

Immune Modulation by Parasites – Th2 Induction by Schistosome Egg Antigen- stimulated Dendritic Cells

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Zusammenfassung

Die Infektion mit *Schistosoma mansoni* resultiert in einer Th2-Immunantwort mit Eosinophilie und erhöhtem IgE-Titer, wobei der wasserlösliche Extrakt der *S. mansoni* Eier (SEA) ausreicht um diese Reaktion auszulösen. In der vorliegenden Arbeit konnte demonstriert werden, dass sich IL-4-produzierende CD4+ T-Lymphozyten in Zellkulturen mit SEA-konditionierten Dendritischen Zellen (DCs) trotz gleichzeitig vorkommenden IFN-gamma entwickeln und SEA die Expression von Faktoren in DCs, die üblicherweise mit einer Th1-Antwort einhergehen, auf Transkriptions- und Proteinebene selektiv hemmt. Um den Faktor aus *S. mansoni* Eiern zu isolieren, der zur Expression von IL-4 in CD4+ Zellen und zur Inhibition von IL-12 in DCs führt, wurde eine Gelfiltrationschromatographie der exkretorisch/sekretorischen Ei-antigene (ES) durchgeführt und die Fraktionen in vitro getestet. Darin wurde gezeigt, dass Fraktionen mit einer Proteinbande von 30 kD die Expression von IL-4 in CD4+ Zellen induzieren. Dieses ES-Protein wurde durch N-terminale Sequenzierung als hepatotoxische Ribonuclease Omega-1 identifiziert, welches ebenfalls die Expression von IL-12 in DCs inhibiert und die Produktion von IL-4 in CD4+ Zellen bei einer 10-fach geringeren Proteinkonzentration als mit dem Kontrollansatz SEA induziert. Zudem sollte untersucht werden, inwieweit Toll-like Rezeptoren in der Generierung einer Th2 Antwort gegen schistosomale Antigene involviert sind. Dazu wurden TLR2-, TLR3-, TLR4- und MyD88-defiziente Mäuse mit *S. mansoni* infiziert und immunologische und pathologische Daten in der akuten und chronischen Phase der Infektion analysiert. Demnach sind TLR2, TLR3, TLR4 und MyD88-abhängige Signaltransduktionswege nicht für eine-Th2 Antwort notwendig, jedoch ist letzteres Molekül in der Ausprägung der typischen Leberfibrose involviert.

Schistosoma mansoni, Th2, Toll-like Rezeptoren, Omega-1

Summary

Infection with *Schistosoma mansoni* results in the induction of a Th2 immune response, eosinophilia and increased levels of IgE. The water-soluble extract of *S. mansoni* eggs (SEA) is sufficient to promote Th2 polarization in a dendritic cell-dependent manner. In this thesis, it was demonstrated that IL-4⁺ CD4⁺ cells emerge in cultures with SEA-conditioned dendritic cells (DCs) in the presence of IFN-gamma and that SEA inhibits selectively the expression of IL-12 and co-stimulatory markers in DCs on the transcriptional and protein level. To identify the putative protein in *S. mansoni* eggs mediating a Th2 induction, a gel filtration chromatography of the excretory/secretory egg antigens (ES) was conducted and the fractions tested in vitro. Fractions containing a single band of 30 kD were sufficient to promote IL-4 induction in naïve CD4⁺ cells. Using N-terminal sequencing this ES-protein was identified as the hepatotoxic *S. mansoni* ribonuclease omega-1 which displayed both biological functions observed with SEA: inhibition of IL-12 in LPS-stimulated DCs and induction of IL-4⁺ CD4⁺ cells at a 10 fold lower protein concentration than SEA. In order to understand, if the innate immune receptors TLR2, TLR3, TLR4 or the TLR adaptor molecule MyD88 are involved in the generation of the Th2 response against schistosomal antigens, the respective knock out mice were infected and immunological and pathological parameters were analyzed during acute and chronic phase of infection. This study showed that during *S. mansoni* infection TLR2, TLR3, TLR4 and TLR activation through the MyD88-dependent pathway are neither required for the induction (priming and polarization) nor for the down-regulation of Th2 responses, however, the fibrotic response against *S. mansoni* eggs was significantly reduced in MyD88-deficient mice suggesting a detrimental role of this pathway in liver pathology.

Schistosoma mansoni, Th2, Toll-like receptors, omega-1

Widmung

Für Doris Steinfelder

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Abbreviations

Ab, antibody

Ag, antigen

APC, antigen-presenting cell

APC, allophycocyanin

BM, bone marrow

BMDC, bone marrow derived dendritic cell

BSA, bovine serum albumin

CD, cluster of differentiation

CLR, cell-surface C-type lectin receptors

d, day

DC, dendritic cell

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

dNTP, 2'-deoxy-nucleotide triphosphate

DTT, dithiothreitol

ELISA, enzyme linked immunosorbent assay

ES, *Schistosoma mansoni* sexcretory/secretory egg antigens

EST, expressed sequence tag

FACS, fluorescent activated cell sorter

FCS, fetal calf serum

FITC, fluorescein isothiocyanate

FSC, forward scatter

g, G-force

GM-CSF, granulocyte-macrophage colony stimulating factor

h, hour

ICE, IL-1beta-converting enzyme

IFN, interferon

Ig, immune globuline

IL, interleukin

kD, kilo Dalton

KO, knock out

LN, lymph node
LPS, lipopolysaccharide
M, (protein) marker
M, molar
mAb, monoclonal antibody
MHC, major histocompatibility complex
min., minute
mLN, mesenteric lymph node
MW, molecular weight
MyD88, myeloid differentiation factor 88
OVA, ovalbumin
PAGE, polyacrylamide gel electrophoresis
PAMP, pathogen-associated molecular pattern
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PE, phycoerythrin
PK, proteinase K
PRR, pattern-recognition receptor
Ps, presenilin
RAG, recombination-activation gene
RNA, ribonucleic acid
RNase, ribonuclease
RPMI, Roswell Park Memorial Institute
RT, room temperature
RT, reverse transcriptase
SDS, sodium dodecylsulfate
SEA, soluble egg antigen
sec., seconds
SSC, side scatter
STAT, signal transducer and activator of transcription
Tab., table
TCR, T cell receptor

Th, T-helper cell

TLR, Toll-like receptor

TNF, Tumor necrosis factor

w, with

w/o, without

WT, wild type

1 Introduction

1.1 General Introduction

Infection with intracellular parasites generally leads to Th1 responses with elevated levels of IFN-gamma while macroscopic pathogens like helminths induce Th2 responses with eosinophilia and increased levels of IgE. The concept that two distinct subsets of murine T effector cells are responsible for these opposing immune responses was introduced in 1986 by Mosmann and Coffman. Briefly, Th1 and Th2 cells represent endpoints of a differentiation process from naïve CD4⁺ T cells to effector cells. Dendritic cells (DCs), as one of the most effective antigen-presenting cells, play a critical role in directing the polarization of T helper cells during their first encounter with antigen. They do this by providing co-stimulatory signals and a specific cytokine microenvironment that result from activation of DCs that carry pathogen-related information (“third signal”) [1]. Third signals result when invariable receptors like Toll-like receptors (TLRs) recognize and bind conserved pathogen-associated molecular patterns (PAMP). Activation of DCs through TLRs leads to their maturation and production of pro-inflammatory factors like IL-12, which promotes the development of Th1 cells. However, the involvement of innate immune receptors in Th2 differentiation by DCs is so far less understood [2]. The recognition of specific pathogen-derived products by DCs is pivotal for the induction of a protective Th subset which supports the elimination or confinement of the pathogen (bacteria, viruses, parasites). These successful immune strategies against pathogens can turn detrimental when directed against non-hazardous environmental or food antigen or self-antigen as in autoimmunity [3]. On the other hand an allergic reaction against harmless antigen that enters the body via the epithelium resembles the Th2 response in helminth infections [4]. The biological heterogeneity of parasitic agents and the chronic infections they often elicit offer important advantages for investigating the function and regulation of Th subsets in mice [5] and humans [4]. Recent advances in DC immunobiology have dramatically improved the understanding of Th2 commitment relative to helminths. However, many key issues still remain unresolved. Of critical importance is the characterization of helminth molecule(s) with Th2-inducing activity, their corresponding DC receptors and signaling pathways. Although distinct in some aspects, allergen and helminth-driven Th2 polarization appear to share many important features. Comparative studies are needed to understand the biological sig-

nificance of Th2 responses and for the rationale design of intervention techniques targeting pathways common to allergic and helminth-induced diseases.

1.2 Life cycle and Epidemiology of *Schistosoma mansoni*

The life cycle of *Schistosoma mansoni* is complex and involves two species as hosts (Fig. 1). The infectious larval stages of the parasite are cercaria, which penetrate the human skin by an active process involving secretion of proteolytic enzymes and shedding of a bifurcated tail [6].

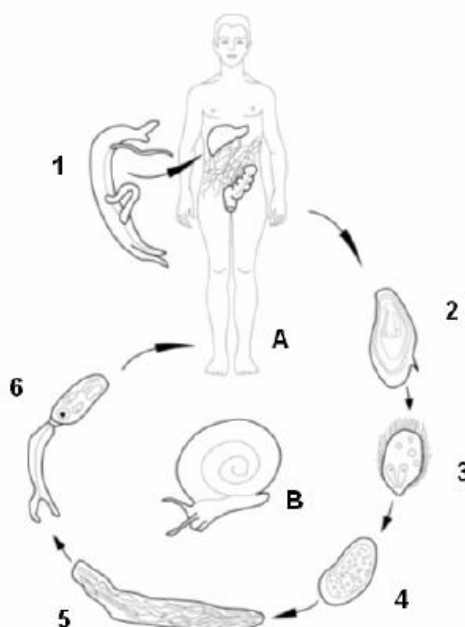


Figure 1: Life cycle of *Schistosoma mansoni*.

(A) final host (B) intermediate host (1) adult worms (2) embryonized eggs (3) miracidium (4) mother sporocyst (5) daughter sporocyst (6) cercaria

In the host they transform into schistosomula that migrate through the lung and heart into the mesenteric venules, where the final maturation into heterosexual adult worms and mating takes place. Paired mature females produce 100-300 eggs daily that mature transverse the gut wall and are released with feces. The majority of the eggs is trapped in the liver, and become principal triggers and targets of strong Th2-type immune responses, which under some conditions may cause typical pathologi-

cal symptoms of schistosomiasis. Two events lead to the hatching of the second stage larva, the miracidium: change in osmotic strength of the environment and exposure to light following release with feces into water. Miracidia actively infect a fresh water snail of the genera *Planorbis* or *Biomphalaria* as the intermediary host. An asexual reproductive phase including the formation of a mother sporocyst and daughter sporocysts takes place inside the snail and mature cercaria leave the snail to infect a definitive host to complete the life cycle [7]. Four important species infecting humans include *S. mansoni*, *S. japonicum*, *S. haematobium* and *S. intercalatum*. *S. mansoni* is endemic in Africa, the Caribbean Sea and the South American east coast. About 200 million people are infected with *Schistosoma spec.* world wide and approximately 0.3 million die each year in sub-Saharan Africa alone [8].

1.3 Immune responses against *Schistosoma mansoni*

Infection with helminths in general and schistosomes in particular lead to Th2 responses, including the production of Th2 cytokines, high serum titers of IgE antibodies and eosinophilia. An insight into the immune response against schistosomes was gained through experimental infection of mice with *Schistosoma mansoni*. The different developmental stages in the mammalian host challenge the host immune response with quantitatively and qualitatively changing antigens (Fig. 2). The immune response to antigens of schistosomula and adult worms during the prepatent period of infection (5 weeks post infection) is weak and primarily Th1 in nature. The onset of egg deposition at five weeks after infection triggers immune responses against egg antigens. This response is CD4⁺-dependent, highly polarized in a Th2 manner and sets in as a consequence of the intrinsic ability of eggs to promote the development of Th2 cells [9], [10], [11]. The continuous production of IL-4 by CD4⁺ cells lead to the expression of IL-5, IL-10 and IL-13. Consequently, high levels of antigen-specific Th2 associated Ig-Isotypes like IgE and IgG1 are found in the serum of infected mice. After about three months of infection, a significant decrease in the magnitude of the Th2 response occurs and an immune hypo-responsiveness prevails during the infection. Since B-cell deficient or Fc-gamma chain-deficient animals display exacerbated pathology and fail to undergo this down modulation [12], a B-cell dependent Fc-gamma receptor mediated mechanism can be implicated.

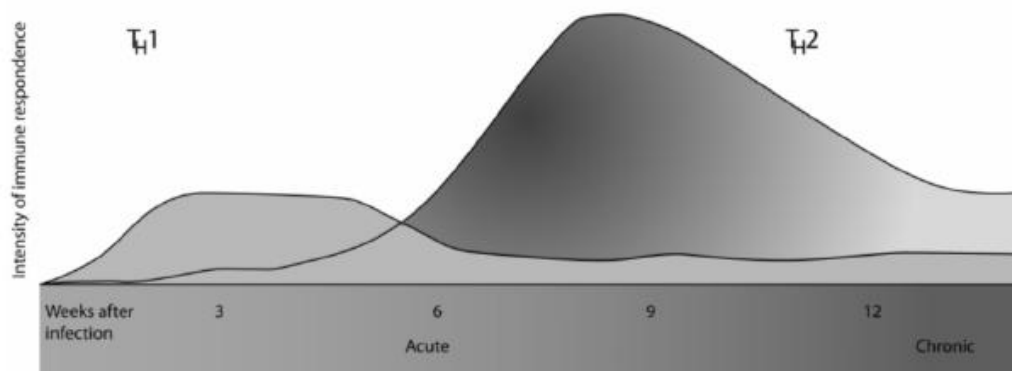


Figure 2: Immune response against *Schistosoma mansoni*

The prepatent period of the infection is a weak Th1 response against worm antigen. After 5 weeks the mature female adult worms start egg-laying and a strong Th2 immune response is established, which gets down-regulated in chronic infection (from Pearce and McDonald 2002)

1.4 Pathology of Schistosomiasis

Early symptoms of schistosomiasis (Katayama's fever) may occur weeks after infection, especially by *S. mansoni* and *S. japonicum*, and is thought to be due to an initial antigen excess as the worms develop and begin to produce eggs. Nevertheless, a large part of infected humans remain asymptomatic. However, with increased egg burden, passage of eggs through the wall of the intestine or bladder may cause bloody diarrhea and hematuria, respectively. The most important pathology induced by schistosomes is that related to eggs trapped in tissues, which induce the development of granulomas. Heavy-longstanding infection causes fibrosis of liver and spleen, which leads to portal hypertension with hematemesis and hepatosplenomegaly. This is complicated by ascites, hepatic failure, oesophageal varices, cystitis and ureteritis (*S. haematobium*), which can progress to bladder cancer, pulmonary hypertension (*S. mansoni*, *S. japonicum*, more rarely *S. haematobium*), glomerulonephritis, and central nervous system lesions. Most of the above pathologies can be successfully reproduced in experimental schistosome infection of mice, wherein a more detailed understanding of the underlying mechanism could be gained. The granulomatous response peaks at 8-9 weeks after infection is dependent on signaling through the IL-4 receptor alpha and STAT6 used by IL-4 and IL-13 [13], [14]. The continuing production of IL-13 from CD4+ Th2 cells results in the deposition

of collagen in the tissue surrounding the eggs, ultimately leading to tissue scarring. This fibrotic response can be inhibited by blocking of IL-13 or deviation to a Th1 response. Although granulomas are detrimental, it is clear that egg-induced lesions also serve an important host-protective function since antigens secreted by schistosome eggs are a continuous stimulus for immune response and need to be effectively sequestered to avoid uncontrolled inflammation [15].

1.5 Schistosoma mansoni egg antigens

The identification of molecules that endow *Schistosoma mansoni* with its intrinsic Th2 promoting ability was focused on the characterization of egg products, while studies aimed at identifying suitable vaccine candidates were focused on the biochemical characterization of various worm antigens. The purified *S. mansoni* eggs alone are able to induce a Th2 response and produce Th2 driven granulomas *in vivo* when injected subcutaneously or intravenously [10], [16]. The fact that similar effects are obtained by the administration of water-soluble extract of schistosomal eggs (SEA) is a proof of principle that the active element is a water soluble molecule. Briefly, SEA is able to induce strong Th2 responses in the absence of additional adjuvant and regardless of the route of injection [17], [18]. Though more than a dozen egg proteins have been identified in the last three decades, information on whether they might directly cause the skewing of the host immune response towards a Th2 phenotype is scant. Interestingly, glycans from parasitic and free-living helminths have also been shown to be potent immune modulators [19], [20], [21]. The *S. mansoni* egg derived Lacto-N-fucopentaose III (LNFPIII) for instance is a human milk sugar containing the Lewis X trisaccharide. This schistosomal glycan was shown to induce a Th2 response and its adjuvant activity has been demonstrated [22].

1.6 Immune induction by Dendritic cells

Dendritic cells have a unique capability of enhanced uptake of antigen and their subsequent presentation to naïve CD4⁺ lymphocytes in stable MHC-peptide complexes. The recognition of pathogen-associated molecular patterns by TLRs can activate dendritic cells directly leading to secretion of pro inflammatory cytokines and che-

mokines. Cytokines produced during the encounter of DCs and T cells are critical in the commitment of the latter towards their specific effector phenotype: Th1, Th2 or regulatory T cells in immune responses against pathogens and the induction of allergy and autoimmunity [23], [24], [25]. This cytokine microenvironment can be influenced by the DC subset, which displays different functions according to their lineage and location since DCs represent heterogeneous cell populations [26], [27]. One initial hypothesis was that activation of different DC subsets leads to differential Th1/Th2 commitment. Work by Maldonado-Lopez *et al.* [28] proposed that, based on the difference in their ability to produce different levels of IL-12, murine CD8alpha+ and CD8alpha- DCs subsets promote the development of Th1 and Th2 cells, respectively. Similarly, human monocyte-derived DCs were found to induce Th1 differentiation, whereas DCs derived from plasmacytoid cells induced Th2 differentiation [29]. Parasites modulate immune responses at different cellular levels in order to evoke an immune response which is conducive for the completion of the parasite life cycle. While fast replicating Th1 pathogens mostly evoke a pro-inflammatory response with high levels of IL-12 and IL-6, most helminths ensure a chronic persistence in their host by immunosuppressive mechanisms [30]. Since DCs function as potent inducers of immune responses, they are potential targets of immune modulation by parasites. Comparative analysis of DC conditioned by pathogen extracts known to prime Th1 and Th2 responses have revealed clear differences in their state of activation. In the former case, DCs rapidly up-regulate the expression of numerous co-stimulatory molecules, cytokines and chemokines [31]. In contrast, Th2 pathogens rather trigger only minimal immune responses in DCs under not-well understood mechanisms [32], [33].

1.7 Factors influencing Th cell differentiation

The list of immuno-modulatory factors described to influence the development of Th1 and Th2 cells over the past decade is extensive, complex and can be divided into pro-Th1 and pro-Th2 elements. Many of the elements display cross-regulatory activities in which they augment the development of one Th subset and suppress the other (Fig. 3).

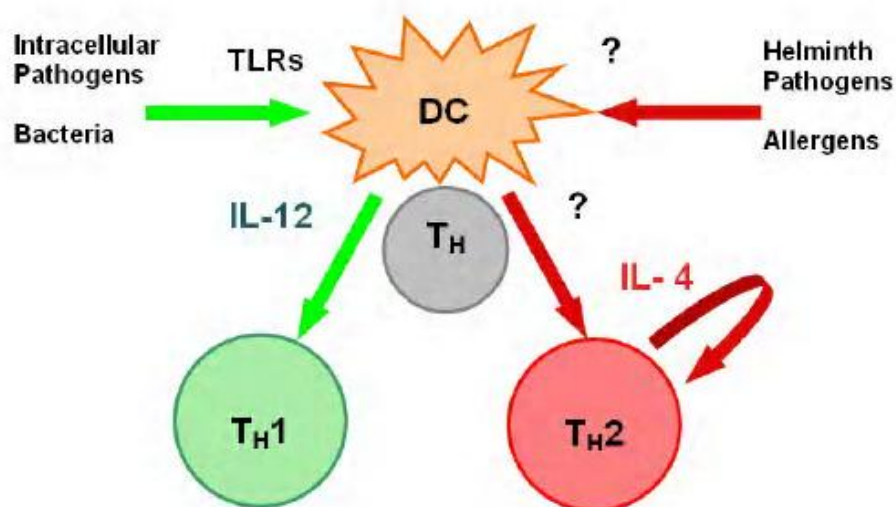


Figure 3: Factors influencing Th1/Th2 polarization

When exposed to parasite extracts from pathogens known to induce polarized Th1 and Th2 responses DCs are able to promote differentiation of the corresponding Th phenotype

IL-12, produced as a result of dendritic cell activation through TLRs, is a key factor in Th1 induction; while IL-4, whose source is poorly defined, is a key regulator in Th2 responses [34]. Depending on the particular setting, IFN-gamma as well as IL-10 can have a Th2 inducing activity, while IL-12 exerts an antagonistic effect on Th2 differentiation [35], [36], [37], [38]. Recently, it has been reported that Notch-signaling not only plays an important role in thymocyte differentiation but also in Th1/Th2 effector choice with the Notch ligand Jagged-2 being critical for Th2 polarization [39]. Several transcription factors have been shown to be involved in Th1/Th2 differentiation (Fig. 4). The transcription factors GATA-3 and T-bet are the intracellular master regulators for the induction of the key cytokines IL-4 and IFN-gamma in effector cells, reflecting the IL-4 / IL-12 dichotomy underlying Th1 and Th2 polarization. GATA-3 is expressed in naïve CD4⁺ and Th2 cells and subsides to a minimal level in Th1 cells. GATA-3 has been shown to be necessary for Th2 cytokine gene expression and sufficient for Th2 polarization; it also down-regulates IFN-gamma in developing Th1 cells and induces IL-4 and IL-5 [40], [41]. On the other hand, T-bet is involved in Th1 development, controls the expression of IFN-gamma and directly represses Th2 responses [42]. To conclude, the outcome of a particular arm of T cell response is dependent on the nature of pathogens and a multitude of cross regulatory cytokines they induce.

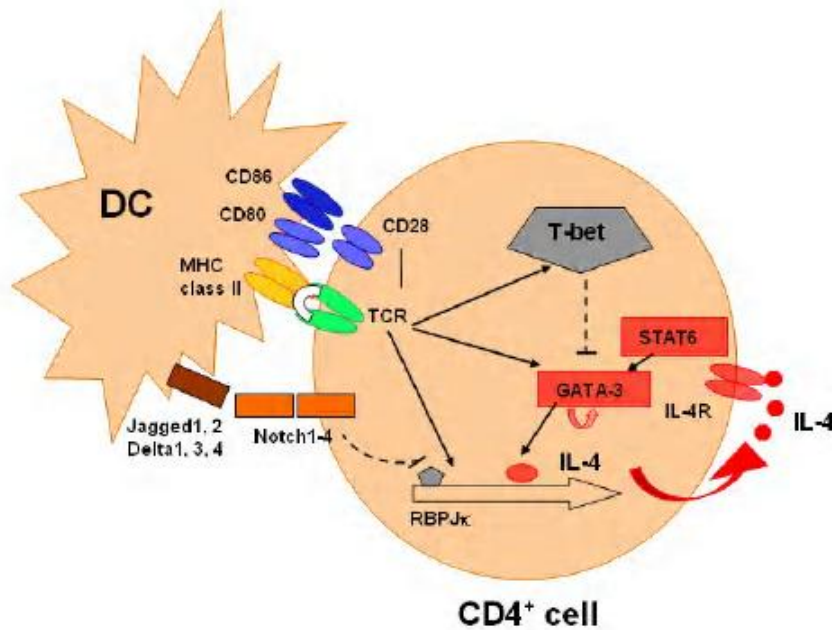


Figure 4: Molecular pathways involved in the expression of Interleukin-4
Transcription of the IL-4 gene is controlled by multiple positive (red) and negative elements (grey).

1.8 Innate Immune receptors

Innate immunity functions as a pathogen sensor and contributes to the eradication of pathogens and contributes to the establishment of adaptive immunity. These functions heavily depend on pattern-recognition receptors (PRRs), which are able to discriminate “self” from “non-self” [43]. The most studied PRRs are a group of transmembrane proteins called Toll-like receptors. Toll-like receptors have a potent “immunoadjuvant” ability to activate antigen-presenting cells (APCs) [44], [45]. TLRs include 10 (TLRs 1-10) and 12 (TLRs 1-9 and 11-13) family members in human subjects and mice, respectively (Fig. 5). Most TLR ligands have been identified as a variety of molecular components derived from microorganisms. They can be categorized based on their chemical structure such as lipids, proteins, and nucleic acids.

