Antagonism between IL-4 and IL-10 in colitis and a helminth infection in *Mus musculus*

Dissertation

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
APC´s	antigen presenting cells
bp	base pair
BSA	bovine serum albumine
CD	clusters of differentiation
cDNA	complementary desoxyribonucleic acid
Con A	concanavalin A
DC	dendritic cell
DEPC	diethylpyrocarbonate
dNTP	deoxyribonucleic triphosphate
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FITC	fluoresceine isothiocyanate
IL	interleukin
IFN	interferon
Ig	immunoglobuline
i.p.	intraperitoneal
i.v.	intravenously
KO	knock out
LPS	lipopolysaccharide
LsAg	*Litomosoides sigmodontis* antigen
µg	microgramme
mg	milligramme
$MgCl_2$	magnesium chloride
mM	millimolar
mAb	monoclonal antibody
MHC	major histocompatibility complex
MF	microfilaria
min	minute
mRNA	messenger ribonucleic acid
ng	nanogramme
OD	optical density
PBS	phosphate buffered saline solution

PCR	polymerase chain reaction
pg	picogramme
p.i.	post infection
rpm	rotation per minute
RAG	recombination activating gene
RT	room temperature
SCID	severe combined immunodeficiency
TBE	Tris-borate-EDTA
T cell	thymus-dependent cell
Th	T helper
TGF	transforming growth factor
TNF	tumor necrosis factor
Tris	2-amino-2-hydroxymethyl-propan-1,3-diol
v/v	volume per volume
w/v	weight per volume
wt	wildtype

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1. Introduction

1.1. Immune responses to invading pathogens - innate and adaptive

Our environment contains a broad variety of microorganisms (viruses, bacteria) and parasites (helminths), which can cause disease and even kill their host if they multiply in an uncontrolled way. The immune system combats infectious agents by eliciting an immune response. This involves recognition of the pathogen as non-self and mounting of a reaction to eliminate it. There are two categories of immune responses: the innate immune response that is non-specific and does not require a prolonged period of induction and the adaptive immune response, which is specific and results in generation of antigen specific effector and memory cells (1).

1.1.2. Innate Immunity

The innate immune system depends upon germline-encoded receptors to recognize a few highly conserved structures present in many different microorganisms. The structures recognized are called pathogen-associated molecular patterns (PAMP) and include LPS, peptidoglycan, mannose and others. Phagocytic cells, such as monocytes, macrophages and neutrophils, can recognize these PAMP´s by receptors and mediate immediate responses in order to attract defensive cells and to prime the adaptive response. Blood vessel permeability is needed to aid the first. Another early defense mechanism is the activation of the complement system, a cascade of plasma proteins. Complement molecules bind to the microorganism and lead to opsonization of the pathogen and the recruitment of other inflammatory cells. These types of responses defend the host against invading pathogens to prevent them from growing freely in the body (1).

1.1.3. Adaptive Immunity

In contrast to the innate immune response, the main function of the adaptive immune response is the generation of antigen specific lymphocytes by clonal selection, leading to long lasting memory. Individual antigen specific lymphocytes are self-tolerant. They proliferate in response to antigen and differentiate into specific effector cells that eliminate the eliciting pathogen, and into memory cells to sustain immunity. All lymphocytes are

derived from bone marrow stem cells, but T lymphocytes develop in the thymus while B lymphocytes (except for B1 cells) develop in the bone marrow. B cells that specifically recognize a particular antigen divide and differentiate into plasma cells, which produce large amounts of antibodies (immunoglobulins). To participate in the adaptive immune response, naïve T cells must first encounter a specific antigen on the surface of professional antigen-presenting cells (APC) that also express effective co-stimulatory molecules. The activated T cells differentiate into effector cells producing cytokines characteristic of the immune response they mediate. Therefore, T cells are subdivided in several different types with a variety of functions (1).

1.1.3.1. Antigen presentation

Antigen presentation is needed to drive naïve T cells into effector T cells. The professional antigen presenting cells are mainly dendritic cells and macrophages. These cells engulf foreign antigens to be degraded into peptides and delivered to the cell surface bound to the major histocompatibility complex (MHC) molecules. Cytosolic peptides are bound to MHC class I and recognized by the T cell receptor (TCR) of $CD8^+$ cells, whereas peptides degraded in intracellular vesicles are bound to MHC class II and recognized by the TCR of $CD4^+$ cells. CD4 and CD8 were known as markers for different functional sets of T cells, but more importantly they are co-receptors, needed for direct interaction with MHC molecules to support T-cell receptor/peptide engagement. The recognition of antigen and activation of the T cells further requires other co-stimulatory surface molecules, such as CD40, CD80 and CD86 on antigen presenting cells and CD40-ligand, CTLA-4 and CD28 on the T cells (1).

1.1.3.2. $CD8^+$ T cells

Upon antigen recognition $CD8^+$ T cells can differentiate into cells either producing IFN-γ (Tc1 cells) or IL-4 (Tc2 cells) (2, 3). Both subsets appear to be equally cytolytic (4). These cells are able to kill target cells that display peptide fragments of cyctosolic pathogens, bound to MHC class I molecules at the cell surface. They release cytotoxic products, such as perforin and granzymes, which induce apoptosis (programmed cell death) of the target cell. Moreover, IFN-γ produced by CD8+ T cells is an important inducer of MHC class I

expression and macrophage activation. Cytotoxic T cells kill infected targets with great precision, sparing adjacent normal cells.

1.1.3.3. CD4$^+$ T cells

CD4$^+$ T cells are restricted to recognizing antigen complexed to MHCII. These cells support T- and B-cells in orchestrating the adaptive immune response. Therefore some subsets are called T helper cells. These cells differentiate and are activated to secrete cytokines (signal peptides), depending on the nature of the pathogen to be eliminated. The cytokines produced whether in mice or humans, characterize the category of helper cells it belongs to and the type of response it mediates (5).The cytokines produced in response to pathogens by the cells of the innate immune system play an important part in shaping the adaptive immune response. If a particular CD4$^+$ T cell subset is activated first or preferentially in a response, it can suppress the development of the other subset. Most responses are dominated by either cell T helper type 1 (Th1) or T helper type 2 (Th2) immunity (1). However, under many circumstances *in vivo*, there is a mixed Th1 and Th2 response.

1.1.3.3.1. Th1 cells

Many pathogens, especially intracellular bacteria and viruses, activate dendritic cells and NK cells to produce IL-12 and IFN-γ. Naïve CD4$^+$ T cells, activated in the presence of these cytokines, are committed to differentiate into Th1 cells. Th1 cells are then able to activate macrophages in the way that they provide two signals after MHC class II engagement. One signal is IFN-γ, which is the most characteristic cytokine produced by Th1 cells, while the other signal needed is the surface molecule CD40 ligand (6), expressed by Th1 cells, to sensitize the macrophage to respond to IFN-γ by contacting the CD40 molecule on the macrophage. These signals as well as other effector molecules such as TNF-α result in the fusion of pathogen containing vesicles with lysosomes within the macrophages and destruction of the intracellular pathogen. Th1 cells can also activate B cells to produce strongly opsonizing antibodies belonging to certain IgG subclasses (IgG1 and IgG3 in humans and their homologues IgG2a and IgG2b in the mouse). Furthermore,

Th1 cells secrete IL-3 and GM-CSF as well as changing surface properties of endothelial cells to direct the migration of phagocytic cells.

1.1.3.3.2. Th2 cells

The induction of Th2 cells occurs mainly by extracellular pathogens including bacteria and helminthic parasites. The strongest polarization signal into Th2 phenotype is given by IL-4 (7). Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 (8). A main function of Th2 cells is to provide two signals as well, but rather for B cell than macrophage activation. The combination of CD40 (B cell)-CD40 ligand (Th2 cell) ligation and secretion of the B cell growth factors IL-4 and IL-5 by Th2 cells leads to B cell proliferation, differentiation into antibody-secreting plasma cells and class-switching from IgM to IgG, IgE and IgA. Plasma cell secreted antibodies bind to the surface of the pathogen, leading to the activation of the complement system, neutralization of toxins, opsonization of the pathogen and the activation and degranulation of mast cells. Furthermore, IL-5 is a potent hematopoietic cytokine that stimulates bone marrow production of eosinophils and basophils (9). These cells infiltrate the tissue, where they degranulate in order to harm the pathogen (10).

1.1.3.3.3. Regulatory T cells

Cells with suppressory function were initially considered within the $CD8^+$ T cell population (11). The suppressive mechanism on $CD4^+$ T cells was suggested to be cytotoxic or lymphokine-mediated (reviewed in (12)). It was later found, that suppressor cell function could be also mediated by $CD4^+$ cells independent of $CD8^+$ cells (13, 14).
Recently the research has focused on the role of $CD4^+$ $CD25^+$ T cells for immuno-suppression. Detectable numbers of self-reactive $CD4^+$ $CD25^+$ T cells escape clonal deletion in the thymus and reach the periphery. This subpopulation of T-cells is essential for suppressing autoreactive responses and constitutes 5% to 10% of peripheral $CD4^+$ T cells. They are able to inhibit both proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells in a contact-dependent, but antigen independent manner. Other subgroups of T cells ($CD4^+CD25^-$) with suppressive activity induced in the periphery have been identified and up to now classified as regulatory by their cytokine profile. T regulatory type 1 (Tr1) cells produce variable amounts of immunosuppressive IL-10 and TGF-ß, but no IL-4. In contrast, T helper type 3 cells (Th3) mediate their suppressive activity by

secretion of IL-10 and TGF-ß together with IL-4 in an antigen specific but contact independent manner in the same way as do Tr1 cells.
Thus, the different subsets of regulatory cells prevent overshooting of proinflammatory responses in infection and the outgrowth or function of reactive T cells against self (autoimmunity) and non-harmful (allergy) antigens.

1.2. An example of dysregulation of immune responses: Inflammatory bowel disease

The adaptive immune response is a critical component of host defense against infection. Unfortunately, adaptive immune responses are also sometimes elicited by antigens not associated with infectious agents and this, too, may cause serious tissue damage. These are unsuppressed adaptive immune responses mounted against self-antigens (autoimmunity) or non-harmful environmental antigens (allergy). Allergic disorders, such as rhinitis, asthma and atopic dermatitis are triggered by Th2 cell-mediated immune responses against allergens (15). One example of Th1 mediated dysregulation of responses against harmless antigens is human inflammatory bowel diseases (IBD) such as Crohn´s disease (CD), one of the major forms of IBD. CD represents a chronic, relapsing and remitting inflammatory condition that affects individuals throughout their lives (16, 17). It is generally described as a condition that occurs in genetically susceptible individuals because of aberrant immune responses to enteric antigens. Although the particular antigens responsible for causing human IBD have remained elusive, enteric flora has proven sufficient to trigger mucosal inflammation in mice deficient for IL-10, IL-2 and TCR-α-chain, as well as in HLA-B27 transgenic rats, since none of these mutants develop intestinal disease under germ-free conditions (reviewed in (18)). While not finally proven in the human diseases, it is generally believed that the major cause of pathology in these conditions results from dysregulation by insufficient mounting of regulatory respectively IL-10 dependent immune responses. A major part of the knowledge was gathered from the model of IL-10 KO mice.

1.2.1. A murine model of colitis: IL-10 deficient mice

IL-10 deficient mice have been proven extremely useful in defining the physiological relevance of the diverse activities of IL-10 (19). These mice, generated by a gene-targeted mutation, develop abnormal immune responses as a result of an exaggerated immune response to the normal enteric gut flora. These mice develop colitis between 7 and 11

weeks of age. Histological evaluation has revealed multifocal lesions throughout the cecum, colon and rectum at 3 month of age. Pathological changes included inflammatory infiltrates in both mucosa and submucosa marked epithelial hyperplasia, and modest numbers of crypt abscesses and focal ulcers. This was accompanied by weight loss and anemia (20). Th1 cells mediate colitis in the IL-10 KO model, as shown by the rapid onset of disease in severe combined immunodeficiency disease (SCID) mice (lacking B and T cells) after adoptive transfer of T cells from IL-10 KO mice (21). To determine which cytokines are responsible for the initiation and/or maintenance of colitis, IL-10 KO mice were treated with neutralizing antibodies. When 3-week-old mutants were given weekly injections of anti-IL-12 for 6-8 weeks, they remained free of disease. Similarly, anti-IFN-γ prevented the disease in 69% of the weanlings (20). In contrast, established colitis in 3- to 4-month-old IL-10 KO mice was unaffected by anti-IFN-γ treatment. These results demonstrated that the uncontrolled production of IFN-γ and IL-12 is responsible for initiating colitis in IL-10 KO mice. Thus, IL-10 has an essential role in the development of normal mucosal immunity and serves as a natural anti-inflammatory agent.

Recently, increasing evidence has shown a potential role for the Th2 cytokine IL-4 in the early induction or sustained production of IFN-γ (22). These findings would suggest that IL-4 does counterregulate the action of IL-10.

1.3. An example of human helminth infections: onchocerciasis

The importance of reduced activity of T regulatory cells (reduced immune suppression), resulting in uncontrolled immune responses, has been emphasized in the section above.

In helminthic infections, where inflammatory responses have been observed to be of Th2 type, the existence of regulatory cells has been described in patients with high parasite loads (23). Interestingly, in some patients the lack of these regulatory cells might lead to hyperreactivity with parasite killing resulting in the observed low parasite load but at the expense of host tissue integrity. This will be now introduced in more detail.

The interplay between host and parasite in filarial infections, which often lasts many decades, suggests an immunosuppressive mechanism induced by the parasite. This long-term peaceful co-existence is of advantage for both host and parasite (24). On the other hand, severe immune reactions were found to take place against filarial species or against the same parasite in other individuals resulting in debilitating manifestations. Moreover,

the existence of individuals living in areas endemic for filariae without showing signs of infection gives evidence for protective immunity against filarial parasites (25). In conclusion, the outcome of infections in human filariasis seems to be associated with the differential ability of individuals to mount a response against the parasite.
In onchocerciasis, caused by the filarial nematode *Onchocerca volvulus*, three clinical groups are classified according to the reaction of the immune system to the infection. In endemic areas, generalized infection with onchocerciasis (GEO) is most frequent and characterized by adult worms in subcutaneous nodules and millions of dermal microfilaria. Despite this high antigen load, individuals with GEO often present weak skin inflammation and exhibit relatively mild alterations, such as atrophy and pigmentary changes. In contrast to GEO, there are a few individuals in hyperendemic areas who remain free from infection despite equivalent heavy exposure to parasites and assumed carriage of L3 (infective larval stage). These people are termed putative immune individuals (PI). Sowda includes the minority of individuals with chronic hyperreactive onchodermatitis presented unilaterally. SOWDA patients suffer from papular dermatitis, hyperpigmented lichenified lesions, pruritus and lymphadenitis. They have low microfilaria loads and low numbers of adult worms resident in large nodules. This condition is assumed to be a result of a hyperreactive inflammatory response, leading to the killing of microfilaria at the expense of skin integrity (26).

1.3.1. *Litomosoides sigmodontis* (Chandler, 1932) is a mouse model for filarial infections

The immunology of filariasis is complex and is complicated by the anatomical compartimentalisation and the differing responses induced by the multiple stages of the parasite. Helminths have been propagated as main examples of Th2-cell inducers in both humans and experimental models. This includes the production of the cytokines IL-4, IL-5, IL-10 and IL-13, as well as IgE and the expansion and mobilization of specific effector cells, such as mast cells, eosinophils, and basophils by the host.
Animal models are powerful tools to investigate the underlying mechanisms. The infection of mice with the nematode *Litomosoides sigmodontis* was recently found to undergo its full developmental cycle in BALB/c mice (27).

1.3.1.1. *Litomosoides sigmodontis* belongs to the phylum nematoda

The Phylum Nematoda Filariae includes cylindrical to threadlike worms that are free-living in water and soil in addition to an impressive number of species that are parasitic in plants and animals. They are sexed, unsegmented and round in transverse sections. The male is generally smaller than the female. They have four main longitudinal chords, a triradiate esophagus and a circumesophageal ring. Nematodes have neither a circulatory nor a respiratory system. They have one or two gonads that open at the vulva in the female and in the rectum of the male. Their exterior is composed of a multilayer collagenous cuticle, which is covered by lipidic epicuticle as well as negatively charged glycocalyx (28).
Of the 16,000 species that have been described, 138 species have been found to infect humans (29). Thus, parasitic nematodes have a substantial impact on human welfare, through corporal damage and diseases of both humans and domestic animals.

1.3.1.2. The life cycle of *Litomosoides sigmodontis*

This rodent parasite lives naturally in the cotton rat (*Sigmodon hispidus*), which can survive very high blood microfilariae levels (up to 10000 MF/µl blood). During the blood meal from an infected rat, the arthropod intermediate host (*Ornithonyssus bacoti* mite) ingests the MF, which migrate through the gut wall into the thoracic muscles where they molt twice to develop into infective L3 larvae within 10-12 days. The L3 migrate to the head and are frequently localized in the blood-sucking mouthparts. L3 are transmitted at the next blood meal with the saliva, make their way through the skin over the lymphatic vessels to the heart, the blood circulation and finally to the lungs, from which they penetrate into the thoracic cavity (30). At day 10 post infection, up to 90% of L4 larvae are detected in the thoracic as well as peritoneal cavities and reach 99% at day 28 p.i. (30). The female and male adult worms mature within 25-33 days and copulate at sexual maturity. The viviparous females release microfilariae into the thoracic cavity. These microfilariae penetrate the heart and eventually reach the blood circulation. MF are detectable in the blood 50-130 days p.i. The infection of BALB/c mice ends with the encapsulation of adult worms by host inflammatory cells and the eventual absorption of dead parasites by the host. Mice are used for immunological studies. The cycle is maintained in cotton rats, due to longer worm survival in the natural host.

