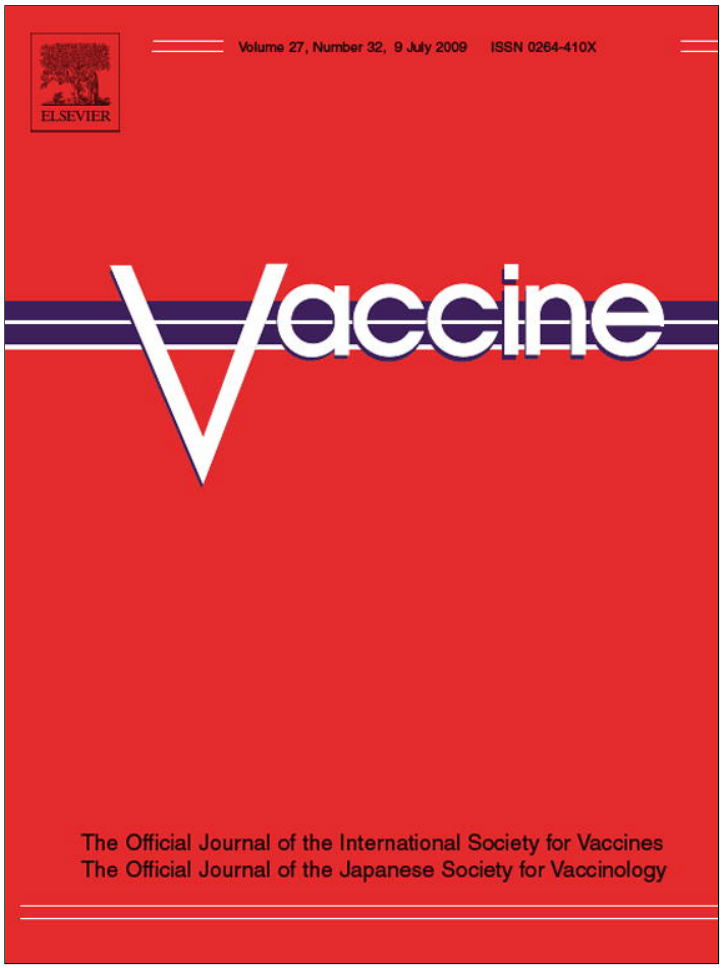

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Immunization with inflammatory proteome of *Brugia malayi* adult worm induces a Th1/Th2-immune response and confers protection against the filarial infection

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ABSTRACT

Mastomys coucha and jirds (*Meriones unguiculatus*) were immunized with four cytokine-stimulating SDS-PAGE resolved fractions F5 (68–84 kDa), F6 (54–68 kDa), F10 (38–42 kDa) and F14 (20–28 kDa) of *Brugia malayi* adult worm to determine which of these fractions has the potential to influence the establishment of subsequently introduced *B. malayi* infection in the animals. The proteins in the fractions were analyzed by 2DE and MALDI-TOF. Immunization with F6 suppressed the establishment of third stage larva (L_3) initiated infection in *M. coucha* (64%; $P < 0.01$) and jird (42%; $P < 0.01$). Survival of intraperitoneally implanted adult worms in *M. coucha* was lowered by F6 (72%; $P < 0.01$) and F14 (66%; $P < 0.05$) but not by F5 and F10. Immunization with F6 intensely upregulated both Th1 (IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IgG1, IgG2a and lymphoproliferation) and Th2 (IgG2b and IL-10) responses and NO release. Immunostimulatory proteins HSP60, intermediate filament protein, and translation elongation factor EF-2 were identified in F6 fraction by 2DE and MALDI. The findings suggest that F6 protects the host from the parasite via Th1/Th2 type responses and thus holds promise for development as a vaccine.

1. Introduction

Human lymphatic filariasis, a mosquito-borne disease of the tropics, is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. The disease is not fatal but responsible for considerable morbidity leading to huge economic loss. In India, more than 500 million people are exposed to infection with 45.5 million asymptomatic carriers harbouring circulating microfilariae (mf) and 22.5 million suffering from chronic manifestations of the disease like hydrocele, lymphoedema and elephantiasis [1].

The infection is initiated by introduction of third stage infective larvae (L_3) of the parasite into the host by the bites of L_3-bearing mosquitoes. The L_3 migrate to the nearest lymphatics and after two molts develop into adult worms, which give birth to mf. The mf enter blood circulation from where they are taken up by mosquitoes during blood meal. Mf develop into L_3 in the mosquitoes.