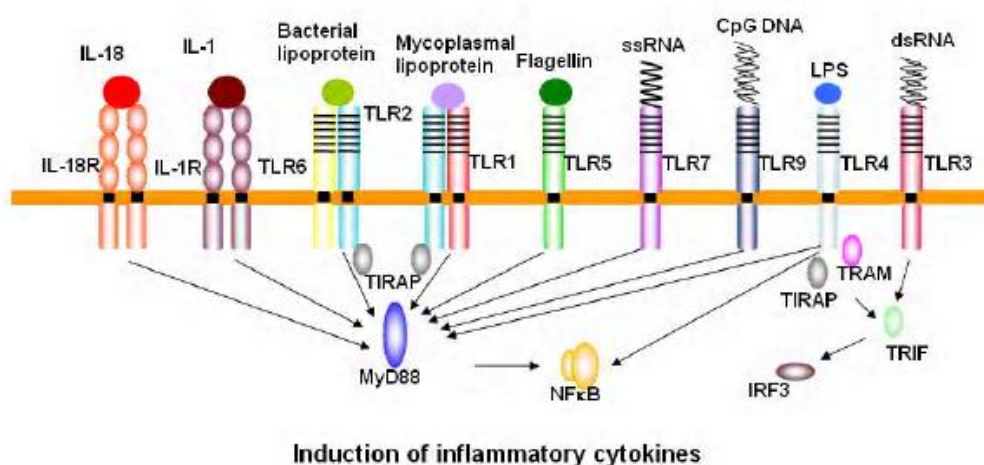


Figure 5: Toll like Receptor signaling in the innate immune response

Toll like receptors are localized in the outer membrane of the cell (TLR 2, 4, 5, 6) or in intracellular vesicles in the cytosol (TLR 3, 7, 9), where they can bind to their specific ligands. Upon ligation of the receptor the down stream signaling is mediated by MyD88 or alternative adaptors and results in transcription of pro-inflammatory cytokines depending on the cell type and combination of TLRs involved.

In general, TLRs activate APCs to support Th1 cell differentiation. Blocking or augmenting TLR function can modify Th1/Th2 balance and manipulate a variety of immune disorders, such as cancer, allergy, and autoimmunity [46]. Studies involving mice deficient in MyD88, a critical adaptor molecule for most TLRs, showed that MyD88 is required for Th1 polarization by pathogen extracts of protozoan or bacteria (e.g. *Toxoplasma gondii*, *Mycobacterium avium*), but not for the generation of SEA-induced Th2 response *in vivo* [18] and *in vitro* [47]. Nevertheless, there are indices that TLR signaling is involved in the recognition of helminth-derived molecules. In particular, schistosomal dsRNA binds to TLR3 on DC and results in the upregulation of type 1 IFN and IFN-responsive gene expression [48], while schistosomal lysophosphatidylserine stimulated IL-10 production in a TLR2 dependent manner [49]. Another study showed that a carbohydrate containing the Lewis X antigen, a major motif of schistosomal egg glycoproteins, leads to Th2 polarization of CD4+ cells [50]. However, a filarial phosphorylcholine-containing glycoprotein mediates low level production of IL-12 and TNF-alpha by macrophages and dendritic cells in a MyD88 and TLR4-dependent manner, while inhibiting IL-12 induction by TLR ligands other than LPS [51]. Other reports support the notion that TLR-triggered immune responses that lead to a classically activated phenotype of dendritic cells and macrophages is rather detrimental for Th2-differentiation: TLR-triggered MyD88-dependent activation of DCs

is a negative signal for Th2 cell development [52], [33], [53]. The question if TLR mediated signaling is required for the Th2 response in the experimental infection with *S. mansoni* remains to be answered to date.

1.9 Aim

The aim of this study was to investigate how parasitic helminths modulate the immune system to generate a Th2 response. Specifically, the mechanism of dendritic cell mediated Th2 differentiation of CD4⁺ cells by helminths was to be investigated by using the human pathogen *Schistosoma mansoni*. Techniques used involved the experimental infection of mice with *Schistosoma mansoni* and an *in vitro* system employing CD11c⁺ splenic dendritic cells and OVA-transgenic DO11.10 CD4⁺ cells in the presence of *Schistosoma mansoni* egg antigen (SEA). To understand DC modulation by SEA and the signal transduction pathways involved in induction by DCs, the gene expression profile of SEA-stimulated bone marrow DCs stimulated was investigated. The Th2 inducing molecule(s) in SEA were characterized and putative candidates were tested *in vitro* to validate their Th2-inducing capacity. To test if immune modulation by helminths is mediated by receptor families involved in Th1 differentiation, mice deficient in the TLR adaptor molecule Myd88 and mice deficient in TLR2, TLR3 or TLR4 were infected with *Schistosoma mansoni* and immunological and pathological parameters were analyzed.

2 Results

2.1 Results - Th2 differentiation by SEA

To investigate the early Th2 induction by SEA-stimulated DCs an *in vitro* system was employed, whereby the polarization Th cells by SEA is measured as the predominant production of IL-4 over IFN- γ in TCR-transgenic CD4+ [47]. To that end, 1×10^5 OVA-specific transgenic CD4+ cells isolated from spleens of DO11.10 mice were co-cultured with CD11c+/B220- splenic DCs in the presence of the nominal OVA-peptide and parasite extracts and cytokine expression measured by intracellular cytokine staining, in the supernatants of the cultures or by RT-PCR.

2.1.1 Th2 induction by SEA is independent of the CD8alpha DC subtype

In order to test if the Th polarization by the Th2 stimulus SEA or Th1 stimulus STAg (soluble *Toxoplasma gondii* antigen) is influenced by CD8alpha+ or CD8alpha- DC subsets, splenic CD11c+ dendritic cells were FACS-sorted according to the expression of CD8alpha and cultured with CD4+ cells in the presence of the parasite extract.

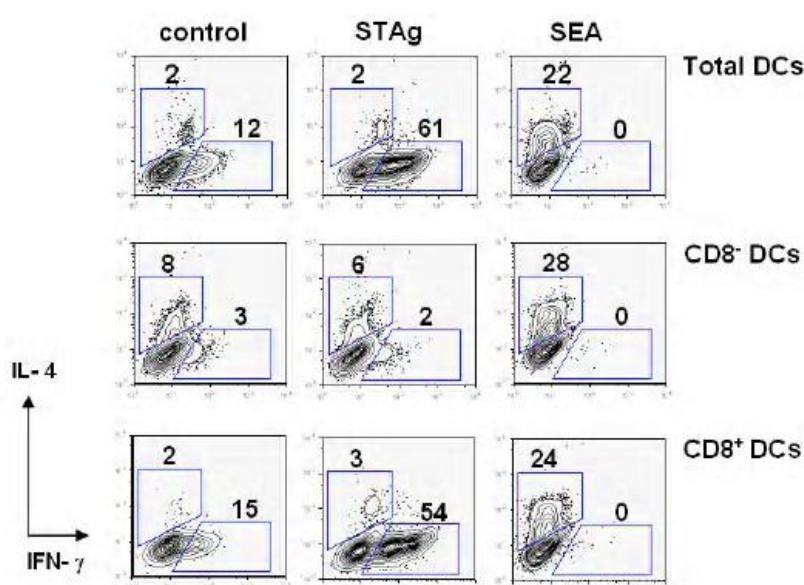


Figure 6: Th2 induction by DC subsets.

1×10^5 CD4⁺ cells were cultured with 5×10^4 CD11c⁺ cells and stimulated with 1 μ M OVA-peptide in the presence of the parasite extracts SEA [40 μ g/ml] or STAg [5 μ g/ml]. Cytokine profiles were analyzed by gating on CD4⁺ cells and frequencies of cytokine positive cells are shown. One representative experiment from three is shown.

2.1.2 Th2 differentiation by SEA is independent of the antigen dose

It was described that higher antigen doses favor the development of Th1 cells, while a lower strength of TCR signaling leads to IL-4 producing CD4⁺ cells [54], [55]. Therefore, the amount of peptide-MHC class II complexes playing a role in SEA-induced IL-4 expression was investigated. To that end, the concentration of OVA peptide was titrated while the number of CD4⁺ DO11.10 cells and total CD11c⁺ DCs remained constant. As seen in Figure 7, higher peptide concentrations resulted in a higher frequency of IFN- γ ⁺ cells, while the reduction to a peptide concentration of 0.01 μ M led to an increase of IL-4⁺ cells in the absence of Th polarizing stimuli (control culture). The addition of the Th1 stimulus STAg resulted in an increased frequency of IFN- γ ⁺ cells compared to the control at each peptide concentration tested, while the tendency to higher proportions of IFN- γ producing cells at higher antigen doses persisted. Similarly, a higher frequency of IL-4 producing CD4⁺ cells were found in SEA cultures compared to the control regardless of the peptide concentration indicating dominance in polarization of the parasite extract over the strength of the TCR mediated signal.

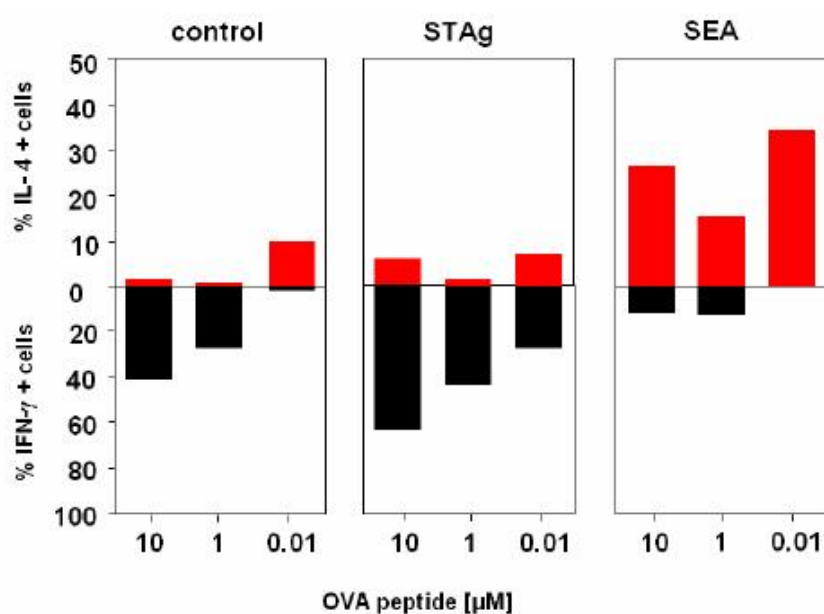


Figure 7: Influence of the antigen dose on the T_H2 induction by SEA.

1x 10⁵ DO11.10 cells were cultivated with 5x 10⁴ CD11c⁺ cells and were stimulated with 10, 1 and 0.01 μ M OVA-peptide. Cytokine profiles were analyzed by gating on CD4⁺ cells and frequencies of cytokine positive cells shown as bar graphs. One representative experiment from three is shown.

2.1.3 Requirements of naive CD4⁺ cells on IL-4 in SEA induced Th2 induction

It has been shown that dendritic cells as a cellular source of IL-4 are dispensable for Th2 induction by SEA in *in vivo* [56] and *in vitro* [47]. Nevertheless, IL-4 is the key trigger for Th2 induction and most likely produced by CD4⁺ cells, themselves. In order to investigate, if IL-4 is required for its own synthesis in the employed assay, endogenous IL-4 was neutralized with antimouse IL-4 mAb at the time of initiation of cultures with SEA and recombinant IL-4. Addition of neutralizing anti-IL-4, but not control mAb at the beginning of the culture period resulted in complete abrogation of the development of the IL-4⁺ cell population in any condition (Fig. 8) suggesting that endogenous IL-4 is indispensable for the development of Th2 commitment.

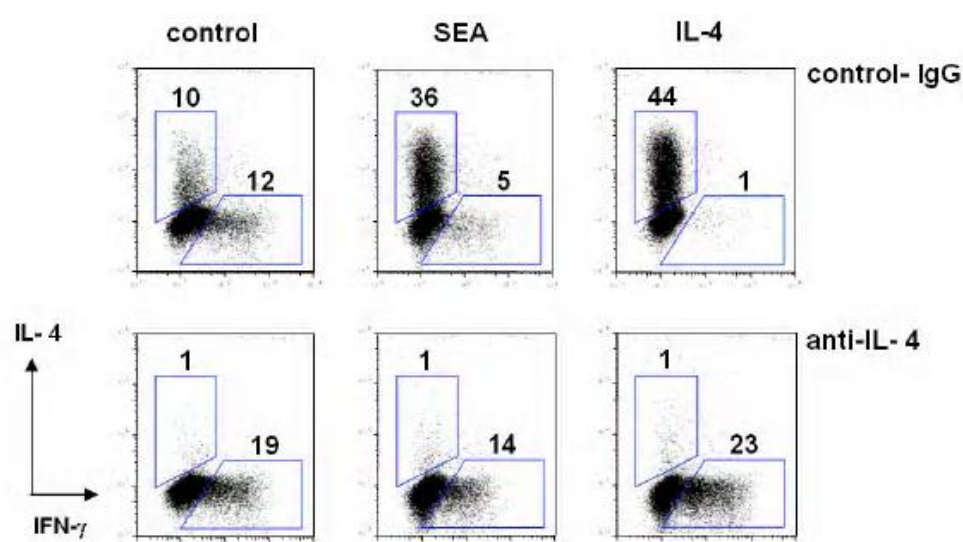


Figure 8: Requirements of IL-4 in Th2 differentiation

Anti-mouse IL-4 or control IgG was added to *in vitro* polarization cultures with SEA [40 µg/ml] or recombinant IL-4 [10 ng/ml]. One representative experiment from three is shown.

To examine how long the IL-4 has to act on antigen stimulated CD4⁺ cells, a kinetic experiment was performed, in which endogenous IL-4 was neutralized at different time points after initiation of cultures with SEA and recombinant IL-4: at 24, 36, 48, 60 and 72 h. On day 7 CD4⁺ cells were restimulated with PMA and Ionomycin and the intracellular production of IL-4 and IFN-gamma measured by FACS-analysis. As can be seen in Figure 9, CD4⁺ cells that have been cultured in the presence of SEA show a cytokine profile with comparable frequencies of cells that produce IL-4 (10 %) and IFN-gamma (11 %), when IL-4 was neutralized after 48 h. The subpopulation of cells

producing IL-4 becomes greater than that producing IFN-gamma, indicating a Th2 phenotype, after 60 h of IL-4 signaling. After this time window a stable IL-4+ population is established and the concurrent IFN-gamma+ population is diminished in comparison with the control culture (peptide alone). The addition of IL-4 to the culture without parasite extracts results in an earlier independence on IL-4 between 24 and 36 h of the culture period indicating an accelerated commitment of CD4+ cells to the Th2 lineage when exogenous IL-4 is supplied.

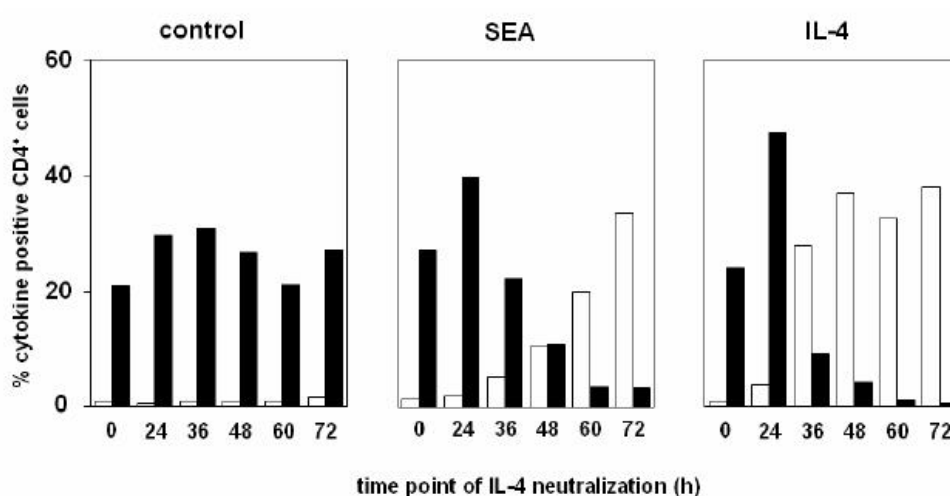


Figure 9: Kinetic of IL-4 requirements in early Th2 induction

Anti-mouse IL-4 antibody was added at the indicated time points and the culture resumed. open bars = IL-4⁺/CD4⁺ cells, closed bars = IFN-gamma⁺/CD4⁺ cells. One representative experiment from three is shown.

2.1.4 Dependence of CD4+ cells on duration of the TCR-stimulation

The central role of dendritic cells in T helper cell differentiation is based on their capability to present antigen to naïve CD4+ cells in the context of the MHC class II molecule while providing the necessary accessory stimuli CD80 and CD86 for their priming. A putative third signal, which may cause the differentiation into the respective effector cell, is thought to act during the time of CD4+ T cell/dendritic cell interaction [57]. In order to define the required time of TCR stimulation to ensure the commitment to the Th2 lineage of naïve CD4+ cells by SEA, the peptide concentration was diluted 1000 fold by washing after 12, 24, 36 and 72 h and the cell culture was resumed in fresh complete RPMI 1640. As seen in Figure 10a, a continued cultivation of 36 h is required to ensure Th2 commitment by SEA as seen by the phenotype of the CD4+ cells (IL-4: 16 % and IFN-gamma: 3 %).

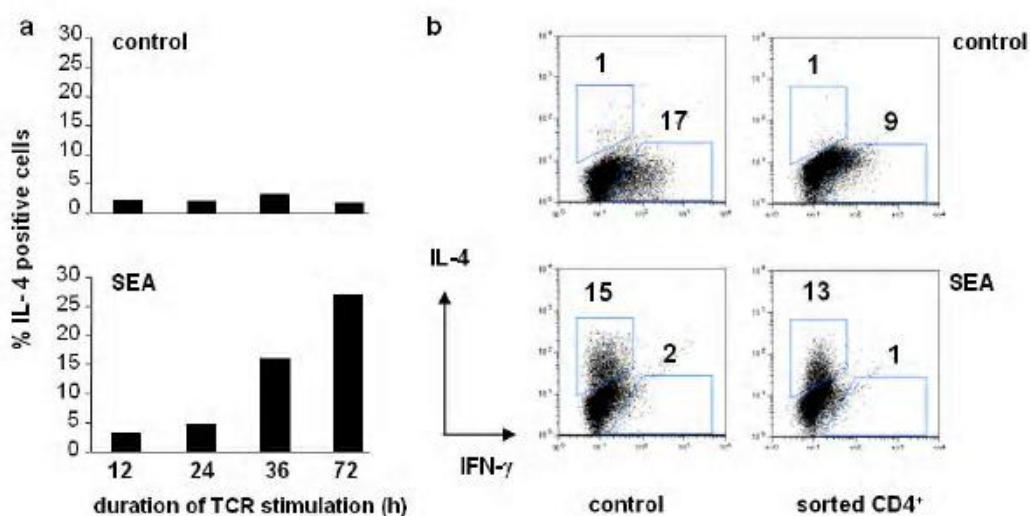


Figure 10: Requirements of CD4⁺ cells on TCR stimulation and DC-contact

a) Cultures with or without SEA were cultured 12, 24, 36 or 72 h before the peptide was removed by extensive washing. b) CD4⁺ cells were sorted at 36 h and culture was resumed. One representative experiment from two is shown.

In addition to TCR-signaling and costimulation, dendritic cells provide an accessory signal to the naïve CD4⁺ cell, which direct the commitment to a Th lineage. To address whether the latter signal is also dispensable after 36 h, CD4⁺ cells were isolated at this time point from the cocultures with DCs by FACS sorting (purity > 98 %, data not shown) and their culture was resumed in fresh complete RPMI 1640. As seen in Figure 10b, unsorted CD4⁺ cells as well as CD4⁺ cells sorted at 36 h from SEA cultures show a similar cytokine profile with 15 % IL-4⁺ and 2 % IFN- γ ⁺ cells and 13 % IL-4⁺ and 1 % IFN- γ ⁺ cells, respectively. The above results imply that Th2 commitment of CD4⁺ by SEA-conditioned DCs is not any longer dependent on DC-derived signals after 36 h.

2.1.5 Influence of IL-10, IL-12 and IFN-gamma on Th2 induction by SEA

Depending on the particular setting, IFN-gamma as well as IL-10 was shown to have a Th2 inducing activity in Th2 differentiation, while IL-12 is known to exert an antagonistic effect. In order to test, if these cytokines are involved in SEA-induced IL-4 ex-

pression, blocking antibodies to IL-10 receptor, IFN-gamma and IL-12 or the respective recombinant cytokines were added at the beginning of the culture period.

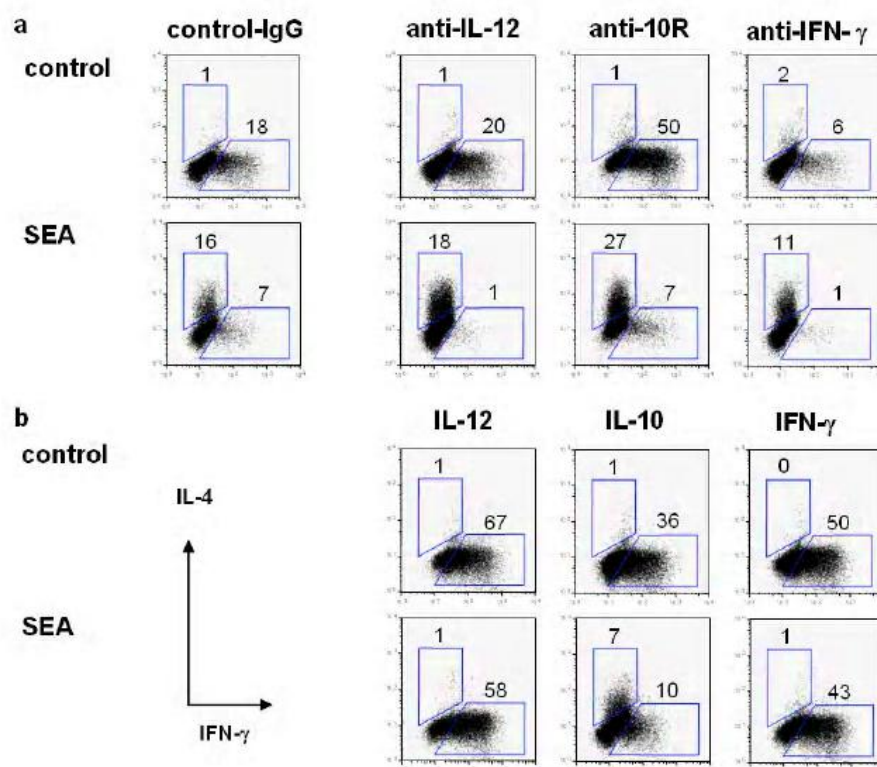


Figure 11: Role of IL-10, IFN-gamma and IL-12 in Th2 induction by SEA

a) Addition of anti-mouse IL-12, IL-10R and IFN-gamma or control IgG, [20 μ g/ml] each, to SEA conditioned cultures b) Recombinant murine IL-12 [10 ng/ml], IL-10 [10 ng/ml] or IFN-gamma [10 U/ml] was added to cell cultures. One representative experiment from three is shown.

As seen in Figure 11a, the addition of anti-IL-12 antibody to cultures with SEA marginally enhanced the induction of IL-4⁺ cells (16 % to 18 %), while shutting off IFN-gamma producing cells, which developed in the control culture. Furthermore, neither anti-IL-10 nor anti-IFN-gamma antibody could prevent the development of IL-4⁺/CD4⁺ cells induced by SEA and no qualitative change in the polarization profile could be observed in comparison with the control in three independent experiments suggesting that these cytokines are not required for SEA-induced Th polarization *in vitro*. In contrast, the addition of 10 ng/ml IL-12 or 10 U/ml IFN-gamma completely inhibited the development of IL-4 secreting cells in SEA cultures from 16 % to 1 %, while the addition of IL-10 did not inhibit Th2 differentiation however resulted in a lower frequency of IL-4⁺ cells compared to the control (16 % vs. 7 %). The addition of

these cytokines to cultures without SEA resulted invariably in an increased population of IFN-gamma⁺ cells (Fig.11b), suggesting a general Th2 inhibiting effect of all three cytokines in this system. Based on the results with neutralizing/blocking antibodies and recombinant cytokines it is apparent that neither IL-10 nor IFN-gamma, even if produced during priming of the naïve CD4 T-cell, is necessary for a SEA induced Th2 differentiation in this assay.

2.1.6 Th2 induction by SEA overcomes the early induction of concurrent IFN-gamma production

To date it is not clear, if SEA induced Th2 cells develop from CD4⁺ cells that produce simultaneously IFN-gamma and IL-4 before commitment to the Th2 lineage. Furthermore, as shown above, IFN-gamma exerts a detrimental effect on Th2 induction by SEA in this *in vitro* assay. In order to investigate if IFN-gamma⁺ is produced and if the production of IL-4 and IFN-gamma is exclusive on the cellular level at early time points, the production of these cytokines and the respective transcriptional factors GATA-3 and T-bet were measured at several time points. Surprisingly, in supernatants of CD4⁺ cells stimulated with SEA-conditioned dendritic cells a comparable amount of IFN-gamma is detected at 72 h as in control cultures (Fig. 12a). This result is mirrored by intracellular cytokine analysis of the same cultures, where a comparable subpopulation of cells is IFN-gamma-positive. Importantly, however, intracellular cytokine staining of SEA-stimulated cultures revealed that IFN-gamma⁺ and IL-4⁺ CD4⁺ cells represent two distinct subpopulations (Fig. 12b). Although not initially altered, the frequency of IFN-gamma⁺ cells in the presence of SEA-conditioned DCs becomes reduced with prolonged incubation to only a few detectable cells on day 7, suggesting that the phenotype of IFN-gamma-positive lymphocytes under these experimental conditions cannot be maintained. The prolonged culture of 7 d in the presence of IL-2 leads to the increase in frequency of IL-4⁺ cells with SEA (48 %), while in the control culture only 1 % of the cells are IL-4-positive.

