

A nematode allergen elicits protection against challenge infection under specific conditions

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Abstract

We describe tropomyosin of the filarial nematode *Acanthocheilonema viteae* as an allergen and study its protective potential in the natural rodent host *Meriones unguiculatus* (jird). Jirds immunized with recombinant *E. coli*-expressed *A. viteae* tropomyosin emulsified in alum were not protected, while immunization with recombinant *A. viteae* tropomyosin or with protein purified from worms together with the adjuvant STP led to reduction of adult worm burdens by 30%. Vaccination with cDNA induced protection of about 30%, while application of cDNA together with aluminium phosphate increased the protection to >40%. Our data suggest that vaccination with tropomyosin under Th1 conditions, which are untypical for nematode infections, induces protection via an antibody independent effector mechanism.

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1. Introduction

Filarial nematodes are endoparasites that dwell subcutaneous tissues or lymphatics of their hosts. Various studies in animal models of filariasis have shown that substantial protective immune responses against these parasitic nematodes are reliably induced by immunization with attenuated infective stage three larvae (L3) or by immunization with ES antigens of L3 [1,2]. In contrast, vaccination with *E. coli*-expressed proteins of filariae has met with difficulties, because the level of protection observed was generally much lower than with irradiated larvae [3,4] and the protective effect of recombinant proteins showed high variability between experiments [5]. Several factors could account for the generally low performance of vaccines utilizing single *E. coli*-expressed proteins as compared to living worms or complex ES-proteins. It is possible that not a single protein, but several antigens in concert might be necessary to induce efficient protective effector mechanisms. In addition, *E. coli* derived proteins might be unsuitable because they lack critical epitopes owing to improper folding of proteins or absence

of post-translational modifications. For example, glycan epitopes were suggested to be targets of protective antibody responses against intestinal nematodes [6,7]. Furthermore, it is conceivable that a particular immunization scheme is necessary to induce relevant immune effector mechanisms.

The type and efficiency of an immune response against an immunizing protein among other factors depend critically on the adjuvant used, the route of immunization and the mode of antigen delivery [8]. The adjuvant aluminum hydroxide (alum) promotes Th2 responses and the production of antibodies, among others high levels of IgE [9] and alum is generally not considered efficient in raising cell-mediated immune responses [10]. In contrast, the adjuvant STP (a mixture of Squalane, Tween and the synthetic Cblock polymer “Pleuronic”) predominantly promotes Th1 responses [11]. The intramuscular immunization with cDNA has been described to induce clear Th1 responses [12], partially due to CpG motifs within bacteria-derived plasmids [13].

To study the influence of such modifying factors we chose filarial tropomyosin as a model antigen, a protein described by several authors to induce protection against nematode L3. Tropomyosin is a microfilament-protein with a α -helical coiled-coil structure, present in all types of eukaryotic cells [14]. It occurs in several tissue specific isoforms, and is most

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abundant in muscles. In both vertebrates and invertebrates it plays a central role in the regulation of cell motility [15]. In filarial nematodes, tropomyosin is not only present in muscles, but was also found in the cuticle of microfilariae and L3 of *Onchocerca volvulus* [16]. Recently, tropomyosin was described as a potent allergen of invertebrates, e.g. shrimps, mites and insects [17,18] that can provoke food and airway allergies.

Previous studies described the protective capacity of filarial tropomyosin [19–21] and tropomyosin of the intestinal nematode *Trichostrongylus colubriformis* [22], but did not address the effector mechanisms leading to protective immunity. Generally, it is believed that filarial nematodes are attacked by mechanisms of antibody dependant cellular cytotoxicity (ADCC). Elegant studies in mouse model systems have described IgE responses as pivotal for protective anti helminth immune responses induced by irradiated larvae [23]. Thus, it appeared attractive to re-evaluate a surface associated, IgE inducing candidate antigen with respect to potential effector mechanisms in a natural host–parasite association.

2. Materials and methods

2.1. Cloning and expression of *A. viteae* tropomyosin

Sequence information from immunoaffinity-purified protein [21] was used to design oligonucleotide primers. A fragment of 612 bp was amplified by PCR from *A. viteae* female cDNA, labeled with digoxigenin (Roche, Mannheim, Germany) and used to screen an *A. viteae* female cDNA library in lambda ZAP. Out of 12 positive clones, one full-length clone of 915 bp was sequenced and subsequently used for further studies. The sequence of the *A. viteae* clone was deposited in GeneBank under the accession number: AF000607. The cDNA sequence was subcloned into the *Bam*HI and *Hind*III sites of the expression vector pQE30 yielding polypeptides with a N-terminal six-histidine tag (Qiagen, Hilden, Germany). The recombinant plasmid was transformed into DHF α competent *E. coli* and screened for expression by SDS-PAGE after induction with 1 mM IPTG. The recombinant *E. coli*-expressed protein, termed rAvTropo, was purified under non-denaturing conditions by affinity chromatography using Ni-NTA resin (Qiagen, Hilden, Germany) and dialyzed against PBS/0.1% Triton X-100, pH 7.4 and subsequently against PBS, pH 7.4.

