *in vivo* asthma model and reduced IgE/Fc ϵ RI mediated histamine release by RBL cells *in vitro*. In contrast, cross linking of Fc α RI led to activation and histamine release [209]. The dual function of the Fc α RI was confirmed by Kanamaru et. al showing that monomeric targeting of Fc α RI trigger a “low-density” signal leading to cell desensitization whereas multimerization gives a “high-density” signal and activates cells [205]. These interesting findings implicate that low concentration of multivalent

slgA induce desensitization and that high concentrations of specific slgA might form immune complexes, induce activation and degranulation of eosinophils respectively. Nonetheless, most studies were done with human blood eosinophils which might substantially differ from activated airway eosinophils in regard to receptor expression. This might lead to the following assumption concerning rAv-TMY treatment in this study:

1. IgA levels reduced by rAv-TMY treatment lead to reduced degranulation of eosinophils
2. Low concentration of rAv-TMY and OVA specific (multivalent) slgA result in a desensitization signal in eosinophils

These conclusions, however, have a weakness as mouse eosinophils do not express Fc α RI, the receptor that is thought to account for most effects in humans. Decot and colleagues found only transferrin receptor 1 (TfR-1) expressed on the surface of mouse eosinophils and the Fc α / μ receptor at the transcriptional level [210]. So far not much is known about the specific interplay between TfR and IgA and the regulatory potential. Arguments for a regulatory role of TfR or unknown IgA receptors in mice, similar to the role as Fc α RI in humans, are provided by the studies of Schwarze and Schneider how both used a mouse model [206,207]. Furthermore, the existence of putative IgA receptors in mice leading to IgA mediated signaling are proposed [204,210,211,212].

4.2.3 Model of rAv-TMY immune modulation

The above discussed effects of rAv-TMY suggest the induction of a modified Th2 response by rAv-TMY [53,93,158]. Such a modified Th2 response is characterized by differential expression of IgE and IgG4, IL-4 / IL-10 production, down regulated IL-5 responses and subsequently reduced involvement of eosinophils. A modified Th2 response was also reported to be protective against OVA induced airway inflammation in schistosoma infected mice. This was a specifically localized pulmonary effect with an “intact” systemic OVA-specific response (IL-4, IL-5, IL-13 response by spleen, sera IgE, IgG). Fallon and Mangan suggested a compartmentalization of immunity allowing a modified Th2 response in the lung with a concurrent “normal” systemic response [158]. The treatment with rAv-TMY might also create compartmentalization of the immune reaction between lung and systemic effects.

The combined expression of IL-4 and IL-10 as well as the modified antibody response involves several implications on mast cell effector functions and T-cell priming (extracted from human and mouse studies):

3. IL-4 and IL-10 have competitive functions regarding IgE expression. IL-4 induce IgE expression whereas IL-10 diminishes it and favor's a switch to IgG4 [213]
4. Both cytokines inhibit the FcεRI expression on mast cells an basophils [154,214]
5. As the increase in FcεRI expression depends on high IgE levels, an environment leading to low IgE supports FcεRI suppression [154,214]
6. Reduced IgE mediated antigen uptake can lead to lowered antigen presentation by APCs (B-cells, DC, macrophages, monocytes) thereby decreasing sensitivity for the antigen by 100- to 1000-fold. Subsequently the production of IL-4, IL-5, recruitment of eosinophils and allergen specific T-cell response are impaired [169].
7. High levels of IgG4 leads to cross linking of the inhibitory FcγRIIB resulting in reduced degranulation and activation of mast cells, although FcγRIIB is a low affinity receptor and actual effects are questionable [70]
8. Cross linking of FcεRI and FcγRIIB by IgG4 antibodies inactivates mast cells [70]
9. Reduced IgA levels lead to reduced degranulation of eosinophils [215]
10. Low concentration of multivariant IgA results in a desensitization signal in eosinophils [205]

Hence, it becomes evident that the combination of low IgE and high IgG1 level and IL-10 expression attenuates the allergic reaction as seen in animals treated with rAv-TMY. Fig. 24 depicts a hypothetical model of rAv-TMY mediated immune modulation.

The observed effects of treatment with rAv-TMY are similar to what is seen in specific immunotherapy (SIT). Specific allergen immunotherapy with allergen extracts is the only causal treatment with long term effects even after treatment discontinuation [169]. The precise mechanisms underlying SIT are not fully understood yet. But what is observed in humans is the induction of immunoglobulin G production, predominantly IgG4 and IgG1, a blunting of seasonal IgE increase and increased IL-10 and TGF-β production by regulatory T cells. Fur-

thermore the eosinophil, mast cell and basophil infiltration is reduced and a decrease in their activation threshold was found [156,157]. There are several mechanisms proposed for the immune modulation by SIT.

First, it was demonstrated that SIT-induced IgG responses inhibited basophil and mast cell degranulation either by direct inhibition of allergen-IgE interaction or by cross linking of FcγRIIB and FcεRI. Second, blocking antibodies induced by SIT inhibited the IgE-FcεR mediated antigen presentation. These effects lowered the dose needed to activate allergen specific T cells by 100- to 1000 fold. The decline in mediator release by effector cells and T cell activation might allow the immune system to recover a more regulatory or Th1 based immune response. The modulation seen by rAv-TMY treatment, however, seems to mimic effects seen by SIT in a much faster progression. The faster progression is probably due to the immediate induction of IL-10 leading to fast isotype switch in the antibody response.