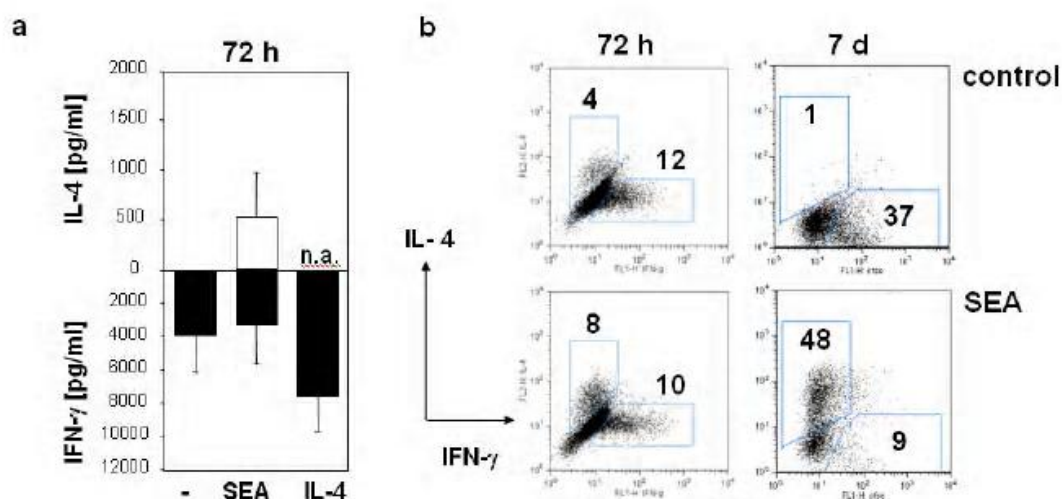


Figure 12: Early concurrent IFN-gamma in cultures with SEA

a) Supernatants of cultures with and without SEA were tested in triplicates for IL-4 and IFN-gamma at 72h (mean \pm SD, n.a. = not applicable) b) FACS analysis of intracellular cytokine staining. One experiment of three is shown

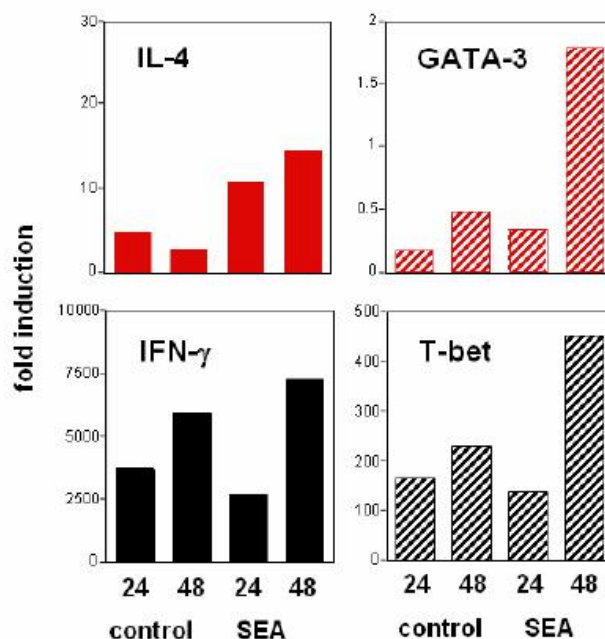


Figure 13: Quantitative RT-PCR of cell cultures stimulated with SEA after 24 and 48 h.

Expression of mRNA was measured as fold induction of the expression of the gene in naïve CD4⁺ cells and normalized to β 2-microglobulin

In order to test, if early cytokine transcription is preceded or accompanied by the expression of their transcription factors, a quantitative Real Time-PCR was conducted

at 24 h and 48 h and the data was normalized to beta-2-microglobulin and expressed as fold induction over naïve CD4⁺ cells (Fig. 13). At both time points, at 24 h and 48 h, the level of induction of T-bet and IFN-gamma mRNA is >100 and >2000 fold, respectively, in cultures with or without SEA. On the contrary, the Th2 master regulator GATA-3 is only up-regulated in cultures with SEA at 48 h compared to naïve CD4⁺ cells, while being down-regulated at 24 h and in control cultures.

2.2 Results - Dendritic cell modulation by *Schistosoma mansoni* egg antigens

2.2.1 Parasite induced Th1/Th2 effector choice is associated with the degree of Dendritic cell activation.

Using parasite extracts from pathogens known to induce highly polarized Th1 or Th2 responses, it was observed that the Th1 stimulus STAg and the Th2 stimulus SEA have opposing effects on CD11c⁺ splenic DC activation. When both parasitic stimuli were added to DCs, a down regulation of MIP-1alpha and MIP-1beta chemokines as well as the activation markers CD40 and CD86 was observed in comparison to DC stimulated with STAg alone [47]. In order to test to what degree a general down regulation of DC function by SEA can be observed on the transcriptional level as observed on the protein level, gene expression profiles using the microarray technique of DCs cultured in the presence of SEA and a Th1 stimulus were generated. Since generation of microarrays demands a large amount of RNA, bone marrow derived dendritic cells (BMDCs) were used as dendritic cells. As a Th1 stimulus, purified lipopolysaccharide (LPS) from *E. coli* was used, since BMDCs are not responsive to the parasitic stimulus STAg (personal communication). First, it was examined if a similar down regulation of activation markers by SEA on LPS-stimulated BMDCs as well as Th2 induction of naïve CD4⁺ cells by SEA-conditioned BMDCs can be observed. To that end, the activation status of BMDCs conditioned with LPS and/or SEA was evaluated by measuring the up-regulation of the co-stimulatory molecules CD40, CD80 and CD86 and MHC class II molecule by fluorocytometry. Results were displayed as mean fluorescence intensity (MFI). As seen in Figure 14a, stimulation of BMDCs with LPS for 24 h resulted in > 2 fold up-regulation of CD40 and CD86 in CD11c⁺ BMDCs in comparison with medium control, while CD80 was only slightly

up-regulated (MFI 193 vs. MFI 278). In contrast, CD11c⁺ BMDCs stimulated with SEA did not display an activated phenotype and showed similar or lower MFI for the measured surface molecules in comparison to nontreated cells. Moreover, when SEA was simultaneously added to LPS-exposed BMDCs, an actual suppression of expression of CD40 in comparison with LPS stimulated BMDCs was detected with > 1.5 fold down regulation of CD40 by SEA. Interestingly, the addition of SEA to LPS-stimulated BMDCs resulted in a dose-dependent suppression of IL-12p40 production as measured in the supernatants of 24 h cultures by ELISA (Fig. 14b). IL-12p40 secretion of LPS-exposed BMDCs [72.4 ± 8.2 ng/ml] was suppressed by SEA by more than 50 % [34.2 ± 3.8 ng/ml].

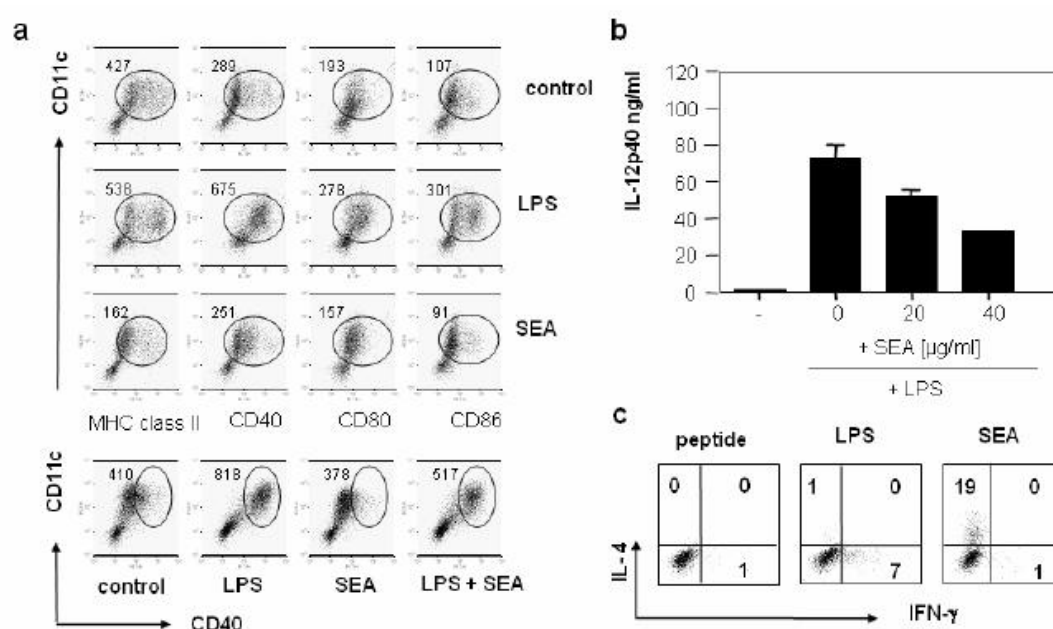


Figure 14: SEA downregulates LPS stimulated DC function

a) BMDCs were incubated for 24h in the presence of SEA, LPS or both stimuli together and stained for CD11c and MHC class II, CD40, CD80 or CD86. Numbers indicate MFI of the gated population. b) Supernatants were tested for IL-12p40 by ELISA. Mean values from triplicates \pm SD are shown. c) Intracellular staining of CD4⁺ cells incubated with BMDCs in the presence of peptide and LPS or SEA. One representative experiment from three is shown.

To ensure that the BMDCs conditioned with the parasite extracts were able to induce T helper differentiation of naïve OVA specific TCR-transgenic CD4⁺ cells, FACS-sorted CD4⁺ DO11.10 cells were co-cultured with BMDCs and OVA-peptide in the presence of 40 μ g/ml SEA or 50 ng/ml LPS. LPS stimulation results in an increase of IFN-gamma producing CD4⁺ cells > six fold as compared to the control (peptide alone), whereas the addition of SEA induces the differentiation of an IL-4⁺ population

(19 %) of CD4⁺ cells that is absent in the control and LPS-culture (Fig. 14c). Taken together, the above results show that BMDCs can mediate Th2 induction of naïve CD4⁺ cells by SEA as well as respond to the down regulatory function of SEA after LPS-stimulation. To understand if the observed down-modulation by SEA on LPS-stimulated BMDCs occurs on the transcriptional level, a cDNA microarray was performed using fluorescent labeled cDNA reverse transcribed from the stimulated BMDCs mRNA. BMDCs were incubated for 6h in the presence of SEA [40µg/ml] and LPS [50ng/ml] total RNA isolated. In order to measure relative gene expression, fluorescent labelled cDNA was synthesized by means of integration of dUTP-Cy3 or dUTP-Cy5 during the reverse transcription step. The Cy5-labeled cDNA from SEA and LPS-stimulated BMDC was then compared to Cy3-labeled cDNA from BMDC stimulated with LPS alone. As additional experimental groups Cy5-labeled cDNA from BMDC stimulated with LPS or SEA only was compared to Cy3-labeled cDNA from untreated BMDCs. The Cy3- and Cy5- labelled cDNA was co-hybridized on a standard mouse genome oligonucleotide microarray (Mmbe) containing 16,600 genes, scanned Axon GenePix 4000B fluorescence scanner (Microarray Research Facility, RTB, NIAID) and analyzed with the mAdb software (NIAID-RTB). The average hybridization rate from three independent experiments was 52.9 ± 15.4 % of genes present on the microarray. In order to estimate the impact of the parasite derived stimuli on global BMDCs transcription, the number of genes that have been up- or down-regulated by more than a twofold was calculated. The stimulation of BMDCs with SEA resulted in the modulation of 313 ± 338 , while LPS-stimulated BMDCs showed a global change of 2212 ± 503 genes due to the microbial stimulus. To compare the down-regulatory effect of SEA on LPS-stimulated gene transcription, BMDCs stimulated with both stimuli were compared to LPS-stimulated BMDCs. Here, the general 2 fold change was 650 ± 699 modulated genes. Figure 15a shows a list of selected genes involved in an inflammatory response from the microarray representing a fraction of the complete list of genes. Here, SEA does not induce the up-regulation of most of the genes induced by LPS indicated by the fold change (as log₂) of the fluorescence signal Cy5 to Cy3. Pro-inflammatory genes like Ccl2 (MCP-1), Ccl4 (MIP-1beta), Ccl5 (RANTES), IL-12p40, IL-1 and TNF-alpha are up-regulated by LPS more than 3 fold, while they remain comparably minimally altered by SEA. Moreover, SEA was found to have a down-regulatory effect on LPS-stimulated DC which is apparent in the suppression of CD40, CD86 and IL-12p40. In conclusion, SEA does result in

the down-regulation of selected genes on the transcriptional level as it has been observed on the protein level, suggesting that the lower levels of activation markers on DCs is not merely the consequence of protein digestion.

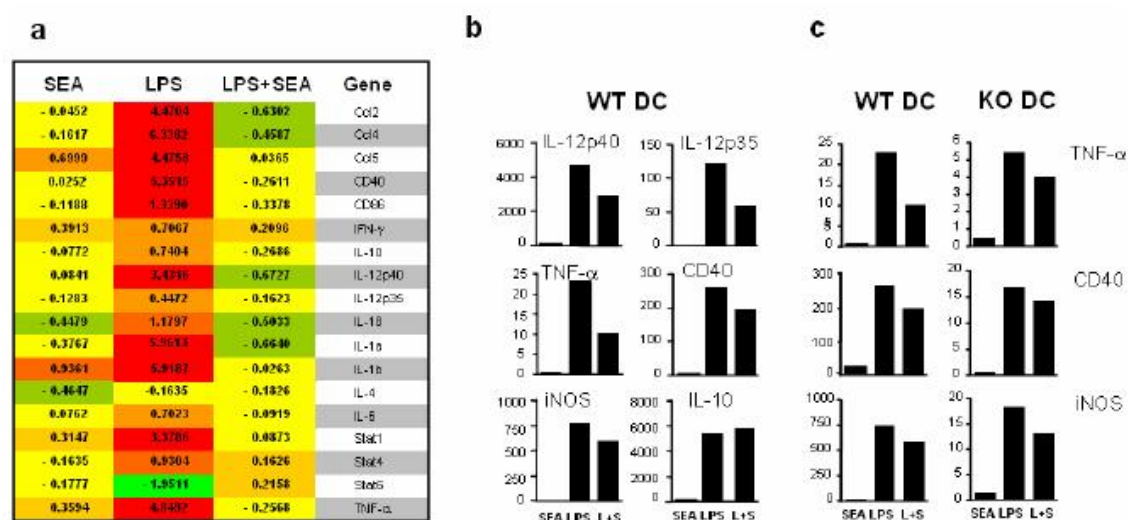


Figure 15: Gene expression profiles of SEA and LPS-stimulated DC

a) Microarray analysis of BMDCs. Colour intensity correlates with fold-change values (log₂). b) Quantitative RT-PCR from wild-type BMDCs. c) RT-PCR from IL-10 deficient BMDCs (KO DC). Values are shown as fold induction over control (BMDC medium). One representative experiment from three is shown.

2.2.2 SEA-induced down-regulation of LPS-stimulated dendritic cell activation occurs in the absence of IL-10

To confirm the above observation a quantitative Real Time PCR was performed for IL-12p35, IL-12p40, TNF-α, CD40, inducible nitric oxide synthase (iNOS) and IL-10. To that end, BMDCs were cultured as described above, total RNA isolated and reverse transcribed in order to obtain cDNA. The Real-Time PCR was done in duplicates for each primer pair and the obtained value normalized to the house keeping gene and compared to the control (untreated BMDCs). As seen in Figure 15b, a down-regulatory effect of SEA could be observed on the expression on all genes selected that are up-regulated by LPS, but not IL-10. In contrary, SEA itself did not induce any of the tested genes in a comparable manner to LPS. IL-10 has been shown to have down-regulatory function on dendritic cells and in some settings Th2 inducing activity. Interestingly, SEA did not up-regulate IL-10 to the degree observed with LPS. To know if the down-regulatory effect of SEA on LPS-mediated induction of pro-

inflammatory genes is IL-10 independent, the experiment was repeated with IL-10-deficient BMDCs stimulated with LPS in the presence or the absence of SEA. As shown in Figure 15c SEA-induced down-regulation of TNF-alpha, CD40 and iNOS mRNA expression was detected when tested on IL-10-deficient dendritic cells stimulated with LPS. Interestingly, the fold induction of genes by LPS in IL-10 ^{-/-} BMDCs was 4 – 45 times lower than in wild-type BMDCs. Taken together, these results indicate that SEA down regulates DC functions associated with a Th1 response in an IL-10-independent manner.

2.2.3 Involvement of Notch-ligands in SEA-induced Th2 induction

It has been reported that Notch-signaling not only plays an important role in the differentiation of thymocytes but also in Th1/Th2 effector choice [58]. In order to examine, if the expression of the Notch-ligands Delta-1, Delta-4, Jagged-1 and Jagged-2 on BMDCs is affected by SEA or LPS, BMDCs were cultured for 12 h with SEA [40µg/ml], LPS [50ng/ml] or both stimuli combined.

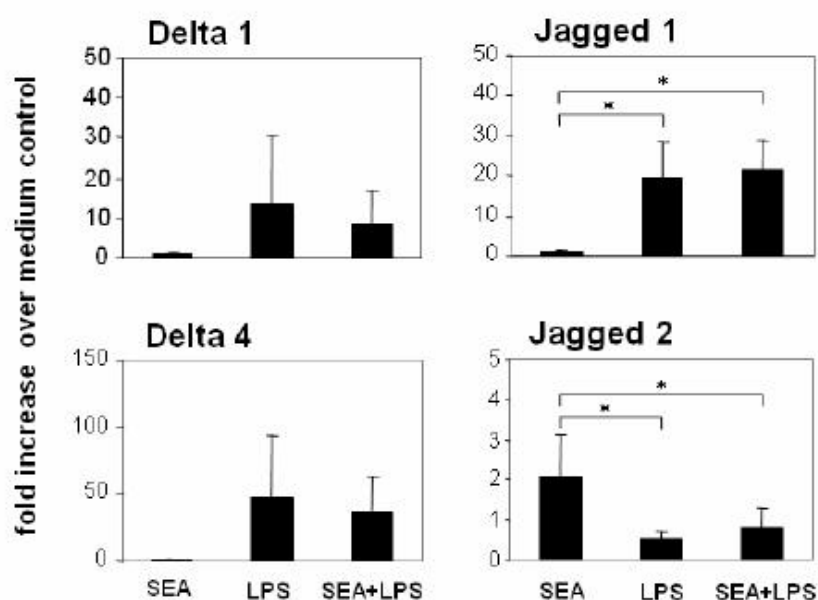


Figure 16: Notch ligands on BMDCs stimulated with SEA and LPS.

Relative expression of genes measured by quantitative RT-PCR. The mean of three independent experiments \pm SD is shown, * indicates a p-value of < 0.05

After isolation of BMDC mRNA, reverse transcribed cDNA was tested for the relative content of transcripts for the Notch ligands by quantitative Real Time-PCR. As seen

in Figure 16, the stimulation with LPS or LPS/SEA lead to the expression of Delta-1, Delta-4 and Jagged-1, whereas the expression of Jagged-2 was repressed compared to the control (0.55 ± 0.15 and 0.83 ± 0.48 , respectively). Especially Jagged-1 was significantly up-regulated in BMDC treated with LPS alone (19.7 ± 8.8) or LPS/SEA (21.6 ± 7.2) over the control (arbitrarily set 1) and over SEA-conditioned DCs (1.1 ± 0.3). Due to the strong variation between the repeated experiments, no statistically differences could be detected for the expression of Delta-1 and Delta-4. However, both Notch ligands were only marginally affected by SEA-treatment (1.01 ± 0.6 and 1.28 ± 0.87 , respectively). Conversely, DCs treated with SEA showed a significant induction of Jagged-2, which could not be seen in both groups of BMDCs stimulated with LPS. Conclusively, Jagged-2, but not Jagged-1, Delta-1 or Delta-4 is exclusively up-regulated in SEA-treated DCs. In order to test, if this selective up-regulation of Jagged-2 is necessary for SEA-induced Th2 polarization *in vivo*, the induction of IL-4 producing CD4 cells was examined in mice deficient in Notch signaling. Mice deficient in Notch-1, Notch-2, Delta-1, Delta-4 and Jagged-1 have a lethal phenotype, while deficiency in Jagged-2 leads to thymus defects [59].

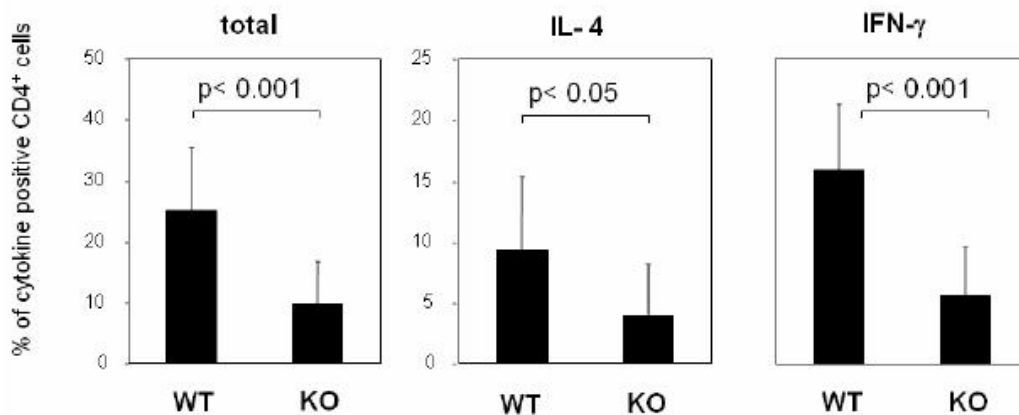


Figure 17: SEA-specific cytokine pattern in Presenilin-deficient CD4⁺ cells

Popliteal lymphocytes were restimulated with SEA and stained intracellularly for IL-4 and IFN-gamma. Data is pooled from 10 wild type (WT) and 14 animals conditionally deficient in presenilin (KO).

To investigate if Notch signaling is involved in Th effector choice in response to SEA, mice conditionally deficient in presenilin, which cannot cleave the intracellular portion of Notch-receptors in CD2⁺ cells, were chosen to investigate its requirement. To test if differentiation of CD4⁺ by SEA is dependent on Notch signaling, SEA was injected in the hind footpad of presenilin-deficient animals. The draining popliteal lymph nodes were isolated 8 days later and popliteal lymphocytes were restimulated with SEA and

stained intracellularly for IL-4 and IFN-gamma. As seen in Figure 17, a significant lower percentage of total (IFN-gamma and IL-4) cytokine producing CD4⁺ cells were observed in the knock out mice (9.5 ± 7 %) in comparison to the wild type animals (25.2 ± 10.4 %). Similarly, also IL-4 and IFN-gamma production was reduced in a significant manner in presenilin-deficient animals, suggesting a clear role of Notch-signaling in priming CD4⁺ cells for cytokine production. However, the polarization of SEA-specific lymphocytes does not seem to be influenced by signaling through Notch, since the ratio of IFN-gamma⁺ to IL-4⁺ cells is similar in wild-type (1.7) and presenilin-deficient mice (1.4). Taken together the above results suggest that in the complete absence of Notch signaling, the activation and generation of CD4 effector cells is compromised. Thus, due to this global defect in CD4 cell priming, the presenilin-deficient animals do not allow to selectively evaluate the role of Notch signaling pathways in SEA-mediated Th2 differentiation.

2.3 Results - Identification of the Th2 inducing molecule in SEA

2.3.1 Biochemical characterization of Th2-inducing molecule(s) in SEA

It has been shown that SEA induces a Th2 response *in vivo* and *in vitro*. To assess if an intact protein structure(s) is responsible for this biological activity, SEA was treated with proteinase K beads and then tested for Th2 polarization of CD4⁺ cells. As seen in Figure 18a, the treatment of SEA for 30 min at 37°C resulted in almost complete degradation of the proteins contained in SEA, since no bands can be revealed in SDS-PAGE of proteinase K-treated SEA. In contrast in mock-treated SEA, a sample that was treated in parallel but without the addition of proteinase K beads, proteins were preserved. However, the same volume of samples applied to the gel resulted in lower protein content in the mock-treated SEA sample as assessed by coomassie blue staining. The differently treated extracts were added to *in vitro* cultures with DCs and CD4⁺ OVA-specific T cells from DO11.10 mice in the presence of the peptide. Only SEA and to a lesser degree mock-treated SEA was able to induce an increase in IL-4⁺ cells, as measured by intracellular cytokine staining (Fig. 18b). While untreated SEA resulted in a population of > 30 % IL-4⁺ cells, proteinase K treated SEA did not increase the frequency of IL-4 producing cells in comparison to the control

(OVA-peptide alone). The result suggests that the intact structure of protein component(s) present in SEA is required for its Th2 inducing activity.