2.2. Preparation of gel-eluted antigen

Worm derived *A. viteae* tropomyosin (wdAvTropo) was prepared by electroelution from SDS-PAGE-separated adult worm extracts. Adult female worm extracts were prepared in PBS, pH 7.4 by homogenization and subsequent sonication for 2 min (Sonifyer Sonoplus HD 200, Bandelin, Berlin, Germany) and centrifugation (10 min, 12,000 $\times$ g). The position of the tropomyosin band was determined by Western blot

analysis of strips of the gel with an anti-*A. viteae* tropomyosin mAb (Sereda et al., unpublished data) and the protein was excised out of the SDS gel after copper staining [24]. Subsequently, the protein was electroeluted with a BioTrap BT 1000 device (Schleicher & Schuell, Dassel, Germany). Eluted native tropomyosin was dialyzed twice against PBS, pH 7.4.

2.3. Preparation of DNA vaccine

The cDNA sequence of *A. viteae* tropomyosin was subcloned into the *Eco*RI/*Apa*I restriction sites of the mammalian expression vector pcDNA 3.1+ (Invitrogen, Karlsruhe, Germany). The recombinant plasmid pcDNA/AvTropo was transformed into competent *E. coli* JM109 and plasmid DNA from a 100 ml liquid culture was purified with NucleoBond Plasmid Maxi Kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The recombinant plasmid DNA was resuspended in TE buffer (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0) and stored at -20°C . Expression of *A. viteae* tropomyosin was analyzed in vitro by transfection of COS7 cells with pcDNA/AvTropo. 2×10^5 COS7 cells/well in a 6-well plate were cultured in 2 ml Dulbeccos MEM medium (Biochrom, Berlin, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and L-glutamine (all from Biochrom, Berlin, Germany). Cells grown to 50–70% confluence were transfected by addition of a solution of 1 μg plasmid pcDNA/AvTropo or pcDNA 3.1+ in 3 μl of FuGENE 6 (Roche, Mannheim, Germany) and 97 μl of serum free MEM medium. After 30 min of incubation the mixture was evenly, drop-wise, added to the cell culture wells and incubated for 48 h after a gentle mix. To determine transfection efficiency, cells were transfected in the same way with a control plasmid pEGFP-N1 (Clontech, Heidelberg, Germany) encoding the EGFP protein and monitored for fluorescence under UV-light. The transcription of AvTropo sequence was analyzed by RT-PCR using specific *A. viteae* tropomyosin primers (Tropo fw 5' ATG GAT GCG ATC AAG AAA AAG 3', Tropo rev 5' ATA TCC AAA AAG TTC TTG GAA GG 3').

2.4. Western blot to detect of *A. viteae* tropomyosin expressed in COS7 cells

Tropomyosin expressed in transfected mammalian cells was detected by immunoblot using a monoclonal antibody specifically recognizing *A. viteae* tropomyosin, but not the vertebrate protein (Sereda et al., unpublished data). A 12% SDS-PAGE was run using lysates of 2×10^5 transfected or untransfected, respectively. Subsequently, proteins were blotted onto Protran[®] nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) for 45 min in a semi-dry blot chamber (LSM, Dossenheim, Germany). Membranes were then trimmed and blocked with 5% skimmed milk in TBS pH 7.4. After washing three times, 5 min with TBST, membranes were incubated 1 h at RT with undiluted hybridoma culture supernatant. After subsequent washing with TBST an

isotype specific secondary antibody (Rockland, Gilbertsville, PA, USA) conjugated to alkaline phosphatase (AP) was added for 1 h diluted 1:5000 in TBS. After another round of washing (3 × 5 min in TBS) the substrate (NBT/BCIP, Roth, Karlsruhe, Germany) was added in AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris, pH 9.5). Reaction was stopped by washing the membrane in tap water. An irrelevant mAb of the same isotype was used as control.

2.5. Vaccination schemes

Groups of 7–10 inbred male jirds (*Meriones unguiculatus*) of 8 weeks age from our own breeding colony were immunized three times in 2-week intervals by subcutaneous injection into the shoulder and flank regions. Animals were immunized each time with 25 µg rAvTropo taken up in a final volume of 250 µl alum (2% aluminium hydroxide; Reheis, Dublin, Ireland) [25] or STP (Tween 80, Pluronic, Squalan) [26] or with 25 µg wdAvTropo in a final volume of 250 µl of STP. The DNA-vaccine was applied three times intramuscularly in the quadriceps muscles of the hind legs, each dose consisting of 50 µg (pcDNA/AvTropo) in 100 µl PBS, pH 7.4 or 100 µl of aluminium phosphate (AdjuPhos®, Brentag Biosector, Elsenbakken, Denmark) [25]. Jirds of the control groups were immunized with the same volume of the corresponding adjuvant (alum or STP), or the vector plasmid without the cDNA insert, respectively. An additional group was immunized with an equivalent amount of PBS to serve as challenge control for viability of L3. Two weeks after the last immunization the jirds were infected with 70 fully motile L3 of *A. viteae* prepared as described before [27]. Animals were challenged by injection under the skin of the neck [28]. To obtain sera jirds were bled under anesthesia by puncture of the retroorbital vein plexus at the start of the experiment (0 weeks), at challenge (2 weeks after last immunization), and at 4, 8 and 11 weeks p.i. Sera were stored as aliquots at –20 °C. Jirds were dissected 11 weeks p.i. and the adult worm burdens and microfilarial loads were determined.