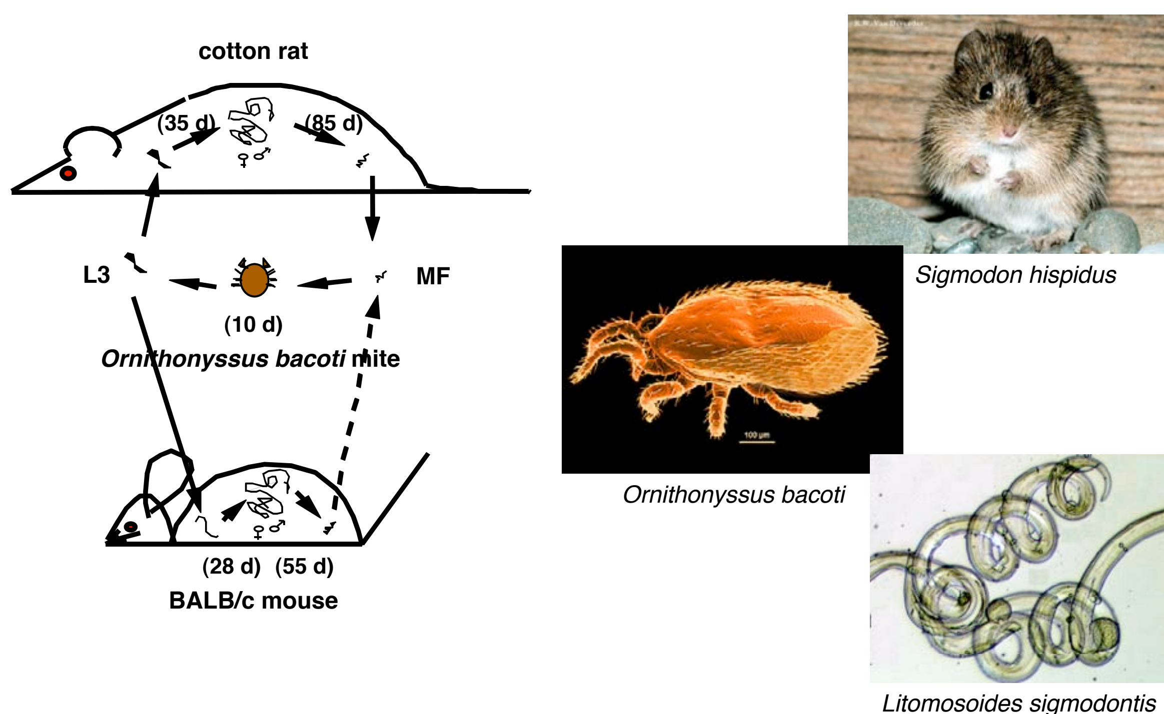


Figure 1: Life cycle of Litomosoides sigmodontis

1.3.1.3. Immunity to *Litomosoides sigmodontis*

To overcome the non-permissivity of inbred mice to filarial parasites and the inaccessibility of the fully permissive to immunological analysis, the *L. sigmodontis* mouse model, originally established by Petit *et al.* (92), was used in these studies. This model accommodates the full cycle from L3 to the release of microfilariae in BALB/c mice, and therefore allows the study and the modulation of distinct states of infection. In microfilaremic BALB/c mice Th2 type cytokine responses predominate together with an impaired cellular proliferative response to filarial antigens (31, 32). Depletion of $CD4^{+}$ T cells results in higher *L. sigmodontis* worm burden and increased and prolonged microfilaraemia (33). IL-5 appears to control adult worm development and microfilaremia in primary infection (34-36). An aquired immunity can be generated by inoculation of irradiated infective L3 larvae resulting in reduction of worm numbers after challenge infection by 50-80% (37), mediated by IL-5 and eosinophils in conjunction with parasite-specific antibodies (38).

While in BALB/c mice full parasite development was observed, in C57BL/6 mice worm development cannot proceed beyond the L4 stage (39). However, infection of IL-4

deficient C57BL/6 mice results in full parasite development and patency, demonstrating that in fact a Th2 response is the key determinant of susceptibility and resistance in these non-permissive mice (39). Furthermore, susceptible BALB/c mice lacking the IL-4-receptor-α chain (unable to respond to IL-4 or IL-13) or IL-5 exhibit levels of MF 100-1000 times higher than wildtype controls (36). Although these data build a convincing picture of Th2 responses in the control of filarial infections, the real picture that emerged proved more complex. Mice, genetically deficient in the type 1 cytokine IFN-γ, contained more adult worms than wildtype controls in murine infection with *Brugia malayi* (40) and *L. sigmodontis* (41), apparently through encapsulation and clearance mediated by neutrophils (35, 41). Furthermore, injection of *B. malayi* microfilariae (MF) into otherwise uninfected mice stimulated the production of IFN-γ (42-45). Thus, under some circumstances both Th1 and Th2 responses can control parasite loads and can even act synergistically as was demonstrated in mice deficient for both IFN-γ and IL-5 (46). This is in contrast to the Th2 type immune responses seen in gastrointestinal helminth infections
Recently, it has been shown that most filariae harbor *Wolbachia* as mutualistic symbionts (47, 48). These bacteria could be possible inducers of innate and Th1 responses. This is supported by ex vivo investigation of the filariae containing nodules in mice (49) and humans (50), where neutrophils but not eosinophils were found directly around the worms in strict dependence on the presence of *Wolbachia* (49). Neutrophils are known to respond to conserved structures as part of the innate immune response (1). These reports underscore that the strict dogma of Th1 and Th2 mediated immunity in filarial infections needs to be reconsidered.
While in human filarial infections regulatory T cells were observed, studies in mice have remained elusive with regard to the presence of suppressive immune mechanisms, such as regulatory T cells. Female worms after implantation have been shown to induce IL-10 mediated immunosuppression leading to prolonged survival of microfilariae (51). This indicates an important role of suppressory mechanisms.

1.4. Autoimmunity, allergy and helminths

An important issue is that of how the presence of ongoing helminth infection affects the likelihood of the development of immunological disorders such as autoimmunity and allergy. Data from studies addressing the issue of autoimmunity are still descriptive but

nevertheless promising. Weinstock and collegues have argued that a failure to acquire helminthic parasites predisposes one to Crohn´s disease and have correlated increased hygiene in Western countries and loss of helminthiases with prevalence of autoimmune intestinal diseases and other forms of autoimmune diseases (52). His group used eggs of the helminth *Trichuris suis* as treatment for patients suffering from CD. 80% of the patients showed improvement of their health status (53).
Helminth infections induce strong IgE responses, which in combination with high antigen-levels would be expected to lead to allergic symptoms and possibly anaphylaxis (54). However, helminth infected individuals appear to suffer less from allergic disorders in general than do helminth-free individuals (55). There are several possible explanations for this paradox, including the production of IgG antibodies that block the access of allergenic Ag to specific IgE (15, 56). Studies correlated increased IL-10 levels resulting from chronic schistosomiasis with reduced expression of house mite allergy in African children (15, 57). Interestingly, antigen-specific T-regulatory-1 clones were found in high frequency in onchocerciasis, in particular in patients with generalized onchocerciasis (high worm load and little pathology) (58). Just recently, it could be shown in an interventional study, that colitis development in IL-10 deficient mice could be blocked by *Heligmosomoides polygyrus* infection (59).
Thus parasitism by helminth, leading to immune suppression in the host, would be beneficial for both, the parasite, which ensures longer survival and for the host, which is protected from overreaction of the immune system with consequenses of self-damage. It is intriguing that more and more studies reveal that the host immunosuppression may not only be restricted towards the inducing helminth but extend to prevent overshooting immune responses that would lead to autoimmunity and allergy.

1.5. Aims and objectives

Since under some conditions (autoimmune diseases, infections), the T cell response (either Th1- or Th2-polarized) may become dangerous for the host, an overwhelming immune response is normally controlled. Recent data suggest that IL-10 dependent, regulatory mechanism exist in prevention of autoimmunity as well as in helminth infections, suppressing Th1 and also Th2 responses. If this is the case, IL-4 and IL-10 must act antagonistically but not synergistically. However, this was never shown in a direct interventional approach. Therefore mice deficient for IL-4 and IL-10 were used in two different mouse models to address the question of the relationship between IL-4 and IL-10.

1) IL-10 deficient mice develop severe colitis in response to their intestinal flora. Does additional knock out of IL-4 increase severity of this disease and therefore suggest a synergism between both cytokines or does it antagonize IL-10 leading do improvement of colitis?

2) IL-4 is a critical determinant of resistance in helminth infections. IL-4 deficiency leads to patency in otherwise resistant C57BL/6 mice, when infected with the rodent filariae *L. sigmodontis*. Does additional knock out of IL-10 synergize with IL-4 leading to even higher parasite loads or does it antagonize and revert susceptibility of IL-4 KO?

2. Material and Methods

2.1. Materials

The specific instruments used in these studies will be mentioned in the corresponding section of the materials and methods. All of the chemicals whose supplier is not specified in this section are either from SIGMA (Munich, Germany), FLUKA (Neu-Ulm, Germany) or Merck (Darmstadt, Germany); the culture medium and its additives are from GIBCO BRL (Eggenstein, Germany). The origin of the antibodies used for ELISA´s and histology studies are R&D (Wiesbaden, Germany) for IL-13 and Pharmingen (Heidelberg, Germany) for all other antibodies.

2.1.1. Animal maintenance

IL-4/10 KO mice on C57BL/6 background were obtained from A. Sher (National Institute of Health, Bethesda, MD, USA). IL-10 KO mice on C57BL/6 background (19) were provided by H. Mossmann (Max-Planck-Institute for Immunobiology, Freiburg, Germany). IL-4 KO and C57BL/6 wildtype (originally from Jackson laboratories) and the knockout strains mentioned above were housed under SPF (specific pathogen free) conditions, in particular free of *Helicobacter hepaticus*. Mice and cotton rats were bred at the animal facilities of the Bernhard Nocht Institute.

2.1.2. Buffers and solutions in alphabetical order

Anesthesia: long-term
Rompun (20 mg/ml, Bayer, Leverkusen, Germany): 20%
Ketanest (10 mg/ml, Parke-Davis, Berlin, Germany): 80%
50 µl-100 µl were used depending on age and size of the mice.

Anesthesia: short-term
Inhalation of Isofluran (Abbott, Wiesbaden Germany)

Cell purification, culture and analysis

Culture medium:	RPMI 1640 (Gibco, Eggenstein, Germany) or ISCOVES supplemented with 50 µg/ml Gentamycine and 2 mM L-Glutamine
Tris-ammonium chloride:	a) 0.1 M NH_4Cl
	b) 0.17 M Tris-HCl, pH 7.65

Working solution: 90 ml of solution a) and 10 ml of solution b) were mixed and the pH was adjusted to 7.2 with 1 N HCl.

Enzyme Linked Immunosorbent Assay (ELISA)

Coating solution:	0.1 M $NaHCO_3$, pH 8.2
Washing buffer:	0.05% Tween 20, PBS (Sigma)
Blocking solution:	1%BSA, PBS
Substrate buffer:	0.1 M $NaH_2PO_4 \cdot 2H_2O$, pH 5.5
Substrate:	6 mg 3,3`,5,5`Tetramethylbenzidine (Roth, Karlsruhe, Germany), dissolved in 1 ml DMSO
Substrate solution:	10 ml substrate buffer
	200 µl substrate
	2 µl H_2O_2 (30 %)
Stop solution:	2 N H_2SO_4

Haemalum (according to Mayer):

1.0 g haematoxiline in 1000 ml distilled H_2O

0.2 g NaIO3 (sodium iodate)

50 g ammonia or potash alum

50 g chloral hydrate

1 g citric acid

Hinkelmann`s solution (Korenaga 1991):

0.5% w/v eosin Y (2.5 g)

0.5% w/v phenol (2.5 ml)

0.185 % v/v formaldehyde (0.925 ml)

ad 500 ml distilled H_2O

LB (Luria-Bertani) medium for agar plates

10 g tryptone

5 g yeast extract

10 g NaCl

15 g Bacto-Agar

ad 1000 ml nanopure H_2O and adjust to pH 7.0

After autoclaving for 20 min at 15 psi, the solution was allowed to cool down to 55°C and 200 µg/ml of ampicillin were added. The solution was then poured into plates and stored at 4°C until usage.

Loading dye (6x)

0.25 % bromophenole blue

0.25 % xylencyanole FF

15 % ficoll

in nanopure H_2O

Monoclonal antibody purification

Protein G sepharose 4 fast Flow (Pharmacia, Freiburg, Germany)

Start buffer:	0.02 M sodium phosphate, pH 7.0 (degassed, filtered)
Elution buffer:	0.1 M glycine-HCl, pH 2.7 (degassed, filtered)
Neutralizing buffer:	1.0 M Tris-HCl, pH 9.0

PBS (phosphate-buffered saline solution)

10 x PBS:	80 g NaCl
	11.6 g Na_2HPO4
	2.0 g KH_2PO4
	2.0 g KCl
	ad 1000 ml nanopure H_2O
	adjust to pH 7.4 when preparing 1 x PBS

TBE (Tris-borate-EDTA)

Solution 1:	108 g Tris
	55 g borate acid
	9.3 g $Na_2EDTA \cdot 2H_2O$
	ad 1000 ml nanopure H_2O
Working solution:	0.5 x stock solution

TBS (Tris-buffered saline solution)

Stock solution:	60.75 g Tris
	87.66 g NaCl
	ad 1000 ml A. dest.
Working solution:	100 ml stock solution
	40 ml 1 N HCl
	ad 1000 ml nanopure H_2O pH 7.6

2.2. Methods

2.2.1. Histological analysis of the intestine

Mice were sacrificed at different stages of colitis. These stages of colitis were defined as follows: pre-disease (4-8 weeks), onset (10-25 weeks), late disease (over 25 weeks). Gut tissue was removed at necropsy from each mouse, flushed with 1x PBS and dissected into duodenum, jejunum, ileum, colon and rectum. These tissue specimens were fixed in 4% formaldehyde-PBS for at least 2 days. According to standard procedures, the samples were then embedded in paraffin using an automated tissue processor (Enno Vieth, Wiesbaden, Germany). In brief, an increasing ethanol series was carried out (50%, 70%, 80%, 2x96%, 2x100%). This was followed by incubation in ethanol/aceton (1:1), 2x aceton and embedded in paraffin. 4 µm sections were cut using a microtome (Reichert-Jung, Hamburg, Germany).

2.2.2. Immunohistochemistry

To detect eosinophils, either antibodies against major-basic-protein (MBP) (60) or FITC (fluorescein isothiocyanate) (61) were used. Deparaffinizing was carried out as follows:

Incubation of the slides in: 4 x 5 min rotihistol
3 x 2 min 100% ETOH
2 x 2 min 96% ETOH
1 x 2 min 80% ETOH
2 x 2 min 70% ETOH
distilled H_2O

MBP (major-basic-protein)-staining: After deparaffinizing and 5 min incubation with proteinase K (Dako), sections were incubated for 1 h with anti-CD16/CD32 (5µg/ml) (Pharmingen, Heidelberg, Germany) in 1 % (wt/vol) bovine serum albumin in 1xTBS to block nonspecific binding to FcεRII or FcεRIII. Sections were incubated either with anti-MBP-antibody (rat IgG) at a 1:1000 dilution or isotype-matched control antibody (5µg/ml) in 1% bovine serum albumin in PBS over night at 4°C. Biotinylated polyclonal goat anti-rat Ig was then added for 60 minutes. After three wash steps, the slides were further incubated with prediluted alkaline phosphate-conjugated streptavidin (1:100) (Calbiochem, San Diego, Calif.) for 30 minutes, followed again by three washings. The color reaction was developed using Naphtol Fast Red and then counterstained with Haemalaun for 10 min and developed with tap water for another 10 minutes. Aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany) was used to mount the coverslips.

FITC-staining: After deparaffinizing and 5 min incubation with proteinase K (DAKO, California, USA), sections were incubated for for 15 minutes with 100 µl 0.01% FITC in PBS. After washing 3x with PBS, slides were covered using fluorescent mounting medium (DAKO). Fluorescent staining was analyzed using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Magnifications are given as that when the photographs were taken and equals objective x ocular.

2.2.3. RNA isolation from gut tissue

Gut tissue samples were collected from colon of all groups, snap frozen in liquid nitrogen and stored at -80 °C until RNA-Isolation. RNA was homogenized in 1 ml TRI-Reagent (Invitrogen, Heidelberg) using a Polytron rotor stator (Kinematica, Lucerne, Switzerland). After 5 min incubation at room temperature to permit the complete dissociation of nucleoprotein complexes, the homogenate was supplemented with 0.1 ml bromo-chloropropane (BCP) per 1 ml TRI-Reagent. Then the samples were shaken vigorously for

15 sec. The resulting mixture was stored at RT for 10 min and centrifuged at 12.000 g for 20 min at 4°C. Following centrifugation, the colorless upper aqueous phase containing RNA was transferred to a fresh tube. 500 µl TRI-Reagent and 100 µl BCP were added and centrifuged again at 12.000 g for 15 min at 4°C. The RNA was precipitated by mixing with 0.8 ml isopropanol. Samples were stored for 5-15 min at –20°C and centrifuged at 12.000 g for 10 min at 4°C. The RNA pellet was washed with 1 ml of 75% ethanol. The ethanol wash was removed and the pellet was dried at 37°C for 3-5 min. Then, the RNA was dissolved in 50 µl DEPC-water (500µl DEPC/500ml H_2O). To remove genomic DNA, 5 µl 10x buffer and 1 µl DNAseI (Ambion, Hamburg, Germany) was added and incubated for 30 min at 37°C.

Quality of extracted RNA was assured by spectrophotometric determination of the OD_{260}/OD_{280} ratio. A ratio of 1.7 to 2.0 was considered to be free of contaminations, such as phenol or proteins. Template-RNA was quantified by the OD_{260} value.

2.2.4. Reverse transcription from mRNA into cDNA

1 µg of total RNA was reverse transcribed with an Omniscript RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with oligo-d(T) primer (Roche, Mannheim, Germany), which bind to the poly-A tail of mRNA and leads to selective transcription.

In brief, reagents listed below were combined and vortexed for 5 sec. Template RNA was added and again vortexed for 5 sec. This was followed by 60 min incubation at 37°C to reverse transcribe the template RNA into cDNA. The cDNA was then stored at –20°C for further analysis.

Table 1: Reaction mix for reverse transcription

component	volume	Final concentration
10 x buffer	2.0 µl	1 x
dNTP mix	2.0 µl	0.5 mM (each dNTP)
Oligo-dT primer (10 µM)	2.0 µl	1 µM
Rnase inhibitor (10 units/µl)	1.0 µl	10 units
Omniscript Reverse Transcriptase	1.0 µl	4 units
Rnase-free water	variable	
Template RNA	variable	
Total reaction volume	20 µl	

2.2.5. Real time PCR

Real Time PCR was performed in a 20 µl reaction volume containing the solutions listed below.

Table 2: Reaction mix for realtime-PCR reaction

component	volume	Final concentration
10 x buffer	2.0 µl	1 x
$MgCl_2$	2.4 µl	3 mM
dNTP	0.1 µl	
Primer 1	1.2 µl	300 nM
Primer 2	1.2 µl	300 nM
BSA (1mg/ml)	0.8 µl	40 µg
Sybr Green	0.2 µl	
Hotstar Taq Polymerase	0.1 µl	
DNA	variable	50 ng
Rnase-free water	variable	
Total reaction volume	20 µl	

Reaction conditions in a LightCycler (Roche, Mannheim, Germany) were 15 min at 95°C followed by 45 cycles of 15 s at 94°C, 20 s at 58°C and 20 s at 72°C. Temperature change rates were 20°C/s. Copy numbers were determined using a plasmid standard and normalized to expression of ß-actin. Primers used are given in table 1.

Primers were designed using the Primer 3 online programm. Primer requirements for realtime PCR in a LightCycler were:

- size: 18-23 bp
- product size: 100 – 200 bp
- gc-content: 30-70%
- primer melting temperature: minimum 58°C, maximum 62°C

To ensure comparable RNA extraction in each sample, the housekeeping gene ß-actin that is known to be expressed constitutively, was also measured. Cytokine values were correlated to their ß-actin values.