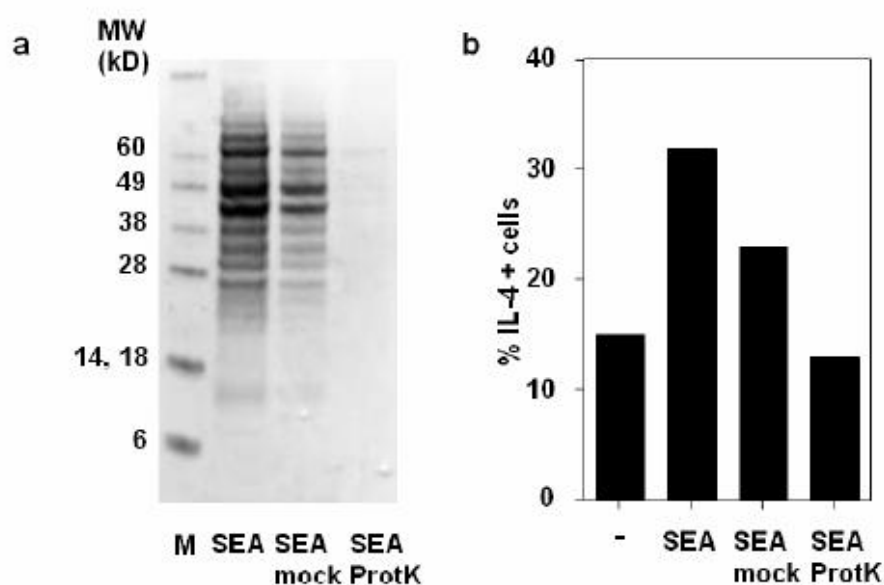


Figure 18: The Th2 inducing activity of SEA is proteinase K sensitive

a) Coomassie stained SDS page of SEA, M = protein marker b) FACS analysis of DO11.10 cells cocultured with proteinase K digested SEA. Data is representative for three experiments.

In order to isolate the putative protein(s), a gel filtration of SEA was performed, where 1 mg of starting material was run over a Sephadex-70 column and eluted in 500 μ l fractions. The fractions were sterile filtered and every second fraction was tested for Th2 inducing activity. As seen in Figure 19b, a complex band pattern is seen in fraction 5-7 with the subsequent fractions displaying fewer bands (8-12) and undetectable protein content in the fraction 13-20 in a coomassie blue stained SDS-PAGE. Tested in coculture with DO11.10 CD4+ cells and CD11c+ dendritic cells in the presence of the OVA peptide, only fractions 7-13 showed an increased population of IL-4+ cells (8.5 % - 14 %) over the background (6 %) as seen in Figure 19a. Based on the molecular weight (MW) markers the active fractions contain molecules with a MW of approximately 10-60 kD. This result was in agreement with studies in which SEA was crudely separated on filter units with membranes with various MW cut-offs (3, 10, 30 and 100 kD). After filtration, pairs of flow-through and retained fractions were tested in the herein used DO11.10 assay. The Th2-inducing activity was recovered in the retained fraction on filters with MW cut-offs 3, 10 and 30 kD and was almost equally distributed among flow-through and retained material when the filter mem-

brane had a cut-off of 100 kD (data not shown). Taken together these results indicated that the SEA-mediated Th2 inducing activity depends on intact protein structure of molecule(s) with a MW between 30 and 60 kD.

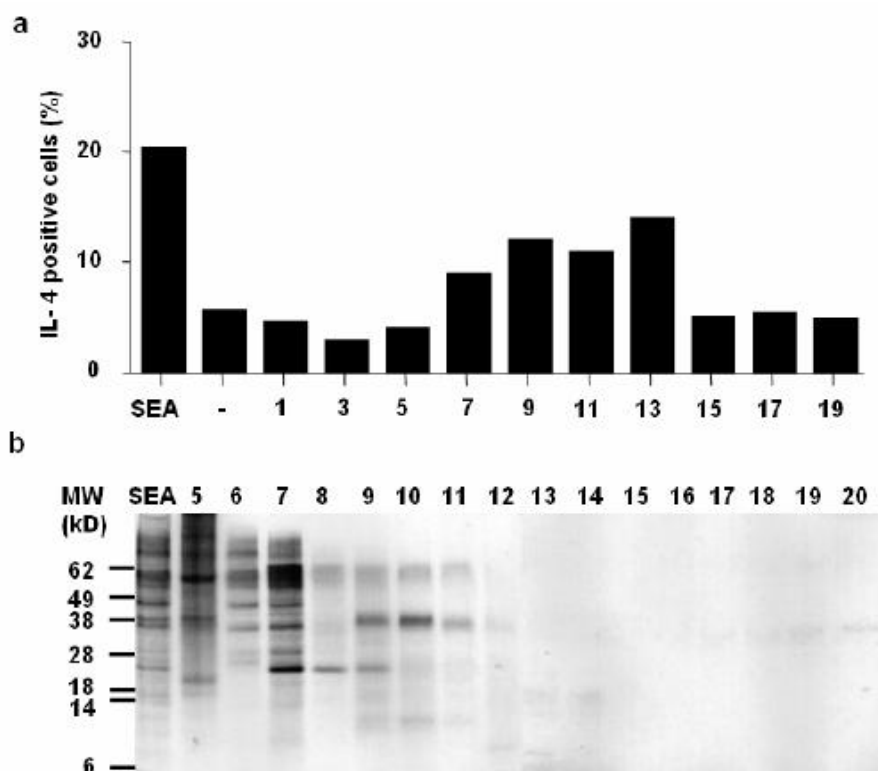


Figure 19: T2 inducing activity of SEA fractions

a) FACS analysis of SEA fractions tested on DO11.10 T cells. b) Coomassie stained SDS-PAGE of the same fractions (performed by J. Andersen, LMVR, NIAID, NIH).

2.3.2 *Schistosoma mansoni* excretory/secretory egg products recapitulate biological activities of SEA

In order to test if excretory/secretory products from *S. mansoni* eggs (ES) have a Th2 inducing activity, ES was prepared from viable *S. mansoni* eggs using a protocol adapted from Ashton *et al.* [60]. Briefly, *S. mansoni* eggs were isolated from livers of chronically infected mice and cultured for 72h at 37°C in minimal 1640 containing additional gentamycin and glutamine. After this culture period, the conditioned medium was filtered and concentrated 30 fold. The same medium without eggs was treated accordingly and used as control. As seen in Figure 20a, SEA and ES display different protein patterns, inferred from a coomassie blue stained gel. In agreement with previous reports in literature [60], ES contains approximately six major proteins

(black arrows), while SEA displays a smear pattern that doesn't allow quantification of the bands.

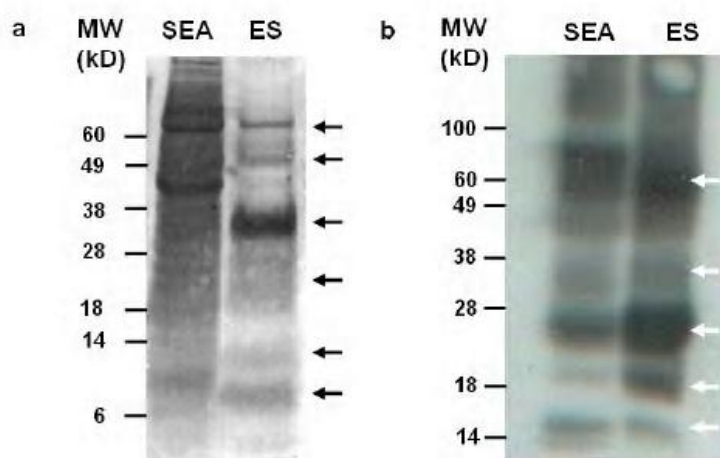


Figure 20: Protein and antigen composition in SEA and ES

SDS-PAGE of SEA (20 μ g) and ES (3 μ g) a) Coomassie stained gel. b) Western blot detected with serum (1:3000) from mice chronically infected with *Schistosoma mansoni*. Detection antibody: HRP-labeled rat anti-mouse IgG (1:3000).

In order to address the question which proteins are recognized by the immune system in experimental infection with *S. mansoni*, both parasite extracts were transferred on nitrocellulose membrane and a Western-Blot with pooled sera from chronically *S. mansoni*-infected mice. Interestingly, a similar pattern of protein bands in SEA and ES was detected (white arrows) irrespective of a greater complexity of SEA, suggesting that immunogenic antigens are similarly represented in ES and SEA (Fig. 20b). To test whether ES is able to induce the production of IL-4 from naïve CD4⁺ cells, it was tested in a Th2 polarization assay parallel to SEA and control medium. *In vitro* Th2 polarization of CD4⁺ DO11.10 cells by ES was dose-dependent and induced an increase of IL-4-producing CD4 cells over the control medium (Fig. 21). As can be seen in the contour plots of the FACS analysis, CD4⁺ cells stained for intracellular IL-4 and IFN-gamma show a Th2 polarization when they have been cultured in the presence of peptide-pulsed DCs and ES to a similar degree as those cultured in the presence of DCs and SEA. The addition of 18 μ g/ml and 36 μ g/ml ES to the culture resulted in an increased proportion of IL-4-producing T cells with 21 % and 40 %, respectively, while the frequency of IFN-gamma⁺ cells was decreased in parallel (Fig. 21). Importantly, when equivalent volumes of the concentrated control medium were

tested in parallel cultures, no increase in frequency of IL-4+ CD4 T-lymphocytes was detected.

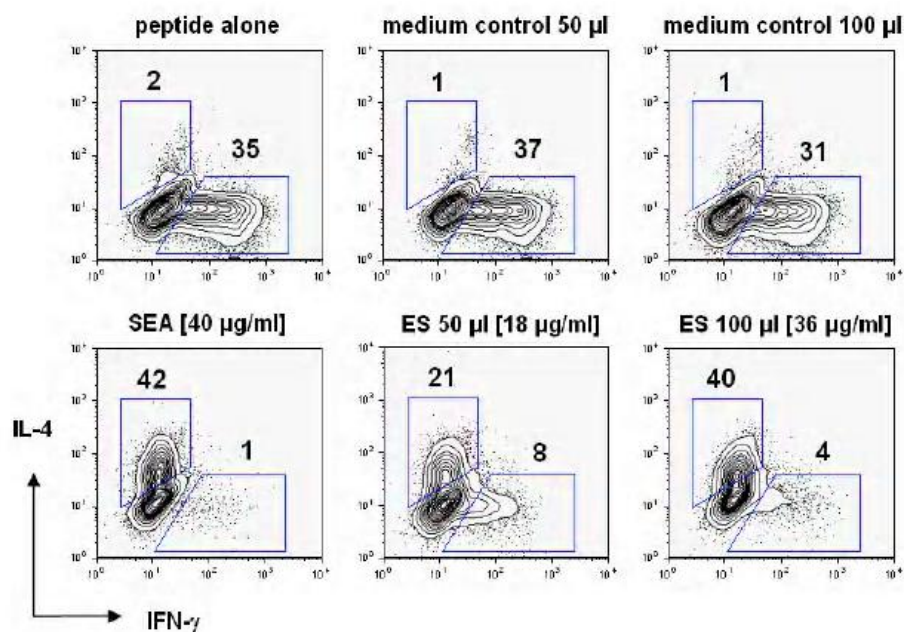


Figure 21: ES induces IL-4 production in CD4⁺ cells

Intracellular cytokine staining for IFN- γ and IL-4. Data is representative for > five independent experiments.

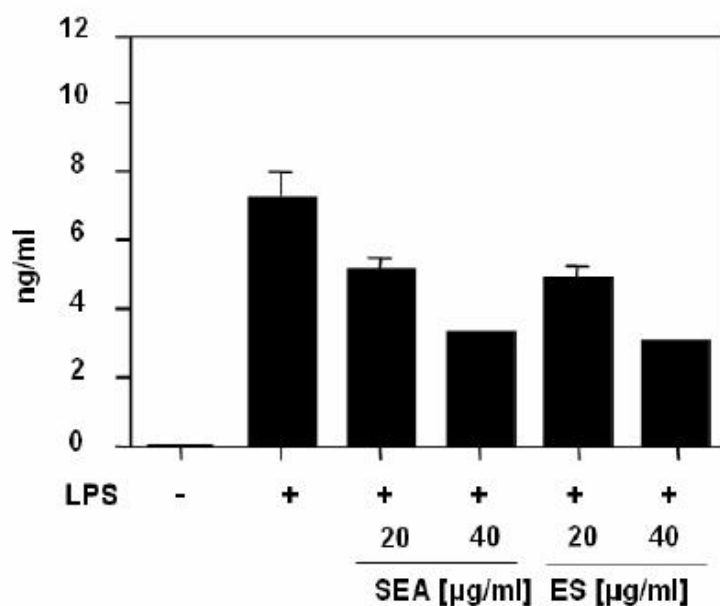


Figure 22: Down regulation of IL-12p40 by SEA and ES

BMDC were stimulated with LPS (50 ng/ml) in the presence or the absence of schistosomal products. The IL-12p40 levels in 24 h culture supernatants were measured by ELISA.

As shown before (Fig. 14), SEA is able to down-regulate IL-12 production by BMDCs treated with LPS. To test if ES too can inhibit the production of IL-12 by LPS-stimulated BMDCs, supernatants of BMDC cultured in the presence of ES and LPS were analyzed by ELISA. At both concentrations tested, ES down-modulated the production of IL-12 by BMDCs in a similar manner to SEA (Fig. 22), while control medium did not inhibit the production of IL-12 (data not shown).

2.3.3 Identification of the schistosomal egg protein omega-1 as a molecule with a Th2 inducing and IL-12 inhibiting activity

To identify the major proteins in ES, N-terminal sequencing and subsequent analysis of three prominent bands was performed by John Andersen, NIH-NIAID-LMVR (Fig. 23).

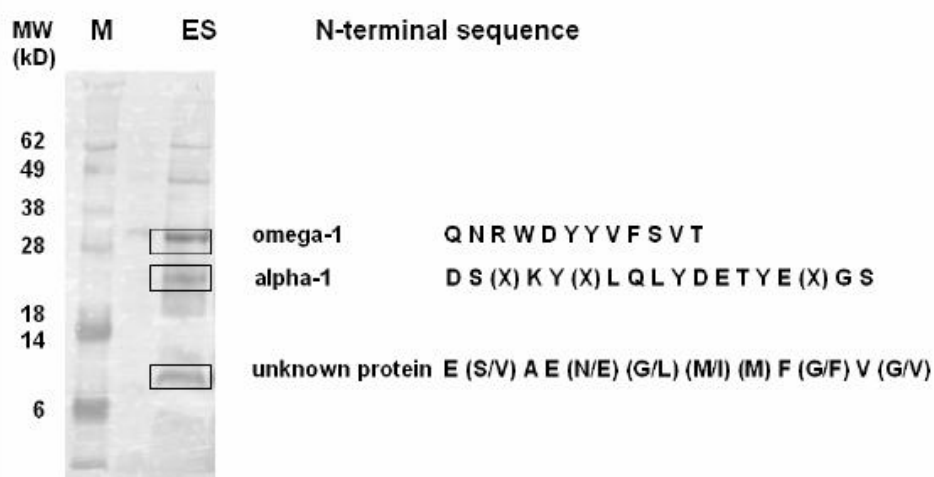


Figure. 23: *Schistosoma mansoni* excretory/secretory products

SDS-PAGE of ES. Boxed protein bands were N-terminal sequenced (performed by J. Andersen, LMVR, NIAID, NIH) and identified as schistosomal proteins. Amino acids in brackets were either not identified (X) or ambiguous (X/Y).

The analysis using annotated EST data banks available from the NCBI and Wellcome Trust Sanger Institute revealed two known proteins and one protein with homology to a *S. japonicum* protein. The band at ~30 kD was identified as omega-1, first described by McLaren *et al.* in 1981 as an antigen of *Schistosoma mansoni* and later characterized as a schistosomal hepatotoxic ribonuclease [61]. The band at ~25 kD

was identified to be the major immune protein alpha-1/IPSE, which was recently shown to induce IL-4 release from human basophils [62]. The band of 10 kD contained a protein with unknown function in *Schistosoma mansoni* (data not shown). To establish which molecule(s) is responsible for the induction of IL-4 producing CD4+ cells and the inhibition of IL-12 by LPS-stimulated BMDCs, a gel-filtration of ES was performed and the fractions were tested in vitro, as had been done with the total water-soluble extract of *S. mansoni* eggs SEA (Fig. 19).

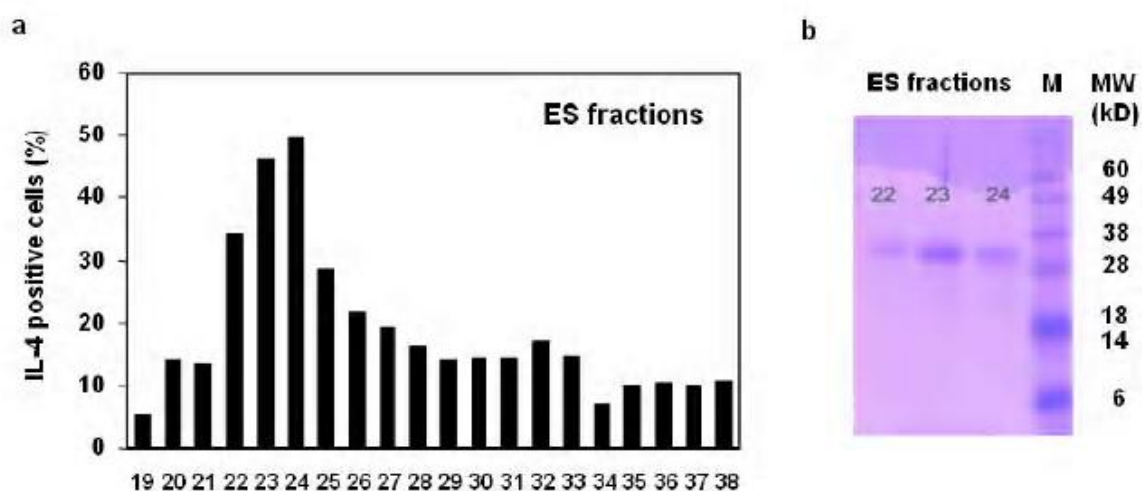


Figure 24: Th2 inducing activity of ES fractions

a) Gel filtration of ES tested for T_H2 polarization of DO11.10 cells. b) Coomassie stained SDS-PAGE of the active fractions 22-24 (performed by J. Andersen, LMVR, NIAID, NIH).

As seen in Figure 24a, the ability to induce higher frequencies of IL-4+ CD4+ cells peaks around the fractions 22 to 24 and gradually decreases. In order to investigate which proteins are contained within these fractions, a SDS-PAGE of fraction 22-24 was performed (Fig. 24b). Interestingly, these fractions contained a single band of ~30 kD, as seen by the coomassie blue stained SDS-PAGE. Since the fractions 22 to 24 with IL-4 inducing activity contain a single band at ~30 kD of molecular weight, it can be concluded that the Th2 inducing protein is omega-1. In order to examine, if native omega-1 results in a similar induction of IL-4 secreting cells at lower concentrations than SEA, the fractions 22-24 were pooled and tested side by side with SEA and recombinant IL-4 for IL-4 induction in CD4+ cells. As seen in Figure 25, a 10-fold lower protein concentration of the 30 kD fractions containing omega-1 protein was able to induce a similar frequency of IL-4 positive cells as SEA (39 % and 40 %, re-

spectively). In addition, native omega-1 was also able to inhibit (up to 24 %) the production of IL-12 by LPS-stimulated BMDCs (Fig. 26).

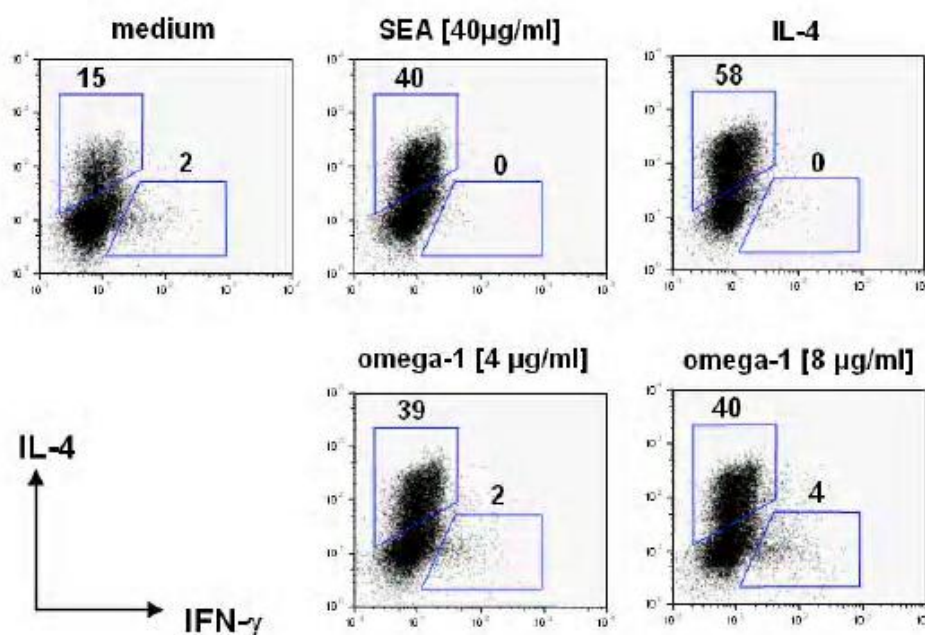


Figure 25: Th2 polarization by native omega-1

Fractions 22, 23 and 24 were pooled and tested in the cell culture. Data is representative for two independent experiments.

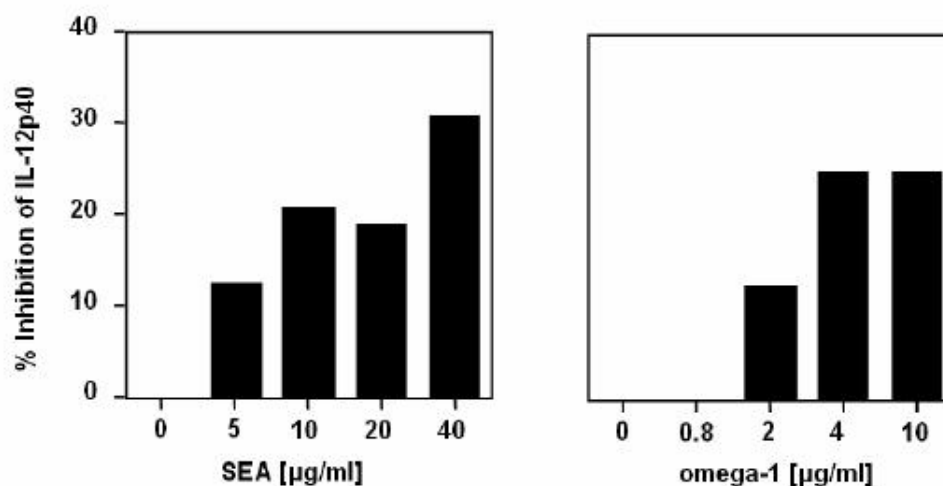


Figure 26: Down regulation of IL-12p40 by native omega-1

BMDCs were stimulated with LPS [50 ng/ml] in the presence or the absence of schistosomal products for 24 h and the concentration of IL-12 was measured by ELISA.

Since the gel filtration experiments of ES demonstrated that fractions containing only one protein with a MW of ~30 kD has two properties: the ability to promote Th2 po-

larization as well as to inhibit IL-12p40 production by LPS-stimulated BMDC, it can be concluded that the schistosomal molecule responsible for both activities is the *S. mansoni* ribonuclease, originally identified as omega-1 antigen.

2.4 Results - Role of TLR in experimental infection with *Schistosoma mansoni*

2.4.1 Experimental infection of TLR-deficient mice with *S. mansoni*

Recognition of pathogens by TLRs is an important feature of host immune responses against bacterial, viral, protozoan and fungal infections. Some TLRs are known to play a role in the generation of Th1 responses. In order to investigate if such TLRs are required for immune responses against *S. mansoni*, TLR2-, TLR4-, and MyD88-deficient mice were percutaneously infected with 35 viable cercariae of *S. mansoni*. To address the question if TLR recognition influences the infection rate or the fecundity of worms, the number of worm pairs and the number of eggs trapped in the liver and gut was measured by perfusion of infected animals and collection of the worms as well as histological examination of sample tissue. As seen in Figure 27, all groups became infected to a similar level.

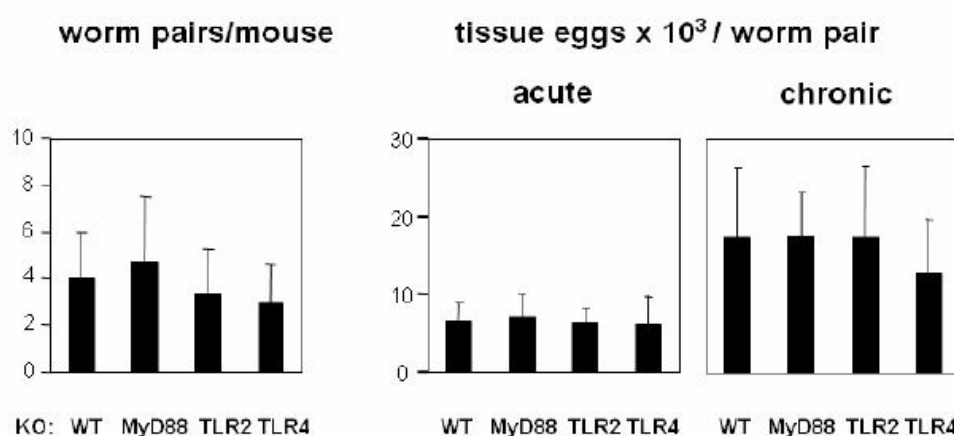


Figure 27: Experimental infection with *Schistosoma mansoni* in MyD88- and TLR-deficient mice
 a) WT-B6 (29), MyD88- (17), TLR2-(12) and TLR4-deficient (18) mice were percutaneously infected with 35 cercariae. The results shown are pooled from two independent infections and represent the mean \pm SD.