2.6. Determination of IgM, IgG and IgG subclasses in sera of immunized jirds

Antibody subclasses in sera of immunized jirds were determined by ELISA. 96-well round-bottom plates were coated with 100 ng/well of rAvTropo in carbonate buffer (13.2 mM Na₂CO₃, 35.7 mM NaHCO₃), pH 9.6 and incubated overnight at 4 °C. After washing three times with PBS, pH 7.4/0.025% Tween 20 (PBST) the wells were blocked for 30 min with 100 µl 3% BSA/PBS at 37 °C. After subsequent washing with PBST, individual sera were added in a dilution of 1:80 in 3% BSA/PBS and incubated for 1 h at 37 °C. Subsequently, the plates were washed again with PBST and incubated with the secondary antibodies: goat anti-mouse IgG or IgM (Jackson ImmunoResearch, West Grove, PA, USA) or rabbit anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Rockland, Gilbertsville, PA, USA) conjugated to alkaline

phosphatase in a dilution of 1:3000 in 3% BSA/PBS. After 1 h of incubation at 37 °C the plates were washed again and the substrate (5 mg *p*-nitrophenyl phosphate disodium (Sigma, München, Germany) dissolved in 10 ml carbonate buffer with 1 mM MgCl₂ was added. The reaction was stopped with 0.5 M NaOH and the absorbance was measured at 405 nm with the additional reference filter at 630 nm. All assays were performed in triplicates.

2.7. Rat basophil leukemia (RBL) cell mediator release assay for the detection of IgE

As there are no antibodies available that detect jird IgE, we determined IgE in the sera of vaccinated jirds with a bioassay as described previously [29]. Briefly, the cell line RBL-2H3 (gift from Dr. I. Pecht, Weizmann Institute, Rehovot, Israel) was maintained in Eagle's minimal essential medium (Biochrom, Berlin, Germany) with 10% fetal calf serum (Biochrom, Berlin, Germany). The cells were sensitized by incubation with jird sera in a final dilution of 1:50 in Eagle's medium. For crosslinking, sensitized RBL cells were incubated in a volume of 100 µl with a final dilution of 10 µg/ml of rAvTropo or total worm extract in Tyrode's buffer (TB) for 1 h in a humidified atmosphere at 37 °C. Controls without antigens were run to measure spontaneous release of the mediator β-hexosaminidase. Total release was measured by lysis of cells with TB containing 1% Triton X-100 (Serva, Heidelberg, Germany). Release of β-hexosaminidase was detected by colorimetric quantification of the enzymatic activity on *p*-nitrophenyl *N*-acetyl β-D-glucosaminide (Sigma, Deisenhofen, Germany) used as substrate. Results were expressed as percentage of the total release after deduction of spontaneous release. The assays were performed in triplicates.

2.8. Antigen-specific proliferation of murine spleen cells

Three BALB/c mice each were immunized three times in intervals of 10 days with 25 µg each of rAvTropo emulsified in alum or STP. In parallel control mice were treated in similar intervals with alum or STP in PBS. Ten days after the last immunization the spleen cells of the immunized and the control mice were isolated and cultured in 96-well round bottom plates (Costar, Corning, Schiphol, NL) at a density of 3.5×10^5 cells/well. The cells were stimulated with 5 µg/ml rAvTropo or a recombinant control protein (rOv33 [30]) of a filarial nematode. Proliferation of the cells was quantified after 5 days by incorporation of ³H-thymidine (1 µCi/well, Amersham, Buckinghamshire, UK) added to the cultures during the last 20 h of incubation. All experiments were performed in triplicates. Arithmetic mean values ± S.D. are shown.

2.9. Statistical analysis

Statistical analysis of the protection was performed with the Wilcoxon test. Protection was calculated according to

the formula: % protection = [(average number of worms in the control group) – (average number of worms in the challenged group)] / (average number of worms of the control group) $\times$ 100. Differences between antibody responses of various groups were also tested with the Wilcoxon test. Differences of $p < 0.05$ were considered as statistically significant. To estimate the statistical significance of correlations between specific anti-*A. viteae* tropomyosin antibody levels of individual jirds and adult worm numbers of the corresponding animals, we used Spearman's rank correlation test.

2.10. Alignment of tropomyosin sequences

For alignment of sequences and calculation of the homology between different tropomyosins we used MacVector™ 7.2.2 software (Accelrys Inc.).