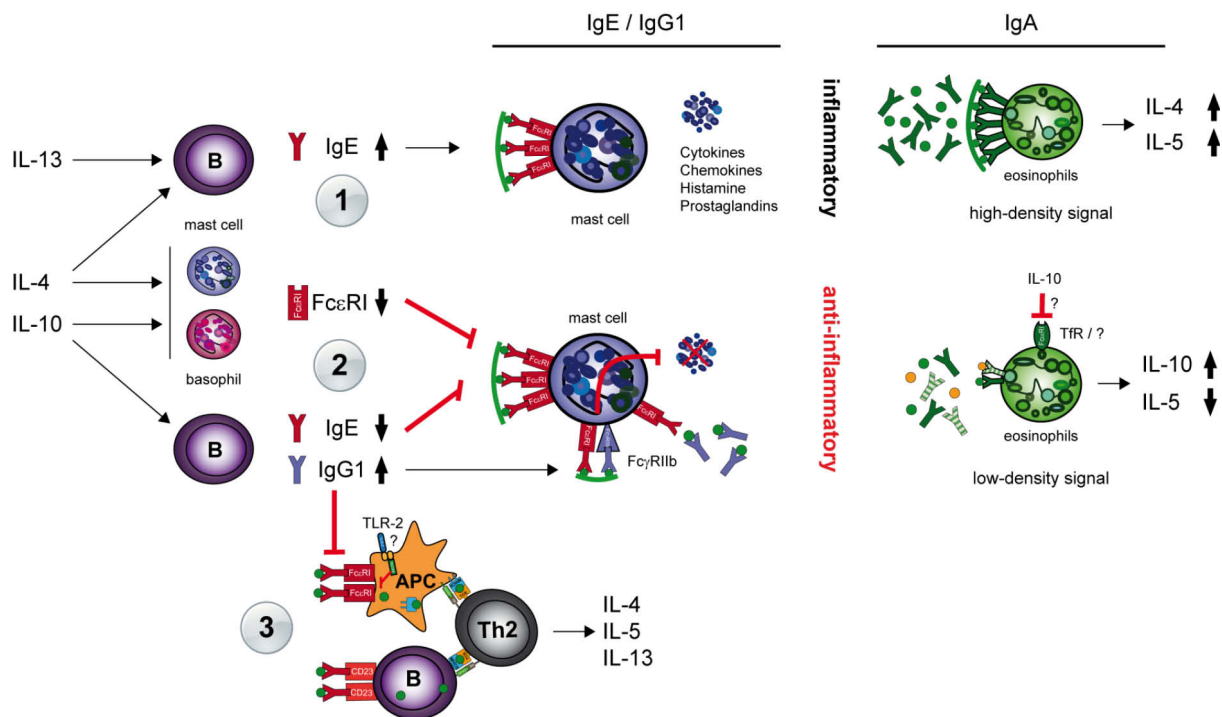


Fig. 24 Potential role of Fc receptors and antibodies in the modulation of allergic airway inflammation by rAv-TMY. ❶ Allergic airway inflammations are characterized by a Th2 dominated environment with high levels of IL-4 and IL-13. The combined action of this cytokines leads to class switch towards IgE producing B cells. High levels of IgE lead to stabilization of FcεRI on mast cells and subsequently to cross linking of FcεRI by antigen specific IgE, resulting in degranulation. High levels of IgA might also contribute to increased IL-4 and IL-5 secretion by eosinophils upon cross-linking of IgA receptors. ❷ Modulation might take place through the combined action of IL-4 and IL-10 induced by rAv-TMY which down regulates FcεRI expression on mast cells and basophils. In addition IL-10 induces the production of blocking IgG1 antibodies. As a result degranulation of mast cells is inhibited by a reduced cross-linking of FcεRI, action of the inhibitory FcγRIIb and antigen dilution by IgG1. As mast cells are crucial for the initiation of inflammation, this mechanism might prevent or diminish antigen induced inflammation. Furthermore, reduced amounts of IgA with different specificities (OVA & rAv-TMY) can lead to a "low-density" signal in eosinophils upon which IL-10 production is increased and IL-5 decreased. IL-10 might also downregulate IgA receptors on eosinophils. As most studies concerning the role of IgA and its receptors are done in the human system, receptors and mechanisms might differ in the murine system. ❸ Moreover rAv-TMY might interfere with the activation of antigen presenting cells. Professional APCs express the high affinity receptor FcεRI and B cells the low-affinity receptor CD23 through which IgE bound antigen is processed for presentation to T cells. IgE mediated antigen presentation can increase sensitivity for the antigen by 100-1000 fold. This process is, however, blocked under conditions with low IgE levels and reduced expression of FcεRI/CD23. It was also shown that TLR-2 on dendritic cells can inhibit the FcεRI expression. B: B cell, APC: antigen presenting cell, TLR-2: toll-like receptor 2, Tfr: Transferring receptor, Th2: T-helper cell 2, MHC class II: Major Histocompatibility Complex II, FcR: fragment crystallizable (region) receptor, Ig: immunoglobulin

4.2.4 rAv-TMY promotes TLR-2 expression on macrophages and their efflux from the peritoneum

Increased TLR-2 expression was found to correlate with decreased susceptibility to asthma [216]. Furthermore, helminth derived products are shown to modulate immunity via TLRs [101,217,218,219,220]. In addition preliminary experiments with rAv-TMY using a TLR-2 reporter cell line revealed that rAv-TMY signals via TLR-2. This findings lead to the speculation that rAv-TMY might also utilize TLR-2 for modulation. To investigate this question rAv-TMY or a control protein were injected into the peritoneum and PEC were isolated 18 hours later for FACS analysis. The analysis revealed a significant efflux of macrophages ($F4/80^{\text{high}}\text{Gr-1}^+$) from the peritoneum. Intriguingly the emigrating macrophage population showed a much higher TLR-2 expression than those of PBS or DHFR treated ones. This hints towards a direct influence of rAv-TMY on macrophages and presumably DCs via TLR-2. A similar effect is described for Lyso-PS, a schistosomal lysophosphatidylserine (Lyso-PS), acting on dendritic cells via TLR-2 and thereby influencing Tregs to produce IL-10. Interestingly, anti-TLR-2 antibody treatment during stimulation of DC with schistosomal PS revealed that the subsequently primed T-cells lose their ability to produce IL-10 but keep their “Th2-phenotype” indicated by a high IL-4 to IFN- γ ratio [218]. Furthermore it was shown that soluble egg antigen of schistosomes suppresses DC activation (MHC class II, CD80, CD86) and IL-12 production in a TLR-2 dependent manner [221]. Other helminth derived products such as ES-62 or dsRNA of schistosome eggs act on TLR-4 and TLR-3 respectively indicating that helminth PAMP recognition by TLRs is a common feature [101,219,220]. TLR-2 is the most promiscuous representative of all TLRs concerning its ligands amongst others lipoteichoic acid of gram-positive bacteria, GPI anchor of *Trypanosoma cruzi* and Lipophosphoglycan of *Leishmania major*. TLR-2 collaborates with TLR-1 /-6 and various other non-TLR PRRs thereby increasing its ligand repertoire and the effector function [222]. The preferential outcome of TLR induction is a Th1 response. Depending on the cell and the engagement of co-receptors TLR stimulation might also lead to a Th2, regulatory or Th17 response [223]. In contrast, TLR-2 stimulation on dendritic cells, macrophages and monocytes preferentially promotes Th2 or regulatory T cell differentiation and induces IL-10 production via the PI-3K-Akt signaling pathway [222]. Recently it has been demonstrated that targeting TLR-2 on human mast cells reduced Fc ϵ RI expression and de-

granulation of *ex vivo* pulmonary mast cells. These effects were specific for TLR-2 and FcεRI but varied between different TLR-2 ligands [224]. Furthermore, stimulation of human mast cells via TLR-2 resulted in IL-10 release and IL-10 reduced the expression of FcεRI on mouse mast cells [214,225]. Hence, a TLR-2 mediated suppression of FcεRI expression on mast cells might be a further explanation how rAv-TMY account for the reduced allergic responses although it might be questionable if the molecule injected into the peritoneum actually reaches the airways. The mechanisms how rAv-TMY might act on TLR-2, if co-receptors are involved, which APC types are targeted and if and how APCs might trigger IL-10 production by T-cells have to be investigated.

5 Summary / Outlook

This study illustrates the limits of RNAi as method for functional gene studies in the parasitic nematode *H. p. bakeri* and, taking the database analysis into account, most probably also in other parasitic strongylids. The development of an invasive delivery method for dsRNA/siRNA and/or *sid-1/sid-2* transgenic parasites might overcome these limits.

Using an *in vivo* model of allergic airway inflammation it could be demonstrated that the muscle protein tropomyosin of *A. viteae* clearly modifies the immune response to an unrelated allergen. Taken together rAv-TMY seems to desensitize the immune system for aeroallergens. This is most likely due to a combined effect of reduced allergenicity of rAv-TMY and the induction of IL-10 leading to a modified Th2 response. However, treatment with rAv-TMY rather ameliorates then abrogates the symptoms of asthma and there are several questions still open. Does a long term treatment with rAv-TMY would increase the observed effects? Does rAv-TMY lead to the same outcome in an invertebrate tropomyosin induced inflammation model? How are local and systemic effects linked? What are the target cells and the signaling pathway leading to the IL-10 production? Nevertheless, despite the many open questions rAv-TMY might be an attractive desensitization therapeutic. In SIT recombinant antigens which have reduced IgE binding capacities or allergoids lacking IgE binding sides but keep their T cell responsiveness are currently under investigation [157]. Helminth derived molecules might reflect this kind of reagents in a natural manner. Furthermore, the comparison of allergenic, non-allergenic and modulatory tropomyosin might put some light on the nature of allergens and their molecular patterns.