The infection of mice resulted in the development on average of 3.8 ± 2.2 worm pairs/mouse, with no significant differences among the groups. Similarly, no signifi-

cant differences were observed in terms of tissue egg burden (liver and gut) between wild type and knock out mice. Naturally, the number of eggs accumulates during the course of infection and at least twice as many eggs are found in the chronic (16 weeks) versus the acute phase (8 weeks). In conclusion, recognition through TLR2, TLR4 and signaling through MyD88 is neither required for successful infection with *S. mansoni* nor natural host resistance. Also, the fecundity of the worms as assessed by the similar number of eggs trapped in the host tissue argues against a role for the TLR pathways in worm development.

2.4.2 Induction and down-regulation of the Th2 response is independent of TLR2, TLR4 and MyD88

Several studies have suggested the involvement of TLR in the development of a Th2 response against egg-derived water soluble products (SEA) of *S. mansoni* *in vitro*. Studies on TLR4-deficient dendritic cells revealed that a carbohydrate (LNFPIII) present in SEA can trigger induction of IL-4 secretion by CD4⁺ cells in a TLR4-dependent manner [50]. On the contrary, *in vivo* studies showed that Th2 priming by SEA can be observed in the absence of the TLR adaptor molecule MyD88 which is indispensable for Th1 induction by STAg (soluble *Toxoplasma gondii* antigen) [63].

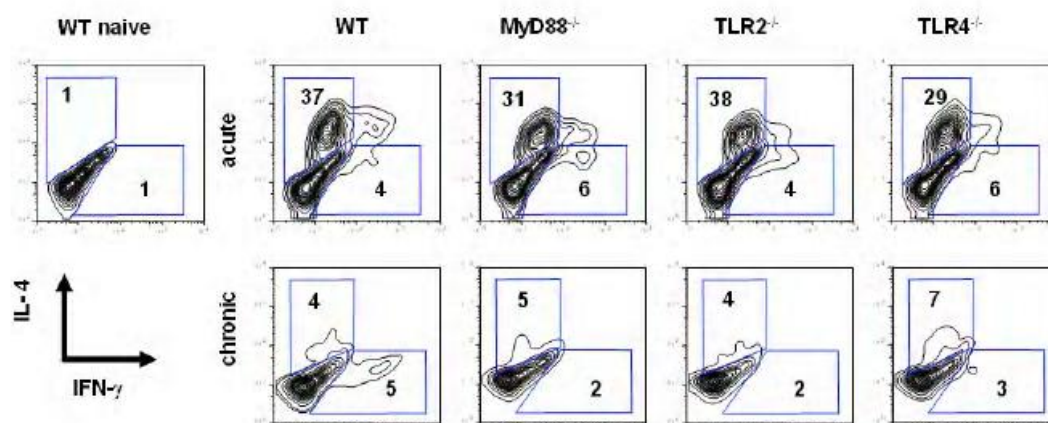


Figure 28: Th2-type cytokine profile of CD4⁺ cells in *Schistosoma mansoni* infected MyD88- and TLR-deficient mice

Intracellular cytokine staining of mesenteric lymphocytes pooled from 3-9 mice. Contour plots are gated on CD4⁺ cells.

To investigate if the development of the typical Th2 immune response during *S. mansoni* infection is dependent on TLR2, TLR4 or MyD88-dependent pathway, mesenteric LN cells from infected mice with corresponding gene deletions were restimulated *in vitro* with SEA at 8 and 16 weeks post infection (p.i.), and cytokine production measured by intracellular cytokine staining. In order to ensure that the absence of these pathways does not result in an aberrant composition of mesenteric LN, cells were stained *ex vivo* using labeled antibodies to known markers (B220, CD4 and CD8). A similar distribution of CD4⁺ cells, CD8⁺ cells and B220⁺ cells was observed in all groups (data not shown). As seen in Figure 28, TLR2⁻, TLR4⁻ and MyD88⁻ deficient mice showed a polarization of their CD4⁺ cells with high frequencies of IL-4-producing cells (29 % – 39 %) and low frequencies of IFN-gamma-producing cells (4 % - 6 %) in the acute phase of infection, similar to the wild type control (37 % IL-4⁺ and 4 % IFN-gamma⁺ cells). This response was a consequence of *in vivo* priming during the infection, since CD4⁺ cells from MLN of uninfected mice tested in parallel displayed only a minor cytokine response, $\leq 1\%$.

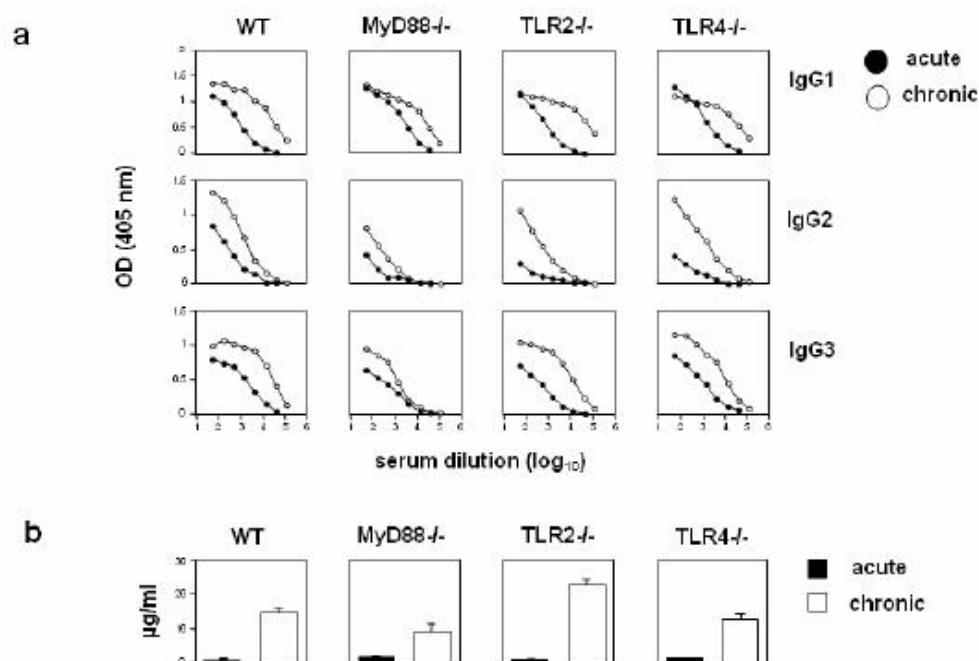


Figure 29: Humoral immune response in *S. mansoni*-infected Myd88- and TLR- deficient mice
a) Sera from 3-9 mice were pooled and the SEA-specific antibody titer of the indicated IgG isotypes were measured by ELISA, SD < 5%. b) Serum concentration of total IgE. Data is representative for two independent experiments, mean \pm SD.

The ability to mount a Th2 response in the absence of these TLR-pathways was also reflected in the humoral response elicited against schistosomal antigens. Pooled sera

of chronically and acutely infected animals were tested for SEA-specific IgG1, IgG2b, IgG3 and total IgE by ELISA. As assessed by serial dilution of serum, the antibody titer of all IgG isotypes was increased in the chronic phase of infection in all groups (Fig. 29a). Interestingly, IgG2b titer was lower in MyD88- and TLR2-deficient mice, while TLR4-deficient animals displayed an antibody titer comparable to that of the wild type in the chronic phase of infection. Furthermore, a high concentration of total serum IgE could be detected in all TLR-deficient mice at 16 weeks p.i. with 9.5 ± 0.2 to 23.2 ± 0.9 ng/ml compared to the wild type mice controls (Fig. 29b). Taken together these data suggest that a typical cellular and humoral Th2 response develops against *Schistosoma mansoni* independent of the Toll like receptor pathways investigated. In infection studies of mice with *S. mansoni*, it has been shown that a strong Th2 immune response observed during the acute phase undergoes a down regulation in the chronic phase despite the Ag persistence.

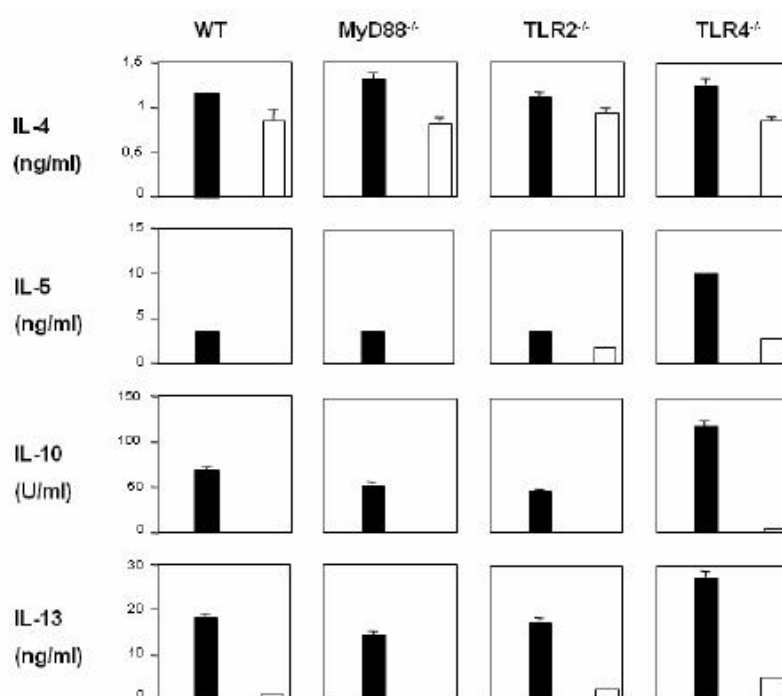


Figure 30: Induction and down-modulation of Th2 cytokines in MyD88-, TLR2- and TLR4-deficient mice

Mesenteric lymph node cells were restimulated with SEA and cytokine secretion after 72 h measured by ELISA. Acute infection (8 weeks p.i.) = black bars, chronic infection (16 weeks p.i.) = white bars. Mean values from duplicates \pm SD are shown. Data is representative for two independent experiments.

Different immuno-modulatory mechanisms have been implicated in this CD4⁺ T cell hyporesponsiveness, including increased function of regulatory T (Treg) cells [64].

Interestingly, TLR2 was shown to mediate the induction of human IL-10 producing Treg cells by DCs conditioned with the schistosomal lipid phosphatidylserine *in vitro*, while Th2 skewing was unaffected by the absence of TLR2 [49]. In order to investigate if the TLR pathways under investigation are involved in the typical down modulation of the immune response, cytokine production and granuloma volumes were measured in the acute and chronic infection. Interestingly, while antibody responses continued to increase as the infection progressed, Th2 CD4⁺ T cell responses decreased. As seen in Figure 30, IL-4 secretion diminished in chronic infection and was independent of TLR2, TLR4 or MyD88. This pattern correlates with a substantial down regulation of IL-5, IL-10 and IL-13 in all groups. To investigate if granulomatous response to *S. mansoni* eggs is also down regulated in a TLR independent manner, histological analysis of paraffin liver sections were stained with hematoxylin and eosin.

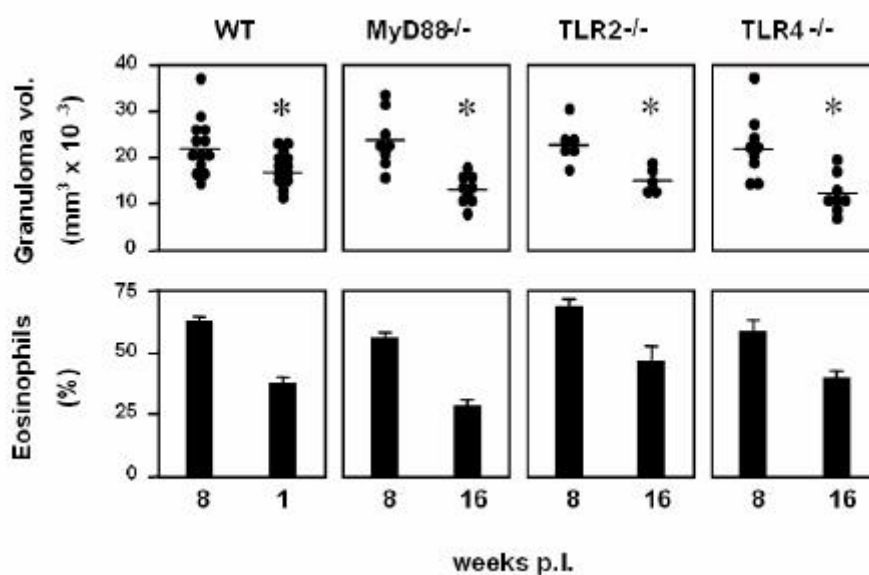


Figure 31: Down-modulation of egg granuloma formation in liver of *S.mansoni* infected mice at 16 wk post infection.

Histological analysis of liver sections of wild type and knock out mice in acute (8 wk) and chronic (16 wk) infection. Data of granuloma volume and eosinophil infiltration were pooled from two experiments.* samples are significantly different between acute and chronic infection, Mann-Whitney U-Test ($P < 0.01$, two-tailed test).

The volume of hepatic granuloma was then calculated by measuring the diameter of granuloma surrounding single viable eggs containing a mature miracidium and assuming a spherical shape. Analysis of TLR2, TLR4 and MyD88-deficient mice revealed that granuloma surrounding eggs trapped in the liver sinusoids are signifi-

cantly smaller in the chronic than in the acute phases of the infection irrespective of the genetic background of the mouse. Furthermore, eosinophil infiltration, a hallmark of a Th2 driven granulomatous response, is significantly reduced at 16 weeks p.i. (Fig. 31). Overall, the TLR pathways investigated do not appear to be involved in down regulation of the Th2 response elicited against schistosomal antigens as well as down-modulation of granuloma size.

2.4.3 Th2 induction in the absence of TLR3

A recent study suggested that *S. mansoni* eggs induce the expression of IFN-responsive genes via stimulation of TLR3. Since TLR3 is MyD88 independent, a possible role for this Toll like receptor cannot be analyzed in MyD88-deficient mice. In order to investigate if TLR3 and therewith IFN-responsive genes are involved in the generation of Th2 responses against *S. mansoni*, TLR3-deficient mice were infected with 35 cercariae and parameter of infection and immunological responses analyzed. As shown for TLR2- and TLR4-deficient mice the absence of TLR3 did not alter infection rate or egg burden in the acute infection in comparison to wild type mice (data not shown).

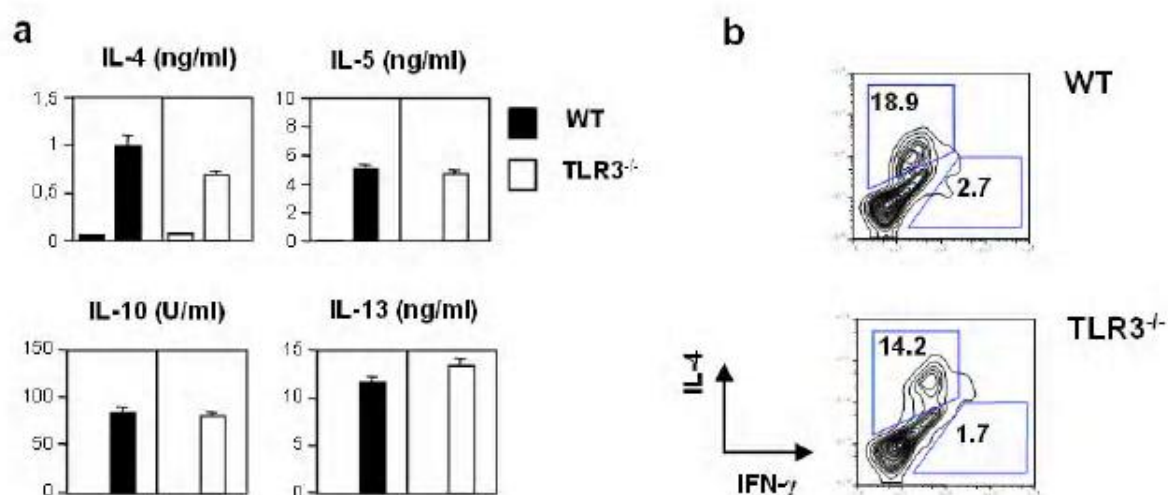


Figure 32: Th2 responses in acutely *S. mansoni*-infected TLR3-deficient mice

Cytokine production from mesenteric lymph node cells at 8 wks post infection. a) Secretion of Th2 cytokines was measured by ELISA in the culture supernatants after 72 h stimulation with SEA. Bars represent mean values \pm SD of duplicate ELISA values. b) FACS analysis of intracellularly stained CD4⁺ cells from the same cultures.

As seen in Figure 32a, the secretion of IL-4, IL-5, IL-10 and IL-13 was induced in TLR3-deficient mice to a comparable level as in wild type mice. Similarly, a clear polarization towards the Th2 phenotype in mesenteric lymphocytes could be observed in both groups as assessed by intracellular staining for IL-4 and IFN-gamma of CD4+ cells restimulated with SEA (Fig. 32b). Thus, neither MyD88-dependent TLR nor MyD88-independent TLR3 signaling pathways are required for the development of Th2 response during acute *S. mansoni* infection.

2.4.4 Liver Pathology during *Schistosoma mansoni* infection in WT vs. TLR-deficient mice

One important sequela of chronic schistosomiasis is liver fibrosis, which leads to portal hypertension and oesophageal varices. Fibrotic tissue results from continuous collagen deposition which is triggered mostly by IL-13 in schistosome-infected mice. In order to examine if MyD88 or Toll-like receptors are involved in a fibrotic response, mice deficient in MyD88, TLR2 and TLR4 were infected with *S. mansoni*. At 8 and 16 weeks p.i., a 200 mg portion of liver was taken and hydrolyzed in 6N HCl and collagen assayed as hydroxyproline by technique B of Bergman and Loxley [65]. In acute infection with *S. mansoni* the degree of fibrosis is comparable between all groups regardless of the background of the mice (Fig. 33).

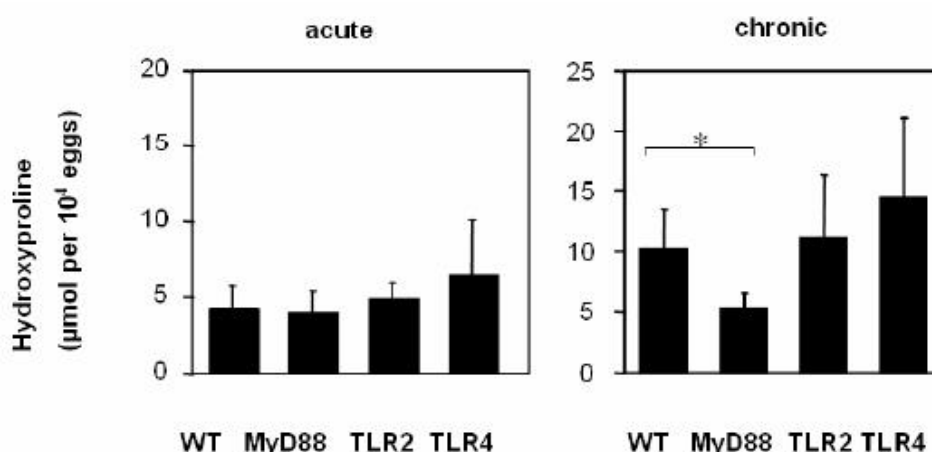


Figure 33: Role of MyD88-dependent pathway in the fibrotic response

Concentration of hydroxy-proline in deficient mice and control mice. Data is pooled from two experiments. Mean \pm SD, * p-value < 0.001.

As the infection progresses liver fibrosis increases in the wild type mice from 4.2 ± 1.6 to 10.1 ± 3.4 hydroxyproline in $\mu\text{mol}/104$ eggs. A similar increase was observed in TLR2- and TLR4-deficient mice. Interestingly, however, MyD88-deficient mice did not display significant increase in liver fibrosis above the level observed in acute phase from 4.0 ± 1.5 to 5.3 ± 1.2 hydroxyproline in $\mu\text{mol}/104$ eggs. Hence, they displayed a significantly lower degree of fibrosis in chronic infection as measured by levels of hydroxyproline in $\mu\text{mol}/104$ eggs (5.3 ± 1.2) when compared to the wild type control (10.1 ± 3.4) (Fig. 33). A direct relationship between MyD88 signaling and collagen deposition is inferred by the following observation: the absence of MyD88 does not result in an altered immune response as shown by cytokine and antibody levels, which thus preclude an effect on the level on IL-13 expression and through FcR-dependent mechanisms, respectively. However, a clear influence in fibrotic response is observed notwithstanding similar rates of infection and egg production. Taken together these results argue that the MyD88-signaling pathway plays a critical role for continued collagen disposition after the acute phase of infection.

3 Discussion

3.1 Discussion - Th2 differentiation by SEA

It has been shown, that SEA stimulated DCs induce the differentiation of naïve human and murine CD4⁺ cells *in vitro* and *in vivo* [66], [47], [67], [18]. The factors leading to early differentiation of naïve CD4⁺ by SEA-stimulated DCs are largely unknown. However, a clear restriction was observed in terms of the type of antigen presenting cell used for T cell priming. While B-cells (CD11c⁻ B220⁺ B cells) were similarly able to induce the proliferation of DO11.10-CD4⁺ cells as measured by CFSE dilution, only CD11c⁺ B220⁻ DCs could mediate an SEA induced polarization of CD4⁺ cells *in vitro* [47]. In order to investigate, which DC-derived stimuli apart from IL-4 may account for the ability of SEA-DCs to promote Th2 differentiation, parameters as CD8alpha DC subtype and antigen-dose were changed in an *in vitro* Th2 polarization assay. To that end, splenic OVA-transgenic CD4⁺ cells were sorted as CD4⁺, DX5⁻ and CD11c⁻ lymphocytes from female DO11.10/Rag-deficient BALB/c mice and cultured in the presence of splenic CD11c⁺ B220⁻ DCs and OVA-peptide. Recently, it was shown that subclasses of DCs induce the development of distinct Th populations. Namely, CD8alpha⁺ CD11c⁺ splenic DCs pulsed with keyhole limpet hemocyanin (KLH) primed lymphocytes to produce IL-2 and IFN-gamma after transfer *in vivo*, while CD8alpha⁻ DCs promoted the secretion of IL-4, IL-5 and IL-10 indicative for a Th2-type immune response [28]. The distinct ability of two subsets to induce a Th1 and Th2 response was paralleled by the production of a high or low amount of IL-12p70 after stimulation with pansorbin, IFN-gamma and GM-CSF and the ability of CD8alpha⁺ CD11c⁺ DCs to promote Th1 cells was dependent on IL-12p40. In addition, Th2 polarization by CD8alpha⁻ CD11c⁺ DCs was shown to be IL-10-dependent [28]. To investigate if Th2 induction by SEA is restricted to either subset, CD8alpha⁺ or CD8alpha⁻ DCs were cultured with CD4⁺ DO11.10 cells in the presence of OVA-peptide. The addition of the Th1 stimulus soluble *Toxoplasma gondii* antigen (STAg) or soluble *Schistosoma mansoni* egg antigen (SEA) resulted as expected in Th1 and Th2 polarization, respectively, when total splenic CD11c⁺ DCs were used ([47] and Fig. 6). Interestingly, SEA induced Th2 polarization could be observed in cultures with both DC subpopulations DCs with 28 % (CD8alpha⁻) and 24 % (CD8alpha⁺) IL-4 positive CD4 cells, while IFN-gamma secreting cells were undetectable. On the contrary Th1 polarization by STAg was only seen by