3. Results

3.1. Molecular cloning and expression of *A. viteae* tropomyosin in *E. coli*

A full-length cDNA clone of 915 bp was isolated from a female *A. viteae* cDNA library. The nucleotide sequence of this clone contained an open reading frame extending from the start methionine at position 29–31 to a stop codon at position 880–882. The clone encodes 284 amino acids with a theoretical molecular weight of 32 kDa. Sequence alignment of *A. viteae* tropomyosin with tropomyosin genes of other nematodes showed a high degree of homology. At the amino acid level AvTropo shows 93% identity (94% similarity) to *O. volvulus* tropomyosin and 87% (92%) identity to *Caenorhabditis elegans* tropomyosin. The homology to chicken cardiac tropomyosin is 52% (70%), to mouse muscle TM1 tropomyosin 54% (70%) and to human TM1 tropomyosin 53% (73%). The amino acid sequence of AvTropo contains two potential Asn-glycosylation sites (aa 136–140, and aa 277–280). The highly conserved, characteristic N-terminal region M-D-X-I-K-K-K-M (aa 1–8) and the tropomyosin signature, L-K-E-A-E-X-R-A-E (aa 240–249), conserved in the C-terminal region, are also present in the sequence of *A. viteae* tropomyosin.

Expression of the AvTropo cDNA in *E. coli* as a polypeptide fused to a C-terminal leader of six histidines yielded a protein with an apparent molecular mass of about 42 kD in SDS-PAGE. The recombinant protein was soluble in aqueous buffers and amenable to purification by nickel chelate affinity chromatography (data not shown).

3.2. Expression of the tropomyosin cDNA in eukaryotic cells

To analyze the expression of AvTropo after delivery as cDNA we transfected mammalian cells in vitro. Transfection of COS7 cells with pcDNA/AvTropo yielded a specific

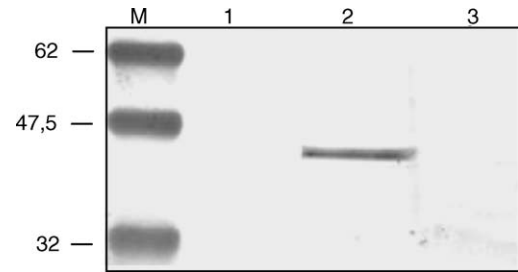


Fig. 1. Expression of AvTropo in COS7 cells. SDS-PAGE-separated lysates of transfected cells were analysed by immunoblotting with a monoclonal antibody specifically recognizing *A. viteae* tropomyosin. COS7 cells were transfected with pcDNA (lane 1), pcDNA/AvTropo (lane 2) and an irrelevant control plasmid (pEGFP-N1, lane 3). M: prestained protein marker.

transcript of 858 bp as shown by RT-PCR with specific AvTropo primers, while transfection with the control plasmid did not. In immunoblots, a monoclonal antibody (mAb) directed against an invertebrate specific epitope of *A. viteae* tropomyosin stained a 42 kD band present in lysates of COS7 cells transfected with pcDNA/AvTropo, but not in lysates of untransfected cells or cells transfected with a control plasmid (Fig. 1). These data show that *A. viteae* tropomyosin was expressed by COS7 cells transfected with the recombinant DNA plasmid.

3.3. Protective capacity of *A. viteae* tropomyosin

To study the influence of the immunological context of an immunization, we vaccinated jirds with *E. coli*-expressed recombinant AvTropo (rAvTropo) together with either alum or STP as adjuvants. After 11 weeks of experimental infection the animals were sacrificed and the adult worm burdens were determined. In two experiments, immunization with rAvTropo induced protection when applied with STP as adjuvant (35.1%, 29.3%; $p < 0.05$), but yielded no protection at all when applied with alum (Table 1).

To test whether worm derived *A. viteae*-tropomyosin (wdAvTropo) has a better protective capacity than *E. coli*-expressed recombinant protein, we immunized groups of jirds with wdAvTropo together with STP. Two experiments showed 29.4% ($p < 0.05$) and 8.1% (not significant) protection, respectively, indicating that possible modifications or other qualities of the worm derived protein did not increase the protective effect (Table 1).

As *E. coli*-expressed recombinant proteins and worm derived proteins denatured by SDS-PAGE might lack certain conformational epitopes, we studied the role of native conformation for the induction of protective immunity by intramuscular injection of jirds with pcDNA/AvTropo. This DNA vaccination led to a consistent reduction of worm burdens by 28.1 and 22.1%, in two consecutive experiments (Table 1). These differences were not statistically significant when the data of the individual experiments were analyzed. However, when transformed data of the two experiments were pooled, the differences of the pcDNA/AvTropo group were

Table 1

Vaccination experiments		Experiment 1 ^a				Experiment 2 ^a			
Antigens used for immunization		No. of animals/surviving	No. of worms in experimental groups	No. of worms in control groups	Protection (%) of worm burden reduction	No. of animals/surviving	No. of worms in experimental groups	No. of worms in control groups	Protection (%) of worm burden reduction
rAvTropo/alum		7/6	23.3 ± 6.3	22.1 ± 7.7	0	7/6	20.5 ± 2.7	20.6 ± 3.6	0.5 ± 8.6
rAvTropo/STP		7/6	13.7 ± 6.7	21.1 ± 7.1	35.1 ± 23.8 ^b	7/6	17.6 ± 7.2	24.5 ± 4.8	29.3 ± 17.8 ^b
wdAvTropo/STP		7/7	17.3 ± 4.9	24.5 ± 4.7	29.4 ± 19.8 ^b	7/6	20.2 ± 2	21.7 ± 8.8	8.1 ± 6.2
pcDNA/AvTropo		7/7	13.6 ± 7.9	18.9 ± 9.5	28.1 ± 17.9	7/6	23.2 ± 11.1	29.8 ± 5.7	22.1 ± 8.4
pcDNA/AvTropo + AdjuPhos		7/5	19.8 ± 5.8	33.8 ± 8.6	41.4 ± 17.1 ^b	7/7	16.9 ± 8.0	29.8 ± 5.7	43.3 ± 16.8 ^b