Several attempts were made to produce protective immunity against L_3 stage in both experimental and human filariasis using irradiated live L_3 [2,3] and DNA vaccine [4], but in vain. On the other hand developing preventive strategies based on both Th1 and Th2 arms of the immune system appears reasonable since putatively immune individuals (true endemic normal) show elevated level of Th1-like responses. Th1-mediated events were also reported to be involved in protective immunity against *Brugia* and *Ascaris suum* in animal models [5,6]. In chronic patients who are mostly amicrofilaremic, there appears to be both antigen specific tolerance and bystander suppression [7–9]. In human filariasis inflammatory cytokines and immunological hyperactivity may, on one hand, promote establishment of the infection [10] and on the other, lead to disease manifestations [11]. Such diverse responses are thought to be due to the ability of live and dead parasite products to stimulate release of either predominantly pro- or anti-inflammatory cytokines under different conditions. Indeed, our recent studies revealed that live stages of the parasites are capable of stimulating release of both pro- and anti-inflammatory cytokines [12]. Maizels and Lawrence [13] also showed that acute exposure to mf induces an inflammatory type 1 response whereas L_3 and adults induce primarily type 2 responses in a mouse model. We have shown that BmAFII, a Sephadex G-200 eluted fraction of *B. malayi* adult worm extract, was predominantly pro-inflammatory and protected the hosts against *B. malayi* in *Mastomys coucha* [12,14] and *Leishmania donovani* in hamsters [15]. The reasons

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for choosing the adult parasite for fractionation are (1) dead adults are believed to contribute to filarial pathological manifestations [16], (2) adult worms have both pro- and anti-inflammatory mediator-stimulating molecules and these molecules are considered responsible for some of the severe and debilitating disease manifestations [17], (3) adult worms provide a large variety of antigens including those common to mf and L_3, and (4) the large size of this stage facilitates availability of adequate quantity of parasite material. Further, for precise identification of fractions inducing pro-inflammatory and anti-inflammatory cytokine release, the adult soluble extract was fractionated by SDS-PAGE and 15 fractions thus resolved were tested for their pro- or anti-inflammatory cytokine-releasing potential using THP-1 cell line [12]. In the present study four of these fractions (three pro-inflammatory cytokine-releasing and one anti-inflammatory cytokine-releasing) were selected to determine which of these has the potential to influence the establishment of human *B. malayi* infection in the rodent models *M. coucha* and jird (*Meriones unguiculatus*) after immunization of the animals with the fraction and to identify the proteins of this fraction by 2DE and MALDI-TOF. The immunological responses of *M. coucha* to this fraction were also studied.

2. Materials and methods

2.1. Isolation of parasite stages

B. malayi infection in jirds was established and maintained as described elsewhere [18]. Adult worms (BmA) were collected from peritoneal cavity of the infected jirds and held in PBS (pH 7.2) at room temperature for about 30 min before use.

B. malayi L_3 were freshly isolated from mosquitoes as described elsewhere [19] and used.

2.2. Preparation and 1D fractionation of adult worm extracts

Soluble somatic extract of freshly collected *B. malayi* adult worms (BmAS) was prepared and resolved by 10% SDS-PAGE as described earlier [20]. Resolved fractions of interest were identified with the help of pre-stained molecular weight markers run simultaneously and cut using sharp and clean scalpel. The bands were designated as F1 to F15. The fractions selected for the present study were F5 (68–84 kDa; stimulates IL-1β release), F6 (54–68 kDa; stimulates TNFα, IL-1β and IL-6), F10 (38–42 kDa; stimulates IL-1β) and F14 (20–28 kDa; stimulates IL-10 release).

Proteins from gel strips were electro eluted (Electroeluter, Millipore, India), concentrated (Centricon of 3 and 10 kDa cut off; Millipore, India), and content estimated [21]. The molecular weight of the proteins in fractions was confirmed in 1D SDS-PAGE as above and then stored in aliquots at −20 °C till use.

2.3. Animals

Healthy 8–10-week-old male *M. coucha* and jird were used in the study. All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines which are similar to those of the Swiss Academy of Medical Sciences, for use and handling of animals. The animals were housed in climate – (23 ± 2 °C; RH: 60%) and photoperiod – (12 h light–dark cycles) controlled animal quarters and fed standard rodent diet pellets with free access to drinking water.