Table 3: primer sequences of primers used in this study

cDNA	Forward	Reverse
ß-Actin	5’ AGA GGG AAA TCGTGC GTG AC 3’	5’ CAA TAG TGA TGA CCT GGC GGT 3’
IFN-g	5’ TCA AGT GGC ATA GAT GTG GAA GAA 3’	5’ TGG CTC TGC AGG ATT TTC ATG 3’
IL-12p40	5’ TGT CCT CAG AAG CTA ACC ATC 3’	5’ TCC AGT CCA CCT CTA CAA CAT 3’
IL-12p35	5´AAC CAG CAC ATT GAA GAC CTG 3´	5´ACA GGG TCA TCA TCA AAG ACG 3´
IL-23p19	5´TGC TGG ATT GCA GAG CAG TAA 3´	5´GCA TGC AGA GAT TCC GAG AGA 3´
MHCII	5’ CTG TGA TCA ACA TCA CAT GGC 3 ’	5’ TTG TGG AAG GAA TAG TCA CGG 3’
IL-10	5´GGT TGC CAA GCC TTA TCG GA 3´	5´ACC TGC TCC ACT GCC TTG CT 3´
IL-4	5’ TGT ACC AGG AGC CAT ATC CAC 3’	5’ GTT CTT CGT TGC TGT GAG GAC 3’
IL-5	5’ AGC ACA GTG GTG AAA GAG ACC TT 3’	5’ TCC AAT GCA TAG CTG GTG ATT T 3’
IL-13	5´AGA CCA GAC TCC CCT GTG CA 3´	5´TGG GTC CTG TAG ATG GCA TTG 3´
Eotaxin	5’ GGC TGA GAT CCA AGC AGT AA 3’	5’ GTG AAG GAA GTG ACC GTG AG 3’
TGF-ß	5’ TGA CGT CAC TGG AGT TGT ACG G 3’	5’ GGT TCA TGT CAT GGA TGG TGC 3’
RANTES	5´TTG CCT ACC TCT CCC TAG AGC 3´	5´GGT TCC TTC GAG TGA CAA ACA 3´

2.2.6. Standardization to plasmids

To design a plasmid for normalization of mRNA, the above PCR products were cloned into TOPO®Cloning vector (Invitrogene, Groningen, Netherlands) according to the maufacturer´s instructions. 4 µl of the PCR product, 1 µl salt solution and 1 µl TOPO® vector was gently mixed, incubated for 5 min at RT and was then placed on ice. 2 µl of this reaction mix was added to chemically competent *Escherichia coli* bacteria, mixed gently and incubated for 5 min on ice. After heatshocking of the cells for 30 sec at 42°C without shaking, they were immediately transferred to ice and 250 µl of room temperature SOC medium was added. 50 µl of the bacteria solution was spread on a prewarmed selection plate and incubated overnight at 37°C. Plasmid positive colonies (white) as determined by blue/white screening were picked and cultered overnight in Circlegrow medium (Q-Biogene, USA) to increase bacteria numbers. 880 µl of the bacteria suspension were supplemented with 120 µl glycerine and stored for further use at –70°C.

Alkaline lysis was performed to isolate the plasmids from bacteria suspension using the NucleoSpin Plasmid kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer´s instructions. 10 µl of this plasmid were than sequenzed to verify insertion of the cDNA (MWG).

These plasmids were used to normalize the copy numbers/µl. In short, the OD was determined at 260 nm, thus giving µg/µl. This was then converted to picomoles/µl, which was then converted to number of molecules/µl.

2.2.7. Gel electrophoresis of PCR-products (DNA and cDNA)

In electrical fields, DNA molecules of different sizes can be estimated and separated. Seperation was carried out on an 1.5% agarose gel, due to the estimated molecule size of 0.2-0.3 kb. Therefore, agarose was dissolved in TBE buffer under heating conditions, 0.2 µg/ml ethidiumbromide added and poured into an electrophoresis chamber (BioRad, Munich, Germany). 5 µl of the PCR product were mixed with 1 µl loading dye (6x) and applied into the gel slots. Electrophoresis was performed at 80 Volt. DNA was then visualized by the activation of the DNA intercalating substance ethidiumbromide by UV light and photographed (Mitsubishi, Japan). The molecular weight was standardized with a DNA-molecular weight marker (1kb, MBI-Fermentas, St. Leon-Rot, Germany).

2.2.8. Infection cycle

The parasite *L. sigmodontis* was maintained by passage through cotton rats (*S. hispidus*) and mites (*O. bacoti*) as described earlier (62). The natural infection of mice with *L. sigmodontis* through the mite vector was carried out as follows. Ten days prior to infection of mice, the mites, which were kept in a glass aquariums filled with about 10 cm high layer of bedding material and covered with a removable sieve, were allowed to have a blood meal for 4-6 hours on microfilaremic (>1000 MF/µl blood) cotton rats. Afterwards, the rat cages were hung on the inside of the cover overnight to allow remaining mites to fall down into the bedding material after the complete suction of blood. The mites that crawled up-ward, were trapped by cotton pieces placed on the upper side of the cover and were returned back to the aquariums. To avoid the contamination of the rooms with mites, the rat cages were placed in a water bath containing detergent for a further 12 hours to guarantee the death of all mites, which remained sticking to the rats. During the 10 days thereafter, the MF (L1) ingested by the mites, develop into infective stage 3 larvae (L3) which then can be transmitted to the mice during a blood meal as that had been described for the rats. All mentioned processes took place under 80-90% air humidity and a room temperature of 26-28°C.

To assure compatibility in all experiments, the genetically different groups of mice were exposed to the same population of mites at the time of infection.

2.2.9. Adult worm recovery and preparation of pleural exudate cells (PLEC) and splenocytes

The number of adult worms in the thoracic cavity of infected mice was assed by flushing the cavity with 2 ml (pleural cavity wash 1) and 8 ml (pleural cavity wash 2) PBS. Subsequently, the worms were allowed to sediment, kept on ice to reduce their motility and then transferred to fresh PBS. Worms were kept at 4°C for further analysis or treatment.
The worm-free thoracic cavity cell suspensions (pleural cavity wash 1 and 2) were centrifuged at 1300 rpm (250 g) for 10 min at 4°C and the supernatant of pleural cavity wash 1 was kept at –20°C for cytokine determination. PLEC were obtained by resuspending cell pellets of pleural cavity wash 1 and 2 in complete medium, pooled and counted in a Neubauer chamber. Spleens were excised and homogenized to single cell suspension. Erythrocytes were removed from splenic cells by incubation for 5 min with Tris-ammonium chloride at RT, and then washed three times in PBS. Cell suspensions were adjusted to $2x10^6$ cells/ml for cell culture.

2.2.10. Assessment of microfilaremia and thoracic cavity microfilariae

Microfilaremia of infected mice was determined during patency at different time points post infection. 50-70 µl blood was collected from the mouse by orbital puncture under full anesthesia using Isoflurane (Abbott, Wiesbaden, Germany). The blood was collected in EDTA containing capillaries and tubes (Kabe, Nümbrecht-Elsenroth, Germany). 50µl blood was diluted in 300 µl Hinkelmann´s solution and then 15 minutes later, after complete lysis of blood cells, centrifuged at 2500 rpm at room temperature. The supernatant was then aspirated and the number of microfilariae in the pellet was determined under a microscope, using 10x magnification. Hinkelmann´s solution was also used to count the thoracic cavity microfilariae at the time of autopsy. 200 µl of 2 ml pleural cavity wash was added to 300 µl Hinkelmann´s solution and treated as described above.

2.2.11. Purification of peripheral blood microfilariae

Since cotton rats display very high microfilaremia, they were used as a source of microfilariae for intraperitoneal injections. Microfilariae were purified from the peripheral blood of cotton rats on a percoll gradient described by (63). Iso-osmotic Percoll (IOP) was

prepared by mixing nine parts of percoll (density 1.13 g/ml) with one part of 2.5 M sucrose. Various dilutions of the IOP in 0.25 M sucrose were made to obtain 25, 30 and 35% solutions. The gradient dilutions were layered in a 15 ml tube then a 2 fold diluted peripheral blood was pipetted on top of the percoll layers. The tubes were than centrifuged at 400 g for 30 minutes at RT. Recovered microfilariae (between 25 and 30% layer) were washed with RPMI-1460-1%FCS and after the assessment of viability, $1x10^6$ MF were injected intraveniously into each mouse.

2.2.12. *L. sigmodontis* antigen

L. sigmodontis extract was prepared from a mixture of adult female and male worms from infected rats. The worms were washed three times in PBS and homogenized in sterile PBS using a homogenizer (B. Braun). The homogenate was centrifuged at 80000 g for 30 min at 4°C. Protein concentration was determined according to Bradford using BSA as a standard. This antigen was diluted as desired for cell culture stimulation in RPMI containing 5% FCS and sterile filtered using a 0.22 µm filter (Schleicher und Schuell, Dassel, Germany).

2.2.13. Cell cultures

PLEC and splenocytes ($2x10^5$ cells/well) were cultured at 37°C and 5 % CO_2 in 96-well microtiter plates, either in complete medium alone, or with 100µl of the following stimuli: 10 µg/ml *L. sigmodontis* soluble antigen and 2.5 µg/ml Con A. Supernatants were removed 96 h after stimulation for cytokine analysis.

2.2.14. Preparation of fecal samples

Fecal contents were removed from the colon, weighed and added to PBS (300µl) containing 10µg/ml each of aprotinin, antipain, chymostatin, leupeptin and pepstatin A (Roche-Boehringer, Mannheim, Germany) and 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Samples were vigorously mixed at 500 rpm for 60 min at 4°C. The homogenate was centrifuged at 10,000 rpm, supernatants were removed and stored at –20°C. For use, fecal samples were diluted 1:320.

2.2.15. Enzyme Linked Immunosorbent Assay (ELISA)

Cytokine concentrations in supernatants of cell cultures, pleura exudates (thoracic cavity wash) and sera were determined by specific sandwich ELISA using Nunc Maxisorp® microtiter plates (Nunc, Roskilde, Denmark) as in Pharmingen standard protocols. The antibody pairs and recombinant proteins as standards used are listed in Table 4. The plates were incubated with 50 µl coating antibody diluted in coating solution overnight at 4°C. After three washes, the plates were incubated with blocking solution, 200 µl/well, for 2 hours at RT and washed again. Samples as well as a series of standards (60 µl/well) diluted in 0.1%BSA, PBS were added and incubated over night at 4°C. After 5 washes, the plates were incubated 60 min with 100µl biotinylated detection antibody solution at predetermined concentrations. The plates were then washed 5 times and incubated with 100 µl streptavidin-peroxidase complex (1:10000, Boehringer, Mannheim, Germany) for 45 minutes at RT. Plates were washed again as previously and 100 µl substrate solution were added. The reaction was stopped after color development using 50µl stop solution. Plates were read at 450 nm using Dynatech MR 5000 reader (Dynatech Hamburg, Germany). The concentration of the samples was determined from the standard curve. Sensitivity was 50 pg/ml for all cytokines.

Table 4: Antibodies used for detection of cytokines and immunoglobulins with ELISA

cytokine	coating antibody	detection antibody	recombinant protein
IFN-γ	R4-6A2, 2µg/ml	XMG1.2, 1µg/ml	19301T
IL-10	JES5-2A5, 2µg/ml	SXC-1, 1µg/ml	19281T
IL-13	MAB413, 2µg/ml	BAF413 2µg/ml	413-ML-005
IgG1	A85-3, 2µg/ml	A85-1, 2µg/ml	MOPC-31C
IgG2a	R11-89, 2µg/ml	R19-15, 2µg/ml	G155-178
IgA	C10-3, 2µg/ml	C10-1, 2µg/ml	

2.2.16. Detection of immunoglobulins using ELISA method

Sample wells were coated over night with anti-mouse IgA, IgG1 and IgG2a (2µg/ml, BD Pharmingen, Heidelberg) in PBS pH 9.6 at 4°C. After blocking with 1% BSA, PBS, faeces supernatants (1:320 dilution in 1% BSA, PBS) or serum samples (1:5 dilution in 1% BSA, PBS) were incubated for 5 h at room temperature. After washing, plates were incubated

with 2 µg/ml isotype-specific anti-mouse immunoglobulin antibodies conjugated with biotin (BD Pharmingen, Heidelberg) for 1 h. ELISAs were developed after incubation with streptavidin-peroxidase complex (1:10,000; Roche-Boehringer,), using 3,5,3`,5`-tetramethylbenzidine (Roth, Karlsruhe, Germany) at 6 mg/ml in dimethyl sulfoxide as substrate). Plates were read at 450 nm in an ELISA reader.

2.2.17. Monoclonal antibody purification

Monoclonal antibody producing hybridomas (XMG1.2) were cultured in ISCOVES medium. Tissue culture supernatants were precipitated using 50% ammoniumsulfate (313 g/l) at 4°C over night, centrifuged at 10,000 g for 30 min, dissolved and dialysed in start buffer for 24 hrs at 4°C. The mouse antibody was further purified using affinity chromatography on protein G sepharose (Pharmacia, Freiburg, Germany) as described by the manufacturer. Briefly, samples were applied to a pre-equilibrated Protein G sepharose Fast Flow column. Unbound proteins were washed using start buffer. The IgG was eluted using elution buffer into a tube containing neutralization buffer. Estimation of protein concentration was performed according to Bradford.

2.2.18. Statistical analysis

Statistical analysis for mRNA expression was performed using ANOVA (analysis of variance) and Student-Newmann-Keuls correction. Furthermore, ANOVA was used to determine differences attributable to the mouse strains independent from the variance of the single experiments. ANOVA was done for parasite loads, MF counts, which had been log transformed to meet parametric assumptions, as well as IL-10 and IFN-γ production of splenocytes. *P* values less than 0.05 were considered significant. Box plots represent median values, 25th and 75th percentiles (boxes) and 10th and 90th percentiles (error bars). The nonparametrical Mann-Whitney-U test, including Bonferroni correction was performed to assess the statistical differences of eosinophil counts in colonic tissue, faecal Ig, parasite loads, microfilarial clearance and humoral IgG production. Kaplan-Meyer test was used to investigate statistical differences in prolapse development.

3. Results

3.1. Impact of IL-4 on the IL-10 deficient mouse model of chronic colitis

Th 1 cells activated by IL-12 and secreting IFN-γ have been described as the main mediators for onset and maintenance of chronic colitis in IL-10 deficient mice. It was therefore surprising that mice deficient for IL-4 in addition to IL-10 showed intestinal pathology very rarely, whereas IL-10 KO mice developed rectal prolapse in most cases (Fig. 1). The first part of this work contains the investigation of ongoing inflammatory processes in mice deficient for IL-4, IL-10 or both cytokines.

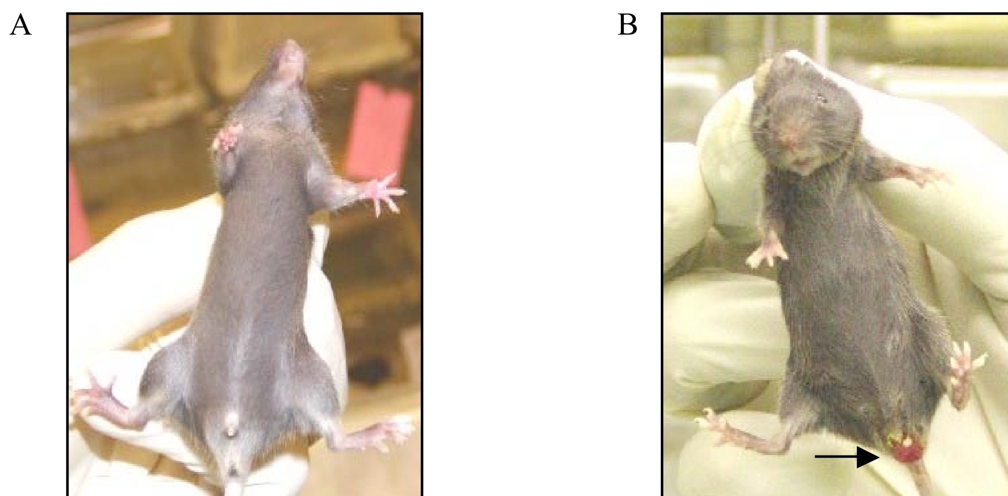


Fig. 2: Mice deficient for both IL-4 and IL-10 (A) or only IL-10 (B). IL-4/10 KO mice developed rectal prolapse only in rare cases, whereas IL-10 KO mice developed prolapse after 10-12 weeks of age (arrow). Rectal prolapse is the most severe form of colitis in the IL-10 deficient mouse model.

3.1.1. Severe colitis is rarely seen in mice deficient for IL-4/10

To investigate the role of IL-4 in a mouse model of chronic enterocolitis, the frequency of induction of severe colitis, i.e. prolapse development, in wild type mice and mice deficient for IL-4, IL-10 or both cytokines was monitored. Wild type and IL-4 KO mice were healthy and without any overt pathology throughout the investigation time. As expected, IL-10 KO mice developed rectal prolapses between weeks 11 (prolapse positive IL-10 KO mice: 27%) and 13 (prolapse positive IL-10 KO mice: 46%) after birth. Figure 3 shows

that in contrast to IL-10 KO mice, IL-4/10 double deficient mice did not develop a rectal prolapse in this experiment (prolapse positive IL-4/10 KO mice: 0.0%).

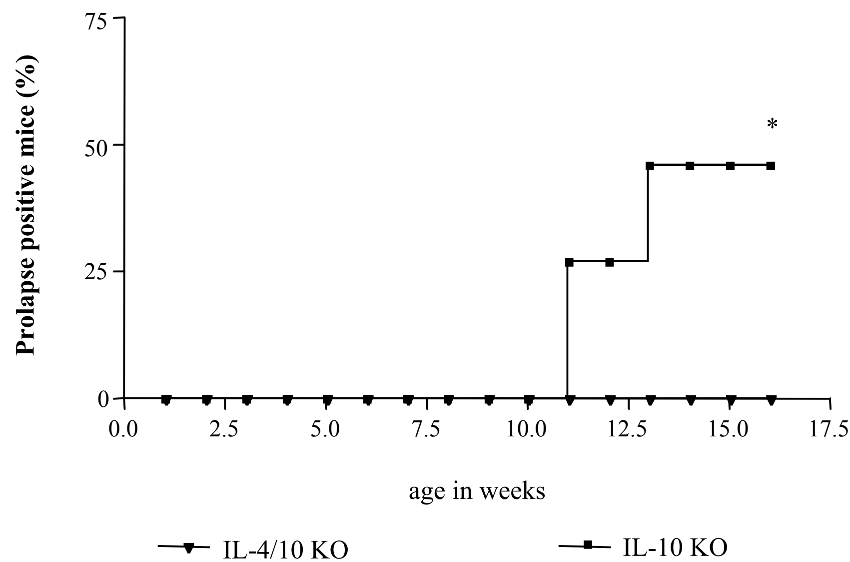


*Figure 3: Percentage of mice with rectal prolapse, indicating severe colitis. Each group consisted of 8-10 animals. IL-10 KO mice developed prolapse in 46% of the cases, whereas none of IL-4/10 KO. IL-4 KO and wild type mice did not develop prolapse and are therefore not displayed in the figure. Asterisks indicate significance (*P *< 0.05, Kaplan-Meier).*

However, in one of three additional independent experiments 13.9% of IL-4/10 double deficient mice did develop prolapse (Table 2). Altogether, a significantly lower incidence of severe pathology ($p< 0.05$, Mann-Whitney U) was observed in IL-4/10 knockout mice. These results give strong evidence that IL-4 is needed for the induction of colitis.