6 Materials and methods:

6.1 Animal models

6.1.1 Animals used for experiments

For asthma experiments female BALB/c mice (Ol1a-Hsd) were purchased from Harlan Winkelmann (Borchen, Germany) or used from the own breeding facility, (HU Berlin) respectively. For asthma experiments mice were kept in individually ventilated cages, for other experiments animals were housed in standard cages. All mice were housed and handled following national guidelines and as approved by the animal ethics committee.

6.1.2 ... in RNAi experiments

6.1.2.1 Feeding of *C. elegans*

Feeding of the nematodes was performed as described before [41]. The worms were allowed to feed for 3 days on recombinant bacteria. Subsequently three hermaphrodites were transferred each to an individual well. Each hermaphrodite was allowed to lay eggs for 24 h and removed afterwards. The resulting progeny was analyzed with regard to phenotypical changes and the total number of worms was determined after three days.

6.1.3 ... in Asthma experiments

6.1.3.1 Model of murine airway inflammation

Female BALB/c mice (Harlan-Winkelmann, Bachem, Germany) were sensitized twice (day 0 and day 14) intraperitoneally with 20 µg OVA (grade VI, Sigma-Aldrich, Steinheim, Germany) emulsified in 2 mg of aluminum hydroxide (Imject[®] Alum, Pierce, Rockford, USA). On days 28 and 29, mice were challenged intranasally with 50 µg OVA (25 µl per nostril). Within the model 20 µg recombinant *A. viteae* tropomyosin diluted in a total volume of 200 µl low-endotoxin PBS or the same amount of control protein DHFR was injected intraperitoneally four times in weekly intervals during the sensitization (see also p. 33 Fig. 11). Naive control animals were treated with PBS in aluminum hydroxide intraperitoneally and challenged with PBS intranasally.

Two days after challenge mice were sacrificed and the following analyses were carried out:

- lavage of the lung to assess BALF and cell numbers and types pointing to inflammation
- removal of spleen to perform cell culture
- removal of peribronchial lymph nodes (PBLN) to perform FACS analysis
- cryoconservation of parts of the lung to perform Real-Time-PCR
- preparation of lungs for histological analysis (formalin)
- assessment of blood to monitor antibody production

6.1.3.2 Bronchoalveolar lavage (BAL)

Animals were sacrificed by cervical dislocation and lungs were lavaged twice using a tracheal drain tube with 0.8 ml of cold PBS/EDTA (proteinase inhibitor tablets, completeTM Mini, Roche). The volumes of the lavages was measured by weighing and after centrifugation (10 min, 2.000 rpm, 4 °C) supernatants of the first lavages were stored at -20 °C for cytokine analyses. The cell pellets of both lavages were resuspended in a total of 1 ml PBS, cell numbers were counted and 100 µl of the cell suspensions was used to prepare cytopspin slides. Cytopspin slides were stained with DiffQuick (Dade Behring AG) and bronchoalveolar lavage (BAL) cells were differentiated by morphological criteria (count of 200 cells under light microscopy), as previously described by Blumchen [226].

6.1.3.3 Histological analysis of lung

Lungs were collected in 3.5 % formalin to be embedded in paraffin later on. Slices of 5 µm were cut and afterwards stained with HE (hematoxylin/eosin) to monitor cells types and infiltration in lung tissue or stained with PAS to visualize mucus production in bronchioles [227]. Slides were analyzed regarding bronchioles and their surrounding by light microscopy with a 40- and 400- fold magnification.

6.2 Molecular biology and biochemistry

6.2.1 ... in RNAi experiments

6.2.1.1 Feeding - Plasmid preparation and transformation

For the RNAi experiments an 855bp cDNA fragment of *H. p. bakeri* encoding tropomyosin (Hp-tmy) was cloned into pGEM-T Easy vector (Promega, Mannheim, Germany). The sequence was analyzed with regard to its GC content to find areas with an increased chance to produce 21 nt fragments by DICER [228]. The full length sequence (designated as *cHp-tmy*) and a 165bp part of the 5' end (designated as *Hp-tmy-1*) of tropomyosin were subcloned into the L4440 vector and expressed in the Escherichia coli strain HT115 (DE3) as described elsewhere [20,229,230]. As control we used the pLT61.1 vector (addgene, Fire Lab *C. elegans* Vector Kit, USA) carrying the *Ce-unc-22* gene encoding for twitchin. *Ce-unc-22* dsRNA results in a clear phenotype in *C. elegans* (positive control) [20,231] and no phenotype in *H. p. bakeri*. As negative control the non-coding maleE part of the maltose binding protein (MBP) was used.

6.2.1.2 Soaking - Target design, plasmid preparation and in vitro transcription

Soaking is described to work most efficiently with sequences of 100 bp to 500 bp [16]. Therefore, *Hp-tmy-1* (bp 1-165) and *Hp-tmy-2* (bp 582-855) were cloned into the L4440 vector between two flanking T7 promoters. The target sequence was amplified with T7 primers and the resulting fragment gel purified. For *in vitro* transcription of dsRNA the MEGAScript RNAi Kit (Ambion, Austin, USA) was used according to the manufacturer's instructions with the following modifications. A single step PCR was used for amplification at 37°C for 4h. After DNA/RNA digestion the dsRNA was purified by ethanol precipitation and dissolved in RNase free water. Purity and integrity was regularly checked on a standard agarose gel. Concentration of dsRNA was measured at 260 nm using a NanoDrop1100 (NanoDrop Technologies, Wilmington, USA) photometer and stored at -20°C until usage.

To analyze its uptake *Hp-tmy-1* dsRNA was labeled with the Cy3 dye using the Silencer® siRNA Labeling Kit (Ambion, Austin, USA) according to manufacturer's instructions.

6.2.1.3 RNA extraction from *H. p. bakeri*

All RNA extractions were performed with the RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the following changes. Adult worms were transferred into Qiagen RNeasy lysis buffer and stored at -80°C. After thawing the supernatant was discharged and 50 µl RLT-β-mercaptoethanol lysis buffer (RLT, Qiagen, Hilden, Germany; β-ME Sigma-Aldrich, Munich, Germany) was added. The worms were squelched and frozen in liquid nitrogen, 150 µl RLT-β-ME was added and eventually remaining worms were ground again. 400 µl RLT-β-ME was used to wash the tube and the suspension was homogenized using QIAshredder columns (Qiagen, Hilden, Germany). The remaining steps were performed following the manufacturer's instructions including a 15 min DNA digestion and RNA concentration determined by photometry on a NanoDrop1100.

6.2.1.4 cDNA synthesis

RNA was reverse transcribed using the TaqMan® Reverse Transcription Reagent (Applied Biosystems, Darmstadt, Germany). The same amount of total RNA for each approach was applied to adjusted reaction volumes. Buffer and cycling conditions were used according to the manufacturer's instructions with oligo hexamers as primers.