CD8alpha+ DCs and total DCs. CD8alpha- DCs could not mediate the Th1 polarizing activity by STAg, since a similar cytokine pattern as with the control culture (peptide alone) was observed. This just recapitulates what has been shown *in vivo*, where the production of IL-12 by STAg is restricted to CD8alpha+ DCs and results in an IL-12 dependent IFN-gamma response [68]. Total splenic CD11c+ DCs comprise up to 15% CD8alpha+ DCs which are sufficient to trigger the STAg-induced Th1 polarization (data not shown). The control cultures without the addition of Th polarizing substances showed a pattern in which CD8alpha- DCs did promote more IL-4 secreting cells and CD8alpha+ DCs more IFN-gamma+ cells, which could result from the intrinsic capacity of the DC subset to promote a distinct Th response under neutral condition, as observed *in vivo* by Maldonez-Lopez *et al.* [69] with KLH, not known to bias Th1/Th2 polarization. As studied extensively in the last decade, the antigen dose, measured as antigen concentration, density of peptide-MHC class II complexes or TCR-affinity is implicated in the outcome the T cell effector choice. In an *in vitro* model system employing naïve TCR-transgenic CD4+ cells specific for moth cytochrome c (pMCC), low peptide concentrations (0.5 ng/ml) resulted in IL-4 secretion, while high peptide doses (50 µg/ml) in IFN-gamma secretion [54]. This finding is supported by another study using DO11.10 cells, where low (0.01 µM) and high (3.7-100 µM) doses of OVA323-339 peptide promoted Th2 polarization while the intermediate doses triggered Th1 development [70]. In order to investigate, if the addition of parasite extract-induced Th development is restricted to a certain peptide concentration and herewith the strength of TCR-signaling, naïve CD4+ cells were stimulated with a range of OVA-peptide. As seen in Figure 7, the addition of SEA and STAg results in the induction of IL-4 and IFN-gamma producing cells, respectively, regardless of the peptide concentration used. A recent study by Boonstra *et al.* [71] showed that splenic DCs (CD8alpha+ and CD8alpha-) can direct the development of naïve Th cells towards either Th1 or Th2 phenotype. The Th differentiation by the two subsets was dependent on the antigen, where gradual lowering of the antigen dose from 10 to 0.01 µM OVA-peptide resulted in reduced IFN-gamma production and enhanced IL-4 secretion. In agreement with the finding from Maldonez-Lopez *et al.* [69] the CD8alpha+ DCs generally induced a stronger Th1 response as measured by the frequency of IFN-gamma+ cells. However, this intrinsic ability of the DC subset was overridden by a superordinate signal such as a pathogen-derived product like CpG [71]. Taken together, the results suggest that SEA-derived stimuli can override sig-

nals provided by the DCs or the TCR strength and seems to convey a third signal to direct T cell polarization. Interestingly, the absence of DC-derived IL-4, the key trigger for Th2 differentiation, did not abrogate the polarization of naïve murine CD4+ cells by SEA *in vitro* [47]. Similarly, SEA-stimulated IL-4 deficient DCs transferred into a wild type recipient induced the development of IL-4 secreting splenocytes to a comparable degree as SEA-stimulated wild type DCs, while the ability of the recipient to produce IL-4 was absolutely required [56]. This finding is in direct agreement with the report by Schmitz *et al.* [72] suggesting that Th2 differentiation is not dependent on IL-4 from non-Th cells. Since it was shown that DC-derived IL-4 is dispensable for the initiation of Th2 differentiation the requirement for CD4 cell-derived IL-4 was investigated *in vitro*. To that end, endogenous IL-4 in cultures containing DO11.10 cells, CD11c+ DC and OVA-peptide with or without SEA was depleted with anti-IL-4 antibody at the beginning of the culture. It was shown that IL-4 is absolutely required for Th2 polarization of CD4 cells in both cultures with SEA and rIL-4, since development of IL-4+ cells is abrogated and a IFN-gamma+ subpopulation emerges, which was not observed in cultures with the control antibody isotype. Since DC-derived IL-4 is not required for Th2 induction in this two-cell system, it can be concluded that CD4-derived IL-4 is absolutely necessary for the initiation of Th2 differentiation *in vitro*. On the contrary, mice deficient in IL-4 and IL-4 signalling (Stat6- and IL-4Ralpha-deficient mice) showed residual Th2 cytokine production, with the latter producing reduced but considerable amounts of IL-4 in infection with *Nippostrongylus braziliensis* or *Schistosoma mansoni* [73], [74], [14]. Further studies corroborated an IL-4R-independent IL-4 induction in CD4+ cells by *S. mansoni* [75], [76] *in vivo*. Mesenteric LN cells isolated from *S. mansoni* infected Stat6-deficient animals displayed a reduced frequency of IL-4+ CD4 cells when restimulated with SEA, while the proportion of IFN-gamma producing cells increased in comparison with wild type mice. Therefore, even though IL-4 is a major factor for Th2 polarization, lack of IL-4R signalling does not completely abrogate induction of IL-4+ CD4+ T cells, but rather hinder the amplification of a Th2 response. This could happen directly by preventing amplification of IL-4 gene transcription through the IL-4R/Stat6 pathway and indirectly by suppressing outgrowth of IFN-gamma producing cells, since IL-4 signalling inhibits the development of Th1 cells [77]. The observed discrepancy from *in vivo* studies might be based on the fact that substantial amounts of IFN-gamma accumulate in cultures with and without addition of SEA *in vitro* (Fig. 12), which will ultimately result in suppression of Th2 devel-

opment in the absence of IL-4. Furthermore, a third cell, producing a Th2 inducing factor other than IL-4 *in vivo* may promote the residual population of IL-4+ CD4 cells. In order to understand, if CD4+ cells become independent of the autocrine action of IL-4 *in vitro*, the cytokine was neutralized at different time points after initiation of the culture. As shown in Figure 9, SEA-cultures become independent of endogenous IL-4 after 60 h as seen by a higher proportion of IL-4+ than IFN-gamma+ cells, while cultures with supplemented rIL-4 become earlier (at 36 h) independent of the cytokine. Since in the latter case, naïve CD4 cells are under the influence of a Th2 promoting factor from the very beginning of the culture, at the time of priming through their TCR, the differentiation process is completed faster than in SEA-cultures, where IL-4 most likely needs to accumulate in order to achieve certain concentration necessary for Th2 priming. IL-4 message can be detected as early as 24 h after priming in cultures without SEA (Fig. 12), but SEA-cultures show an increased level of IL-4 mRNA at both 24 and 48 h, which might explain why only in the latter cultures a Th2 differentiation process sets off. Early Th2 differentiation by SEA was shown to be independent of IL-4 after 60 h of culture, but was indispensable at earlier time points. A possible explanation of this discrepancy could be that IL-4 is required to downregulate a concurrent Th1 response and that in the *in vitro* system used a substantial amount of Th1 cells develop before SEA-induced Th2 cells expand and suppress IFN-gamma+ cells through IL-4 signaling as discussed above. To investigate further, at what time point T cells become independent of signals obtained directly from dendritic cells to become Th2 effector cell, T cells were sorted from dendritic cells and their culture resumed in the absence from parasite stimulus, peptide and antigen-presenting cell. In a preliminary experiment, where the TCR-Peptide-MHC class II interaction was disrupted through extensive washing of the culture, it could be found, that T cells seem to become independent from DC-derived signals at 36 h (Fig. 10a). Indeed, as seen in Fig. 10b, CD4+ cells primed by peptide-loaded DC in the presence of SEA, become independent of DC-derived stimuli after 36 h, since they show a similar frequency of IL-4 than the unsorted control group. As it was shown before, DC need to be cultured with SEA for at least 10 h in order to be conditioned by the parasite extract to convey a Th2 inducing signal to the naïve CD4+ cell [Jankovic *et al.* 2004]. Thus, it can be assumed, that the processes involved in converting a DC to become DC2 and receiving the third signal by the CD4+ cell are occurring between 10 and 36 h after beginning of the culture. One important factor in the development of effector

CD4 cells is the cytokine milieu during their first encounter with antigen. In order to understand which cytokines are involved in the early priming of a Th2 response, the effect of IL-10, IFN-gamma and IL-12 were investigated, since they have been shown to direct Th1/Th2 polarization *in vivo* and *in vitro*. Here, their role in SEA-induced Th2 polarization was investigated by neutralization or supplementation in the described two-cell model. Transfer studies in mice suggested that IL-10 bears a Th2 promoting activity, where KLH-pulsed DC were treated with IL-10 *in vitro* and transferred into syngeneic mice. The restimulation of lymphocytes revealed that IL-10 treated DC primed for Th2 polarization as measured by the production of IL-4, IL-5 and IL-10, while IFN-gamma was reduced in comparison with the control [37]. In order to test if SEA-induced Th2 polarization requires IL-10, the cytokine was neutralized with 20 µg/ml anti-IL10R antibody at the beginning of the culture. As seen in Figure 11a, blocking of IL-10 signaling did not result in abrogation of Th2 polarization but rather expanded the population of cytokine secreting cells in both control cultures and SEA-cultures. Similarly, the addition of IL-10 to both cultures lead to a reduced number of IL-4+ CD4 cells suggesting that IL-10 is neither necessary nor supporting IL-4 induction in naïve CD4 cells (Fig. 10b). In line with this data is a report showing that IL-10, while being produced by human Th2 clones themselves inhibits their antigen-specific proliferation and cytokine production [78]. It could be argued that IL-10 acts on DC to become DC2-type cells, which are prone to prime for Th2 polarization, while IL-10 receptor signaling in naïve T cells inhibits their cytokine production. The importance of IL-10 in *S. mansoni* infection was inferred from studies in IL-10 KO mice, where a mortality of up to 30 % was associated with increased granulomatous inflammation and higher levels of Th1 cytokines, while Th2 cytokines still persevered as compared to control mice. Therefore, IL-10 is rather involved in the regulation of liver pathology in acute infection and down-modulation of a concurrent Th1 response [79]. *In vitro* analysis of SEA-conditioned BMDC revealed that IL-10 could not be detected on the protein level [67], which supports the notion that DC stimulated with SEA induce Th2 polarization in an IL-10 independent manner. A recent report investigated the role of IFN-gamma in Th2 differentiation and concluded a positive role of IFN-gamma in a strictly IL-4 dependent manner in that it was blocked with anti-IL-4 antibody [36]. On the contrary, the addition of IFN-gamma at a concentration of 10U/ml to SEA-cultures resulted in complete abrogation of development of IL-4 secreting cells (Fig. 11b) and the expansion of IFN-gamma+ cells (7 % to 43 %). This data suggests that IFN-

gamma is not required for Th2 induction by SEA in agreement with *in vivo* studies using IFN-gamma deficient mice where *S. mansoni* infection lead to Th2 differentiation independent of this gene [80]. IL-12 is a key cytokine in Th1 induction and is secreted by dendritic cells following numerous pathogen-derived stimuli such as LPS, STAg or CpG. On the other hand, the addition of exogenous IL-12 suppresses Th2 cytokines in mice infected with *Schistosoma mansoni*. However, IL-12 does not result in suppression of Th2 responses in mice lacking the IFN-gamma gene [81], [35] suggesting an indirect role of IL-12 in suppression of Th2 cytokines through its induction of IFN-gamma. In agreement with the latter study, here the addition of IL-12 resulted in complete abrogation of SEA-induced IL-4 induction and promotion of IFN-gamma+ cells (Fig. 11b). Even though, IFN-gamma was shown to be detrimental for the induction of IL-4, SEA induced Th2 development occurs in the presence of IFN-gamma as measured at 72 h in the supernatant of SEA-cultures (Fig. 12a). This data suggests, that SEA exerts a stimulus on DC that biases T cell effector choice, but it cannot act on DCs that are activated through IL-12/ IFN-gamma at the time of T cell priming. Interestingly, as measured by Real Time PCR, in cultures with SEA, both, message for IFN-gamma and its transcription factor T-bet could be detected at 24 and 48 h of culture to a similar degree as in control cultures. T-bet is expressed in Th1 clones and in primary Th1 cells that were generated under Th1 priming conditions [82] and T-bet-deficient mice fail to develop a Th1 response but possess an overexpanded Th2 compartment, which leads to a spontaneous asthma-related phenotype [42], [83]. A recent study showed that T-bet reduces IL-4 production, presumably through physical interaction with GATA-3, a major transcription factor of the IL-4 gene [82]. As seen in Figure 13, GATA-3 is exclusively over expressed at 48 h in SEA-cultures compared to unprimed naïve CD4+ cells. Moreover, a population of IL-4 positive IFN-gamma negative CD4 cells exists in cultures with and without SEA at 72 h, but only in SEA-cultures an IL-4+ CD4+ T cell population is established as detected on d 7 (Fig. 12b). This upregulation of GATA-3 could be the driving force of Th2 polarization, since it was shown that IL-4-secreting Stat6-deficient Th2 cells stably express GATA-3 and that the introduction of GATA-3 into Stat6-deficient T cells completely restores Th2 development and Th2 cytokine expression, while suppressing Th1 development in an IL-4 independent manner [84], [85]. Together these findings indicate that early Th2 polarization of CD4+ cells by SEA-conditioned DC involves an active induction of IL-4 producing CD4+ T lymphocytes that is able to overcome the simultaneous induc-

tion of IFN-gamma⁺ cells in the same CD4 T cell population. This however is only ensured as long as the initial transcription of the IFN-gamma gene and its secretion from unpolarized T cells is not supported through factors directly or indirectly supporting the differentiation of Th1 cells, since both the addition of IL-12 and STAg result in abrogation of a Th2 response (Fig. 11 and [47]). Furthermore the processes involved in converting DCs to DCs priming type 2 responses (the third signal) seems to occur between 10 and 36 h after beginning of the culture. Moreover, the induction of Th2 polarization by SEA appears to be completed by 60 h.

3.2 Discussion – Dendritic cell modulation by SEA

Of all immune cells expressing the MHC class II molecule, dendritic cells are thought to be the most important antigen presenting cells to initiate a primary immune response of T helper cells. They not only present the processed antigen, but also provide the necessary co-stimulation of the naïve CD4⁺ cell through CD80 and CD86. In addition, a third signal in form of a cell-cell contact dependent or soluble factor influences the type of immune response that will result from the encounter of DCs and CD4⁺ cell specific for the presented antigen. For the initiation of a TH1 response, not only the up-regulation of co-stimulatory molecules, but also the secretion of pro-inflammatory signals like IL-12 is thought to be pivotal to instruct effector cells. For the induction of a Th2 response a similar scenario was not proposed. In contrary to DCs that are prone to favour the commitment of naïve CD4⁺ cells to the Th1 lineage, DCs that have encountered helminth-derived antigen do not display a full activated phenotype as measured by the up-regulation of CD80, CD86 and CD40 and secrete only low levels of IL-12 [86], [67],[33]. Microarray-based data demonstrated that Th1 stimulation upregulated multiple genes in DCs, while stimulation of DCs with the helminth *Brugia malayi* do not lead to significant gene upregulation suggesting minimal activation [32]. In this regard, *Nippostrongylus brasiliensis* excretory/secretory antigens primed mice for Th2 responsiveness, while selectively up-regulating CD86 and OX40 ligand together with IL-6 on murine BMDCs, and blocking the production of LPS [33]. In conclusion, immune modulation by helminthes seems to favor minimal activation or suppression of dendritic cell function. In accordance with these results, BMDCs stimulated with the schistosome egg antigen (SEA) in contrary to LPS-conditioned DCs do not respond with enhanced expression of CD80, CD86 or CD40. Neither did they produce IL-12p40 as measured in the supernatant by ELISA (Fig.

14). However, they were still able to induce a higher frequency of IL-4 producing OVA-specific TCR transgenic T cells in vitro than in cultures with the nominal peptide alone. It has been shown recently, that SEA stimulated splenic CD11c⁺ DCs also do not up-regulate co-stimulatory markers as observed with splenic DC cultured in the presence of the Th1 stimulus soluble *Toxoplasma gondii* antigen (STAg) [47]. Moreover, SEA could down-regulate the STAg-induced up-regulation of CD86 and CD40 and significantly inhibit the secretion of the two chemokines MIP-1alpha and MIP-1beta. Here, SEA could down modulate the LPS-induced expression of CD40 and IL-12p40, when BMDCs were cultured in the presence of both stimuli on the protein level (Fig. 14b). Furthermore, this down modulation of pro-inflammatory genes extends on a variety of molecules that are typically up-regulated by LPS as measured by Microarray technique and quantitative Real Time PCR (Fig. 15). These findings suggest that a down modulation by SEA observed on the protein level is not merely the effect of enzymatic digestion by proteases known to be present in SEA [87] as it has been shown for the proteolytically active house dust mite allergen Derp1. This cleaves CD40 on DCs and renders them less responsive to stimulation through the CD40L-CD40 pathway. Der p 1 pretreated dendritic cells produce significantly less IL-12 and induce the production of significantly less IFN-gamma but more IL-4 by CD4 T cells [88]. The role of co-stimulation of naïve CD4⁺ cells by DCs through CD80(B7-1)/CD86(B7-2) and signaling through CD40 ligation for an immune response against *Schistosoma mansoni* in vivo was addressed by Hernandez *et al.* [89] and MacDonald *et al.* [90], respectively. Firstly, CD4⁺ Th cells from B7-1/2 double-deficient mice displayed a dramatic loss of proliferative capacity upon stimulation with schistosomal egg Ag and secreted only IFN-gamma, but not IL-4 and IL-10, a pattern entirely opposite to that displayed by wild-type controls [89]. And secondly, even though studies from MacDonald *et al.* [90] could show that CD80, CD86 and CD40 are not up-regulated in the infection with *Schistosoma mansoni* to a degree observed in the acute infection with *Toxoplasma gondii*, the expression of CD40 is absolutely required for a Th2 response against schistosome antigens as inferred from CD154 (CD40 ligand) deficient mice. One possible explanation for these apparently conflicting observations regarding the costimulatory molecules requirement for induction of Th2 responses in vitro and in vivo could be that expression of CD80, CD86 and CD40 at the basal level is absolutely necessary but that an enhanced expression is not required. Moreover, increase in expression in costimulatory molecules may pre-

vent Th2 polarization, e.g. CD40 is a major stimulus for the induction of microbial dependent production of IL-12p70 by DCs [91]. It has been shown that bioactive IL-12p70 can inhibit the development of Th2 cells by SEA in vitro and schistosomal antigen in vivo (Fig. 11 and [81]). Interestingly, even though IL-12p40 production by DCs can be measured in some models for Th2 development, i.e. DCs exposed to secreted proteins from *Nippostrongylus brasiliensis*, the production of IL-12p70 by these DCs co-stimulated with LPS is inhibited [33]. The down regulatory function of DCs conditioned with helminth-derived products or other molecules known to induce a Th2 response is a widely observed phenomenon and is thought to be conducive for Th2 development. The filarial glycoprotein ES-62 from *Acantocheilonema viteae* acts to bias the immune response towards an anti-inflammatory/Th2 phenotype, while inhibiting IL-12 induction by TLR ligands such as bacterial lipoprotein and CpG [51]. Similarly, a recent report showed, as in this work, that SEA inhibits an array of signature genes of a pro-inflammatory response against LPS [52]. In this report, it was shown that SEA inhibits LPS-stimulated secretion of IL-12p40 and p70 by BMDCs. A similar down-regulation was observed with other TLR ligands such as CpG and PolyI:C suggesting that SEA does not exclusively interfere with the TLR4 pathway. Interestingly, SEA-conditioned BMDCs stimulated with LPS also secreted higher levels of IL-10 in the supernatant than LPS-stimulated BMDCs in the absence of SEA. This could not be confirmed in this work as measured by IL-10 message (Fig. 15b). A possible explanation could be the method of detection (Real-Time PCR vs. ELISA) and time point of data collection (6 h vs. 24h). The anti-inflammatory cytokine IL-10 was reported to be Th2 inducing in some instances [37] and able to down-regulate immune functions [92]. However, a suppression of DC function was detected even in the absence of IL-10 as inferred from IL-10-deficient BMDCs by quantitative Real-Time PCR (Fig. 2b) and Affymetrix Microarray analysis [52]. Interestingly, IL-10 deficient DCs were less responsive towards stimulation with LPS in terms of upregulation of the genes tested in Real-Time PCR in comparison with wild type DCs. A possible explanation for this contradictory result could be that IL-10 deficiency leads to an overactivated state of DCs, which results in a reduced fold induction of pro-inflammatory genes above the basal expression level in unstimulated cells. The question, if other molecules known to exert an anti-inflammatory effect on DCs such as TGF-beta, PGE2 [1] or other soluble factors as well as a direct effect of molecules contained in the water soluble schistosome egg extract could not be answered in this

work. The importance of a down regulation of DC function for a Th2 response is in line with the observation that TLR-activated DCs are not able to prime naïve T cells for Th2 development, as shown by Sun *et al.* [53]. There, DCs primed with *Propionibacterium acnes* induced maturation of DCs and failed to stimulate OVA-specific TCR transgenic CD4⁺ cells to become Th2 cells, which was observed in cultures with untreated DCs. The inhibitory effect was shown to be MyD88-dependent and supernatants of DC cultured with *P. acnes* could transfer the inhibitory effect on untreated DCs. Even though the supernatant contains an inhibitory activity, no single cytokine could be found to be sufficient for inhibiting Th2 development. Furthermore, the supernatant alone could only mediate a partial inhibitory activity, while *Propionibacterium acnes* treated DCs themselves exert an inhibitory activity to a greater extent suggesting a cell-contact dependent inhibition. Possible candidates could be Notch-ligands and their receptors. Previously found to be involved in early lymphopoiesis and T cell lineage commitment, the Notch pathway was recently shown to influence also the fate of effector T cells. In mammals there are four receptors (Notch-1-Notch-4) and five ligands: Jagged-1, Jagged-2, and Delta-1, Delta-3 and Delta-4 [58]. Notch signalling is initiated through ligand-receptor interactions, leading to proteolytic cleavage of the receptor, whose intracellular domain (NIC) translocates to the nucleus and can interact with its target genes. The cleavage is dependent on the gamma-secretase activity of presenilin, which is responsible for cleaving NIC on all forms of Notch. The report by Maekawa *et al.* [93] could show that the Notch-ligand Delta-1 suppresses Th2 development even in the absence of IL-12. As shown in Figure 3, Delta-1, Delta-4 and Jagged-1 are increasingly expressed by BMDCs stimulated with LPS or LPS and SEA, while SEA-conditioned BMDCs only marginally expressed these ligands. Interestingly, while BMDCs stimulated with SEA are able to augment the frequency of IL-4-producing CD4⁺ lymphocytes (Fig. 14c), the addition of LPS does suppress development Th2 cells (data not shown). The expression and signalling through Delta-1-Notch-3 furthermore promotes the development of IFN-gamma secreting CD4⁺ cells and is supposed to be important for Th1 development [93]. On the other hand, Jagged-2 is not only up-regulated in BMDCs treated with PGE2 and cholera toxin - both Th2 promoting stimuli - but also sufficient for IL-4 transcription [39]. As seen in Fig. 16, Jagged-2 is exclusively up-regulated by SEA and significantly increased over Jagged-2 expression in BMDCs treated with LPS or LPS/SEA. The increased or decreased expression of Jagged-2 on BMDCs treated

with Th2- or Th1-stimuli compared to untreated BMDCs therefore clearly correlates with their ability to induce Th2 or Th1 polarization, respectively. As discussed above, helminth-induced Th2 development can occur in the absence of the IL-4 signaling [75], [76]. A possible factor that can compensate for the lack of the IL-4 trigger early in T cell effector fate could be Jagged-2, since it was shown to be an instructive signal for Th2 differentiation independent on IL-4 or Stat6 [39]. Similarly, a more recent report underpinned the importance of Notch signalling in type 2 immunity. The expression of a dominant negative form of MAML, a protein required as a co-activator of NIC, prevents the transcriptional activation by all four forms of Notch. Here, the mutant mice failed to develop a protective Th2 cell response against the gastrointestinal helminth *Trichuris muris* and in the same instant exhibit a functional Th1 response against *Leishmania major* [94]. In order to understand, if Notch signalling is required for SEA-induced Th2 development in vivo, mice deficient in the gamma-secretase presenilin (Ps) were injected with SEA in the hind foot pad. Lymphocytes of the popliteal lymph nodes were restimulated with SEA and the cytokine profile of CD4+ cells determined by intracellular cytokine staining. The expression of IL-4 and IFN-gamma of Ps-deficient CD4+ cells is significantly lower than in CD4+ cells able to respond to Notch ligation. Since both cytokines are affected by the inability of Ps-deficient mice to cleave the intracellular domain of Notch receptors, it cannot be determined if Notch signalling is exclusively required for the induction of a Th2 response. A recent report stated the importance of Notch-signalling, especially via Notch-1, in the development of Th1 cells [95]. As seen in wild type animals (Fig. 17), the injection of SEA into the footpad also elicits the generation of Th1. Since Ps-deficient animals are generally deficient in Notch-signalling, the development of both Th cells seems to be effected in this model. To understand the specific requirement of Jagged-2 in the induction of a Th2 response against schistosomal egg antigens or its importance in other Th2 conditions should be investigated in mice lacking only the notch receptor Jagged-2.