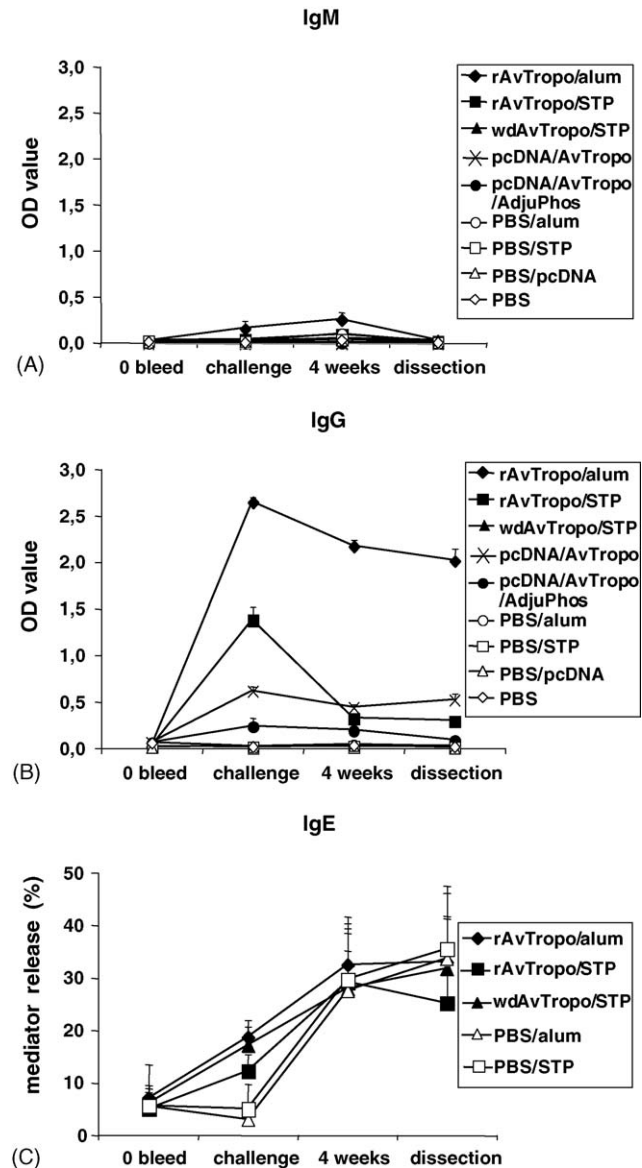
^a Values represented are means ± standard deviations.^b Significantly different ($P < 0.05$) from values obtained for corresponding control group.

Fig. 2. Comparison of the recognition of *Acanthocheilonema viteae* tropomyosin by IgM, IgG and IgE of jird sera. Groups of jirds were immunized with rAvTropo or wdAvTropo together with alum or STP or pcDNA/AvTropo, and sera were checked by ELISA (A and B) or by RBL assay (C) for specific antibodies against rAvTropo. Each point represents a value obtained with pooled sera of six or seven animals. Mean and S.D. of a triplicate determinations are shown.

statistically significant in comparison to the pcDNA/PBS control group ($p < 0.05$). Thus, vaccination with a DNA construct that expresses *A. viteae*-tropomyosin in a supposedly native conformation does not impart stronger protection than vaccination with *E. coli*-expressed or gel-eluted tropomyosin.

When jirds were immunized with pcDNA/AvTropo together with aluminium phosphate as adjuvant, the worm burdens of the animals were reduced by 41.4 and 43.3% ($p < 0.05$) in two experiments, when compared with the animals of the corresponding pcDNA/PBS control groups (Table 1). The observed protection did not extend to micro-

filariae, as immunization with different forms of rAvTropo and wdAvTropo did not have significant impact on the levels of microfilariae of the animals.

3.4. Analysis of the IgM and IgG responses of immunized jirds

The analysis of serum antibodies by ELISA showed that the different modes of immunization induced different patterns of antibody responses against rAvTropo. While application of rAvTropo together with alum resulted in low IgM levels and a strong IgG response ($p < 0.002$) at all three time points, immunization with rAvTropo and STP induced very low IgM responses and moderate IgG responses (Fig. 2A and B). The PBS-treated challenge control group showed low antibody responses like the adjuvant control groups (Fig. 2A and B). Application of tropomyosin as a DNA-vaccine resulted in only moderate IgG and IgM antibody responses (Fig. 2A and B). An analysis of IgG subclasses revealed that the non-protected animals immunized with rAvTropo/alum had consistently higher titers of IgG1, IgG2a, IgG2b and IgG3 against rAvTropo than the rAvTropo/STP-immunized protected animals (Fig. 3). IgG3 was the most abundant subclass. The most conspicuous difference between the groups was an almost three times higher IgG1 level in rAvTropo/alum animals as compared to rAvTropo/STP animals ($p > 0.008$).