6.2.1.5 qPCR TaqMan® relative gene expression assay for RNAi experiments

The sequence of Hp-tmy cDNA was aligned against the respective *C. elegans* genomic sequence to determine the putative exon-exon borders to prevent amplification of genomic DNA. Primers and probes (Tab. 2) were designed with the PrimerExpress® software (Applied Biosystems, Darmstadt, Germany) spanning a putative exon-exon region.

Tab. 2 Primers and probes used in the qPCR TagMan® assay

Target Gene	Accession No.	Primer / Probe Name	Sequence (5' – 3')
<i>Hp-18s</i>	AY542283	f: HP_18S-T2F	GCACGCGCGCTACAAT
		r: HP_18S-T2R	AACGGTTTACCAATGCCTTTCG
		p: HP_18S-T2M2	FAM-CCAGCTGATTCTTCC-NFQ
<i>Hp-tmy</i>	EU131541	f: HP_TROPO-T3F	GCCAACTGGAGGAGAAAGACAAG
		r: HP_TROPO-T3R	GGTTCAGGGCAGCTACCT
		p: HP_TROPO-T3M2	FAM-TCAGCCTCCTGGACTTT-NFQ

To prevent amplification of *Hp-tmy* template DNA the primers were located between *Hp-tmy-1* and *Hp-tmy-2* (p. 24, Fig. 5). 18s RNA of *H. p. bakeri* served as endogenous control. Amplification efficiency of the primers was tested by a standard curve assay. Linear regression analysis showed similar slopes for all tested primers (Tab. 3).

Tab. 3 Slope and coefficient of determination (R^2) of the standard curve assay; A slope of -3,32 represents a PCR reaction of 100% efficiency.

Primer	Slope	R^2
<i>Hp-tmy</i>	-3,1392	0,9944
<i>Hp-18s</i>	-3,0686	0,9919

The Real-Time PCR was performed on an AB7000 (Applied Biosystems, Darmstadt, Germany) using the conditions as presented in Tab. 4. Values were normalized to *Hp-18s* RNA as endogenous control. Relative gene expression was calculated using the $-\Delta\Delta C_t$ formula [232].

Tab. 4 Reaction and cycling conditions used for a 20 μ l qPCR TaqMan® approach

Gene Target	Primer [nM]	Probe [nM]	cDNA 1:10 [μ l]	Cycling Conditions
<i>Hp-18s</i>	900	250	1	10 min @ 94°C; 40x (15 sec @ 95°C, 60 sec @ 60°C)
<i>Hp-tmy</i>	900	250	6	10 min @ 94°C; 40x (15 sec @ 95°C, 60 sec @ 60°C)

6.2.2 ... in Asthma experiments

6.2.2.1 Plasmid preparation and transformation

For Asthma experiments Av-TMY was cloned into a pQE30 expression vector (Qiagen, Hilden, Germany) and expressed in *E. coli* XL-1 blue as described elsewhere [15].

6.2.2.2 RNA extraction from lung tissue

For RNA preparation tissues were frozen in liquid nitrogen and then ground with ice-cold mortar and pestle. The powdered material was then treated according to manufacturer's in-

structions of RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by digestion of DNA using the RNase-free DNase-Set (Qiagen) according to the manufacturer's instructions.

6.2.2.3 *cDNA synthesis*

RNA was reverse transcribed using the TaqMan® Reverse Transcription Reagent (Applied Biosystems, Darmstadt, Germany). The same amount of total RNA for each approach was applied to adjusted reaction volumes. Buffer and cycling conditions were used according to the manufacturer's instructions with oligo hexamers as primers.

6.2.2.4 *SYBR green relative gene expression assay for asthma experiments*

The sequence for the Primer were obtained from Primer Bank (see Tab. 5) and validated in a melting curve assay for specificity [233,234]. Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH), β -actin, peptidylprolyl isomerase A (PPIA) and hypoxanthine guanine phosphoribosyl transferase (HPRT) served as endogenous control. The Real-Time PCR was performed on an AB7000 (Applied Biosystems, Darmstadt, Germany) using FastStart universal SYBR green master mix (Roche 04913850001) and the conditions as presented in Tab. 6. Values were normalized to endogenous control RNA and relative gene expression was calculated using StatMiner software.

Tab. 5 Primers used in the qPCR SYBR green assay. Primers were obtained from the Primer Bank database (<http://pga.mgh.harvard.edu/primerbank/index.html>); f: forward, r: reverse

Target Gene	PrimerBank ID	Primer name	Sequence (5' – 3')
<i>CCL2</i>	6755430a1	f: CCL2_fw	TTAAAAACCTGGATCGGAACCAA
		r: CCL2_rv	GCATTAGCTTCAGATTACGGGT
<i>CCL5</i>	7305461a1	f: CCL5_fw	GCTGCTTGCCTACCTCTCC
		r: CCL5_rv	TCGAGTGACAAACACGACTGC
<i>CCL11</i>	6755418a1	f: CCL11_fw	GAATCACCAACAACAGATGCAC
		r: CCL11_rv	ATCCTGGACCCACTTCTTCTT
<i>CCL12</i>	6755420a1	f: CCL12_fw	ATTTCACACTTCTATGCCTCCT
		r: CCL12_rv	ATCCAGTATGGTCTGAAGATCA
<i>CCL24</i>	9625035a1	f: CCL24_fw	ATTCTGTGACCATCCCCTCAT
		r: CCL24_rv	TGTATGTGCCTCTGAACCCAC
<i>CCR3</i>	31542354a1	f: CCR_fw	TCAACTTGGCAATTCTGACCT
		r: CCR_rv	CAGCATGGACGATAGCCAGG
<i>VCAM-1</i>	31981430a1	f: VCAM1_fw	AGTTGGGGATTGCGTTGTTCT
		r: VCAM1_rv	CCCCTCATTCCTTACCACCC
<i>GAPDH</i>	6679937a1	f: GAPDH_fw	AGGTCGGTGTGAACGGATTTG
		r: GAPDH_rv	TGTAGACCATGTAGTTGAGGTCA
<i>PPIA</i>	6679439a1	f: PPIA_fw	GAGCTGTTTGCAGACAAAGTTC
		r: PPIA_rv	CCCTGGCACATGAATCCTGG
<i>HPRT</i>	7305155a1	f: HPRT_fw	TCAGTCAACGGGGACATAAA
		r: HPRT_rv	GGGGCTGTACTGCTTAACCAG
<i>β-actin</i>	6671509a1	f: bAct_fw	GGCTGTATTCCCCTCCATCG
		r: bAct_rv	CCAGTTGGTAACAATGCCATGT

Tab. 6 Reaction and cycling conditions used for a 20 µl qPCR SYBR green approach

Primer	PCR-Mix	cDNA 1:10	Cycling Conditions
[nM]	[µl]	[ng / µl]	
			10 min @ 95°C
300	10	1,25	40x (15 sec @ 95°C, 60 sec @ 60°C)
			1x (15 sec @ 95°C, 60 sec @ 60°C, 15 sec @ 95°C)

6.2.2.5 Purification of recombinant Av-TMY

A pQE30::Av-TMY clone was incubated overnight at 37°C in 30 ml LB medium containing 100 µg/ml Ampilicin and 20 µg/ml tetracyclin. 10 ml of overnight culture was used to start large scale cultures (300-500 ml), in which expression of the protein was induced by addition of 1 mM IPTG isopropyl β-D-thiogalactoside for 3 h when cultures reached an optical density (OD₆₀₀) of 0.6. After centrifugation (4 °C, 6,000 rpm, 15 min) pellets were resuspended in 20-30 ml lysis buffer A1 containing lysozym and incubated 30 min on ice while constantly shaking. Lysates were sonificated (2x 80 sec., 20 %) and centrifuged again (4 °C, 12,000 rpm, 15 min) to get protein containing supernatants. Supernatant was loaded on HiTrap FF columns of a FPLC (fast performance liquid chromatography). rAv-TMY was bound to the columns via the attached His-tag followed by a washing step with buffer A2 and a fractionized elution with buffer B. Fractions were loaded on a SDS-PAGE to determine expression, purity and size of purified proteins. Fractions with highest protein concentration and purity were pooled and used for endotoxin removal. After endotoxin removal flow through was dialyzed against low endotoxin PBS using centrifuge concentration units to an approximate concentration of 200 µg/ml. Actual concentrations was measured in a bicinchoninic acid test and rAv-TMY stored at -20°C.