Table 2: IL-4/10 KO mice show low to absent rectal prolapse development.

Exp. No	IL-4/10 KO	IL-10 KO
2	13.9 % (5/36)	43.8 % (21/48)
3	0.0 % (0/21)	66.7 % (10/15)
4	0.0 % (0/23)	38.1 % (8/21)

3.1.2. Th1 type cytokine mRNA levels are increased during onset of colitis in IL-10 KO mice but remained unchanged in IL-4/10 double deficient mice

To further characterize the inflammatory process, taking place in IL-10 deficient mice at the onset of colitis but not in IL-4/10 deficient mice, mRNA expression in colonic tissue was investigated. RNA was recovered using TRIzol, reverse transcribed and screened for several markers of Th1 and Th2 type responses (Figure 4).

IFN-γ mRNA was clearly upregulated in IL-10 KO mice at the time of onset of colitis, whereas all other strains expressed relatively low levels IFN-γ mRNA (Figure 4A). Another important cytokine, which has been shown to be relevant for induction of IFN-γ in murine enterocolitis, is IL-12. The IL-12p40 subunit of IL-12 has been reported to reflect the biologically active IL-12p70 heterodimer (64). Therefore, the expression of IL-12p40 was measured. IL-12p40 expression correlated with that of IFN-γ, being significantly higher in IL-10 KO mice (Figure 4B). The same pattern could be found for MHCII expression (Figure 4C). MHCII, a molecule that binds extracellular antigens taken up through phagocytoses and presenting them to T cells, was expected to be highly expressed during ongoing inflammatory processes. It is known to be up-regulated not only by IFN-γ but also by IL-4 (20, 65, 66).

In order to investigate a possible Th2 induction, IL-4 expression in wildtype and IL-10 KO mice, and IL-13 in all mouse strains were measured. IL-4 was expressed at low levels with no differences between wild type and IL-10 KO (Figure 4D), whereas IL-13 was at detection limit (Table 6).

These results agree with previous reports of increased IFN-γ and IL-12 expression in IL-10 deficient mice at the time of disease onset. However, this Th1-mediated induction process is lacking in IL-4/10 KO mice. Even though no induction of Th2 response could be observed at the mRNA level in IL-10 KO mice, IL-4 appears to be necessary for mounting the Th1 response responsible for pathology of the disease as seen in prolapse development.

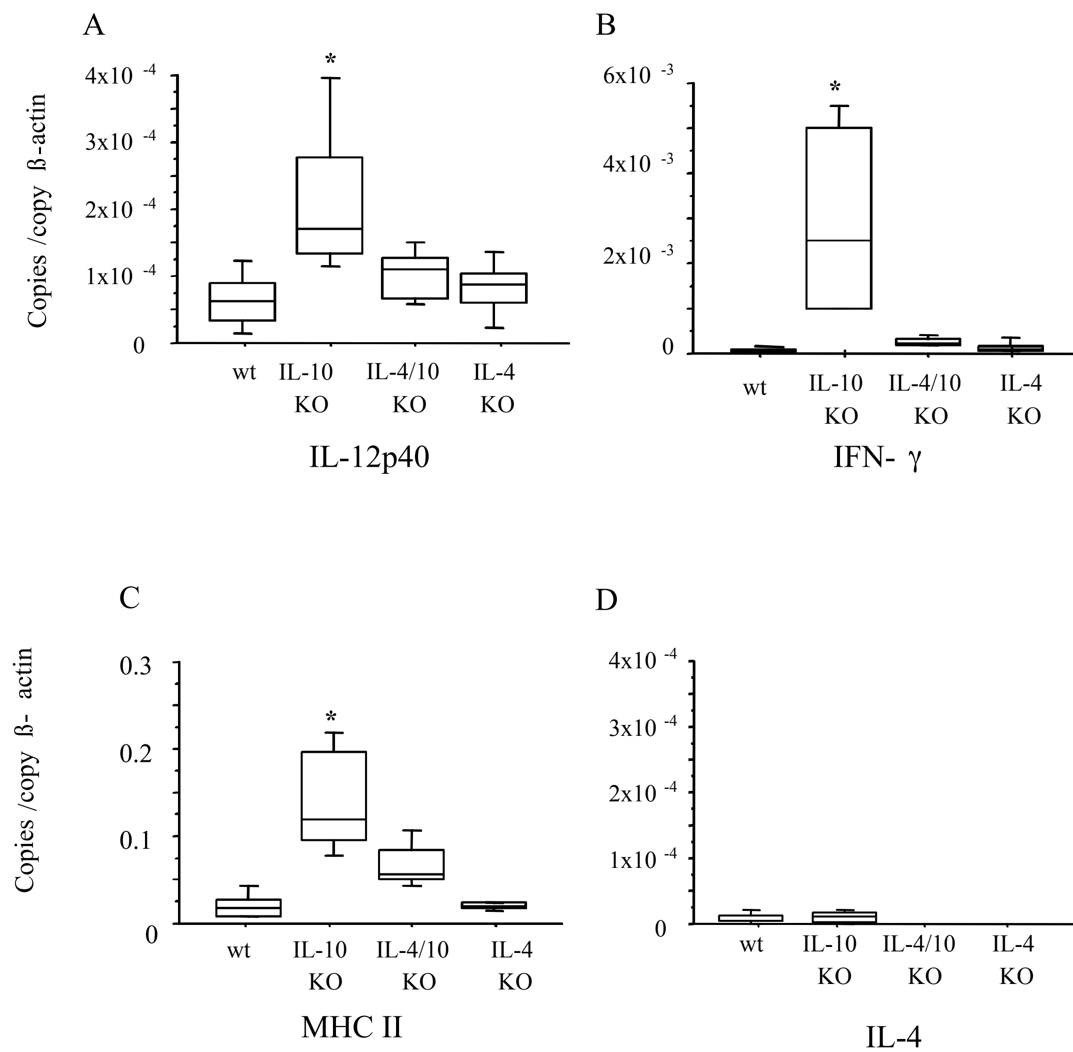


Figure 4: Th1-associated gene expression is increased only in colon of IL-10 deficient mice. Data represent copies of cytokine mRNA / copy ß-actin (n=5-8). Significant differences (as indicated by asterisks, ANOVA and Student-Newman-Keuls, $P < 0.05$) between IL-10 KO and all other strains could be observed for IL-12p40 (A), IFN-γ (B) and MHC II (C), but not IL-4.

3.1.3. Longitudinal study of inflammatory mediators in IL-4/10 KO vs IL-10 KO mice

To investigate the role of IL-4 in disease progression, mRNA expression in the colonic tissue of mice of different ages and disease progression/status was analyzed. Mice were divided into three groups. In addition to the mice analyzed at onset of colitis (10-25 weeks), mRNA expression was determined over the course of time at pre- (4-8 weeks of age) and late (>25 weeks) disease (Table 6).

In the early phase of disease cytokine expression with no differences between mouse strains could be observed, even though the absolute baseline levels were different for the tested cytokines. In contrast during onset the expression of IFN-γ, IL-12p40 and MHCII was significantly elevated in IL-10 deficient mice compared to the other strains. In detail, a 60-fold, 2-fold and 10-fold increase in IFN-γ, IL-12p40 and MHCII expression in IL-10 KO was observed in contrast to the early phase. These differences were significant when compared to all other mouse strains.

Finally, in the late phase, levels of IFN-γ, IL-12p40 and MHCII mRNA were decreased to baseline in IL-10 KO mice. However, these parameters as well as IL-10 were elevated in IL-4 KO in the late phase of the disease.

Expression of the suppressive cytokine TGF-ß did not show group specific differences at any of the investigated time points, even though their baseline levels do vary longitudinally. Furthermore, comparison of IL-4 expression in wild type and IL-10 KO mice reveals no significant differences at any of the time points.

Altogether, these data (Table 6) show a strong correlation between disease induction and up-regulation of IFN-γ, IL-12p40 and MHCII and therefore suggest that the combination of all three parameters at the same time may contribute to the development of pathology.

Table 6: mRNA expression in colon of genetically deficient mice. Only IL-10 deficient mice show Th1 type cytokine expression. mRNA, depict as x10^{-4} copies/ß-actin, are given. Bold numbers indicate significance compared to all other groups (ANOVA, Student-Newman-Keuls post hoc).

	disease status	wt	IL-10 KO	IL-4/10 KO	IL-4 KO
IFN-gamma	pre	0.8 ± 0.5	0.5 ± 0.2	2.2 ± 0.6	0.9 ± 0.4
	onset	0.7 ± 0.2	**30 ± 10**	2.6 ± 0.4	1.4 ± 0.6
	late	2.1 ± 1.6	1.7 ± 1.2	2.8 ± 1.6	10 ± 1.3
IL-12p40	pre	0.5 ± 0.3	0.9 ± 0.5	0.5 ± 0.2	0.1 ± 0.06
	onset	0.6 ± 0.2	**2.0 ± 0.4**	1.0 ± 0.2	0.8 ± 0.2
	late	2.4 ± 1.2	1.4 ± 0.6	3.2 ± 1.5	9.7 ± 2.0
MHCII	pre	620 ± 60	260 ± 50	430 ± 100	280 ± 2.0
	onset	200 ± 50	**1360 ± 180**	670 ± 110	190 ± 20
	late	260 ± 80	510 ± 250	830 ± 27	**1300 ± 280**
IL-4	pre	0.2 ± 0.2	0.3 ± 0.2	ND	ND
	onset	0.08 ± 0.02	0.1 ± 0.03	ND	ND
	late	0.8 ± 0.6	4.5 ± 4.3	ND	ND
IL-13	pre	0.08 ± 0.07	0.08 ± 0.03	0.13 ± 0.05	0.02 ± 0.006
	onset	0.02 ± 0.009	0.002 ± 0.002	0.009 ± 0.008	**0.05 ± 0.02**
	late	0.29 ± 0.16	0.18 ± 0.18	0.017 ± 0.008	0.46 ± 0.31
IL-5	pre	0.02 ± 0.01	0.005 ± 0.002	0.04 ± 0.04	0.002 ± 0.001
	onset	0.01 ± 0.005	0.02 ± 0.006	0.01 ± 0.003	0.04 ± 0.02
	late	0.01 ± 0.01	0.0 ± 0.0	0.04 ± 0.02	0.07 ± 0.06
eotaxin	pre	2.8 ± 1.1	2.0 ± 1.0	2.1 ± 0.4	1.5 ± 0.5
	onset	19.1 ± 10	11.0 ± 2.7	9.8 ± 1.8	10.1 ± 2.7
	late	10 ± 4.5	4.1 ± 2.1	3.2 ± 0.6	3.9 ± 1.0
TGF-ß	pre	2180 ± 630	3300 ± 560	3380 ± 990	5090 ± 1420
	onset	490 ± 60	550 ± 60	630 ± 50	1560 ± 950
	late	2740 ± 560	1310 ± 110	1530 ± 150	2490 ± 290
RANTES	pre	0.16 ± 0.08	0.24 ± 0.08	0.25 ± 0.01	0.12 ± 0.04
	onset	0.08 ± 0.05	**0.72 ± 0.15**	0.23 ± 0.06	0.09 ± 0.04
	late	0.11 ± 0.06	**3.5 ± 1.4**	0.22 ± 0.07	0.09 ± 0.04
IL-10	pre	0.18 ± 0.13	ND	ND	0.10 ± 0.02
	onset	0.16 ± 0.04	ND	ND	0.29 ± 0.04
	late	0.09 ± 0.03	ND	ND	**2.23 ± 0.6**

3.1.4. Faecal IgG 1 is increased in IL-10 KO

A shift in IgG subclasses is an important indicator, more stable than the actual cytokine measurement. It reflects long-term induction of a particular T helper response. To investigate the immunoglobulin profile at the site of disease, proteins from faeces of mice at the onset of colitis were isolated. Sandwich ELISA was performed to detect total amounts of IgG1, IgG2a and IgA. No IgG2a was detectable in any of the strains (not shown), whereas the highest amount of IgG1 was present in IL-10 deficient mice (Figure 5A). Weak IgG1 expression was seen in both strains deficient for IL-4. There were equivalent levels of IgA in all strains (Figure 5B). The higher presence of IgG1 indicates

that IL-4 dependent mechanisms are elevated locally in the gut tissue of IL-10 KO, a phenomenon that may not have been detectable when analyzing IL-4 mRNA expression.

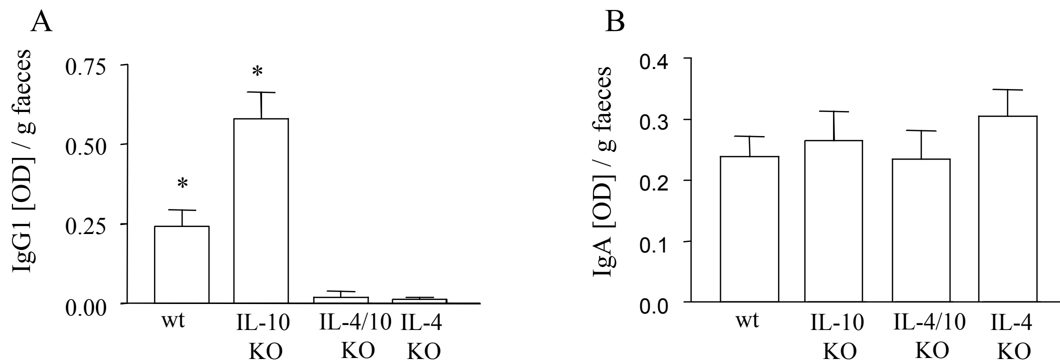


Figure 5: Faecal immunoglobulin levels of wild type, IL-10 KO, IL-4/10 KO and IL-4 KO. For each isotype, colon contents were treated with proteinase inhibitors. Sandwich ELISA was performed on total soluble protein from faeces to determine IgG1 / g faeces (A) and IgA / g faeces (B). (*) indicates significant difference in IgG1 concentration (Mann-Whitney-U test and Bonferroni correction, $P < 0.05$)

3.1.5. IL-4/10 KO mice develop less colonic eosinophilia compared to IL-10 KO

Colitis in IL-10 KO mice is characterized as overwhelming Th1 response. Eosinophils were observed in colon tissue but not further discussed when the IL-10 knockout strain was first described (19). However, eosinophils are a hallmark of ongoing Th2 responses. Since they can be immunostained in tissue, they offer another means to visualize the presence of possible Th2 responses in intestinal tissue. Eosinophil numbers were assessed in different compartments of the intestine of naive wild type mice, IL-4 KO, IL-10 KO and IL-4/10 double deficient mice at the time of onset of colitis. The analysis of eosinophils was carried out on formaldehyde fixed tissue, using either specific anti-MBP antibodies (Figure 6A) or unspecific with FITC (Figure 6B) that binds almost exclusively to positively charged proteins such as those present in the eosinophilic granule.

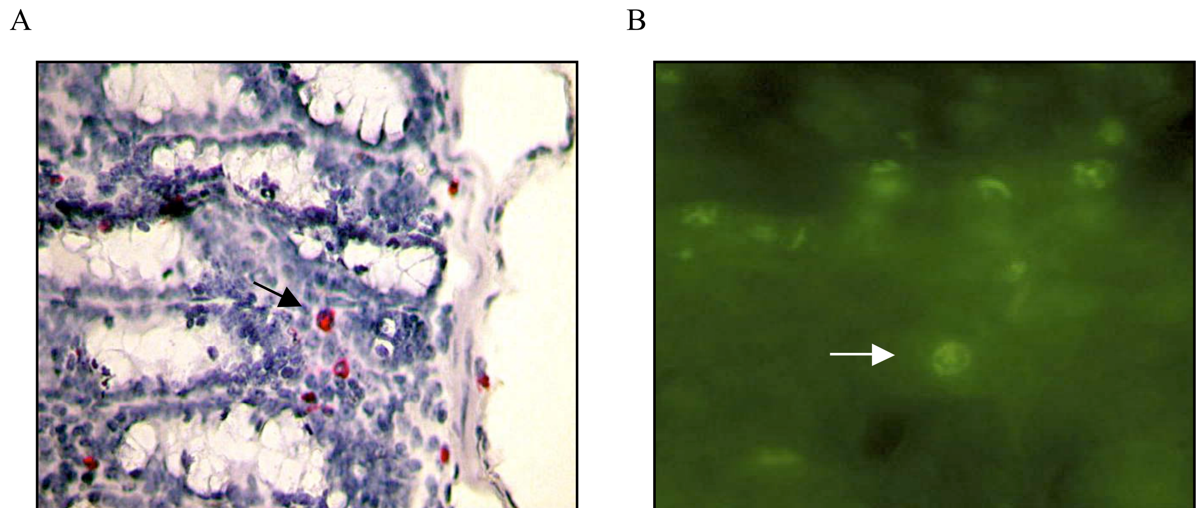


Figure 6: Arrows depict eosinophils in colon sections either stained with anti-MBP antibodies (A, 400 x magnification) or FITC (B, 1000 x magnification).

To assess the eosinophilia throughout the small intestine, eosinophils of the duodenum, jejunum and ileum were stained with FITC and counted. Significant differences in eosinophil counts could only be observed in the jejunum of the small intestine (Figure 7B).

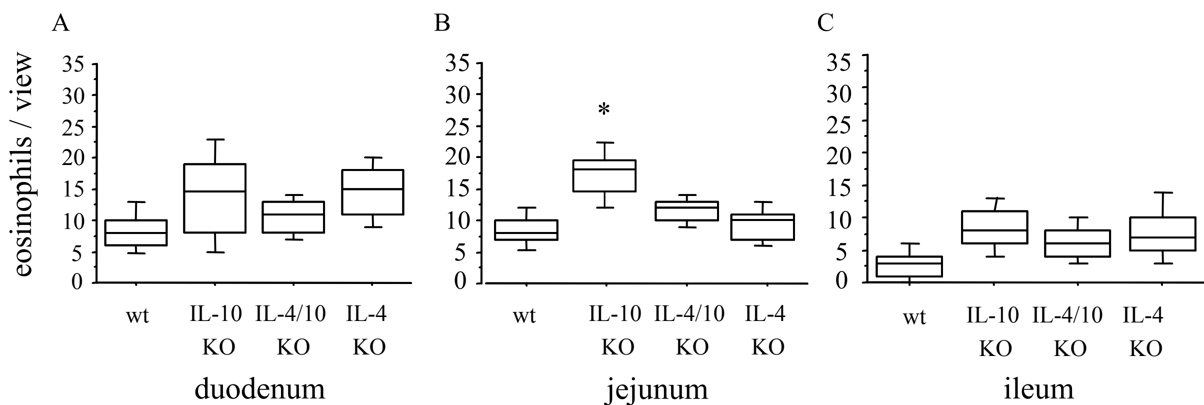


Fig. 7: Eosinophil quantification of sections from three parts of the small intestine (duodenum (A), jejunum (B), ileum (C)) of C57BL/6 wt, IL-10 KO, IL-4/10 KO and IL-4 KO at the onset of disease. Data represent eosinophils/view in 8 random sections per mouse (n=5-8).