Jirds immunized with wdAvTropo had no detectable antibody responses against rAvTropo indicating distinctive differences in epitope composition or/and epitope accessibility of the two antigens. However, sera of jirds immunized

with rAvTropo did recognize native *A. viteae* tropomyosin suggesting that common epitopes exist. Sera of animals vaccinated with pcDNA/AvTropo contained no IgM, and only marginal amounts of IgG against rAvTropo (Fig. 3C). The antibody response was even lower in the group immunized with pcDNA/AvTropo together with aluminium phosphate (data not shown). Levels of IgG and IgM antibodies in the groups did not increase after challenge infection (see Figs. 2 and 3A–C), suggesting that tropomyosin set free by worms in the context of the infection did not restimulate the IgM and IgG antibody responses induced by the recombinant vaccine. Animals of the challenge control group did not develop IgM antibodies (data not shown) or IgG responses against recombinant AvTropo during infection (Fig. 3D). However, sera of challenge control animals did recognize wdAvTropo and reacted with worm extracts in ELISA (data not shown). This supports a lack of certain antibody-inducing B cell epitopes in recombinant tropomyosin.

3.5. Allergenic potential of native and recombinant *A. viteae* tropomyosin in jirds

To study the possible role of IgE antibodies for protection, serum IgE was measured with an in vitro-assay based on the degranulation of IgE-sensitized cells of a rat basophil cell line. Immunization of jirds with rAvTropo/alum induced the formation of specific IgE antibodies that led to degranulation of cells when cross-linked with *A. viteae* female antigen (Fig. 4). The quantity of antibodies rose during immunization, increased sharply after the challenge infection and then

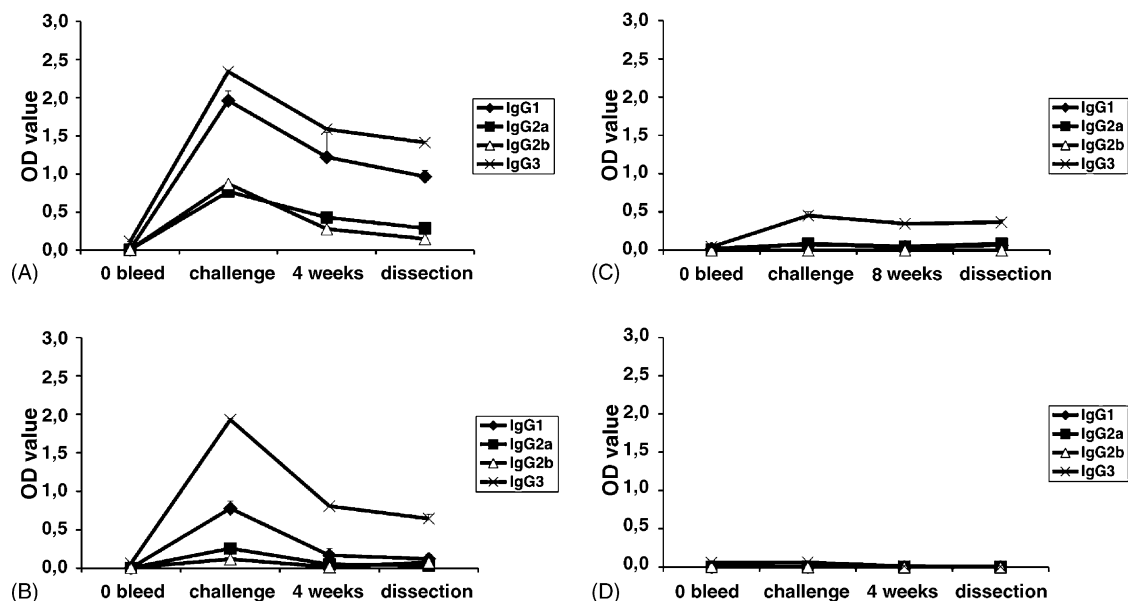


Fig. 3. Kinetics of IgG antibody subclasses in sera of jirds immunized with *Acanthocheilonema viteae* tropomyosin. Groups of jirds were immunized with rAvTropo with alum (A), rAvTropo with STP (B), pcDNA/AvTropo in PBS (C) or injected with PBS alone (D) and challenged with *A. viteae* L3. IgG1, IgG2a, IgG2b and IgG3 antibodies were checked by ELISA for specific antibodies against rAvTropo. Each point represents a value obtained with pooled sera of six or seven experimental animals. Mean and S.D. of triplicate determinations are shown.