6.2.2.6 Endotoxin removal by EndoTrap

To remove endotoxin contaminations from rAv-TMY purifications, EndoTrap blue columns were used according to the manufacturer's instructions (Profos, Regensburg, Germany) with following modifications: columns were equilibrated with buffer B and both buffer B and Protein solution were substituted with 100 µM Ca²⁺.

For rDHFR decontamination EndoTrap red columns were used according to the manufacturer's instructions (Profos, Regensburg, Germany).

6.2.2.7 *Limulus amoebocyte test (endotoxin measurement)*

Final endotoxin concentrations were detected by limulus amoebocyte lysate (LAL) test (Cambrex, Bio Sciences, Walkersville, USA) according to manufacturer's instructions.

6.2.2.8 *Quantification of protein*

Protein concentrations were measured with the bicinchoninic acid test using BCA protein assay kit (Pierce) according to manufacturer's instructions (Pierce, USA).

6.2.2.9 *SDS-PAGE (sodium-dodecyl-sulfate polyacrylamid gel electrophoresis) and coomassie staining*

SDS-PAGE was used to determine expression, purity and size of induced proteins. SDS-PAGE separates proteins according to their size. Differences in polarization are mainly eradicated by denaturation with β -mercaptoethanol and heat and binding of anionic SDS to the proteins in constant ratios. According to the expected size of the wanted protein separation gels of 10-14 % polyacrylamid were used, always with a 6 % stacking gel. Gels were run at 120V/400mA. To visualize proteins gels were stained 30 min at 60 °C with coomassie staining solution. Unbound coomassie was washed away with de-staining solution.

6.3 Parasitological methods

6.3.1 Parasite maintenance

6.3.1.1 *Life cycle of H. p. bakeri*

H. p. bakeri was maintained by serial passage in BALB/c mice. Mice were infected with approx. 200 L3 using a feeding tube. Infective larvae were obtained from fecal cultures. Feces were collected from infected animals, washed in distilled water and plated in Petri dishes on humid blotting paper for 7 days. On day 7, L3 were washed from the plate and kept in distilled water at 4 °C after extensive washing. L3 were used for infection up to 8 weeks after fecal culture.

6.3.1.2 Life cycle of *A. viteae*

The life cycle of *A. viteae* was maintained essentially as described by Lucius and Textor [235]. Briefly, the infective L3 stages of *A. viteae* were obtained from infected *Ornithodoros moubata* ticks and used to infect *Meriones unguiculatus* subcutaneously with app. 70 L3. The L3s develop to the L4 and subsequently to the adult male and female stages. The female worms release microfilariae into the peripheral blood. The peripheral blood of infected *Meriones* was used to infect ticks where the microfilariae develop to the L2 and subsequently to the infective L3 stages.

6.3.2 ... in RNAi experiments

6.3.2.1 *E. coli* HT115 as food source

To prevent an excessive grow of bacteria *H. p. bakeri* larvae are usually fed on the uracil auxotroph *E. coli* strain OP50 preventing an overgrow of the larvae. To test the *E. coli* strain HT115 as food source 3400 *H. p. bakeri* eggs were seeded on HT115 or OP50 lawns on NGM agar plates [236]. The proportion of larvae that had developed to L3 was calculated in relation to the applied egg number after six days.

6.3.2.2 Preparation of eggs and feeding of *H. p. bakeri*

Feces from *H. p. bakeri* infected BALB/c mice were collected for a time period of 8-12 h. *H. p. bakeri* eggs were obtained by flotation with saturated sodium chloride solution [237]. Bacteria and fungi were removed by treatment with bleaching solution (1,67 M NaOH + 3,34% NaOCl, added to a final concentration of 30% (v/v) for 10 min) and eggs were washed twice with M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 17 mM NaCl, 1 mM MgSO₄) supplemented with tetracyclin (20 µg/ml), ampicillin (100 µg/ml) and counted using a MacMaster chamber [237].

Bacterial lawns of recombinant HT115 on NGM agar containing tetracyclin (20 µg/ml), ampicillin (100 µg/ml) and 1 mM IPTG were prepared in 12 well plates and allowed to express dsRNA for 12 h at RT. Subsequently, each well was seeded with 1000 *H. p. bakeri* eggs and left at RT for 7 days to finally determine the proportion of eggs that had developed to viable L3.

6.3.2.3 Feeding on bacteria expressing the green fluorescent protein (*gfp*)

Recombinant *E. coli* BL21 containing the pET28b-EGFP plasmid were grown in LB medium with kanamycin (100 µg/ml) and seeded on NGM plates containing kanamycin (100 µg/ml) and 1mM IPTG. After 12 h of induction eggs of *C. elegans* or *H. p. bakeri* were transferred to the plates. Hatched L1 larvae were examined under a fluorescent invert microscope for up-take of bacteria.

6.3.2.4 Electroporation of adult worms and larvae

For electroporation a BTX ECM830 square wave electroporator with a 2 mm cuvette and a commercial electroporation buffer (Ambion, Austin, USA) as well as a trehalose buffer (272 mM trehalose, 7 mM KH₂PO₄ pH 6, 1 mM MgSO₄) [37] was used for transfection with Cy3-siRNA (Ambion, Austin, USA). Conditions for voltage and pulse time are presented in Tab. 7.

Tab. 7 Electroporation conditions

	No.	Voltage [V]	Time [ms]	Buffer
Larvae	500	100-1000	0,1 / 1 / 5 /10 / 15 / 20	Trehalose
	500	500	3,5 / 5,4 / 8,9 / 10 / 30 / 50	Ambion / Trehalose
Adults	10-40	100-3000	0,1 / 1 / 5 /10 / 20 /30 / 40	Trehalose

6.3.2.5 Soaking of *H. p. bakeri* adult worms

Adult worms were recovered from the small intestine of *H. p. bakeri*-infected BALB/c mice and washed several times in 0,9% NaCl solution followed by an incubation in RPMI 1640 (Gibco, Invitrogen, Karlsruhe, Germany), supplemented with 1% penicillin/streptomycin at 37°C for 1-2 h. Subsequently, 20 adult worms each were kept in 260 µl RPMI 1640 (w/o FCS) supplemented with 1% penicillin/streptomycin, 8 U RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen, Karlsruhe, Germany) and 0,5 µM of *Hp-tmy-1* Cy3-dsRNA and incubated

at 37°C under 5% CO₂ for 18 h. Worms were washed with prewarmed RPMI 1640 and analyzed for dsRNA uptake using a fluorescent microscope.