Importantly, the number of eosinophils in the colon was significantly increased in IL-10 KO mice, whereas additional knockout of IL-4 reversed this finding (Figure 8). This observation was confirmed over the time of disease progression (Fig. 9A-C).

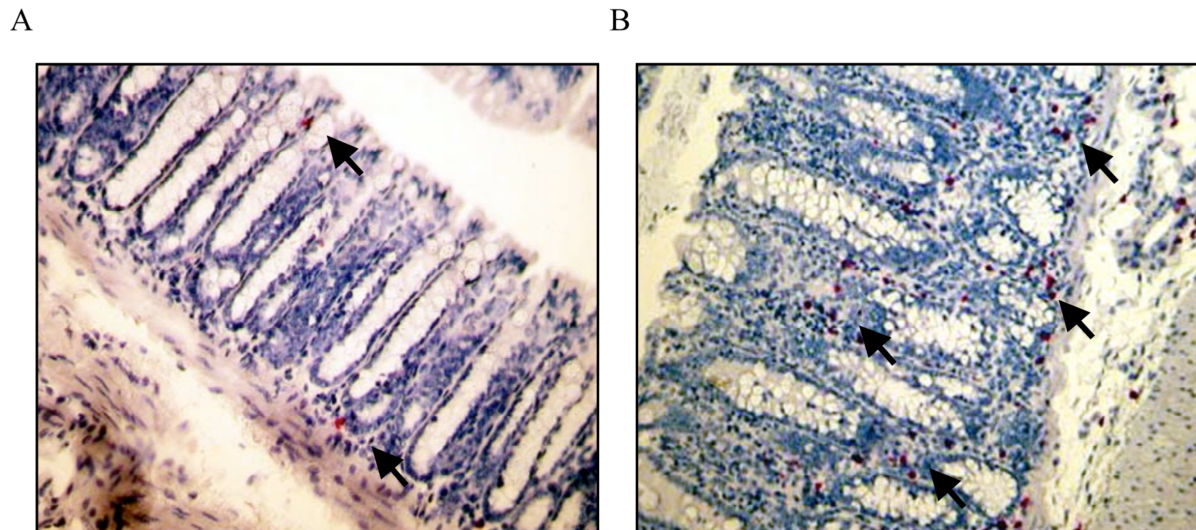


Figure 8: Histological sections of the colon of IL-10 KO and IL-4/10 KO mice. Sections were stained with anti-MBP and counterstained with heamalum. Arrows depict representative eosinophils in IL-4/10 KO (A) and 10 KO (B). Original magnification was 200x.

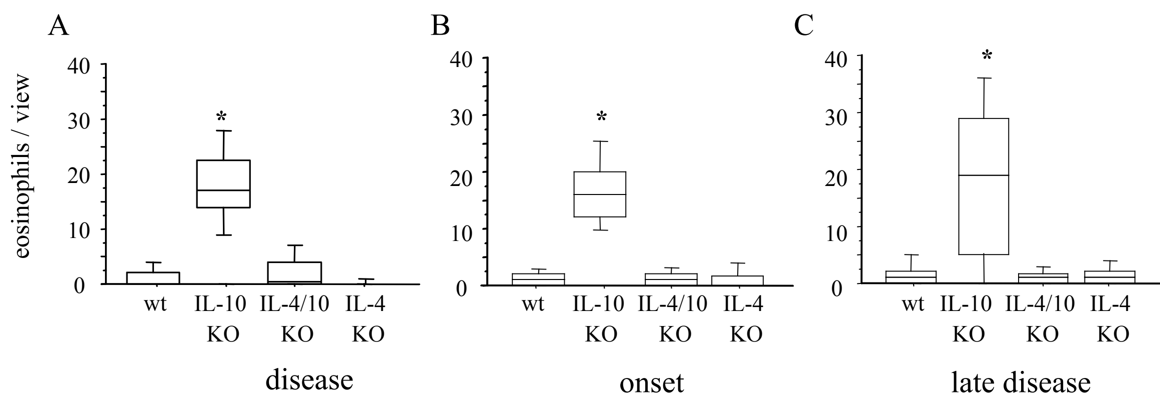


Figure 9: Eosinophil quantification in sections of colon from wild type, IL-10 KO, IL-4/10 KO and IL-4 KO mice at pre-disease (A), onset (B) and late disease (C). Data represent eosinophils/view in 8 random sections per mouse (n=5-8). Significant differences between IL-10 KO and all other strains could be observed (as indicated by asteriks, Mann-Whitney-U test and Bonferroni correction, $P < 0.05$).

To better define eosinophilia in inflammed regions, in IL-10 KO mice aged 10-25 weeks, eosinophil infiltration between mice with prolapse and those without were seperated. In IL-

10 KO mice not yet suffering from severe pathology, increased levels of eosinophils could be found in the colonic tissue (Figure 10). Compared to IL-10 KO mice without severe pathology (median of eosinophils per section: 13), prolapsed mice show even significantly higher levels of eosinophils (median of eosinophils per section: 20) in colonic tissues.
The fact that colonic eosinophil infiltration precedes the onset of colitis may indicate a role of eosinophils in the development of this disease.

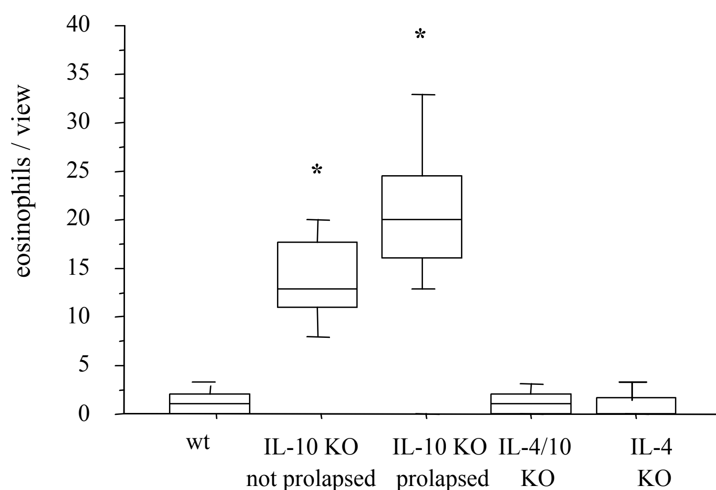


Figure 10: Number of eosinophils is strongly increased in sections of colon tissue of IL-10 KO, but not IL-4/10 KO and IL-4 KO mice. IL-10 KO mice were subdivided at onset of disease into prolapse negative (not prolapsed) and positive (prolapsed) mice. Data represent eosinophils/view in 8 random sections per mouse (wt, IL-4 KO and IL-4/10 KO (n=5-8), IL-10 KO (n=3)). Significant differences are indicated by asterisks, Mann-Whitney-U test and Bonferroni correction, $P < 0.05$*) between IL-10 KO and all other strains.*

3.1.6. Inflammation in colon tissue

In order to investigate pathological changes in colon tissue, histological examination was carried out (Figure 11). Histological sections of IL-10 KO mice revealed extensive lymphoplasmocytic and histiocytic infiltration of the lamina propria and lamina muscularis mucosae, which was strongly extended (Figure 11 B). These abnormal alterations were not seen in colon tissue of all other mouse strains. An increase of mucin in lamina propria goblet cells was observed in mouse strains deficient for IL-4, when compared to wildtype. In particular, the additional knockout of IL-4 in the IL-4/10 KO mice resulted in a prevention of the colonic inflammation seen in IL-10 single KO mice, clearly arguing for

antagonistic effects between IL-4 and IL-10. However, the reason for mucin increase was not further investigated, because it was not the focus of this study.

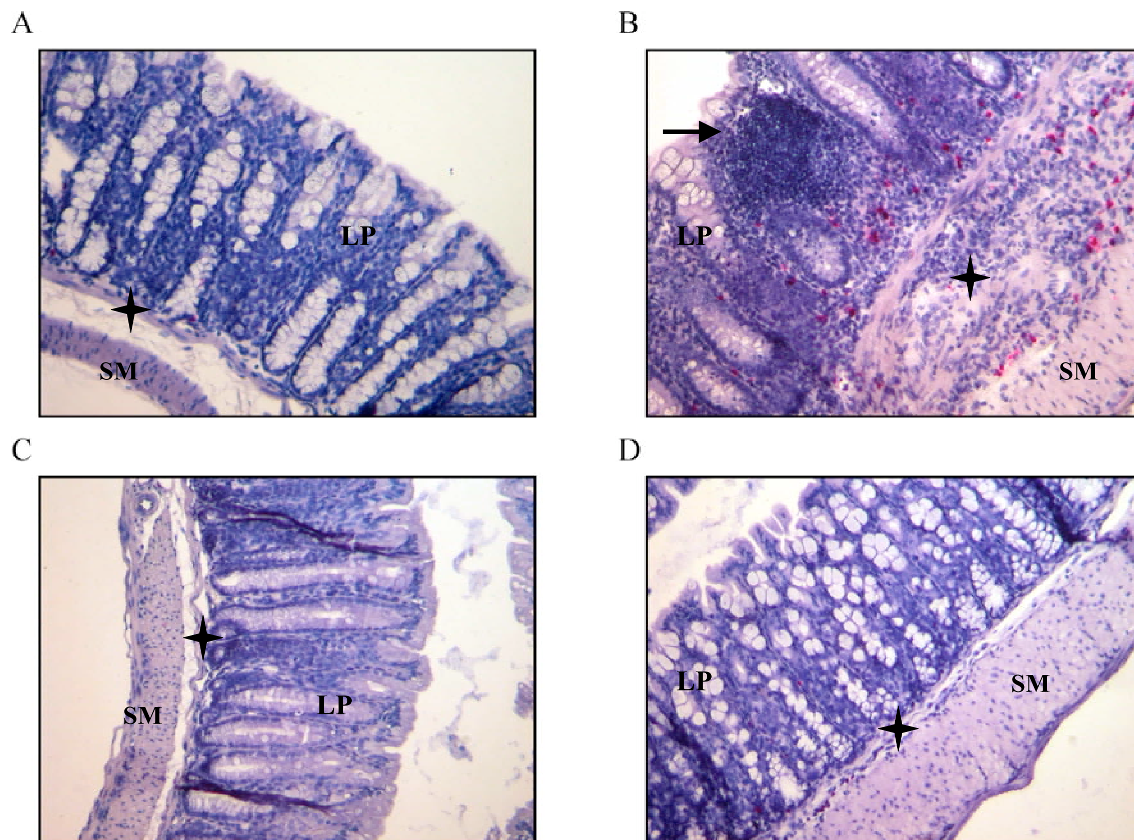


Figure 11: Histological sections of the colon of IL-4/10 KO (A), IL-10 KO (B), wildtype (C) IL-4 KO (D) mice. Sections were stained with anti-MBP and counterstained with heamalum. Original magnification was 200x in A, B, D and 140x in C. Stars depict lamina muscularis mucosae, SM = submucosa, LP = lamina propria. The arrow in (B) points to a lymph follicle.

3.1.7. RANTES is expressed in IL-10 KO mice

To find possible inductors for the attraction of eosinophils into the colon, mRNA levels of IL-5, eotaxin 1 and RANTES were analysed. While IL-5 expression was at the detection limit and eotaxin was expressed at similar levels in all groups, a significant increase in RANTES expression could be observed in IL-10 deficient mice (Table 6). Correlating this to eosinophilia levels in old mice, RANTES expression stays upregulated over time and even increased (Figure 12 B, C). However, in young mice no significant differences in

RANTES expression exist between the mouse strains, suggesting that at early timepoints another eosinophil chemoattractant than RANTES could be active.

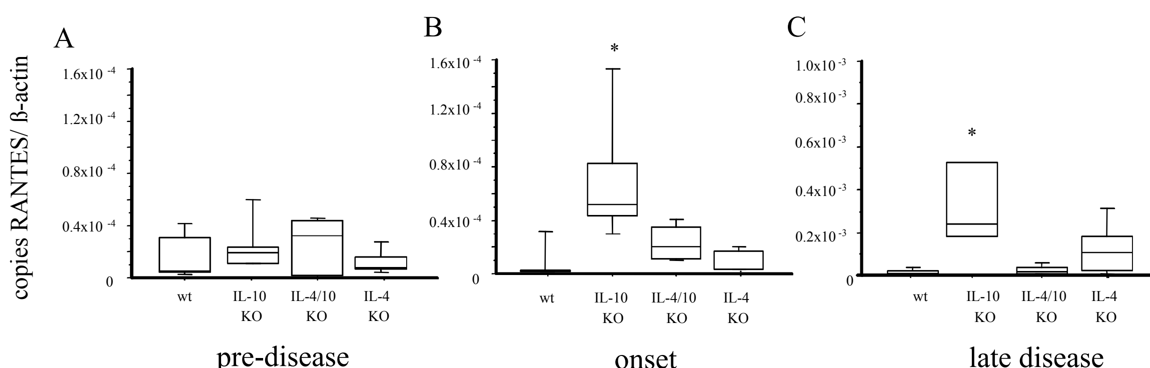


Figure 12: The mRNA of the eosinophil chemoattractant RANTES is increased only in colons of IL-10 deficient mice during onset and late disease. Data represent copies of mRNA / copies ß-actin (n=5-8). Significant difference between IL-10 KO and all other strains was indicated by asterisks (ANOVA with Student-Newman-Keuls, $P < 0.05$*).*

3.2. Antagonism of IL-4 and IL-10 in a mouse model of helminth infection (*Litomosoides sigmodontis*)

The second part of this study tries to add proof to the concept that has arisen in human filariasis, namely that IL-10 (produced by T cells and also a variety of non T cells) dependent responses which are associated with patency are antagonistic to bona fide Th2 responses that control parasite loads (26, 58, 67). However, antagonistic activity between IL-4 and IL-10 has not been directly assessed. To test this hypothesis in vivo, mice deficient for IL-4, IL-10 or both cytokines were infected with the rodent filaria *L. sigmodontis*.

3.2.1. Susceptibility of IL-4 KO to *L. sigmodontis* infection is reversed when IL-10 is also knocked out

The course of infection was followed in C57BL/6 wildtype, IL-4 KO, IL-10 KO and IL-4/10 double KO mice. At day 65 p.i., all worms had been cleared in wildtype mice, whereas IL-4 KO still harbored a significant number of worms (Figure 13). Interestingly,

the additional deficiency of IL-10 in IL-4/10 double knockout mice reversed the susceptibility seen in IL-4 KO. Only two out of six double knockouts harbored 3 to 5 adult worms, whereas all six IL-4 KO contained adult worms (range 16-147). IL-10 KO mice showed worm levels equivalent to IL-4/10 KO mice. This finding was reproduced in six additional experiments. ANOVA testing confirmed that parasite loads are independent from experiment to experiment variation and strictly depend on the single (IL-4 KO) versus double (IL-4/10 KO) deficiency ($p < 0.0001$).

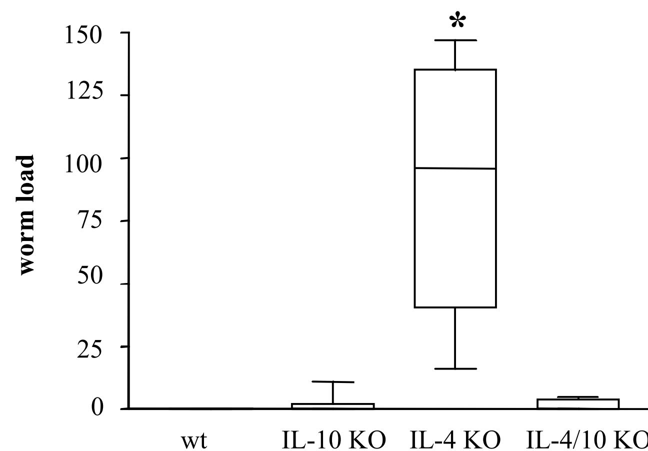


Fig. 13: IL-4 KO mice contained significantly more adult parasites compared to C57BL/6, IL-10, IL-4/10 KO mice, infected with L.sigmodontis day 65 post infection. Viable parasites were obtained by pleural lavage and counted. One of six consistent experiments is shown, comprising six to eight animals per group, P = 0.0024, Mann-Whitney-U test and Bonferroni correction

Worms in IL-4 knockout mice also reached sexual maturity and released microfilariae into the thoracic cavity and eventually in the blood.
No microfilariae were detected in the pleural cavity or blood of wildtype, IL-10 KO and IL-4/10 double knockout mice (Table 7).

Table 7: Development of patent infection in IL-4 KO mice, but not IL-10 KO strains. Blood and pleural cavity microfilarial status was assessed at day 65 p.i. in L. sigmodontis *infected C57BL/6, IL-10, IL-4 and IL-4/10 deficient mice.*

	No. of MF in [a]	
no of microfilariae	**200μl pleural lavage fluid**	**50μl blood**
C57BL/6	**0-0**	**0-0**
IL-4 KO	**31 [1-1982] [b]**	**0 [0-113] [c]**
IL-10 KO	**0-0**	**0-0**
IL-4/10 KO	**0-0**	**0-0**

[a] medians [10-90% percentiles] are given

[b] P<0.0001 using ANOVA test of log-transformed pleura MF counts

[c] P=0.0198 using ANOVA test of log-transformed blood MF counts

Day 42 p.i. was selected for analysis on the basis of the available literature (39). Two experiments were carried out, one of which is shown in Figure 14. There is a clear trend towards higher worm loads in IL-4 KO mice at this time point. This is due to the onset of worm destruction in all other mouse strains. This experiment also confirmed the result seen in Figure 12 day 65 p.i., where higher worm load in IL-4 KO reached significance compared to all others. Absolute worm numbers varied considerably between experiments (compare Figure 13 and Figure 14). This is due to natural variation of infective larvae in the mite population used for infection. However, the differences between IL-4 KO and the other groups remained over that range of worm load.