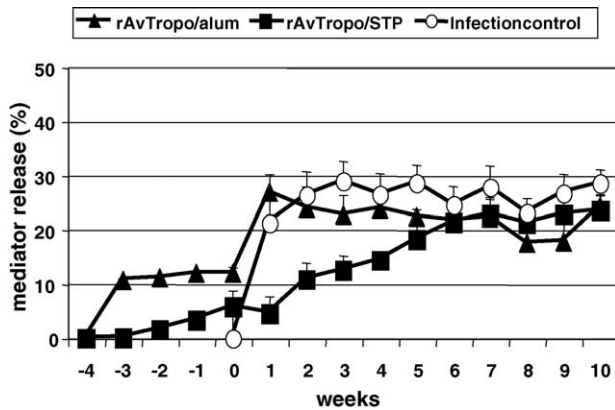


Fig. 4. Analysis of the kinetics of IgE antibodies of jirds immunized with rAvTropo. Jirds were immunized four times in 10-day intervals with rAvTropo/alum or rAvTropo/STP. The challenge infection with *A. viteae* L3 was given 14 days after the last immunization at Day 0. Sera were collected weekly and analysed for tropomyosin specific IgE by RBL assay. For cross-linking of reactive IgE antibodies RBL cells were incubated with female worm extract at a concentration of 5 µg/ml. Mean and S.D. of triplicate determinations of sera pooled from four animals are shown.

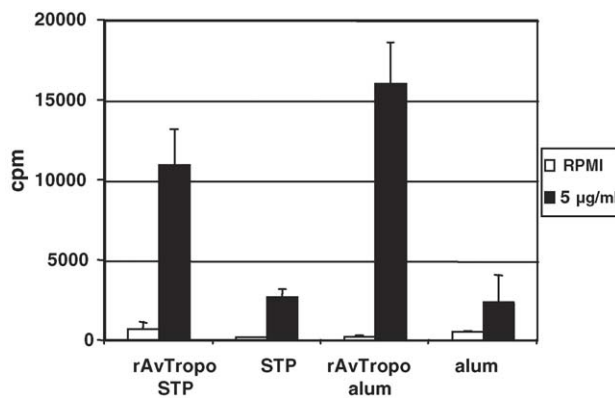


Fig. 5. T cell proliferation in presence of *A. viteae* tropomyosin. Three BALB/c mice each were immunized with rAvTropo/STP, rAvTropo/alum or with the adjuvant in PBS. Spleen cells (3.5×10^5) of each group of mice were restimulated with a constant amount of rAvTropo (5 µg/ml). Proliferation of spleen cells was quantified by thymidine incorporation. Mean and S.D. of triplicate determinations of cells pooled from three animals are shown.

reached a plateau. Immunization with rAvTropo/STP and subsequent challenge infection induced consistently lower quantities of IgE antibodies, but the antibody levels rose steadily, reaching high levels at the end of the period studied. Jirds of the control groups that had been infected without previous immunization developed the highest levels of anti-tropomyosin IgE, which rose sharply after the onset of infection.

Thus, judged by its potential to induce IgE antibodies during a natural infection and after immunization with the recombinant protein, *A. viteae* tropomyosin is an allergen. Sera of all groups immunized with *A. viteae* tropomyosin consistently contained specific anti AvTropo IgE antibodies. In contrast to the IgG and IgM antibodies, IgE responses against rAvTropo

were restimulated by the challenge infection (Figs. 2C and 4), implying that dominant IgE-inducing epitopes were common to recombinant and to worm derived tropomyosin. The quantity of tropomyosin-specific IgE in sera of immunized animals did not correlate with protection against challenge, given the fact that the rAvTropo/STP-immunized animals, which had relatively low IgE levels, were protected, whereas the rAvTropo/alum-immunized animals were not.

3.6. T cell responses induced by *A. viteae* tropomyosin

To determine whether AvTropo stimulates T cell responses we used BALB/c mice that were immunized with rAvTropo together with alum or STP. Fig. 5 shows that rAvTropo has T cell stimulatory capacity as spleen cells of mice immunized with rAvTropo mounted a proliferative response upon restimulation with rAvTropo, whereas control mice did not. The T cell proliferation was specific for AvTropo as a recombinant filarial control antigen (rOv33) failed to restimulate spleen cells of mice immunized with rAvTropo (data not shown). Immunization of mice using alum as adjuvant resulted in a stronger T cell response as compared to immunization with rAvTropo emulsified in STP (Fig. 5).

4. Discussion

The present study shows that vaccination with recombinant filarial tropomyosin imparts partial protection in jirds against challenge with L3 of *A. viteae* only when applied with a protocol that favours the development of Th1 responses. Immunization with recombinant or native, gel-eluted tropomyosin induced about 30% protection when the Th1-inducing adjuvant STP was used [11]. Similarly, intramuscular injection of AvTropo DNA—a route of immunization that elicits a Th1-polarized immune response [12] induced protection against challenge infection, and this effect was increased by the inclusion of aluminium phosphate as an adjuvant. These schemes induced low or moderate quantities of serum IgM, IgG and IgE. In contrast, no reduction of worm burdens was seen when animals were immunized with tropomyosin in conjunction with alum, a treatment inducing high levels of antibodies, in particularly elevated IgG1 and IgE. This differential induction of antibody responses by adjuvants follows the pattern observed in mice [31] and demonstrates that the skewing towards Th2 and Th1 responses by alum and STP, respectively by STP also operates in jirds.