The knock down experiments were carried out as stated above with six groups each consisting of two males and three females kept in 50 µl RPMI supplemented with 2 µM of the respective dsRNA. After 24 h or 6 days of dsRNA soaking, the adult worms were examined with regard to morphological changes by light microscopy. The tropomyosin mRNA level was determined by qPCR. Alternatively adults were soaked in dsRNA as described and subsequently kept for further 48 h in dsRNA free medium

6.4 Immunological methods

6.4.1 Enzyme-linked immunosorbent assay (ELISA)

6.4.1.1 Serum levels of IgE (total and OVA-IgE), OVA-IgG1, OVA-IgG2a

Total IgE levels were measured by sandwich ELISA using anti-IgE antibody as catcher, 3% BSA in PBS to block unspecific binding, dilutions of sera (1:10, 1:100, 1:1000, 1:10000 in blocking buffer) containing induced antibodies and anti-IgE antibody coupled to Biotin as detection antibody. HRP (horseradish peroxidase) labeled streptavidin was applied in a 1:10000 dilution to detect Biotin-labeled antibodies and binding was visualized by application of the HRP-substrate TMB. After stopping the reaction with 1M H₂SO₄ plates were measured at 450/630 nm in a plate reader. IgE-standard solution was analyzed in accordance to samples to calculate IgE amounts.

OVA-specific antibodies were measured in an analogical. After application of diluted samples 3 µg/ml biotinylated ovalbumin was used to detect exclusively OVA-specific IgE antibodies. To calculate amounts, purchasable IgG1-standards or IgE-and IgG2a standard sera were used, respectively. OVA-specific IgE and IgG2a are therefore calculated as lab units (LU) and not in µg [238].

6.4.1.2 Serum levels of IgG subclasses specific for OVA or rAv-TMY

200 ng/well of the referring protein was coated in carbonate buffer overnight and plates were blocked with 3 % BSA / TBS. Sera were applied in 1:50 dilution (in 1 % BSA / TBS) and antibody subclasses were detected by use of subclass-specific AP (alkaline phosphatase)-

labeled detection antibodies and AP-substrate (0,1 mg/ml p-Nitrophenylphosphat, 1 mM MgCl₂ in carbonate buffer). Reaction was abrogated with EDTA and measured at 405 / 630 nm in ELISA reader.

6.4.1.3 Serum / BALF levels of total IgA

96 well plates were coated with 50 µl anti-IgA antibody catcher in carbonate buffer over night at 4°C. Wells were blocked with 200 µl of 3% BSA in TBS for 30 min at RT. 50 µl of standard, sera or BALF per well was incubated for 2h at RT followed by an incubation with 50 µl of AP labeled detection antibody for 1h at RT. 50 µl substrate (0,1 mg/ml p-Nitrophenylphosphat, 1 mM MgCl₂ in carbonate buffer) were added and incubated for 30 min at 37°C. Reaction was abrogated with EDTA and measured at 405 / 630 nm in ELISA reader.

6.4.1.4 Cytokine detection

IL-4, IL-5 and IL-10 measurements were performed using OptEIA ELISA kits according to manufacturer's instructions, but using 50 µl of samples and standards / well. IL-13 was analyzed by R&D IL-13 ELISA kit also according to manufacturer's instructions, but using 50 µl of samples and standards / well.

6.4.2 Cytometric Bead Array

In several experiments cytokine bead arrays were performed to analyze IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, IFN- γ , TNF- α simultaneously according to manufacturer's instructions (BD™ Cytometric Bead Array Flex Set) but using 10 µl of capture beads and detection reagent. Briefly, Sera or BALF of mice were incubated with cytokine specific antibodies bound to beads with a distinct fluorescent intensity for one hour in 96 well plates. PE labeled cytokine specific antibodies were added and incubated for another hour. Plates were centrifuged and supernatant discarded. After one washing step beads were resuspended in wash buffer and analyzed by flow cytometry. Bead-populations were discriminated by the FL3 and FL4 channels and cytokine concentration calculated by the mean fluorescent intensity of the PE channel according to the standard curve.

6.4.3 Flow cytometric analysis

Surface staining of lymphocytes using mAb was performed in PBS / 0.2 % BSA / 5 mM EDTA / pH 7,4 on ice and in the dark for 15 min. Cell suspensions (1×10^6 total cells) were washed in PBS/BSA and stained with α CD4, α CD25 and α CD103 to detected Treg cells.

Macrophages in peritoneal exudate cells (PEC) were stained for α CD11b-FITC, α F4/80-Cy5 and α Gr-1-biotin/SA-PE-Cy7.

TLR-2 expression on macrophages was measured by labeling PECs with α F4/80-Cy5 and α TLR-2-PE. Unspecific binding of the mAbs was blocked by the addition of α Fc γ RII/III (20 μ g/ml). Cytometric analysis was performed using FACS Calibur or LSRII and FlowJo software.

6.5 Cell culture techniques

6.5.1 Preparation of splenocytes and peribronchial lymph node cells (PBLNC)

PBLN and spleen were isolated aseptically from euthanized mice. Organs were dissociated bypassing them through a steel mesh in PBS pH 7.4 containing 0.2% BSA to obtain single cell suspensions. Erythrocytes were removed by resuspension of washed cells in erythrocyte lysis buffer for 5 min on ice. After washing, cells were adjusted to desired concentrations in cRPMI for culture or PBS/BSA for surface stainings and flow cytometry analysis. In the asthma model spleen mononuclear cells were isolated by density gradient centrifugation (Lympholyte-M, Cedarlane Laboratories, Hornby, Ontario, Canada). Cells were cultured with a density of 1×10^6 cells/well in RPMI 1640 (10 % FCS, Pen/strep, L-Glu and fungi) for 96 hours in the presence of 10 μ g/ml OVA, 10 μ g/ml rAv-TMY, 10 μ g/ml DHFR or 2,5 μ g/ml concanavalin A (ConA). Cell culture supernatants were stored at -20 °C until performance of cytokine ELISA.

6.5.2 Peritoneal lavage and preparation of peritoneal exudate cells (PEC)

To obtain PEC the peritoneal cavity of sacrificed mice was washed 4 to 5 times with 2 ml ice-cold RPMI. Cells were centrifuged and lysis of erythrocytes was performed. After counting cells were centrifuged and resuspended in PBS / 0,2% BSA for FACS staining.

6.6 Statistics

Statistics were performed using analysis of variance (ANOVA) to prove significant differences between more than two groups. ANOVA was followed by a post hoc test to prove significant differences between single groups (Dunn's or Mann-Whitney-Test). Post hoc test was only performed if ANOVA resulted in $p \leq 0.05$. Differences between individual groups were tested to be significant for $p < 0.05$, $p < 0.01$ or $p < 0.001$. Statistics were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

6.7 Database analysis

The sequences of 18 *C. elegans* proteins involved in RNAi were used to search for orthologs in different databases using a BLAST. Sequences were aligned against the EST database of *H. contortus* (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus, assembled contigs 27 / 01 / 06, TBLASTN, low complexity filter), the whole genome shotgun (WGS) database of *B. malayi* (<http://tigrblast.tigr.org/er-blast/index.cgi?project=bma1>; tblastn, default settings) and the genome assembly database version 3 (GAV3) of *Schistosoma mansoni* (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni, TBLASTN, low complexity filter). Sequences with a p-value below e^{-5} were considered as homologous. Homologous sequences were subsequently used to search for conserved domains in the conserved domain database (CDD) available on NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, threshold 0.01, low complexity filter) that matches known domains of the blasted proteins.