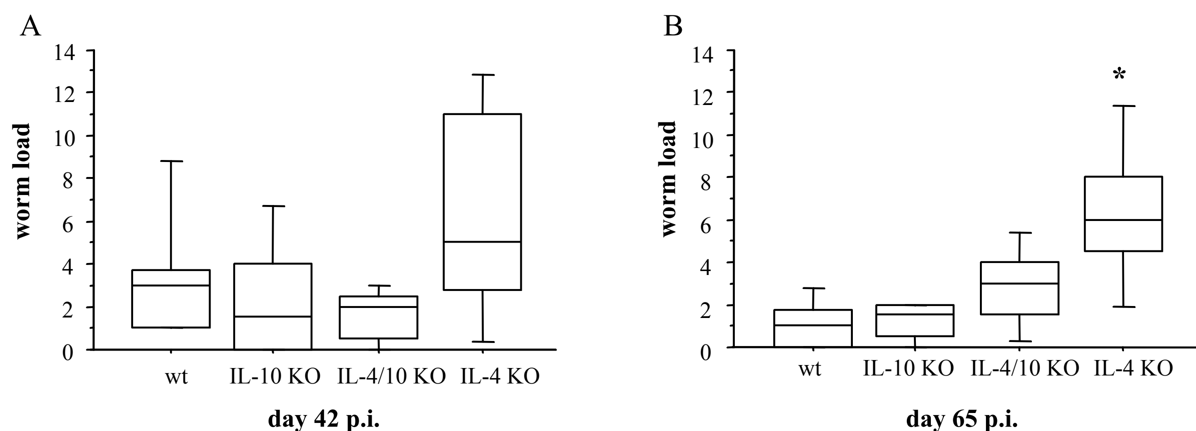


Fig. 14: IL-4 KO mice show a tendency towards higher worm load compared to C57BL/6, IL-10, IL-4/10 KO mice, infected with L.sigmodontis *day 42 post infection. At day 65 post infection worm load in IL-4 KO*

increased to significance (P < *0.05). One of two consistent experiments is shown, comprising six to eight animals per group.*

3.2.2. Equivalent clearance of intravenously injected microfilariae in all mouse strains

In order to monitor clearance of MF without interference of adult worms, 100,000 MF of *L. sigmodontis* were injected intravenously. IL-10 KO mice were not avaliable for this experiment, but are known to show equivalent declines of MF as wildtype C57BL/6 mice (51). All mouse strains cleared the MF with similar kinetics (Figure 15). A 50% drop of microfilaraemia was seen on day 1 following injection in all strains. No microfilaraemia was detected past day 6. This indicates that there is no direct effect of cytokine deficiency on MF containment.

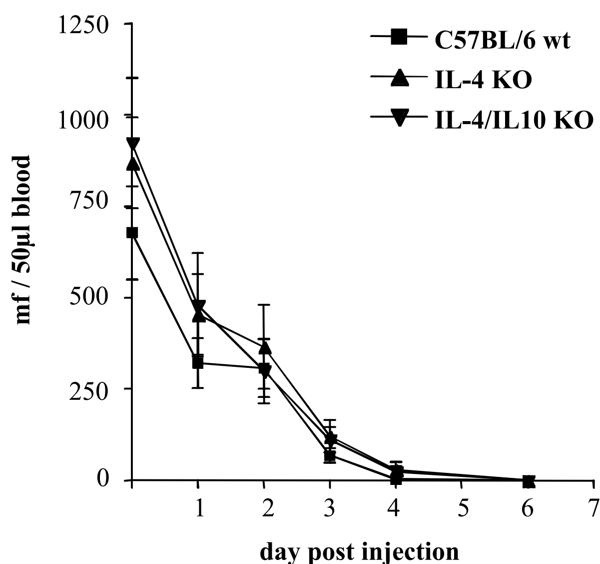


Figure 15: *There is no difference in the clearance of microfilariae (mean values ± SEM) following intravenous injection of 100,000 MF in C57BL/6, IL-4 and IL-4/10 KO mice (n=8 in each group). One of two consistent experiments is shown.*

3.2.3. Filarial specific antibody responses do not correlate with the parasite load

L. sigmodontis antigen specific antibodies in the sera on day 65 p.i. were investigated. Consistent with the fact that IL-4 is a major factor of class switching to IgG1, the two strains deficient for IL-4 produced none to low amounts of filaria specific IgG1 (Figure 16A). Conversely, these strains displayed elevated levels of IgG2a (Figure 16B). The fact that immunoglobulin subclass production followed the pattern of IL-4 expression but not

that of parasite loads suggests that antifilarial antibodies of these subclasses do not correlate with parasite containment in these mouse strains.

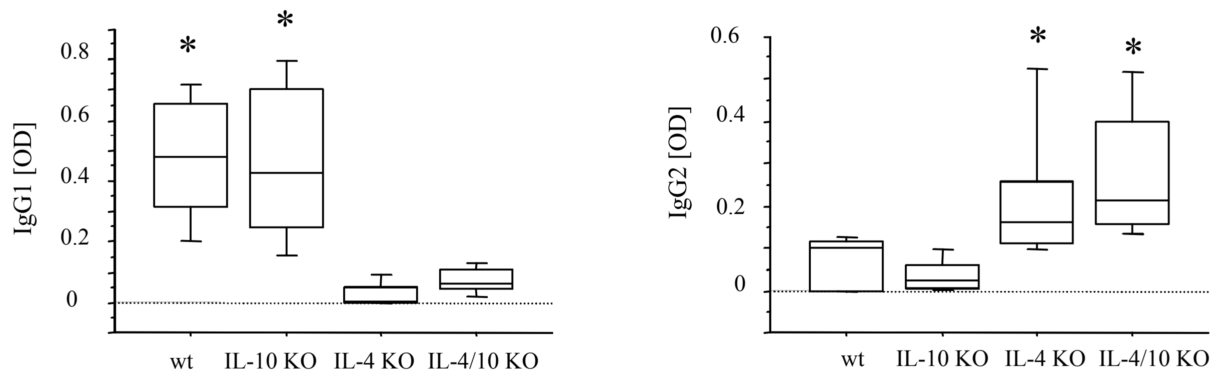


Figure 16: Humoral analysis of C57BL/6, IL-4 KO, IL-4/10 KO and IL-10 KO following infection. Results for filarial specific IgG1 (A) and IgG2a (B) are showns. For each isotype, four to five mice were bled at day 65 p.i. by retro-orbital-vein puncture. Sandwich ELISA was performed to determine filarial specific serum concentration of the relevant isotype. (*) Represents a significant difference of IgG concentration (Mann- Whitney-U test and Bonferroni correction; P *= 0.0044). Figures are one of two representative experiments.*

3.2.4. Anti-IFN-γ antibody does not alter parasite loads in IL-4/10 KO mice

Given that in BALB/c mice an impact of IFN-γ on the control of adult worms could be shown earlier (41), it was of interest to monitor IFN-γ production in the different mouse strains after *L. sigmodontis* infection. At day 65 p.i., splenocytes of KO strains in comparison to wildtype mice displayed increased levels of IFN-γ in vitro upon stimulation with mitogen or *L. sigmodontis* antigen (Figure 17) with strongly higher amounts being produced by the IL-4/10 double knockout compared to IL-4 single knockout mice. In IL-4/10 KO and IL-10 KO mice, elevated basal secretion of IFN-γ was observed.

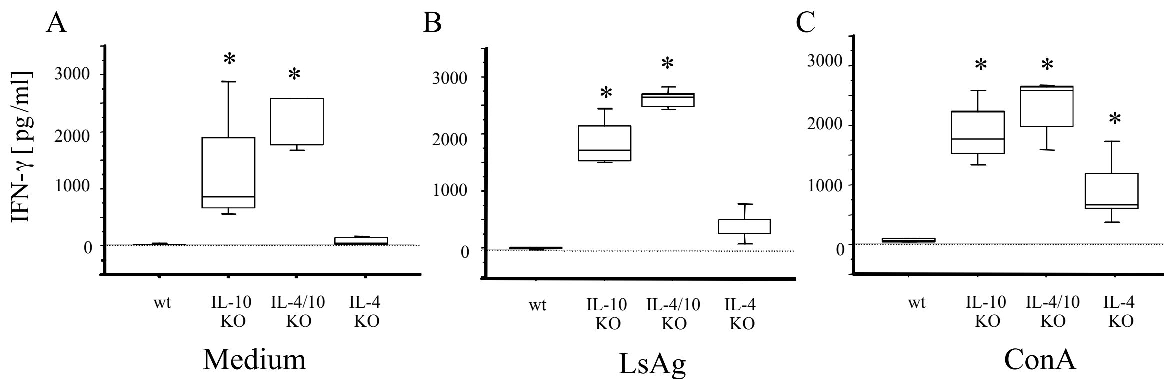


Figure 17: Splenocytes of infected mice with IL-10 deficiency show higher IFN-γ production at day 65 p.i. unstimulated (A) as well as after restimulation with filarial antigen (B) and ConA (C). Sandwich ELISA was performed to determine murine IFN-γ concentration. Two consistent experiments, comprising 4-5 animals per group, were combined using ANOVA, showing that this difference is not due to variations from infections. The asterisks denote significantly higher IFN-γ levels in IL-10 and IL-4/10 KO strains compared to wildtype and IL-4 KO ($P < 0.0001$*; A, B, C) and in IL-4/10 KO, when compared compared to IL-10 KO (*$P < 0.05$*; A, B).*

To find out whether the increase in IFN-γ production in IL-4/10 KO double knockout mice was responsible for their resistance to a patent infection, anti- IFN-γ antibody was injected into the peritoneal cavity of these mice at weekly intervals from day 20 to day 41 p.i. in a separate experiment.

Mice were sacrificed and analyzed for their worm containment at 65 days p.i. Consistently, the number of worms found in the thoracic cavity of IL-4 KO was significantly higher than all other strains. However, in two separate experiments no difference was observed between anti-IFN-γ treated and control-Ig treated IL-4/10 KO mice (Figure 18). There was also no significant difference when data from the two experiments were combined (ANOVA). An insufficient efficacy of the anti-IFNγ antibody treatment is not likely since rectal prolapse development in the IL-4/10 KO strain (observed not spontaneously but after infection in approximately one third of cases) was reversed each time after the repeated injections. In addition, analysis of IFN-γ levels at day 65 p.i., i.e. 23 days after the last antibody administration, showed that IFN-γ levels are highly elevated in IL-4/10 KO mice (420 ± 253 pg/ml, SE), but reduced to the level of IL-4 KO (11 ± 9 pg/ml, SE) in anti-IFN-γ treated IL-4/10 KO (7 ± 6 pg/ml, SE) mice.

As a further control experiment IFN-γ KO mice on C57BL/6 background were infected with *L. sigmodontis*. The course of parasite infection appeared equivalent to that of

C57BL/6 wt mice in that at day 65 p.i. (i.e. at the time point when IL-4/10 KO treated with anti-IFN-γ were assessed) neither strain contained adult worms (not shown). This further suggests that in mice on C57BL/6 background, IFN-γ is not a major mediator of parasite control.

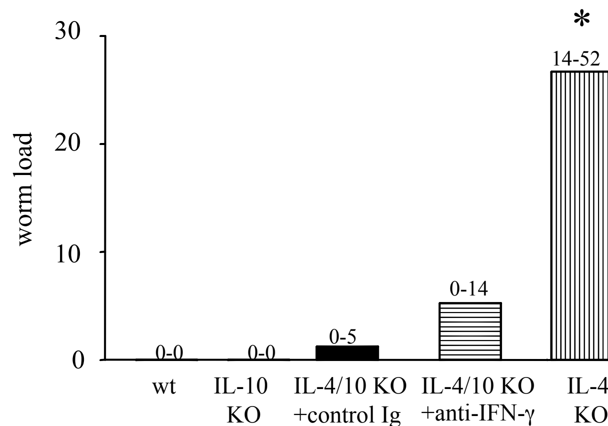


Figure 18: Treatment of infected IL-4/10 KO mice with anti-IFN-γ antibodies (horizontal stripes) has no effect on worm load compared to IL-4/10 mice treated with control Ig (black bars). C57BL/6, IL-10, IL-4 and IL-4/10 KO mice were naturally infected with L. sigmodontis and worm load was analyzed 65 days later. (*) Represents significant increase in IL-4 KO mice compared to all other genotypes of mice ($P < 0.05$), comprising 4-7 mice per group (4 in the antibody treated groups).

3.2.5. IL-4 KO mice permit worm embryogenesis, while IL-4/10 KO mice do not

In order to monitor the effect of IL-4 and IL-10 on worm fertility, adult female worms recovered from the thoracic cavity were subjected to an analysis of embryogenesis, using light microscopy to observe developmental status of the microfilariae directly in the uteri. To confirm these observations, the squeeze preparation technique was also performed. Development from oocytes to stretched and motile micofilariae was accomplished only in IL-4 KO (Figure 19A, B). However, embryos prepared from worms recovered from IL-4/10 double KO mice failed to develop past the morula stage (Table 8). These data suggest that IL-4 and IL-10 exert contrasting effects on embryogenesis in murine filariasis.

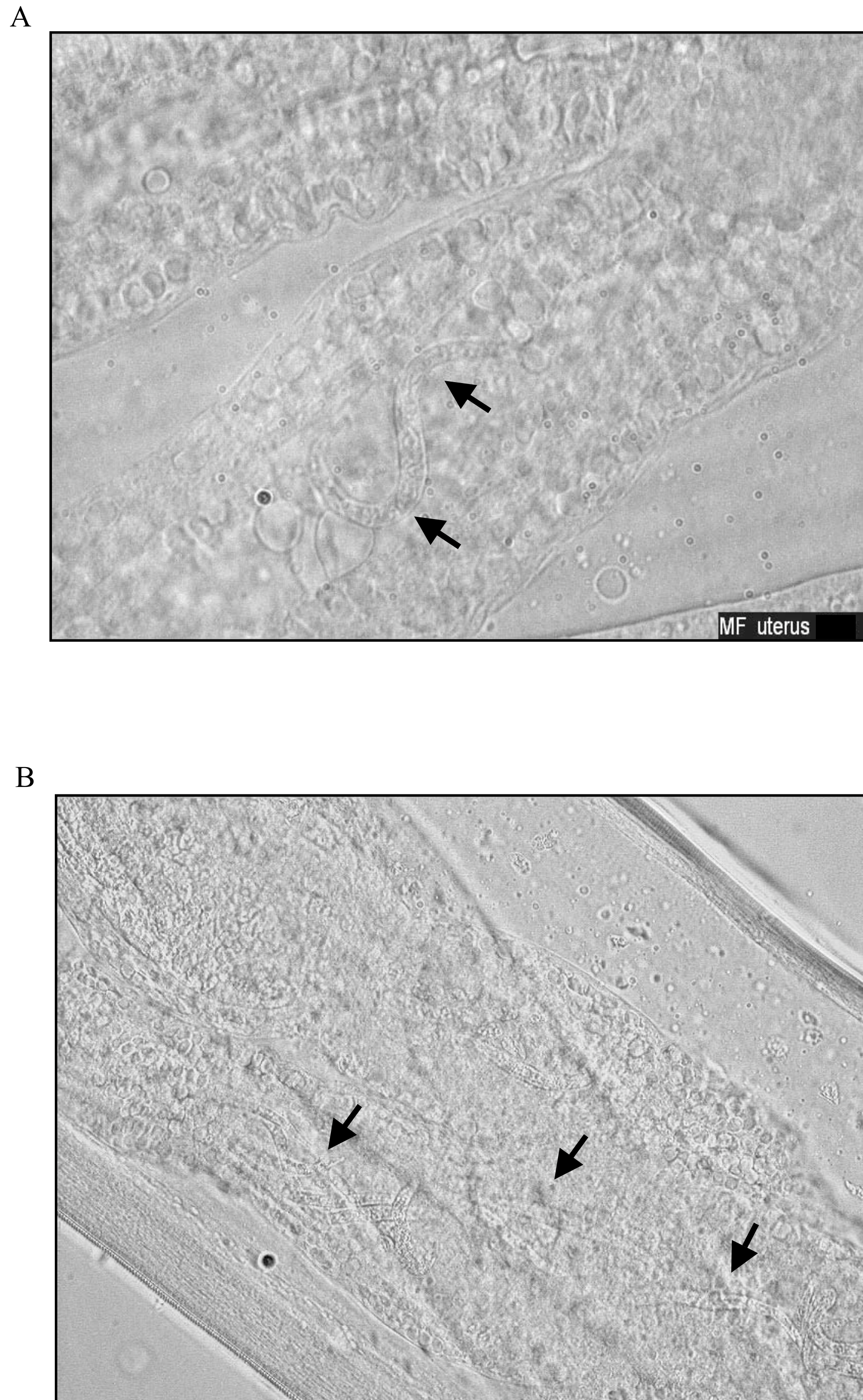


Figure 19: Microfilariae within the uterus of an adult female worm recovered from IL-4 KO mice. Arrows depict the microfilariae (A,B). Original magnification was 400x (A) and 200x (B).

Table 8: Fertility of L. sigmodontis *worms, as determined by squeeze preparations*

Day 65 p.i.	Embryo development in female worms No. of mice	No. of worms	Worms positive for morula stage	Worms positive for stretched mf
C57BL/6	**7**	**2**	**0**	**0**
IL-4 KO	**8**	**31**	**23**	**17**
IL-10 KO	**8**	**7**	**0**	**0**
IL-4/10 KO	**8**	**9**	**9**	**2**

3.2.6. IL-10 levels in sera are elevated in IL-4 KO mice

The above data suggested that IL-10 might be involved, probably indirectly, in the embryogenesis and thus in permissivity towards filarial infection in IL-4 KO mice. In support of this, blood was drawn from the animals of three experiments and then analyzed collectively by student´s t-test. Sera from IL-4 KO mice showed elevated levels of IL-10 at day 65 p.i. compared to C57BL/6 wt mice (Figure 20).

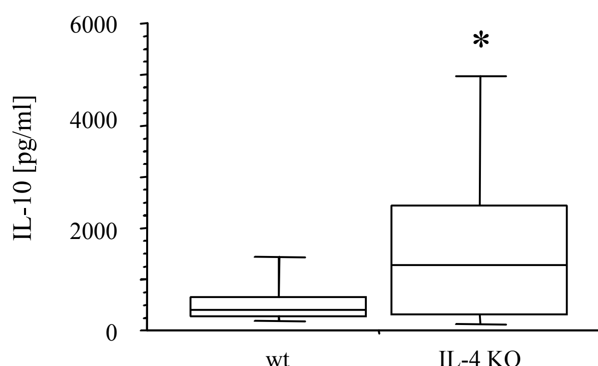


Figure 20: IL-10 levels in sera of mice infected naturally with L. sigmodontis. *Significantly elevated levels were observed in IL-4 KO. The data represent the combined analysis of sera from animals of three experiments and analysed with student´s t-test (*P *= 0.0347). ANOVA confirmed that the significant difference did not arise from variation in the natural infection model.*

3.2.7. IL-13 is not elevated in IL-4/10 KO mice

In order to look for other possible mechanisms of parasite control in IL-4/10 KO mice, IL-13 levels were analysed in serum and pleural lavage fluid at day 65 p.i. No significant difference between mouse strains was observed, when analysing 4 independent experiments in sera (Figure 21A, ANOVA, $P > 0.05$) and 8 independent experiments in pleura lavage (Figure 21B, ANOVA, $P > 0.05$). This argues against the hypothesis that

elevated IL-13 mediates resistance in IL-4/10 KO compared to full susceptibility in IL-4 KO.