Protective immune responses against filarial L3, elicited by immunization with filarial tropomyosin have been described previously [19–21]. All these studies employed immunization schemes that drove immune responses against tropomyosin into Th1 direction, either by using Freund's complete adjuvant, STP or intramuscular DNA vaccination. Thus, our own observations are in line with the published data and support the link between protective immunity and Th1

immune responses against L3. Interestingly, filarial infections are described to induce a polarization towards Th2 responses [32–34]. Thus, tropomyosin is only protective when used for immunization under conditions that do not correspond to the natural infection.

This situation is reminiscent to immunizations with so called “novel antigens” or “concealed antigens”, i.e. antigens that do normally not induce immune responses, but can be protective when applied in a suitable form. Such antigens are typically part of structures to which the host immune system does not have access, e.g. the gut of blood feeding parasites. Novel antigens have successfully been used as experimental vaccines against a gastrointestinal nematode [6] and form the basis of a commercialized anti-tick vaccine [35]. It has been hypothesized that the efficiency of novel antigens is owing to the fact that the worms did not develop specific evasion mechanisms during their co-evolution with the host that would counteract the immune effector mechanisms induced by such components. By analogy, an antigen that displays protective properties only when applied in a context untypical for an infection can also be considered as novel antigen. Therefore, our study shows a potentially important principle, and novel antigens of the tropomyosin type might have potential as anti-helminth vaccines. The relatively low levels of protection observed here could possibly be increased by combination of several molecules or improvements of the vaccination scheme.

The inverse correlation of antibody response and protection of jirds, shown in all our experiments, suggest that protection in our model is due to immune effector mechanisms that operate independently of antibodies. This is in contrast to the prevailing view that considers ADCC mechanisms as the most important immune effector mechanism against parasitic nematodes [23,36–38]. However, our data do not generally exclude antibodies as important effector molecules for three reasons: First, immunity against large metazoan parasites like filarial L3 probably requires cooperation of different immune effector mechanisms, directed against various antigens, at different time points and sites to limit infections [8]. Thus, immune effector mechanisms like the antibody-independent response described here should co-exist with ADCC in an infected animal. Second, ADCC might be the prevailing type of effector mechanism in a natural infection, whereas antibody independent responses could have escaped the attention. Third, all articles cited above analyze the reaction of mice against filarial parasites that are not adapted to this host species. Therefore, it cannot be excluded that studies with such non-natural host–parasite associations are biased with regard to immune effector mechanisms [39]. As the natural parasite–host association studied here is very limited with regard to immunochemical reagents and genetically deficient host strains do not exist, a more detailed analysis of the possible effector mechanism is restricted. It is important to note that generally microfilariae are more susceptible to ADCC responses than L3 as shown by various studies in mouse models and in natural parasite/host associations [36].

Paramyosin of the blood flukes *Schistosoma mansoni* and *Schistosoma japonicum*, a prominent vaccine candidate antigen [40,41], has properties very similar to tropomyosin. The protective effect of paramyosin was described to rely on killing of schistosome larvae by activated macrophages [42]. Activation of macrophages would occur through IFN- γ produced by antigen specific T cells that are triggered by paramyosin released during early infection [43]. Mice vaccinated against *S. mansoni* were best protected when native paramyosin was intradermally injected together with bacillus Calmette-Guerin (BCG) an immunization scheme driving immune responses towards Th1 responses [44,45]. Paramyosin has certain traits common with tropomyosin, both being muscle proteins with a coiled-coil structure and containing repetitive elements [46]. As another trait in common with paramyosin, *A. viteae*-tropomyosin is a potent T cell antigen. Interestingly, our experiments show that the degree of T cell proliferation was independent of the usage of alum or STP as adjuvant. Thus, it could be a qualitative factor, e.g. the cytokine pattern of activated cells that determine the difference between protective and non-protective type of immune response. Furthermore, paramyosin of mites and the parasitic nematode *Anisakis simplex* are potent allergens [47–49]. These striking parallels suggest that the immune effector mechanisms operative in animals immunized with tropomyosin and with paramyosin could be very similar.

Tropomyosin has been described as a “panallergen” of invertebrates, whereas vertebrate tropomyosins are considered non-allergenic [18,50,51]. Interestingly, *O. volvulus* tropomyosin has also been suggested to be an allergen [16]. We show that *A. viteae*-infected jirds developed high levels of IgE. And wdAvTropo as well as rAvTropo induced high levels of specific IgE in immunized animals. Moreover, the degranulation assays show that *A. viteae* tropomyosin is also a functional allergen that leads to crosslinking of surface bound IgE. However, the data presented here suggest that the allergenic qualities of tropomyosin in our model are not crucial for the protective effect. In the contrary, protection is induced when the allergen is applied in a context that minimizes its IgE-inducing properties. Further studies have to show whether such a constellation can be exploited for the rational design of anti-helminth vaccines.

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