3.3 Th2 inducing molecules in SEA

The Th2 inducing stage in the *Schistosoma* life cycle is the egg [24], and the water soluble extract off eggs (SEA) alone is sufficient to induce a Th2 response *in vitro* and *in vivo* [66], [18]. To date no egg component has been identified which is neces-

sary and/or sufficient to induce Th2 differentiation. The aim of the present study was to identify the molecule(s) in SEA able to promote bystander Th2 polarization of CD4⁺ cells by dendritic cells *in vitro*. In order to determine, if the IL-4 inducing activity in SEA requires an intact protein structure, SEA was digested with proteinase K and tested in an *in vitro* assay using OVA-specific TCR-transgenic DO11.10 CD4⁺ cells and splenic CD11c⁺ DCs in the presence of OVA peptide. Treatment of SEA with proteinase K resulted in complete digestion of proteins, as inferred from a coomassie blue stained SDS-PAGE (Figure 18a). Importantly, the same sample has lost completely the Th2 inducing activity, as measured by the frequency of IL-4 producing CD4⁺ cells (Figure 18b). The observation, that the mock-treatment resulted also in a slight reduction in IL-4 secreting cells, could be explained by the fact that SEA contains numerous proteases [87]. Thiol proteases and leucine aminopeptidases in the eggs were shown to originate from the glands of miracidia and are released at the time point of hatching. Being present in the homogenate of eggs they could account for the partial digestion of SEA incubated at 37°C even in the absence of exogenous proteinase K as seen in Figure 18a. The results shown suggest that the induction of IL-4 in native CD4⁺ cells *in vitro* by SEA-conditioned DCs is dependent on intact protein structures, and thus, imply that the IL-4 promoting molecule(s) contained in SEA is a protein. However, studies by Okano *et al.* [17, 96], demonstrated that carbohydrates on SEA are required for the observed adjuvant activity and Th2 inducing activity by SEA *in vivo*. The repeated intranasal sensitization with native SEA in mice led to IgE production and production of IL-4, IL-5 and IL-10 by restimulated nasal lymphocytes. The treatment of SEA with sodium metaperiodate resulted in abrogation of the observed effects arguing that intact carbohydrate structures are required for Th2 induction in this model. Sodium metaperiodate is used to open saccharide rings between vicinal diols leaving two aldehyde groups. This results in a modified form of carbohydrates while not altering the protein composition in SEA based on coomassie stained SDS-PAGE of SEA [17]. One explanation for the apparent discrepancies in the requirement of unmodified glycans or intact protein structures in SEA for Th2 induction might be that periodate treatment affects protein integrity indirectly, such as proper folding of the glycosylated protein. To characterize the active molecules present in SEA, a gel filtration of SEA was performed and the fractions were tested in the *in vitro* assay described above. As seen in Figure 19a, only fractions 7-13 lead to an increase in the frequency of IL-4 producing CD4 cells. When tested for their pro-

tein composition in gel electrophoresis and subsequent staining, the fractions 9, 11 and 13 revealed not only fewer bands than in SEA but also seemed to contain similar proteins based on their molecular weight. The elution of the activity in a single broad peak further supported the concept that the Th2 inducing activity is unlikely due to a carbohydrate component, often commonly shared by many proteins, but rather a specific protein(s). Preliminary attempts to identify the Th2 inducing proteins in these active fractions using N-terminal sequencing were not successful because they lead to identification of proteins without signal-peptide and that were not expected to be released from the egg, most likely due to contaminations with structural components present in egg extracts (data not shown). Although currently most of the studies with schistosome eggs employ different types of extracts containing mixtures of structural and secreted molecules, excreted/secreted antigens of the eggs are likely to be the first Ag encountered by the host immune-system and as such the crucial mediators of the immune modulation, as it has been shown for numeral other secreted products from helminthes [97]. The rationale that a Th2 inducing molecule needs to be secreted by the schistosome egg in order to encounter and modulate cells of the immune system in vivo lead to the following hypothesis: Excretory/secretory products of *S. mansoni* eggs (ES) are sufficient to induce Th2 polarization of CD4+ cells. In order to test this hypothesis, a protocol described by Ashton *et al.* [60] was adapted to isolate ES. As seen in Figure 20a, ES contains fewer protein bands than SEA. Nonetheless, a similar band pattern is observed in the Western blot using pooled serum of mice chronically infected with *S. mansoni*, suggesting that indeed these few proteins are the ones the host immune system responds to in terms of antibody production. More importantly, ES was able to induce a Th2 polarization similar to SEA (Figure 21). Moreover, the LPS-induced secretion of IL-12p40 by BMDCs could be suppressed by ES in a comparable manner to SEA (Figure 22). These results suggest that the same active molecule(s) present in SEA are contained in ES. In order to identify the proteins, N-terminal sequencing of three prominent bands in ES of 30, 25 and 10 kD was performed, and the resulting sequences were compared to EST data banks available from the NCBI and the Wellcome Trust Sanger Institute. The analysis revealed two known schistosomal proteins, omega-1 (30 kD) and alpha-1 (25 kD), and a protein of unknown function (Figure 23). Omega-1 and alpha-1 were originally identified as two major SEA molecules based on their positive charge and recognition by sera from mice with chronic infections [98]. Initially, it was shown that omega-1 is a

31 kD monomer from live *S. mansoni* eggs and a glycoprotein with an isoelectric point greater than 9 [99]. The full biochemical characterization of this molecule as a hepatotoxic ribonuclease is however very recent [61]. Furthermore, it has been shown to be restricted to the egg stage of the parasite, since no transcripts were found in cercariae, miracidia or adult worms [61]. The physiological role of omega-1 is not clear to date, but the present study suggests an as of yet unexpected role in modulation of the host immune response in that the native form is able to induce a Th2 response in a two-cell model involving DCs and naïve CD4⁺ cells. Native omega-1 was shown to induce a comparable increase in the frequency of IL-4⁺ cells at a 10 fold lower protein concentration as the total extract SEA, suggesting that omega-1 is a more potent inducer of Th2 responses than SEA. Omega-1 contains two potential N-glycosylation sites (residues 71 and 176) each within Asn-X-Ser/Thr sequons [61]. The expression of glycans on parasite derived molecules was shown to be involved in the modulation of the host response in a variety of models. Among them alpha3-fucose/beta2-xylose [100], LDN-DF [49] and Lacto-N-fucopentaose III (LNFPIII) [50] from schistosomes modulate cells of the immune system. In filarial nematodes intact glycans are required to induce a type 2 response *in vivo* [19]. However, non-glycosylated proteins from *Schistosoma mansoni* and *Dirofilaria immitis* show similar ability to skew the immune response [62], [101]. The schistosomal derived glycan LNFPIII induces a Th2 response in a two-cell model involving naïve T helper cells (DO11.10) and DCs in a TLR4 dependent mechanism [50], even though the dendritic cell-specific C-type lectin DC-SIGN was shown to recognize the Lewis X motif as well [102]. Interestingly, in the light of the recent report that some complex carbohydrates may be potent inducers of Th2 responses, cell surface molecules on APC that are in nature lectin receptors, able to bind a specific type of sugar, became subject of intensive investigation. Biological effects of carbohydrate antigens are dependent on recognition of these antigens by carbohydrate-binding proteins (lectins). Cell-surface C-type lectin receptors (CLRs), like DC-SIGN, L-SIGN and the mannose receptors that recognize particular glycan Ags of schistosomes and other parasites, may contribute to orchestrate Th2 responses, since it was shown that binding to CLRs can lead to down-regulation and modulation of DC-mediated immune responses favoring a Th2 response [103], [104]. The question, if the glycosylation of omega-1 is required for its ability to down-regulate IL-12 production or promote IL-4 induction, was not addressed in this work. The second protein identified in ES is al-

pha-1, variously known as IPSE [62], SmCKBP [105] or SmEP25 [106]. Alpha-1 was shown to be glycosylated, as expressing the Lewis X motif [107] and as having the ability to bind IL-8 and thus prevent neutrophil migration [105]. However, alpha-1 does not seem to require glycosylation for all of its biological functions, since alpha-1 recombinantly expressed in *E. coli* has been shown to display immunomodulatory activities in the absence of glycosylation: By binding to the Fc portion of IgE molecules, alpha-1 can trigger IL-4 release from basophils isolated from non-infected humans [62]. Alpha-1 also represents the dominant immune Ag for CD4⁺ cells during infection with *S. mansoni* [108], [106]. Even though alpha-1 was shown to trigger IL-4 release from basophils, it does not seem to be involved in DC-mediated Th2 induction of CD4⁺ cells, since in positive fractions of the ES fractionation only the 30 kD band of omega-1 could be detected [Figure 24b]. However, in an infection with *S. mansoni* different factors could synergize to generate a well orchestrated and amplified Th2 response against the parasite eggs. Certain antigens (such as omega-1) initially may bias the immune response towards a Th2 type by modifying the signals provided from DCs in the interaction with naïve CD4⁺ cells. Other schistosomal antigens (such as alpha-1) might serve as a target of an already biased immune response, due to their abundance among antigens or due to their facilitated uptake in APCs. Once an initial population of IL-4 from CD4⁺ cells is established, a further amplification of a Th2 response involving IgE mediated mechanism and other cell types (such as basophils) could be gained [109]. Omega-1 shares sequence homology with members of the RNase T2 family, as inferred from its two site consensus sequences CAS-1 and CAS-2, and its ribonuclease activity was experimentally confirmed by negative staining zymography [61]. Ribonucleases are divided into several families according to their sequence specificity and pH optimum: RNase T1, RNase A and RNase T2 [110]. The RNase T2 family consists of enzymes with a MW in the range of 24 to 36 kD and are either non-specific or adenylic acid preferential. RNase T2 family members are particularly widespread within animal and plant kingdoms, but their biological function is best studied in gametophytic self-incompatibility in plants [111]. Interestingly, different ribonucleases were shown to display various functions in immune responses against viruses and tumors. Amphibian onconase (RNase A family) and human RNase T2 (RNase T2 family) are endowed with an anti-tumor function and are both resistant to ribonuclease inhibitors, suggesting that other properties than the enzymatic activity are involved in their antitumor effect [112], [113]. Other

RNase A family members, the secretory effector proteins of eosinophils ribonucleases eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP), have ribonuclease activity-dependent antiviral effects [114], [115]. The question, whether the ribonuclease activity of omega-1 is required for the induction of IL-4 producing CD4⁺ cells or the inhibition of IL-12p40 production by CD11c⁺ DCs, has yet to be confirmed. Former studies in mice, however, suggest that there is no equivalent hepatotoxin or cross-reacting antigen in other schistosome species [116], [99]. Furthermore, the analysis of the homolog mRNA in *S. japonicum* revealed that it encodes a protein which would not contain the complete RNase T2 consensus sequences. If omega-1 would be sufficient for Th2 skewing in *S. mansoni* and other *Schistosoma* species, it would be likely that molecular properties other than its ribonuclease activity, were responsible for the observed immune-modulating activities [61]. In order to test formally if the nuclease activity is required for the Th2 skewing function of omega-1, modification of the active site of the enzyme by site-directed mutagenesis or deletion of the conserved sequences CAS-1 and CAS-2 should be performed. In addition, to understand the mechanism of action of omega-1 in DCs, it remains to be determined whether omega-1 is taken up specifically by binding to a certain receptor (and possibly signaling through it) or is nonspecifically endocytosed.

3.4 Discussion – Role of TLR in experimental infection with *S. mansoni*

The recognition of conserved molecular patterns of pathogens enables the immune system to select an evolutionary successful effector mechanism to eliminate the perpetrator or control its spreading within the host. Among pathogen recognition receptors (PRR) a group of molecules were studied extensively within the last decade, the toll-like receptors (TLR). This protein family is expressed on a variety of cell types, including dendritic cells, and can bind to distinct molecular features of mostly non-self ligands (Fig. 5). In a plethora of diseases associated with protective type 1 immune response the characterization of pathogen-associated molecular patterns (PAMP) fit to be recognized by TLR and subsequent up-regulation of pro-inflammatory genes on dendritic cells led to the hypothesis that a similar scenario will hold true for pathogens eliciting type 2 immune responses. In numerous cases, ligation of one of these receptors, TLR4, on DC results in upregulation of costimulatory markers and IL-12 secretion leading to an induction of Th1 responses. However, there are also instances

in which the activation through TLR4 is necessary for a type 2 immune response. For example, the presence of TLR4 is necessary for the development of an OVA-specific Th2 response in a mouse model of allergic sensitization with low dose LPS [23] and in the absence of LPS [117]. In addition, there are reports where helminth-derived molecules drive a Th2 response *in vitro* dependent on TLR4 specifically. The human milk sugar lacto-N-fucopentaose III (LNFP III) containing the Lewis X sugar moiety found in SEA was shown to induce the production of IL-4 by CD4⁺ cells when cultured with DC. Dendritic cells deficient in TLR4 mediated signalling could not mediate such an effect. In order to test, if this glycan is responsible for the observed Th2 response *in vivo*, mice were infected with *Schistosoma mansoni* and immunological parameters analyzed. As seen in Fig. 28, a similar proportion of mesenteric CD4⁺ lymphocytes do produce IL-4 as compared to the wild-type control (C57BL/6). Similarly, other Th2 cytokines such as IL-5, IL-10 and IL-13 are produced by lymphocytes restimulated with SEA, even to a higher degree as from TLR4 competent cells. The ability of TLR4 knock out mice to display all pathological and immunological features present in the wild type mice argues against a non-redundant role of LNFP III in Th2 polarization. As described by Schramm *et al.* [62] a protein isolated from SEA induces the release of pre-stored IL-4 from basophils in an IgE-dependent manner. The Interleukin-4 inducing factor from schistosome eggs (IPSE/alpha-1) was shown to trigger basophils from naive human donors to degranulate, release mediators, and express IL-4 and IL-13 via cross-linking of FcRI-bound IgE. Interestingly, it was later found to contain the Lex motif in glycans N-linked to IPSE. However, since unglycosylated recombinant IPSE exerts the same effect, the mechanism, by which it induces the degranulation of human basophils seems to rely on the protein structure solely [107]. The finding that FcRI-deficient mice mount normal Th2 responses [118], when they are infected with *Schistosoma mansoni* suggest that also the Th2 inducing activity of IPSE can be compensated by other Th2 promoting factors in the absence of IgE signalling. An additional Toll like receptor that was reported to be involved in the development of IL-10 and IL-4 producing T cells is TLR2. The TLR2 ligand Pam3Cys was reported earlier to elicit low IL-12(p70), but abundant IL-10, and favour a Th2 response [119]. A recent finding suggested that the yeast cell wall component zymosan binds to TLR2 in conjunction with dectin-1 and induces DCs to secrete abundant IL-10 but little IL-6 and IL-12(p70) [120]. Moreover, the schistosomal lipid lyso-phosphatidyl serine (PS) is signalling through TLR2 and supposed to be critical

for the induction of IL-10 producing T cells, when human DC and T cells are cultured in the presence of PS [49]. In order to understand, if TLR2 is involved in the development of a Th2 response against schistosomal egg antigens or the subsequent down-regulation observed in the chronic phase of the infection TLR2-deficient mice were tested in parallel to TLR4- and MyD88-deficient animals. As it was shown mice lacking TLR2 are able to mount a similar degree of a Th2 response as the wild type control animals based on FACS analysis of intracellular staining of mesenteric CD4⁺ lymphocytes (Fig. 28a), production of IL-4, IL-5, IL-10 and IL-12 (Fig. 30) and antibody production (Fig.29). Moreover, the absence of TLR2 does not interfere with a down-regulation of this response as measured by the cytokine response and granuloma volume at the chronic phase of the infection with *S. mansoni*. A TLR2 mediated emergence of IL-10 producing T cells was not shown for the murine system and a biological relevance for IL-10 production by human T cells cultured with PS-conditioned DC cannot be inferred directly from the data gained from TLR2-deficient mice. However, it can be argued that any TLR2-dependent effect of the schistosomal lipid PS on IL-10 synthesis was not detected *in vivo*, because putative deficiency might have been compensated by the induction of IL-10 producing Th2 cells triggered by other factors present in SEA (e.g. water soluble components). As it was shown before, MyD88 is not required for the development of a Th2 response triggered by SEA, when injected multiple times intra peritoneally, whereas it was indispensable for an IFN-gamma driven response after STAg administration with the same protocol of immunization or after i.p. infection with *Toxoplasma gondii* [18], [63]. Similarly, a characteristic response against *Schistosoma mansoni* was measured in the absence of MyD88 in the acute and chronic infection as measured by antibody titer and cytokine levels (Fig. 28a, 29a and 30). There are only few *in vivo* helminth-infection studies in TLR-deficient mice. A recent report by Layland *et al.* [121] studied as well the role of MyD88 in the infection with *Schistosoma mansoni*. Their findings support the notion that MyD88 is not required for the development of a Th2 response as measured by the secretion of IL-4 and IL-10 from restimulated total splenocytes from infected mice as well as IL-13 production in the liver. Another study addresses the role of TLR4 and MyD88 in the immune response against *Trichuris muris*, a gastrointestinal nematode, whose expulsion is mediated by a Th2 response involving IL-4 and IL-13. Here, the Th1 response-associated susceptibility in mice is dependent on activation signals mediated by MyD88 and TLR4, since TLR4- and MyD88-

deficient mice are highly resistant to chronic *T. muris* infection and develop strong antigen-specific Th2 responses in mucosa-associated lymphoid tissues [122]. Activation signals through MyD88 are reported to be not only conducive for the development of a protective type 1 immune response, but are also thought to counter regulate Th2 responses [53], [123]. Signal transduction through TLR originates from their intracellular Toll/interleukin-1 receptor (TIR) domain, which binds to MyD88, the common adaptor protein containing a TIR domain, thought to be obligatory for the signalling of most Toll like receptors (TLR1, TLR2, TLR5, TLR6, TLR7 and TLR9), while for TLR4 and TLR3 also MyD88-independent signalling pathways have been described [124], [125], [46]. Since mice deficient in TLR4 and MyD88 were shown to develop a full Th2 response against *S. mansoni* it is not expected to find other MyD88-dependent TLR being involved in the immune response against *S. mansoni*. However, the role of TLR3 as being able to signal via TRIF (Fig. 5) was addressed in a further experiment. As shown in Figure 31, TLR3-deficient animals are able to mount a type 2 response in the acute infection with *S. mansoni* as shown by their cytokine production. Recent studies suggested a TLR3-dependent induction of IFN-responsive genes in dendritic cells by schistosomal double stranded RNA. The report from Trottein *et al.* [126] showed that live eggs from *S. mansoni* induce the up-regulation of pro-inflammatory genes such as IL-12p40, TNFalpha, MIP-1beta and IFN-beta in myeloid DCs in vitro. This finding stands in contrast to the results observed with SEA which is not able to induce IL-12p40 or MIP-1beta in DCs [47], [52]. Moreover, the production of IL-12p40 by live eggs was partially mediated by MyD88, while secretion of IL-12p40 by dsRNA purified from eggs was abolished in TLR3-deficient DCs in vitro [48]. Since neither MyD88- and TLR3-deficient nor IL-12-depleted mice have an impaired Th2 response (Fig. 28, Fig. 31 and [127]) the putative production of pro-inflammatory genes by live eggs and dsRNA from *S. mansoni* in vivo does not seem to be relevant for Th2 differentiation in the experimental infection with *S. mansoni*. Hallmarks of chronic schistosomiasis are down-regulation of the Th2 response and the size of granulomatous lesion on the one hand and increased liver collagen deposition on the other hand [11]. As it was shown, the down-regulation of SEA-specific cytokine production by CD4+ cells of the MLN (Fig. 28 and Fig. 30) was drastically reduced or undetectable in the chronic phase of the infection independent of TLR2, TLR4 and MyD88. As expected the reduced ability of CD4+ cells to secrete cytokines in the chronic phase of infection correlated with a lower granulomatous re-

response around newly deposited eggs (Fig. 32). The granuloma volumes are significantly reduced at 16 weeks p.i. towards 8 weeks p.i. (p-value: 0.01) in all groups. The mechanism for a down-regulation was attributed to B cell dependent signalling [12] and IgE/FcεRI interaction [118] since infected mice deficient in FcγRI and FcεRI-deficient mice showed enhanced egg granuloma formation. Neither of these two pathways appears to be affected in MyD88-, TLR2- and TLR4-deficient infected mice, because all three groups show high titers of SEA-specific IgG1 and total IgE antibodies in the chronic phase comparable to the levels in infected wild type animals (Fig. 29). The lower proportion of infiltrated eosinophils seems to follow a lower concentration of IL-5 in chronic schistosomiasis (Fig. 30 and Fig. 32). This cytokine was shown to be necessary for the emergence of eosinophils in blood and granulomas [5]. Since IL-5 is down-regulated in the absence of TLR2, TLR4 and MyD88, a subsequent lower infiltration of eosinophils in the granulomas is expected. Taken together, the TLR-pathways investigated do not seem to be involved in the natural resistance against *S. mansoni* and are not required for the induction or subsequent down regulation of a Th2 response. The question remains if other receptors than members of the TLR family might be critically involved in a Th2 response against *S. mansoni* or helminths in general such as receptors recognizing glycans [21]. A unique difference between the TLRs under investigation is the fibrotic tissue response in the chronic stage of the infection. Only MyD88-deficient animals displayed an aberrant response in comparison with the wild type mice in that the concentration of hydroxyproline/μmol per 104 eggs as an indicator for collagen [65] content was significantly lower (Fig. 33). This effect is surprising since the collagen deposition surrounding the eggs is assumed to be driven by Th2 cytokines like IL-4 and IL-13 [128], [129] and MyD88 is not involved in the upregulation of these cytokines as discussed before. Moreover, a recent study suggested that MyD88-deficient animals display smaller granuloma that are less cellular and contain more collagen than wild type animals in the acute infection with *S. mansoni* (9 weeks p.i.). This was not observed in the present study since both, wild type and knock out mice, showed similar granuloma volumes in the acute infection (8 weeks p.i.). A possible explanation for this discrepancy could be the higher infection rate (100 cercariae) resulting in detectable levels of IFN-γ which is abrogated in MyD88-deficient animals. Apart from TLRs, also IL-1 and IL-18 signal through MyD88 [130]. Since IL-1 was shown to be both, involved in Th2 responses [131] and fibrogenesis [132] it could be

the factor mediating fibrogenesis in chronic infection when levels of IL-13 are decreased.

4 Methods

4.1 Methods in cell culture

4.1.1 Isolation of CD4+ cells

Naïve OVA specific TCR-transgenic CD4+ lymphocytes were obtained from lysed spleen cell suspensions from female DO.11.10 mice (Rag-/- background). Total splenocytes were stained with CyChrome anti-mouse CD4 (1:1000), FITC anti-mouse CD11c (1:100), and FITC anti-mouse DX5 (1:100) mAb and single positive CD4+ cells were positively sorted (>99% purity) on a FACStar flow cytometer.

4.1.2 Isolation of CD11c+ splenic dendritic cells

Spleen cell suspensions of female BALB/c mice were prepared after digestion in 0.2 mg/ml Liberase for 30 min. at 37°C in 5 % CO₂ in a humidified incubator and lysis of erythrocytes by osmotic treatment with ACK-lysing buffer. Total splenocytes were stained with PE anti-mouse CD11c (1:200) and FITC anti-mouse B220 mAb (1:100) and sorted as single positive CD11c+ cells. The purity of CD11c+ DCs was >98%. To obtain CD8-/CD11c+ and CD8+/CD11c+ DCs total splenocytes were additionally stained with APC-anti-mouse CD8 (1:300) before sorting. All sorting procedures were performed on a FACStar flow cytometer.

4.1.3 Generation of bone marrow derived dendritic cells (BMDCs)

This protocol was adapted from Lutz *et al.* [133]. Bone marrow cells were harvested from femurs of female BALB/c mice and washed with PBS. The cells were centrifuged and the pellet resuspended in 1 ml of ACK lysing buffer for 2 min. at RT. Cells were washed and resuspended at 2×10^5 cells/ml in complete RPMI 1640 containing 15 % cell culture supernatant from CHO cells transfected with the plasmid pCDNA3 containing the recombinant mouse GM-CSF gene (kind gift of R. Goldszmid, [134] and incubated in 100-mm diameter plates as 10 ml cultures at 37°C in 5 % CO₂ in a humidified incubator. After 72 h the cells were refed with fresh complete RPMI 1640. After 5 days of culture immature DCs were harvested and routinely analyzed by phenotypic FACS-analysis and used in the assays as described.

4.1.4 Culture condition for in vitro Th differentiation

CD4⁺ lymphocytes (1×10^5 /ml) were incubated with DCs (5×10^4 /ml) in complete RPMI 1640 in the presence of 1 μ M OVA 323–339 in 0.2 ml vol. in round-bottom 96-well plates in 5 % CO₂ in a humidified incubator if not stated otherwise. When indicated, IL-4 [10 ng/ml], STAg [5 μ g/ml], SEA [40 μ g/ml], LPS [50 ng/ml] was added to the wells. After 72 h of culture, supernatants were replaced with fresh complete RPMI 1640 containing 10 U/ml IL-2, and after an additional 2–3 days, intracellular cytokine staining was performed as described below.

4.1.5 Culture condition for expression of mRNA, cytokines and surface molecules of BMDCs

For the expression of surface markers and cytokine production, BMDCs (2×10^6 cells/ml) were cultured in 48 well plates in 1 ml in the presence or absence of LPS [50 ng/ml] and/or SEA [40 μ g/ml] for 6 h (for mRNA) or 24 h (for protein).

4.1.6 Culture conditions for ex vivo cytokine measurement

Mesenteric lymph node (LN) or popliteal LN (3×10^6 /ml) cells from infected or foot-pad-injected animals were cultured in 24-well plates in 1 ml/well at 37°C in 5 % CO₂ in a humidified incubator. After 72 h, supernatants were collected for cytokine determinations and cultured for additional 24 h in fresh complete RPMI 1640 before intracellular cytokine staining.

4.2 Immunological methods

4.2.1 Cell surface staining for fluorocytometric analysis

BMDCs were incubated with anti-mouse CD16/32 mAb (1:200) and then stained with PE anti-mouse CD11c (1:200) and FITC anti-mouse MHC class II (1:500), FITC anti-mouse CD40 (1:500), FITC anti-mouse CD80 (1:500), or FITC anti-mouse CD86 mAb (1:500) for 15 min. at 4°C. Fluorocytometric analysis was performed using a

FACScan flow cytometer and data was collected using CellQuest software (BD Biosciences) and analyzed using FlowJo software (TRiStar).

4.2.2 Intracellular cytokine staining of lymphocytes

Cells were stimulated with PMA [10 ng/ml] and Ionomycin [1 µg/ml] for 4.5 h with addition of Brefeldin A [10 µg/ml] during the last 2 h. Cells were washed once in minimal RPMI 1640 and stained with Cy-chrome anti-mouse CD4 mAb (1:1000) in the presence of purified anti-mouse CD16/32 mAb (1:200) and fixed for 15 min. in 2 % paraformaldehyde at RT. Fixed cells were then stored at 4°C in PBS with 10 % heat-inactivated FCS for up to 48 h or processed for permeabilization. For permeabilization, cells were incubated for 30 min. in permeabilization buffer at 4°C, afterwards cells were stained for 30 min. with PE anti-mouse IL-4 (1:100) and FITC (1:250) anti-mouse IFN-gamma at 4°C, washed twice with permeabilization buffer, and resuspended in PBS with 0.5 % heat-inactivated FCS. Cell fluorescence was measured using a FACScan flow cytometer. Data was collected using CellQuest software (BD Biosciences) and analyzed using FlowJo software (TRiStar) by gating on the CD4+ population and their cytokine-positive subpopulations.