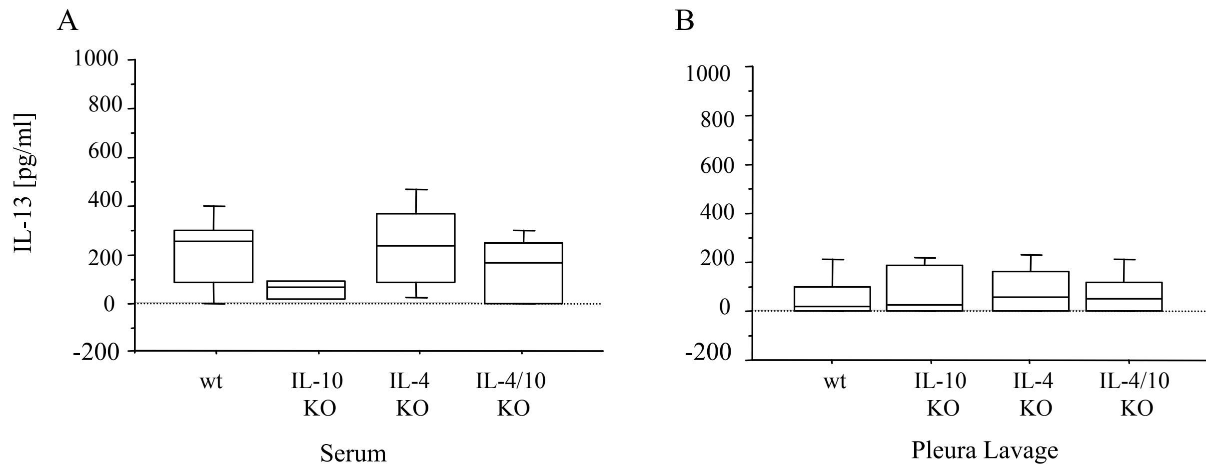


Figure 21: IL-13 levels in sera (A) and pleural lavage fluid (B) of mice naturally infected with L. sigmodontis. No significant differences could be observed between C57BL/6, IL-4 KO, IL-10 KO and IL-4/10 KO mice, when combining analysis of four (A) and eight (B) experiments and analysing with student´s t-test. ANOVA confirmed that no variation in the natural infection model existed.

3.2.8. Transfer of splenocytes from IL-4 KO mice does not increase susceptibility in IL-4/10 KO mice

In order to investigate, if cells with the capability to produce IL-10 are able to reverse the decreased susceptibility in IL-4/10 KO mice, $1x10^7$ splenocytes from day 40 infected IL-4 KO mice were injected i.v. into IL-4/10 KO mice, either prior to infection (Figure 22 A) as well as 20 days post infection (Figure 22 B). Mice were sacrificed day 65 p.i. In both settings the donor cells were not able to change parasite loads in IL-4/10 KO mice, however donor cells were still detectable.

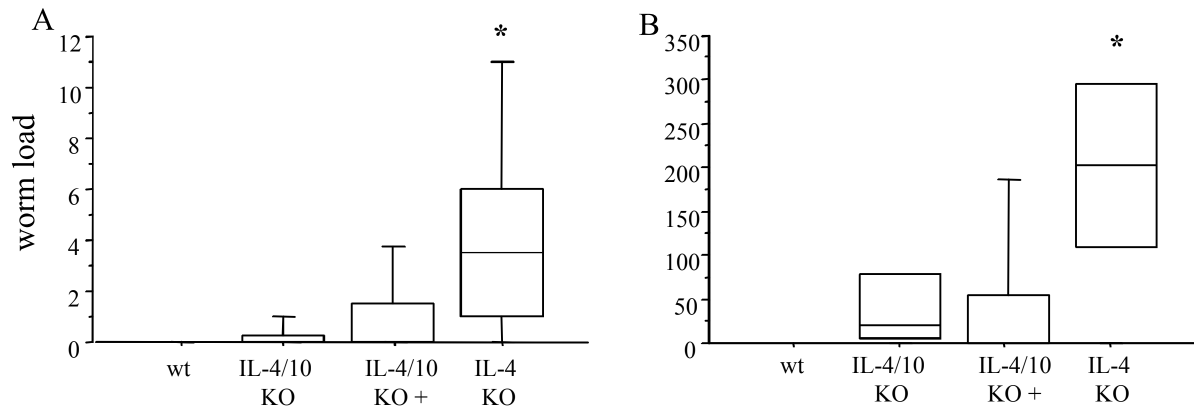
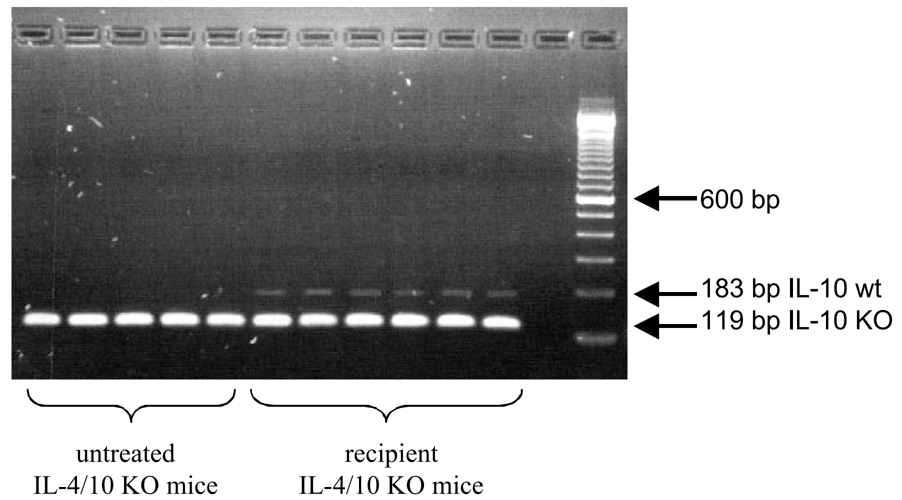


Figure 22: 1x10^7 splenocytes from infected IL-4 KO donor mice transferred into IL-4/10 KO mice (IL-4/10 KO +) did not lead to increased worm burden in IL-4/10 KO mice, when transferred prior to (A) or 20 days post infection (B). Worm load was significantly increased only in IL-4 KO mice ($P < 0.05$*). Mice were sacrificed day 65 post infection. Figures are one of two representative experiments*

The presence of these donor cells could be confirmed by realtime PCR, detecting genomic DNA of IL-4 KO mice with an intact IL-10 sequence in IL-4/10 KO with a disrupted IL-10 gene (Figure 23 A). Donor cells comprised 1-3 % of the total cell number in the pleura of IL-4/10 KO recipient mice (Figure 23 B).

A



B

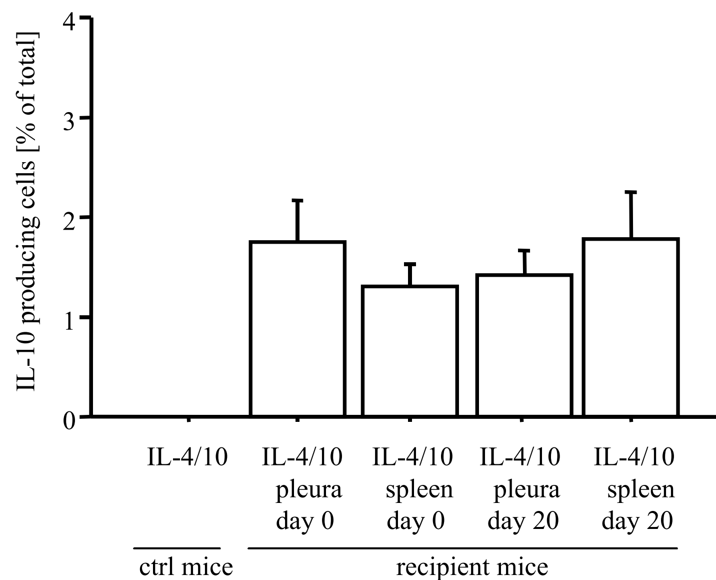


Figure 23: Splenocytes (IL-10 producing cells) from day 40 infected IL-4 KO mice transferred into IL-4/10 KO mice prior to infection (day 0) with L. sigmodontis *were detected in the pleural cavity of recipient mice 65 days post infection by PCR for the IL-10 gene. Cells were detected also in the spleen of these mice as well as in pleural cavity and spleen of mice that received donor cells 20 days post infection (B).*

4. Discussion

The reason for the increasing prevalence of not only Th2-mediated allergic disorders but also of some Th1-mediated autoimmune or chronic inflammatory disorders in Western countries is a highly debated issue. Since individuals infected with helminth parasites appear to suffer much less from these disorders (52, 68, 69), it has been proposed that helminth infection can protect against unsuitable immune responses against self or harmless antigens by the production of regulatory cytokines, such as IL-10 (70) or strong Th2 responses (59).

The original paradigm of the Th1/Th2 dichotomy required that there be hallmark cytokines which were associated with either subtype, such as IL-2 and IFN-γ with Th1 and IL-4/5 and IL-10 with Th2. Cytokines of one subtype would act synergistically to antagonize the other T helper subset (71). Therefore, IL-10 was also considered a Th2 marker, at least in mice, due to its profound antagonistic effects on Th1 responses. This paradigm was supported by several animal models, in particular by the infection of mice with *Leishmania major*. In this model, resistance was associated with a pattern of gene expression of both IFN-γ and IL-2 whereas $CD4^+$ T cells from susceptible mice co-expressed IL-4 and IL-10. Treatment with anti-IL-4 antibody resulted in protection and at the same time in reduction of both IL-4 and IL-10 concomitant with increased IFN-γ (72). In this model a delayed-type hypersensitivity (DTH) response to leishmanial antigens was found dependent on Th1, and its maximum inhibition required the combination of both IL-4 and IL-10 (73).

However, over the recent years it has become clear that the Th1/Th2 paradigm was not applicable for situations, where strong Th2 responses had to be counter-regulated. This was particularly true for allergic disorders, where sensitization was associated with a strong IL-5 response leading to high IgE levels and eosinophilia, while therapeutic hyposensibilisation led to the appearance of IL-10 production by allergen specific T cells (74). These data, as well as results from a mouse model of allergy (75), strongly suggested that IL-10 would act antagonistically to IL-4; however there were no direct interventional studies, e.g. using IL-4/10 double deficient mice, that could prove this hypothesis.

Also in human helminth infection, such as onchocerciasis, an antagonism between IL-4/5 and IL-10 is suggested by the existence of mutually exclusive polar forms of disease, such as the hyperreactive Sowda form characterized by high IL-4 and IL-5 but low IL-10 production, high IgE and eosinophil attack of microfilariae in the skin, in contrast to the generalized form of onchocerciasis characterized by the opposite cytokine pattern, low IgE

and absence of eosinophil attack of undamaged microfilariae permitting high parasite loads. Again, a direct proof of counter-regulatory activity of IL-4 and IL-10 in filarial infections has been lacking.

Therefore, the main focus of the thesis was to provide direct evidence for such a counter-regulatory activity of IL-4 and IL-10. To answer this question in a direct interventional manner, IL-4/10 double deficient mice were used in two different experimental settings.

In a murine model of IBD, mice deficient for IL-4 and IL-10 did develop colitis to a much lower extent than did IL-10 single deficient mice. An induction of an inflammatory and damaging Th1 response seen in IL-10 KO did not take place in IL-4/10 KO. Therefore, IL-4 is needed for the induction of Th1 mediated colitis development.

In a mouse model of helminth infections, causal evidence for an antagonism could be directly demonstrated for parasite loads. Thus, while IL-4 deficiency permitted persistence of filarial worms and microfilarial production, this permissivity was reversed in mice deficient in both IL-4 and IL-10. The presence of IL-10 and the absence of IL-4 in this model were associated with embryo development.

The results clearly show that IL-4 does counteract IL-10 in both experimental setups.

4.1. The role of IL-4 in chronic colitis in a model of IL-10 deficient mice

Chronic enterocolitis in IL-10 deficient mice is known to be dependent on a Th1 type response (19, 20, 76, 77). However, in several infection models IL-4, produced not only by T cells, plays an important role in the induction of Th1-mediated immunity, which appears to be in conflict with the dogma of antagonistic Th1 and Th2 type immunity. For example, in an experimental model of candidiasis, protection correlated with occurrence of a Th1 response, whereas Th2 responses were associated with disease exacerbation and pathology. Interestingly, IL-4 KO mice controlled the infection, whereas the wildtype mice did not. It was further demonstrated that IL-4 was required for the induction of protective $CD4^{+}$Th1 anti-*Candida* responses. Application of recombinant IL-4 in early infection but not at later time points was sufficient for the restoration of protective Th1 cells in this model (78).

Another study reported protection of otherwise progressive leishmaniasis in BALB/c mice when applying recombinant IL-4 to these mice. IL-4 being present at the time of DC priming instructed the DC to develop into type 1 cells and to produce IL-12, which in turn

activated T helper cells to develop into Th1 type cells with IFN-γ production. Application of IL-4 at later time points, e.g. T cell priming, led to development of Th2 cells and progressive leishmaniasis (79). These findings would suggest that IL-4 does counterregulate the action of IL-10.

Therefore, it was of interest for the present thesis to adress this question using mice deficient for both IL-4 and IL-10 in comparison to single deficient mice. A synergism would imply that mice deficient for both IL-4 and IL-10 develop more severe colitis than do single IL-10 deficient mice. The data of this thesis give strong evidence for the opposite, an antagonism between IL-4 and IL-10.

IL-4/10 deficient mice developed chronic colitis to a much lesser extent than IL-10 single deficient mice (Figure 2, 3). Therefore IL-4 appears to be necessary for the inductive process of IL-10 mediated colitis. This supports earlier work in another model by Fort *et al.* (65). This group could exacerbate colitis by application of recombinant IL-4 when inducing colitis by transferring $CD4^{+}CD45RB^{high}$ cells into RAG deficient mice (lacking B and T cells).

However, in *Helicobacter hepaticus* -induced colitis, which appears to be a strong Th1 inductor, IL-4/10 deficient mice did develop severe inflammation similar to IL-10 deficient mice (80). Consistent with these data, the animals used in this study were free of *Helicobacter* species as tested by PCR. However, using IL-4/10 KO mice of the same breeding site, these mice developed a higher rate of colitis, when infected with the filarial nematode *Litomosoides sigmodontis*, which induces both a Th1 and Th2 proinflammatory response (see below). These data and the work by Kullberg *et al* (80) suggest that stimuli do exist, which induce a higher degree of colitis development in IL-4/10 KO mice that are otherwise more resistent than IL-10 single KO mice. However, the findings presented here demonstrate an important role also of IL-4, a major component of Th2 type responses, for colitis development. Thus, IL-4/10 double knockout mice developed rectal prolapse, a marker for severe colitis, only in 0-13%, compared to 38-67% of IL-10 KO mice (Table 5).

4.1.2. IL-4 is essential for the increased mRNA expression of IL-12p40, IFN-γ and MHCII at the onset of IL-10 KO-induced colitis

Based on the results in the previous section the question arose, whether differences in T helper responses do exist, leading to severe inflammation in IL-10 KO but not in IL-4/10 KO mice. It has been shown that transfer of $CD4^{+}$ T cells from spleen, mesenteric lymph

nodes and lamina propria of IL-10 KO mice into RAG KO mice caused severe colitis within 8 weeks after transfer (76). In addition, the cytokine profile of IL-10 KO T cells reconstituting the intestines of the RAG KO mice remained that of a Th1 response (high IFN-γ but no IL-4). However, it is well recognized that the overproduction of a given cytokine is not a proof of a pathogenic role. Various anti-cytokine treatments have been carried out in neonatal IL-10 KO mice, but only anti-IL-12 and anti-IFN-γ mAb prevented colitis (20, 76, 81). It is generally assumed that IL-12 produced by accessory cells such as macrophages and dendritic cells stimulates the generation of Th1 cells and, if not suppressed by IL-10, converting protective immunity into pathology.

Interestingly, in this study IL-4 was found to be essential for the significant increase in mRNA expression of IL-12p40 and IFN-γ at the onset of IL-10 KO-induced colitis (Figure 4). The molecular mechanisms of IL-4 in induction of IL-12-mediated Th1 responses have partially been elucidated. Accordingly, IL-12 increases with application of IL-4 (22), because IL-4 promotes IL-12p70 heterodimerisation and inhibits the generation of IL-12p40 homodimers, which also bind to the IL-12 receptor, but inhibit IL-12p70 function. Furthermore, an IL-10-deficient/IL-12p40-transgenic mouse is likely protected from colitis development due to homodimerisation of the subunits and therefore inhibition of IL-12p70 (82). Therefore, IL-12p40 and p35 were measured (Table 6). Increased expression of IL-12p40 could be observed in IL-10 KO mice, whereas p35 was expressed at levels too low to detect differences between mouse strains. However, the increased levels of IFN-γ in IL-10 KO mice support the presence of bioactive IL-12.

T cells require a set of signals for full activation. One of the most important signals is the recognition of antigen presented on MHC molecules. Intestinal MHCII mRNA expression was upregulated in IL-10 KO but not IL-4/10 KO mice (Figure 4). While this could have been an effect of IFN-γ, IL-4 itself was described earlier to induce MHCII up-regulation (65). MHCII upregulation could be responsible for further stimulation of pathogenic immune responses.

Even though it was not possible to detect increased amounts of IL-4 mRNA in colon of IL-10 KO mice, prolapse development and Th1 responses only in IL-10 KO mice and not in IL-4/10 KO, supports the importance of IL-4 for colitis development. This is consistent with studies where an induction of IL-4 in colon explants (83) or in lamina propria $CD4^+$ T cells (20) of patients suffering from crohn´s disease was not observed. Unselected lamina propria mononuclear cells of IL-10 KO mice have been described to not produce IL-4

spontaneously, but upon ConA stimulation (77), arguing for a preceding priming event for these cells.

In the present study, increased amounts of IgG1 in faeces only in wt and IL-10 KO (Figure 5A) mice indicated that IL-4, an IgG1 inducing cytokine (84), had been effective even when present at low amounts. IL-4 is not the only mediator that induces class switching to IgG1. IL-13 has also been shown to be important for IgG1 induction in atopic disease (85). Therefore one could discuss in principle a role for IL-13 in the induction of faecal IgG1. This is however unlikely because IL-13 mRNA expression from colonic tissue was not detected (Table 6). The primary lack of IL-13 gene expression also makes it unlikely that varying amounts of the inhibiting, soluble IL-13Rα2 ("decoy receptor"), which is known to be upregulated by IFN-γ (86), could have influenced IL-13 bioactivity.