6.8 Material

6.8.1 Laboratory equipment

Flow cytometer:	LSRII BD Biosystems, San Jose, CA
ELISA-reader	Dynatech, Denkendorf, Germany BioTek, Vermont, USA
Ultrasound-disintegrator	Heinemann, Schw. Gmünd, Germany
Real-time PCR system	7300 Applied Biosystems, Foster City, CA
Cytocentrifuge (cytospin)	ThermoShandon, Frankfurt, Germany
Square wave electroporator	BTX ECM830, BTX, Holliston, USA
FPLC	ÄKTA (UPC-900, P-920, Frac 900), Amersham Pharmacia Biotech, Uppsala, Sweden

6.8.2 Buffers and media

Bleaching solution	1,67 M NaOH 3,34% NaOCl Added to final concentration of 30% v/v
M9 buffer	22 mM KH_2PO_4 42 mM Na_2HPO_4 17 mM NaCl 1 mM MgSO_4
Electroporation buffer	Ambion, Austin, USA
Trehalose buffer	272 mM trehalose 7 mM KH_2PO_4 (pH 6) 1 mM MgSO_4
Coomassie	20% EtOH 10% acetic acid 2 tabl. PhastGel BlueR (Amersham Pharmacia Biotech, Uppsala, Sweden) Add to 1 l dest. Water
De-staining buffer	20% EtOH 10% acetic acid
FACS staining buffer	0.2 % BSA in PBS
FACS fixation buffer	0.2 % BSA

	2 % paraformaldehyde in PBS
Erythrocyte lysis buffer	0.01 M KHCO_3 0.155 M NH_4Cl 0.1 mM EDTA pH 7.5
Cell culture medium (cRPMI)	RPMI-1640 5- 10 % FCS 20 mM L-glutamine 100 U/ml penicillin 100 $\mu\text{g}/\text{ml}$ streptomycin all from Biochrom, Berlin, Germany
Electroporation buffer	Ambion, Austin, USA

6.8.3 Protein purification buffers

Lysis buffer A	300 mM NaCl 50 mM NaH_2PO_4 20 mM Imidazol pH 7,4
Wash buffer A2	300 mM NaCl 50 mM NaH_2PO_4 30 mM Imidazol pH 8,0
Elution buffer B	50 mM NaH_2PO_4 300 mM NaCl 250 mM Imidazol pH 8,0
BAL-solution	10 ml PBS (Dulbecco's w/o) 1 tabl. complete mini protease inhibitor (Roche)

6.8.4 Chemicals and biologicals

Recombinant RNase inhibitor	RNaseOUT, Invitrogen, Karlsruhe, Germany
RNA stabilization reagent	RNAlater, Qiagen, Hilden, Germany
RLT- β -mercaptoethanol lysis buffer	RLT, Qiagen, Hilden, Germany; β -ME Sigma-Aldrich, Munich, Germany
BSA, fraction V	AppliChem, Darmstadt, Germany
Proteinase inhibitor cocktail	Roche, Mannheim, Germany
Paraformaldehyde	Sigma, München, Germany

6.8.5 Commercial Kits

pGEM-T Easy vector kit	Promega, Mannheim, Germany
<i>C. elegans</i> vector kit	Addgene, Fire Lab, USA
Silencer siRNA labeling kit	Ambion, Austin, USA
MEGAScript RNAi kit	Ambion, Austin, USA
OptEIA ELISA kits	BD Biosciences, Heidelberg, Germany
IL-13 ELISA-set	R&D Systems, Minneapolis, MN
BCA kit	Pierce, USA
TaqMan primer and probes	Applied Biosystems, Darmstadt, Germany
TaqMan cDNA synthesis kit	Applied Biosystems, Germany
TaqMan PCR master mix	Applied Biosystems, Germany
RNeasy Mini kit	Qiagen, Hilden, Germany
QIAshredder spin columns	Qiagen, Hilden, Germany
LAL QCL-1000	Cambrex, USA
Cytokine bead array flex sets	BD Biosciences, Heidelberg, Germany
EndoTrap system blue	Profos, Regensburg, Germany

6.8.6 Software

FlowJo (Tree Star, Inc., Ashland, USA)
 BD FACSDiva (BD Biosciences, Heidelberg, Germany)
 FCAP Array Software (Applied Biosystems, Darmstadt, Germany)
 Prism (GraphPad Software, San Diego California USA, www.graphpad.com)
 PrimerExpress (Applied Biosystems, Darmstadt, Germany)
 StatMiner (Integromics)

6.8.7 Antibodies and secondary reagents

6.8.7.1 FACS reagents

Specificity	fluorochrome	clone	Purchased from
α CD4	FITC	RM4-5	BD Biosciences
α CD25	PE	PC61	BD Biosciences
α CD103	(biotin)	M290	BD Biosciences
α F4/80	Cy5	F4/80	DRFZ*
α CD11b	FITC	M1/70	BD Biosciences
α Gr-1	Biotin		DRFZ*
α TLR-2	PE	6C2	eBioscience
α Fc γ R		2.4G2	DRFZ*
Secondary reagents			
SA-PE-Cy7	PE-Cy7		BD Biosciences

* mAb was a kind gift of the German Arthritis Research Centre.

6.8.7.2 Antibody detection

Immunoglobulin	Capture ab	Standard	Detection ab
total IgE	α IgE 10 μ g/ml	IgE 250ng/ml	α IgE-Bio 2,5 μ g/ml
	PC284 The binding site	557079 Pharmingen	553414 Pharmingen
OVA-IgE	α IgE 2 μ g/ml	OVA standard serum #67	OVA-Bio 3 μ g/ml
	553413 Pharmingen	1:64	
OVA-IgG1	α IgG1 2 μ g/ml	OVA-spec. IgG1 340ng/ml	OVA-Bio 3 μ g/ml
	553445 Pharmingen	A6075 Sigma	
OVA-IgG2a	α IgG2a 2 μ g/ml	OVA standard serum #67	OVA-Bio 3 μ g/ml
	553387 Pharmingen	1:64	
total IgA	α IgA 10 μ g/ml	mouse IgA isotype control	α IgA-AP 1:3000
	104001 Southern Biotech	1250-19,5 ng/ml	104004 Southern Biotech
		eBioscience 14-4762-85	

7 Abbreviations

A.D.	Atopic dermatitis
Ab	Antibody
Ag	Antigen
AHR	airway hyperresponsiveness
AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammoniumperoxydisulfat
Av	<i>Acanthocheilonema viteae</i>
Av17	<i>Acanthocheilonema viteae</i> 17kDa immunomodulator (cystatin)
BAC	bacterial artificial chromosome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BCIP	5-brom-4-chlor-3-indolylphosphat
Bio	biotin
Bm	<i>Brugia malayi</i>
BSA	bovine serum albumin
CCL	C-type chemokine ligand
CCR	C-type chemokine receptors
CDD	conserved domain database
COPD	chronic obstructive pulmonary disease
CTLA-4	cytotoxic T-lymphocyte antigen 4
Cy3	Cyanine 3 (~550 nm excitation, ~570 nm emission)
Cy5	Cyanine 5 (~650 nm excitation, ~670 nm emission)
DAPI	4',6-diamidino-2-phenylindol
DC	dendritic cell
DHFR	dihydrofolat reductase
DMF	dimethylformamid
DMSO	dimethylsulfoxid
dsRNA	double stranded RNA
e.c.	epicutaneous
E/S	excretory/secretory
EAE	experimental autoimmune encephalomyelitis
EAR	early asthmatic response
Ec	Escherichia coli
ECP	extra-cellular matrix protein