4.2.3 Enzyme-linked immuno-sorbent assay (ELISA)

To measure the concentration of cytokines in supernatants of cell cultures a standard enzyme-linked immuno-sorbent assay was performed using Nunc Immunolon-2 plates for IL-4, IL-5, IL-12p40, IL-13 and IFN- γ and Immunolon-4 plates for IL-10. The coating Ab were added as 100 µl/well in coating buffer (IFN-gamma) or PBS (all others) at 4°C ON, the supernatants were added as 100µl/well in dilution buffer at 4°C over night, the detection Ab and horseradish peroxidase coupled streptavidin (1:1000) were added in 100 µl/well in dilution buffer at 37°C for 2 h. The ABTS substrate solution was added in 100 µl and incubated for 30 min. protected from light, the reaction was stopped by addition of 10 % SDS [10 µl/well]. All washing steps were performed in washing buffer. The standards (recombinant mouse) were diluted in dilution buffer and blanks were included in each experiment. Absorbance was measured at 405 nm.

The following antibodies were used in ELISA:

IL-4:

To coat: purified rat anti-mouse IL-4 (BVD4-1D11), [1µg/ml]

To detect: biotin rat anti-mouse IL-4 (BVD6-24G2), [0.5µg/ml]

IL-5:

To coat: TRFK-5, [5µg/ml]

To detect: biotin TRFK-4, [1:1000]

IL-10:

To coat: purified rat anti-mouse IL-10 (JES5-2A5), [5µg/ml]

To detect: biotin anti-mouse IL-10 (SXC-1), [1 µg/ml]

IL-13:

To coat: rat anti-mouse IL-13 clone # 38213 [5µg/ml]

To detect: biotin polyclonal goat anti-mouse IL-13, [5µg/ml]

IL-12 (p40):

To coat: anti-mouse IL-12 (C17.15), [5µg/ml]

To detect: biotin anti- mouse IL-12 (C6.7.6), [1:1000]

IFN-gamma:

To coat: purified rat anti-mouse IFN-gamma (R4-6A2), [2µg/ml]

To detect: biotin rat anti-mouse IFN-gamma (XMG1.2), [1µg/ml]

4.2.4 Western Blot

4.2.4.1 Transfer of proteins onto nitrocellulose membranes

Proteins separated by SDS-Page were washed for 10 min. in 20 % methanol and immobilized onto nitrocellulose membranes by electrophoretic transfer [135]. A transfer cassette was assembled in transfer buffer using a sponge followed progressively

by a 3 MM Whatman paper, nitrocellulose membrane, SDS-Page gel, and another Whatman paper and finally a second sponge. The cassette was introduced into a Blot module (Semidry Blot) between electrodes so that proteins are transferred towards the nitrocellulose membrane at the positively charged anode. The outer chamber was filled with H₂O and the inner chamber with transfer buffer. The transfer was performed at 20 V for 1.5 h at RT.

4.2.4.2 Immunodetection of immobilized proteins.

Nitrocellulose membranes with immobilized proteins were incubated with 5 % blocking solution for 1 h at RT and rinsed with washing buffer. The membrane was incubated over night at 4°C with polyclonal serum (1:3000) from mice chronically infected with *S. mansoni*. The membrane was washed three times extensively with washing buffer and incubated with the secondary conjugate antibody (HRP-coupled goat anti-mouse IgG, 1:3000) for 1 h at RT followed by washes as above. Detection was done by using ECL Western Blotting substrate according to manufacturer's recommendation. The Signal was detected using an autoradiographic film exposed 10 sec. to the ECL soaked membrane and subsequently developed.

4.3 Molecular biology techniques and biochemical methods

4.3.1 Proteinase-K treatment of SEA

For enzymatic hydrolysis of SEA proteinase K coupled to acrylic beads were used according to the manufacturer's instruction. The amount of 1g of proteinase K beads was allowed to rehydrate at 4°C for 2 h in 10 ml sterile H₂O. Subsequently, the beads were washed three times by brief centrifugation in PBS for removal of stabilizers. The beads were resuspended in 5 ml of PBS and the solution was added in a ratio of 1:2 to the parasite extract. The enzymatic reaction was performed at 37°C for 30 min. As a mock treatment, SEA was diluted 1:2 in PBS and incubated together with the reaction. After that, the suspension was briefly centrifuged and the supernatant filtered with 0.2 µM sterile filter units to remove residual beads and applied as such.

4.3.2 Isolation and Purification of total RNA from mouse cells

BMDCs or T cell cultures were lysed in RLT-Buffer containing 1 % beta-mercaptoethanol and passed over a Qias shredder column following the manufacturer's instructions. Total RNA was then extracted by using the RNeasy mini Kit for isolation of RNA from animal cells according to the manufacturer's instructions. Total RNA was eluted from the column and the concentration of total RNA was determined by measuring the absorbance at 260 nm (A₂₆₀) using a spectrophotometer. Only samples with an A₂₆₀/A₂₈₀ ratio of 1.9 - 2.1 were used for further experiments. Samples were adjusted at a concentration of 0.1 µg/µl (RT-PCR) or 5 µg/µl (microarray) in RNase free water and used immediately or stored at -80°C until further usage.

4.3.3 Gene profiling by Microarray analysis

Fluorescent labeled cDNA of two samples to be compared were co-hybridized on mouse standard microarrays (RTB-NIAID-NIH) based on long oligonucleotide sets produced by Qiagen. The sets represent predicted genes based on RefSeq and UniGene cluster assignments. Hybridization was performed using the Microarray Hybridization Service of the RTB-NIAID-NIH. The two-dye microarrays were scanned using an Axon GenePix 4000B fluorescence scanner and the signal was calculated as mean intensity–median background, normalized using the 50th percentile (median) and expressed as the ratio of Cy5/Cy3. The data extraction was performed using the mAdbe software and ratios are shown as log₂ values, where positive values indicate a relative over-expression and negative values indicate a relative under-expression of the experimental sample compared to the control sample. For fluorescent labelling of RNA, oligo dT primer were added to individual RNA samples and the mixture was heated at 70°C for 5 min. and subsequently chilled on ice:

Tab. 1:

Reagent	µl
RNA sample (30 µg for Cy3, 40 µg for Cy5)	13.25
Oligo dT primer	1.0
DEPC water	2.0
Total	16.25

To the RNA sample was then added:

Tab. 2:

Reagent	µl
RT buffer, 5x	6
DTT (0.1M)	3
dATP (100 mM)	0.15
dCTP (100 mM)	0.15
dGTP (100 mM)	0.15
dTTP (100 mM)	0.15
dUTP-Cy3 or dUTP Cy5	2
SuperScript II RT	1.5
RNAse out	0.25
DEPC-water	0.52
Total	13.75

The RT reaction was carried out at 42°C for 90 min. and subsequently stopped by addition of 10 µl of 1M NaOH and heating at 70°C for 15 min. The sample was neutralized with 10 µl of 1 M HCl. In order to remove excess dye the samples were washed twice using TE-buffer and VIVAspin concentrator tubes (500 µl) at 12.000 g for 8 min. Sample volume were readjusted to 30 µl and combined accordingly:

Sample 1: Cy5-BMDC-SEA + Cy3-BMDC-control

Sample 2: Cy5-BMDC-LPS + Cy3-BMDC-control

Sample 3: Cy5-BMDC-SEA/LPS + Cy3-BMDC-LPS

To 19 µl of combined labeled sampled was added 1 µl poly (A) [1 µg/µl], 1 µl Cot-1 DNA [10 µg/µl] and 1 µl yeast t-RNA [4 µg/µl] to reduce non-specific hybridization.

4.3.4 Reverse Transcription of RNA into cDNA

Individual sample RNA (1 µg) was reverse transcribed into cDNA using Superscript II Reverse Transcriptase. Per sample the master mix contained:

Tab. 3:

Reagent	µl
dNTP (10 mM)	0.625
RT buffer (first strand)	5.0
DTT (0.1 M)	2.0
RNAse OUT	0.25
Random Hexamer	2.0
DEPC water	4.625
SuperScript II RT	0.5
Total	15.0

10 μ l of each RNA sample [0.1 μ g/ 10 μ l] was heated for 5 min. at 70°C and quickly chilled on ice before the master mix was added to the sample. The reverse transcription was carried out for 60 min. at 37°C. After an additional step of 5 min. at 90°C the sample was diluted by addition of 175 μ l DEPC water. cDNA samples were stored at -20°C.

4.3.5 Quantitative Real-Time PCR

Real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Relative quantities of mRNA for several genes was determined using SYBR Green PCR Master Mix and by the comparative threshold cycle method as described by Applied Biosystems for the ABI Prism 7900HT Sequence Detection Systems. In this method, mRNA levels for each sample were normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) or beta2-microglobulin (b2m) mRNA levels and then expressed as a relative increase or decrease compared with the level of expression in control cells: untreated BMDCs or unstimulated CD4+ cells.

Tab. 4:

Reagent	μ l
SYBR Green PCR Master Mix, 2x	2.5
forward primer [5 μ M]	2.5
reverse primer [5 μ M]	2.5
cDNA	5.0
DEPC water	2.5
Total	25.0

RT-PCR was performed in 96-well plates for 40 cycles including blanks and water controls. Each sample was assayed in triplicate. For a given cDNA, relative abundance of each target was normalized to the control gene (HPRT/beta2-microglobulin according to the formula: $2^{-\Delta\Delta CT}$, where $\Delta CT = C_T^{\text{target gene}} - C_T^{\text{control gene}}$. Normalized

target signals in the sample were expressed relative to the expression in control cells (untreated BMDCs or CD4+ cells) according to the formula: $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T^{\text{sample}} - \Delta C_T^{\text{control cell}}$.

4.3.6 Isolation of ES (*Schistosoma mansoni* egg excretory/secretory products)

Freshly isolated *Schistosoma mansoni* eggs from trypsinized livers from chronically infected mice (Fred Lewis, BRI) were washed three times in RPMI 1640 with 300 U/ml penicillin, 300 µg/ml streptomycin, 500 µg/ml gentamicin and 200 mM glutamine. To obtain *Schistosoma mansoni* egg conditioned medium, 2×10^5 eggs/ml were incubated for 3 days in 30ml tissue culture flasks at 37°C in 5 % CO₂ in a humidified incubator. The suspension was centrifuged at 100 g for 5 min. and the supernatant concentrated 10-30 fold using centrifugal filter units with a 10 kD cut-off (Centriplus). Protein concentration was tested regularly using the Bradford procedure.

4.3.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Analytical polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as described by Laemmli [136]. Samples were heated for 5 min. at 100°C in NuPAGE LDS sample buffer according manufacturer's instructions. Samples were applied at volumes of 30 µl to a 12 % Bis-Tris Minigel. The electrophoresis was performed at 20 mA with running buffer MES including 0.25 % NuPAGE Antioxidant. The run was stopped when the bromophenol dye front reached the lower end of the gel. Protein bands were stained with coomassie Simply Blue and prepared for permanent record.

4.3.8 Determination of protein concentration

Protein concentration was determined by the coomassie (Bradford) Protein Assay Kit (Pierce, Rockford, USA). A linear standard curve was made with 0.075 to 1 mg/ml BSA. Samples were measured in quadruplets at 595 nm.

4.3.9 Gel filtration chromatography of SEA and ES

For gel filtration chromatography 1 ml of starting material was run over an equilibrated Superdex 75 column (size 10/300) in 20mM Tris HCl with 150 mM NaCl (pH 7.4) at 0.5 ml/min and a fraction size of 0.5 ml. Each fraction was combined with the same volume of complete RPMI 1640 and sterilized using Ultrafree-MC centrifugal filter (pore size 0.22 μ m).

4.4 Methods in Parasitology and Histopathology

4.4.1 Experimental infection with *Schistosoma mansoni*

Cercariae from *S. mansoni*- Puerto Rican strain (provided by BRI, Rockville, MD) were counted and diluted to 35 cercariae per 5 ml tube filled with water (snail-water provided by BRI, Rockville, MD). Mice were experimentally infected with 35 cercariae by bathing the tail of the mouse for 30 min. in tubes with cercariae. Infected mice were maintained at an American Association of Laboratory Animal Care accredited facility at the National Institute of Allergy and Infectious Diseases (Bethesda, MD).

4.4.2 Assessment of histopathological data

The sizes of pulmonary and hepatic granulomas were determined on histological sections that were stained with Wright's Giemsa stain (provided by Histopath of America, Millersville, MD). Around 30 granulomas per mouse were included in all analyses. A skilled pathologist evaluated the percentages of eosinophils, mast cells, and other types of cells in the same sections. The number of schistosome eggs in the liver and the gut and the collagen content of the liver, as measured by hydroxyproline levels,

were determined as previously described [128]. Hepatic collagen was measured as hydroxyproline by the technique of Bergman and Loxley [65] after hydrolysis of a 200 mg portion of liver in 5 ml of 6 N HCl at 110°C for 18 hours. The increase in hepatic hydroxyproline was positively related to egg numbers in all experiments, and hepatic collagen was reported as the increase above normal liver collagen in μmol per 10,000 eggs ($[\text{infected liver collagen} - \text{normal liver collagen}]/\text{liver eggs} \times 10^{-4}$). The same individual scored all histological features and had no knowledge of the experimental design.

4.4.3 Footpad injection of parasite antigen in mice

Presenilin-deficient and wild type mice were injected with 50 μl SEA [1.8 mg/ml] in both hind footpads and popliteal lymph nodes harvested at day 8. Popliteal lymph nodes of single mice were pooled and single cell suspensions were prepared and tested for cytokines *ex vivo*.

4.5 Statistical analysis

Statistical analysis was performed using the Mann-Whitney U-Test.

5 Materials

5.1 Animals and parasites

Female BALB/c DO.11.10 TCR Tg animals on the RAG-2^{-/-} background BALB/c and C57BL/6 mice were provided by the National Institute of Allergy and Infectious Diseases Animal Supply Contract at Taconic Farms (Germantown, NY). MyD88-, TLR2-, TLR3- and TLR4-deficient mice were backcrossed for four to five generations onto the C57BL/6 background and were maintained at an American Association of Laboratory Animal Care accredited facility at the National Institute of Allergy and Infectious Diseases (Bethesda, MD). Presenilin-deficient mice were kindly provided by K. Laky (NIAID-NIH). Mice 8- to 12-wk-old were used in all experiments. Cercariae and purified eggs from *Schistosoma mansoni* (Puerto Rican strain) was provided by the BRI, Rockville, MD.

5.2 Laboratory equipment

Electrophoresis apparatus, Blot module (Semidry blot) (all Invitrogen, Carlsbad, CA)
PCR machine (MJ Research, Waltham, MA)
Flowcytometer (analysis), FACScan, Flowcytometer (sorting) FACStar, (all BD Biosystems, San Jose, CA)
ABI Prism 7900HT (Applied Biosystems, Foster City, CA)
Axon GenePix 4000B (Molecular Devices, Union City, CA)
ELISA scan washer 300 (Skatron, Lier, Norway)
EISA V-max kinetic microplate reader (Molecular Devices, Union City, CA)
Superdex-75 column (Amersham Bioscience, Piscataway, NJ)

5.3 Plastic ware and consumables

Cell culture plates, tubes and flasks (Corning Inc., Acton, MA)
ELISA plates (Nunc, Rochester, NY)
96-well RT-PCR reaction plates 0.1ml (Applied Biosystems, Foster City, CA)
Nitro cellulose membrane, Bis-Tris Minigel 12% (all Invitrogen, Carlsbad, CA)
Whatman paper (3MM) (Whatman, Florham Park, NJ)

Qiashredder (Qiagen, Valencia, CA)

Centrifugal filter units (Millipore, Billerica, MA)

Vivaspin concentrator 500 µl (Sartorius, Edgewood, NY)

Autoradiographic film (CL-XPosure) (Pierce, Rockford, IL)

5.4 Commercial kits

RNeasy Mini Kit (Qiagen, Valencia, CA)

Protein Assay Kit (Pierce, Rockford, IL)

5.5 Antibodies and cytokines

antibody pairs for ELISA:

rat anti-mouse IL-4 (BVD4-1D11), biotin-rat anti-mouse IL-4 (BVD6-24G2), rat anti-mouse IFN-gamma (R4-6A2), biotin-rat anti-mouse IFN-gamma (XMG1.2), rat anti-mouse IL-10 (JES5-2A5), biotin anti-mouse IL-10 (SXC-1) (all BD pharmingen, San Jose, CA)

rat anti-mouse IL-13 clone # 38213, biotin-polyclonal goat anti-mouse IL-13 (all R&D systems, Minneapolis, MN)

anti-IL-5 (TRFK-5), biotin anti-IL-5 (TRFK-4), anti-IL-12p40 (C17.15), biotin-anti-IL-12p40 (C6.7.6), (all home made, LPD-NIAID-NIH)

cytokines for ELISA standards:

recombinant mouse IL-4, recombinant mouse IL-5, recombinant mouse IL-10, recombinant mouse IL-13, recombinant mouse IL-12, recombinant mouse IFN-gamma, (all BD pharmingen, San Jose, CA)

antibodies for FACS:

PE-Cy5 rat anti-mouse CD4, FITC hamster anti-mouse CD11c, PE hamster anti-mouse CD11c, FITC rat anti-mouse CD49b/Pan-NK cells (DX5), APC rat anti-mouse CD8alpha, FITC rat anti-mouse CD45R/B220 (B220), rat anti-mouse CD16/32, FITC rat anti-mouse I-A/I-E (MHC class II), FITC rat anti-mouse CD40, FITC hamster anti-

mouse CD80, FITC rat anti-mouse CD86, PE rat anti-mouse IL-4, FITC rat anti-mouse IFN-gamma (all BD pharmingen, SDan Jose, CA)

for IL-4 neutralization:

Isotype control (rat IgG2b NA/LE), rat anti-mouse IL-4 NA/LE (BVD4-1D11)

(all BD pharmingen, San Jose, CA)

recombinant human IL-2, Cetus Oncology Corp., Emeryville, CA

5.6 Biologicals and chemicals

STAg from *T. gondii* tachyzoites (RH) (home made, LPD-NIAID-NIH)

SEA from *S. mansoni* eggs (BRI, Rockville, MD)

LPS E. coli K12 ultra-pure (Invivogen, San Diego, CA)

Liberase, BSA, fraction V, ABTS (all Roche, Indianapolis, IN)

Normal mouse serum (Jackson, West Grove, PA)

Proteinase K beads, Paraformaldehyde, PMA, Ionomycin, Brefeldin A, DMSO (all Sigma-Aldrich, St. Louis, MO)

Saponin (Calbiochem, La Jolla, CA)

HRP-streptavidin, HRP-coupled goat anti-mouse IgG (all R&D systems, Minneapolis, MN)

ECL Western Blotting Substrate (Pierce, Rockford, IL)

PBS w Ca²⁺ and Mg²⁺ (BioSource Int., Carnarillo, CA)

Sybr Green PCR Master Mix (Applied Biosystems, Indianapolis, IN)

RPMI-1640, PBS w/o Ca²⁺ and Mg²⁺, Penicillin/Streptomycin, L-Glutamine, Pyruvate, MEM non-essential AS, Fetal calf serum, Protein marker (SeeBlue plus 2), MES buffer (20x), Antioxidant, Reducing agent (10x), LDS Sample buffer, Coomassie (Simply Blue) SuperScript II RT, dNTP, Dithiothreitol, RT-buffer (first-strand buffer), RT-buffer (5x), RNase Out, Random Hexamer, Oligo-dT, Mouse Cot-1 DNA, Yeast tRNA (all Invitrogen, Carlsbad, CA)

dATP, dGTP, dCTP, dTTP, poly(A) 40-60, Cy3-dUTP, Cy5-dUTP (Amersham Bioscience, Piscataway, NJ)

ACK cell lysing buffer, DEPC H₂O, 10 % SDS, Tween-20, HEPES, TE-buffer, Trypan Blue 0.4 % (Cambrex, Baltimore, MD)

5.7 Synthetic oligonucleotides

Primer pairs were designed using Primer Express software (version 2.0; Applied Biosystems and synthesized at Lofstrand, Gaithersburg, MD.

Tab. 5:

gene	forward sequence	reverse sequence
Jagged-2	gct gtc acc gag gtc aag gt	gtt ctt tcc tgc gct ttc gt
Jagged-1	cag ctt cgg ctc agg gtc ta	acg atg cga tta cgg tcg tt
Delta-1	tgg ctt ctc cgg gag gta ct	tgc agt tct tgc ccg tgt ag
Delta-4	ctg cag gag ttc gtc aac ca	aag gca aat gcg gaa gaa ag
CD40	gct tgt tga cag cgg tcc at	ctt cct ggc tgg cac aaa tc
Tnf-alpha	aaa att cga gtg aca agc ctg tag	ccc ttg aag aga acc tgg gag tag
IL-12p40	ctc aca tct gct gct cca caa g	aat ttg gtg ctt cac act tca gg
IL-12p35	acg cta cct cct ctt ttt ggc	ggg act ggc taa gac acc tgg
iNOS	tgc ccc ttc aat ggt tgg ta	act gga ggg acc agc caa at
IL-10	att gca tgg cct ctg taa cc	tgg gct act tcg att ttg gt
HPRT	gcc ctt gac tat aat gag tac ttc agg	ttc aac ttg cgc tca tct tag g
IL-4	aca gga gaa ggg acg cca t	gaa gcc cta cag acg agc tca
IFN-gamma	cat tga aag cct aga aag tct gaa taa c	tgg ctc tgc agg att ttc atg
GATA3	gca ggt cac acg cct cct	gat tcc ttg gcg ctc aga ga
T-bet	cct gtt gtg gtc caa gtt caa c	cac aaa cat cct gta atg gct tgt
b2m	tga ccg gct tg tat gct at	cac tgt gac cca gga tat a

5.8 Buffer, media and stock solutions

cell culture buffer:

complete RPMI 1640: RPMI 1640 w/o glutamine with phenol red, 10 % heat-inactivated FCS, 4 mM L-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin, 20 mM HEPES, 1 mM Pyruvate, 100 µM MEM non-essential AS, 150 µl β-mercapto ethanol

minimal RPMI 1640: RPMI 1640 w/o glutamine with phenol red, 20 mM HEPES, 200 U/ml penicillin, 200 µg/ml streptomycin

permeabilization buffer: PBS w Ca^{2+} and Mg^{2+} , 0.1 % Saponin, 0.1 % FCS, 20 mM HEPES, add fresh 10 % NMS and anti-CD16/CD32

ELISA buffer:

dilution buffer: PBS w/o Ca^{2+} and Mg^{2+} , 5 % BSA, 0.25 % Tween20

washing buffer: PBS w/o Ca^{2+} and Mg^{2+} , 0.25 % Tween 20

blocking buffer: PBS w/o Ca^{2+} and Mg^{2+} , 5 % skimmed milk powder

coating buffer: 0.05 M carbonate-bicarbonate buffer, 7.95 g Na_2CO_3 , 14.65 g NaHCO_3 , 1.0 g NaN_3 , add up to 500 ml with dH_2O and adjust to pH 9.6.

Buffer for Western Blot:

transfer buffer: 50 ml NuPAGE Transfer Buffer (20X), 50 ml Methanol, add H_2O to 500 ml

washing buffer: PBS w/o Ca^{2+} and Mg^{2+} , 0.02 % Tween 20

blocking buffer: PBS w/o Ca^{2+} and Mg^{2+} , 5 % skimmed milk powder

5.9 Software, Data banks and web-based programs used

Primer Express software (Applied Biosystems), FlowJo software (Tristar), CellQuest software (BD Biosciences), RTB-NIAID-NIH: <http://madb-niaid.cit.nih.gov/>, Wellcome Trust Sanger Institute: http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_mansoni, NCBI: <http://www.ncbi.nlm.nih.gov/blast/>

Publications and conference abstracts

Publications:

Svenja Steinfelder, Richard Lucius, Gisela Greif and Thomas Pogonka.

Treatment of mice with the anticoccidial drug Toltrazuril does not interfere with the development of a specific cellular intestinal immune response to *Eimeria falciformis*. Parasitology Research (2005)

Dragana Jankovic, Svenja Steinfelder, Marika C Kullberg and Alan Sher.

Mechanisms underlying helminth- induced Th2 polarization: default, negative or positive pathways? Chemical Immunology and Allergy (2006)

Svenja Steinfelder, Dragana Jankovic, John Andersen, Manju Joshi and Alan Sher
Characterisation of the ribonuclease omega-1 as the Th2 inducing protein in schistosomal egg antigen. (in preparation)

Conference abstracts:

Svenja Steinfelder, Alan Sher and Dragana Jankovic

Activation of dendritic cell gene expression by Th1-inducing stimuli is selectively impaired in the presence of *S. mansoni* egg extract (SEA). WHIP 2004, Woods Hole, USA

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Early dynamics of INF- γ and IL-4 expression by CD4 T cells during Th2 polarization by soluble schistosome egg Ag (SEA). WHIP 2005, Woods Hole, USA

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Th2 biasing of the immune response by schistosome egg antigen. EMDS 2005, Amsterdam, Niederlande

Svenja Steinfelder, Alan Sher and Dragana Jankovic

Are dendritic cell chemotactic factors responsible for the adjuvant activity of *Schistosoma mansoni* egg products? WHIP 2006, Woods Hole, USA

Eidesstattliche Erklärung

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

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