Even though IgA^+ B cells are known to infiltrate colon tissue (87), an increased level of excreted IgA could not be measured in faeces of IL-10 KO mice (Figure 5B). It may have been necessary to determine IgA in colonic mucosa histologically as done by Kuhn *et al.*(19).

Immunoglobulins are markers for a shift in the Th1/2 balance, but they do not seem to play a major causal role in colitis. The absence of B cells on IL-10 deficient background did not hinder or alter the course of colitis development (21). Therefore, (auto-) antibodies that are produced in human disease (88, 89) and in murine colitis (21) appear not to be the reason for IBD.

4.1.3. IL-12p40, IFN-γ and MHCII mRNA is only induced at the onset of colitis in IL-10 KO mice

To follow the expression kinetics of cytokines that are involved in colitis induction, colon tissue of the different knockout strains at different time points (pre-disease, onset, late disease) according to Spencer *et al.* (77), were analyzed with real-time PCR. In the pre-phase of the disease, i.e. week 4–8, no differences in cytokine expression between mouse strains could be seen (Table 6), consistent to the above report (77). In another study where minimal patho-histologic alterations of the intestine at 3 weeks of age could be observed, cytokine expression was not determined (20). The increase of IL-12p40, IFN-γ and MHCII seen in IL-10 KO mice during onset of colitis was not seen in the late phase. This supports the finding that IL-12 establishes early colitis in IL-10 KO mice via IFN-γ, but plays little, if any, role in late disease (77). Interestingly, in IL-4 deficient mice, an increase of IL-

12p40, IFN-γ and MHCII was observed in the late phase, although without apparent induction of colitis. The fact that in IL-4 KO mice, development of colitis has not been observed by us nor by others, may be partially explained by the elevated expression of the down regulatory cytokine IL-10 in colon tissue of IL-4 KO mice (Table 6). This phenomenon, that both pro- and anti-inflammatory cytokines are strongly upregulated but mutually inhibit one another, could reflect an aging immune system (77).
Taken together, the investigation of different time points showed that IL-4, even though not beeing highly expressed, is needed at the early timepoints of colitis development to induce Th1 inflammatory response. Thus all proteins, IL-12, IFN-γ, and MHCII, could have exerted synergistic effects in this study.

4.1.4. Th2 induction in chronic colitis

The results further argue for an association of intestinal eosinophilia with colitis (Figure 8, 9), which had been noted in the original report on colitis in IL-10 KO mice but were not further discussed (19). The fact that increased tissue eosinophilia preceded the onset of colitis (Figure 9), as well as the presence of eosinophils in higher numbers in non-prolapsed IL-10 deficient mice compared to prolapsed ones (Figure 10), argues against eosinophils being a mere epiphenomenon of inflammation. Looking at molecules attracting eosinophils to the site of infection, RANTES but not IL-5 nor eotaxin were associated with intestinal eosinophilia (Table 6, Figure 12). The induction of intestinal eosinophilia, as well as the fact that IL-4 is essential for colitis, also suggests that the normal intestinal flora contains not only Th1- but also Th2-inducing stimuli which in wild type mice are counter-regulated with IL-10 but lead to exacerbation in IL-10 KO mice. Recently, LPS of *Porphyromonas gingivalis* has been reported to polarize dendritic cells to induce Th2 responses (90), supporting the above findings. Further studies are suggested to elucidate possible Th2 inductors amongst bacterial species.

4.2. Antagonism of IL-4 and IL-10 in a murine model of filariasis

The second aim of this study was to investigate whether IL-4 and IL-10 are synergistic or antagonistic in a murine model of filariasis. Deficiency of IL-4 drastically increases susceptibility to filariasis, and in particular to *L. sigmodontis* infection in mice of both susceptible BALB/c and resistant C57BL/6 backgrounds. Microfilaremia is enhanced and

extended (36, 39, 91) while in IL-4 KO on C57BL/6 background the life span of adult worms also appears prolonged (39), demonstrating that as for gastrointestinal nematodes, IL-4 is a critical determinant of resistance. The precise mechanism of IL-4 action however remains unclear.

In agreement with the observations mentioned above, deficiency of IL-4 permitted persistance of filarial worms and microfilarial production in otherwise resistant C57BL/6 background in this study. If a synergism between IL-4 and IL-10 would exist, parasite loads in IL-4/10 KO mice would be even higher. However, permissivity was reversed in mice deficient in both IL-4 and IL-10 (Figure 13). The presence of IL-10 and absence of IL-4 in this system was associated with embryo development (Table 7). The results on the role of IL-10 in compromising Th2 cytokine mediated protection differ from data in murine *T. muris* infection where both deficiency of IL-10 or IL-4 leads to a failure to eliminate larvae (92). In this model, IL-4/10 double KO mice neither showed increased nor reduced larval burden in comparison to single knockout mice. The differences to murine filariasis may be due to i) the fact that IL-10 per se is essential for worm control in *T. muris* and ii) the fact that Th1 responses promote susceptibility to *T. muris* infection and are similarly induced in mice deficient for IL-10, IL-4 or both cytokines, while in murine filariasis Th1 responses either do not influence parasite loads (C57BL/6 background) or are associated with protection (41). One may conclude from this comparison that different mechanisms of parasite control are effective in different helminth infections. Accordingly, in experimental murine schistosomiasis, mice deficient for IL-10, IL-4, or for both cytokines did not display parasite loads that were different from that of wildtype mice (93-95).

4.2.1. Role of Th1 type responses

Differences to schistosomiasis were also found regarding infection-induced IFN-γ production in IL-4/10 KO mice. While IL-4/10 KO mice had an excessive Th1 response to schistosomiasis and died significantly earlier than did single KO mice, in the present work IL-4/10 KO mice did not succomb to infection, although IFN-γ levels were strongly elevated compared to wildtype and IL-4 KO and to a lesser extent compared to IL-10 KO (Figure 17). Surprisingly, the ratio of IFN-γ induction by mitogen or specific antigen over medium in splenic cell cultures was rather low. This correlated with high baseline secretion by cells from IL-10 and IL-4/10 KO strains. This may be due to the existence of T cells,

which are already stimulated in vivo by the *L. sigmodontis* infection, or reflect the fact that sources other than T cells contribute to IFN-γ production in these mice.

In addition the role of IFN-γ in parasite control seems to differ between schistosomiasis and filariasis: in BALB/c mice the complete absence of IFN-γ resulted in increased loads of adult filariae, whereas only small amounts of IFN-γ, as observed in IL-12 KO mice, were sufficient to limit adult worm loads to the level of BALB/c control mice (41). This is different on C57BL/6 background, where IFN-γ deficiency does not result in elevated worm loads (3.2.4.).

Still, the control experiment with C57BL/6 IFN-γ KO mice does not exclude a protective role for IFN-γ in IL-4/10 KO mice, all the more since elevated IFN-γ levels were observed in IL-4/10 KO mice. Therefore, neutralizing anti-IFN-γ mAb was administered at weekly intervals from day 20 to 41, when maturation of worms and embryogenesis takes place. While the injections did reverse rectal prolapse development, thus demonstrating in vivo efficacy, they did not influence *L. sigmodontis* infection. Although it cannot be fully excluded that the antibody reached the colon tissue and prevented rectal prolapse but did not affect the local inflammatory environment that controls the worms, these data argue against IFN-γ being the major factor of resistance in IL-4/10 KO mice.

4.2.2. Role of Th2 type responses

The question arose, if other molecules would substitute for IL-4 action leading to resistance in IL-4/10 deficient mice. IL-13 is such cytokine that could be responsible for the reversal of the susceptibility in IL-4/10 compared to IL-4 KO mice, because IL-13 signals through the IL-4Rα (96) and induces similar effects as IL-4. Therefore, the levels of this cytokine were determined in the sera and thoracic cavity fluids of the mice (Fig. 21), but no significant differences were found. This result however does not fully exclude a biological role for IL-13 because its functional efficiency is controlled by expression of the IL-13 decoy receptor (sIL-13Rα2-Fc). Since IFN-γ was found (via IL-12) to downregulate decoy receptor expression and thus to increase the functional effect of IL-13 (97), it is possible that elevated IFN-γ levels in IL-4/10 KO mice might have led to increased function of IL-13. An elegant way to prove such a compensatory effect of IL-13 would have been treatment with the recombinant IL-13 decoy receptor (formerly from Wyeth, Cambridge, MA), which unfortunately is no longer available. The finding that IL-13 does not seem to substitute IL-4 is supported by the fact that mice deficient for IL-4Rα (unable

to respond to IL-4 and IL-13) do not show increased susceptibility, when compared to IL-4 deficient mice (unpublished observation). In infections with *Schistosoma mansoni* IL-13 has been shown to be involved, inducing IL-4 independent granuloma formation. A conclusion made from the results presented in this thesis is that IL-13 does not have a major effect on parasite development in *Litomosoides sigmodontis* infected mice.

IL-4 is known as an inductor of IgG1 and IgE responses, while IFN-γ would induce IgG2a. When IgG1 and IgG2a were measured in this study (Figure 16), it was observed that these subclasses were regulated according to the literature knowledge, i.e. IgG1 was reduced in IL-4 deficient mouse strains, whereas IgG2a was increased. Apperently, immunoglobulin subclass levels were not correlated parasite loads. This supports earlier findings in the murine filariasis model. It was shown that antibodies do not play a major role given that μMT KO mice, which have no functional B cells, do not display an altered course of parasite infection (91, 98).

4.2.3. Role of regulatory mechanisms

The finding of elevated levels of IL-10 in sera of IL-4 KO mice (Figure 20) in the present study may indicate that IL-10 is promoting patency in these mice. This would be in line with our finding of patency reversal by additional deficiency of IL-10, and with an earlier study in *L. sigmodontis* infection, where it was found that i.v. injected MF survived significantly longer when female worms were transplanted into the peritoneal cavity prior to MF injection. Interestingly, this facilitation of patency by adult female worms required IL-10, as it was not seen in IL-10 KO mice (51).

While the present study indirectly confirms the published finding, albeit by a different approach, it additionally reveals the importance of IL-10 for embryogenesis. An elevated level of IL-10 in sera of IL-4 KO mice (Figure 20) correlated with the appearance of MF in the pleural cavity and blood of these mice (Table 7). Interestingly, in mice lacking IL-10 in addition, embryogenesis did not exceed the morula stage within the uteri of the few developed worms. Further, adult worm development itself was influenced by IL-10, as the worm load, when recovered 42 days p.i. was similar in wildtype, IL-10 KO and IL-4/10 KO, whereas a trend to higher worm load was seen in IL-4 KO. Direct effect of the different knockouts on microfilarial stage of the parasite itself could be excluded, since i.v. injected MF are cleared from the blood at the same rate in all mouse strains (Figure 15).

To investigate if patency of IL-4 KO can be transferred into non-patent IL-4/10 KO mice, splenocytes of day 40 infected IL-4 KO were transferred into IL-4/10 KO recipient mices at the day of infection (Figure 22 A) or 20 days p.i. (Figure 22 B). However, the patency as observed in IL-4 KO could not be regained in IL-4/10 recipient mice.
The reason for this is unclear at present. Premature death of effector cells was excluded by PCR (Figure 22). 40 and 60 days after transfer, cells from donor mice were still detectable in recipient mice (Figure 23). Transfer of splenocytes includes mostly T cells and B2 cells, i.e. rather poor producers of IL-10, but only small numbers of B1 cells and macrophages, both known to be prominent IL-10 producers. This might explain the incapability to produce enough IL-10 for the induction of patency in IL-4/10 KO. This suggests that not only suppressory T cells could be a source of IL-10 but also macrophages and other cells.

Data, extending this thesis, i.e. that IL-10 from macrophages is more important than that of T cells, have been obtained. Mice overexpressing IL-10 under the IL-2 promotor or under the CD68 promotor restricting IL-10 secretion to T cells respectively macrophages were infected with the rodent filaria *L. sigmodontis*. Interestingly, IL-10 transgenic mice under the IL-2 promotor cleared the infection like wildtype controls, whereas IL-10 transgenic mice under the CD68 promotor showed increased susceptibility. This strongly supports the importance of IL-10 produced not only by T cells for worm development.

Evidence for antagonistic effects of IL-4 and IL-10 can also be found in human filarial infection. In onchocerciasis, patients with generalized disease and high microfilarial skin loads have PBMC that show low proliferation to onchocercal antigen but produce high amounts of IL-10. This is in contrast to the hyperreactive Sowda form of onchocerciasis, where microfilariae are effectively reduced by Th2 mediated mechanisms and where PBMC proliferate vigorously and produce high amounts of IL-5 but little IL-10 upon antigen specific stimulation (23, 99, 100). In another large survey, IL-5 production in whole blood of patients was inversely related to MF skin loads (101). At least part of the IL-10 response is driven by regulatory T cells (Tr-1), which could be cloned from PBMC as well as from onchocercomas. These Tr-1 cells were shown to inhibit Th1 and Th2 clones in vitro (58). Also in PBMC from patients with lymphatic filariasis, high MF load was positively associated with IL-10 and correlated negatively with IL-5 (102-104). While a direct interventional study in humans to prove these associations is ethically not possible, the results given in this study using the well defined murine filariasis model of *L.*

sigmodontis infection provide to date the first causal evidence for an antagonism between IL-4 and IL-10 in filariasis control. There are several potential pathways of IL-10 downregulating parasite control mechanisms. While direct action of IL-10 on worm fertilty can in principle not be excluded, there exist several other proven avenues of IL-10 downregulating the function of effector cells such as macrophages, eosinophils and/or neutrophils (105-108).

Increasing evidence for an antagonism between IL-4 and IL-10 comes from the field of allergy. Transfer of Tr-1 cells inhibited antigen specific serum IgE responses in OVA immunized mice (75). In humans, antigen specific desensitization was associated with an increase of antigen specific production of IL-10, suggesting that antigen specific IL-10 induction is key to a novel concept of treatment of allergy and asthma.
Given that protective immune mechanisms against helminth, as well as allergic responses, have a common evolutionary basis, it seems logical that the antagonistic down regulatory mechanisms are similar. Thus, the genetic predisposition to allergy can be interpreted as an evolutionary advantage in the defense against helminths. Rather, the same genetic variants that are associated with atopy (109) are also linked to hyperreactivity against onchocerciasis, which is harmful to the host (110). The elucidation of antagonistic effects between IL-4 and IL-10 therefore improves our understanding of both helminth infection and allergy. In as much as IL-10 induction may prevent allergy, neutralization of IL-10 may improve vaccination against helminths as has been shown for schistosomiasis (94).

5. Summary:

The dichotomy of T helper cell immune responses developing into the Th1 type and Th2 type, has been a very powerful means to understand the different pathways that the immune system uses to defend the host against different pathogens and tumors. Intracellular pathogens are usually controlled by Th1 type responses, while extracellular pathogens such as helminths are generally believed to be controlled by Th2. The majority of autoimmune diseases is thought to be due to a Th1 type over-reaction, while allergic diseases are seen as Th2 response against non-harmful environmental antigens. Over the last years, it has become clear that this dichotomy was not sufficient to explain the phenomena of suppression against autoimmunity or tumor immunity on one hand, and of immunosuppression against helminths or the mechanisms of hyposensibilisation against allergens on the other hand. In addition, one has to consider the so-called regulatory T cell responses. Regulatory T cells are characterized by low proliferation and production of IL-10 or TGF-beta. While not all subtypes of regulatory T-cells need IL-10 for their immunosuppressive action, in many situations IL-10-dependent regulatory pathways inhibit both Th1 and Th2 type immune responses.

These hypotheses would require that in some situations of autoimmunity, allergy and helminth infections, when Th2 responses are suppressed by regulatory pathways, the respective hallmark cytokines IL-4 and IL-10 will have antagonistic effects. However, direct evidence of such an antagonism has so far not been provided. In the work that led to the present thesis, antagonistic effects of IL-4 and IL-10 in two immunological model situations could be demonstrated, by using mice that have a double deficiency for both IL-4 and IL-10, in comparison to the single knockout mice.

In the first model, murine colitis that regularly develops in IL-10 knockout mice, the previous assumption that this pathology is Th1-driven would have argued that the additional knockout of IL-4 in the double knockout mice leads to a worsening of pathology. However these data clearly show that the opposite is the case: IL-4 and IL-10 double deficient mice develop colitis significantly less frequently then do IL-10 single KO mice. Using real time PCR to quantify cytokine mRNA expression from colon tissue, it could be shown that only in IL-10 knockout but not in the double deficient mice, levels of IL-12, interferon gamma, and MHCII, which are associated with the Th1 responses, were elevated at the time of onset of colitis. Additional deficiency of IL-4 in the IL-10 knockout

mice prevented the expression of these genes. Immuno-staining for eosinophils revealed that this cell type is strongly increased in frequency in the colitis of IL-10 knockout mice, arguing that the pathology is not only Th1-mediated. In IL-4 and IL-10 double knockout mice, the eosinophil influx was not different from those of wildtype mice. The eosinophil influx in IL-10 knockout mice seemed to be mediated by the chemokine RANTES. Together these data show IL-4 acts antagonistically to IL-10, promoting the onset of colitis in this model.

Infection with the rodent filaria *Litomosoides sigmodontis* was used as the second model to demonstrate antagonistic effects of IL-4 and IL-10. In this infection, mice on the C57BL/6 background do not allow a full maturation of the infective larvae into fertile adult worms, which would produce microfilariae. Rather the worms do not develop beyond the L-4 stage/juvenile adult stage. In IL-4 knockout mice, earlier data could be confirmed that adult worms mature and release microfilariae into the bloodstream, similar to susceptible BALB/c mice. Therefore, the cytokine IL-4 is essential for the control of fertility on the C57BL/6 background. If IL-4 and IL-10 showed synergistic effects in that model, this would require that additional knockout of the IL-10 gene would lead to an even higher parasite load. However in this thesis it could be shown that the opposite is the case: additional knockout of IL-10 reverts the susceptibility, seen upon single knockout of IL-4, with no fertility of adult worms nor microfilaremia detected in the double knockout mice. Although production of interferon-gamma was increased in IL-4/10 KO mice, depletion of gamma interferon by monoclonal antibodies did not influence parasite elimination. Taken together, the results of the study add proof to the concept that has arisen for human filariasis, that IL-10 dependent responses, which are associated with patency, are antagonistic to bona fide Th2 responses, which control parasite loads.

It can be concluded from this work that situations in autoimmunity and infections diseases exists where IL-10 acts antagonistically to IL-4. This result increases our understanding of the interplay of immune responses and will hopefully be of help for the further development of immune-intervention strategies against autoimmune diseases and for vaccine design against helminth infections.

6. Literature

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Hiermit versichere ich, daß ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfaßt, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Ferner versichere ich, daß ich diese Dissertation an keiner anderen Universität eingereicht habe, um ein Promotionsverfahren eröffnen zu lasse.

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The linguistic correction of the present work
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