EDTA	ethylenediaminetetraacetic acid
ES-62	excretory-secretory product (ES-62) of <i>Acanthocheilonema viteae</i>
EST	expressed sequence tag
EU	endotoxin unit
FACS	fluorescence activated cell sorter
Fc	fragment crystallizable (region)
FcR	fragment crystallizable (region) receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box transcription factor p3
FSC	forward scatter
Gal	galactosidase
GAV3	genome assembly version 3
GFP	green fluorescent protein
GITR	glucocorticoid-induced TNF-receptor-related protein
GM-CSF	granulocyte-macrophage colony stimulating factor
HDM	house dust mite
HELICc	Helicase conserved C-terminal domain
Hp	<i>Heligmosomoides polygyrus bakeri</i>
i.n.	intranasal
IFN	interferon
Ig	immune globuline
IL	interleukin
IL-10R	interleukin-10 receptor
ILN	inguinal lymph node
ILNC	inguinal lymph node cell
IPTG	isopropyl-thio-b-D-galactopyranosid
kan	kanamycin
KO	knock out
L3, L4	larvel stage 3, larvel stage 4
LAR	late asthmatic response
LB	Luria Bertani
LPS	lipopolysaccharides
LT	leukotriene
LU	lab unit
mAb	monoclonal antibody
MØ	macrophage

MBP	major basic protein
mf	microfilariae
MHC	major histocompatibility complex
MIF	macrophage-migration inhibiton factor
min	minute
ml	milliliter
MLN	mesenteric lymph node
MLNC	mesenteric lymph node cell
MLV	multilamellar vesicle
MMP	matrix metallopeptidase
mRNA	messenger RNA
NBT	nitro blue tetrazolium
NC	nitrocellulose
ng	nanogram
NGM	nematode growth medium
NK	natural killer cell
NKT-cells	natural killer T-cells
NO	nitric oxide
Ov17	<i>Onchocerca volvulus</i> 17kDa immunomodulator (cystatin)
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PAR	protease activated receptors
PAS	200-kDa protein component of <i>Ascaris suum</i>
PAZ	Piwi Argonaut and Zwillie domain
PBLN	peribronchial lymph node
PBLNC	peribronchial lymph node cell
PE	phycoerythrin
PEC	peritoneal excudate cells
Penh	pause enhanced
PGE	prostaglandin
PHA	phytohaemagglutinin
Piwi	P-element induced wimpy testis
PRR	pattern recognition receptor
PSTG	post-transcriptional gene silencing
RANTES	acronym for Regulated on Activation, Normal T Expressed and Secreted
RdRP	RNA dependent RNA polymerase

RISC	RNA induced silencing complex
RNAi	RNA interference
RNase	ribonuclease
RT	room temperature
SA	streptavidin
SCF	stem cell factor
SDS	sodium dodecylsulfate
SID	systemic RNAi deficient
siRNA	small interfering RNA
SIT	allergen-specific immunotherapy
SOCS	suppressor of cytokine signaling
Taq	<i>Thermus aquaticus</i>
TGF	transforming growth factor
TGH	TGF- β homologue of <i>Brugia malayi</i>
Th1	T helper cell 1
Th17	T helper cell 17
Th2	T helper cell 2
TLR	toll-like receptor
TM	tropomyosin
TNF	tumor necrosis factor
Tr1	Type 1 regulatory (Tr1) cells, also known as inducible Treg cells
Treg	regulatory T cell
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol
TSLP	thymic stromal lymphopoietin
VEGF	vascular endothelial growth factor
WGS	whole genome shotgun
X-Gal	5-brom-4-chlor-3-indolyl-b-D-galacto-pyranosid

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7 Publications and Presentations

Presentations:

Matthias Lendner (2009). Pitfalls on the way to RNA interference in parasitic nematodes – The example of *Heligmosomoides polygyrus*. Presented at the BSP Spring Meeting 2009

Matthias Lendner (2008). RNA interference - A Challenge in Parasitic Nematodes. Presentation at the biennial symposium of the German Society for Parasitology.

Matthias Lendner (2007). RNA interference - A Challenge in Parasitic Nematodes. PhD work presented at the annual retreat of the GRAKO-1121

Matthias Lendner (2006). Establishment of RNA interference in the parasitic nematode *Heligmosomoides polygyrus*. PhD work presented at the annual retreat of the GRAKO-1121

Publications:

Paper: Rausch S, Held J, Stange J, Lendner M, Hepworth M, Klotz C, Lucius R, Pogonka T, Hartmann S; A Matter of Timing: Impact of an acute and chronic intestinal nematode infection on a concurrent protozoan infection”, in preperation

Paper: Lendner M, Sereda M, Schnöller C, Lucius R, Hamelmann E, Hartmann S, “Role of a nematode pan-allergen in the modulation of unrelated allergic reactions”, in preparation

Paper: Hartmann S, Schnoeller C, Dahten A, Avagyan A, Rausch S, Lendner M, Bocian C, Pillai S, Loddenkemper C, Lucius R, Worm M, Hamelmann E. Gastrointestinal nematode infection interferes with experimental allergic airway inflammation but not atopic dermatitis. Clin Exp Allergy. 2009 Jun 8. [Epub ahead of print] PubMed PMID: 19508324.

Paper: Lendner M, Doligalska M, Lucius R, Hartmann S. Attempts to establish RNA interference in the parasitic nematode *Heligmosomoides polygyrus*. Mol Biochem Parasitol.

2008 Sep;161(1):21-31. Epub 2008 Jun 18. PubMed PMID: 18606194.

Poster: Lendner M, Lucius R, Hartmann S. (2009) "Tropomyosin of parasitic nematodes – Functional analysis of its immunomodulating properties". DFG/GRAKO-1121evaluation, (Humboldt-University, Berlin, Germany)

Poster: Lendner M, Lucius R, Hartmann S. (2007) "RNA interference – A Challenge in Parasitic Nematodes". ZIBI / Sackler Institute Symposium: Host Pathogen Interactions (Humboldt-University, Berlin, Germany)

Poster: Lendner M, Lucius R, Hartmann S. (2006) "Establishment of RNA Interference in the Parasitic Nematode *Heligmosomoides polygyrus*". 22. Jahrestag der Deutschen Gesellschaft für Parasitologie e.V. (Vienna, Austria)

Poster: Lendner M, Lucius R, Hartmann S. (2005) "Functional analysis of immunomodulatory proteins of parasitic nematodes". ZIBI / Sackler Institute Symposium: Host Pathogen Interactions (NYU School of Medicine, New York City, U.S.A).

Diploma Thesis: Lendner M. (2005) "Studien zur Transfektion von Nematoden mit Wolbachien und Plasmiden".

Eidesstattliche Erklärung

Hiermit erkläre ich, Matthias Lendner, geb. am 23.10.1976, an Eides statt, die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben.

Berlin, den

Matthias Lendner