2.4. Immunization of animals with fractions and exposure to infection

2.4.1. M. coucha

The procedure described by Dixit et al. [12,14] was used for immunization of animals and their exposure to the parasite life stages. Groups of 6–8 *M. coucha* in two independent experiments received three subcutaneous injections (once weekly) of F5 (Groups IF5, IF5-A, IF5-L), F6 (Groups IF6, IF6-A, IF6-L), F10 (Groups IF10, IF10-A, IF10-L) and F14 (Groups IF14, IF14-A, IF14-L) in PBS. The first injection contained the fraction (∼10 μg protein/animal) mixed with Freund's complete adjuvant (FCA) and the subsequent two injections contained half the amount of protein mixed with Freund's incomplete adjuvant (FIA). Groups S, S-A and S-L received the injections of PBS in place of fractions; on day 21 post first injection (p.f.i.), S-A, IF5-A, IF6-A, IF10-A and IF14-A received intraperitoneal instillation of freshly isolated 10 female and 5 male adult worms per animal [18] while S-L, IF5-L, IF6-L, IF10-L and IF14-L received s.c. injection of L_3 (100/animal) [19]. Thus, Group S served as control for Groups IF5, IF6, IF10 and IF14; Group S-A served as control for Groups IF5-A, IF6-A, IF10-A and IF14-A and Group S-L served as control for IF5-L, IF6-L, IF10-L and IF14-L.

Animals were sacrificed under deep ether anaesthesia on day 28/30 post adult instillation (p.a.i.) or day 150 post L_3 inoculation (p.l.i.); Groups S, IF5, IF6, IF10 and IF14 were sacrificed on day-21 p.f.i.

2.4.2. Jirds

Groups of jirds (6/group) were immunized with F6 (JIF6-L) or injected PBS (JS-L) in place of F6 as above and given i.p. inoculation of L_3 (100/animal). Animals were killed as above on day-90 p.l.i.

2.5. Parasite burden

2.5.1. M. coucha

Microfilaraemia was assessed in L_3-exposed animals on day 90 p.l.i and thereafter at weekly intervals till termination of the experiment (day 150 p.l.i). 10 μl of tail blood was drawn from each animal between 12 noon and 1.00 pm and smeared on clean glass slide. Dried smears were dehaemoglobinized, air dried and stained with Leishman stain. Mf was counted under microscope [19]. On autopsy, the heart, lungs and testes were isolated, teased, examined, parasite recovered counted and recorded [19]. Similarly, adult worms from peritoneal cavity of *M. coucha* transplanted with the worms were collected, examined for abnormality if any, and counted [12,14]. During autopsy, mf in peritoneal fluid of the animals was counted in 5 μl of peritoneal fluid using counting chamber method [22]. Condition of adult worms (calcification, etc.) including their uterine contents was examined as per method described by Gaur et al. [23].

2.5.2. M. unguiculatus

The jirds were killed and parasite burden (both mf and adult worms) was assessed as described above.

2.6. Two-dimensional electrophoresis and MALDI-TOF analysis

For 2D electrophoresis (2DE), BmAS was extracted in lysis buffer containing 20 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, 1 mM EDTA and protease inhibitor. About 70 μg of BmAS was loaded in immobiline Dry Strip (pH 4–7; 7 cm; Amersham Biosciences, GE healthcare) with overnight rehyration. Isoelectric focusing (1st dimension) was performed using the Ettan IPGphor 3 at 20 °C for a minimum of 14 kVh. After IEF, the strip was double equilibriated with DTT and iodoacetamide in equilibriation buffer (0.05 M Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% Bromophenol blue) for 15 min each at room temperature. Second dimension was run by transferring IPG strips to 12.5% SDS-PAGE. The gels were stained with Coomassie brilliant blue (Sigma, St. Louis, USA) and resolved protein spots of interest were excised manually.

The in-gel digestion of proteins and purification of peptides from spots was carried out according to the manufacturer's instructions (ABI). Briefly, after excision, protein spots were washed in deionized water and 50% (v/v) acetonitrile (ACN) in 25 mM ammonium bicarbonate (pH 8.0), dehydrated in ACN and were allowed to dry in vacuum. Dried gel spots were hydrated in digestion buffer containing sequencing grade modified trypsin (12 μg/ml; Promega, Madison, USA). Peptides were extracted in 100 μl of 0.3% TFA in 50% ACN, vacuum dried and re-dissolved in 10 μl of 0.1% TFA in milli-Q water. Then peptides were purified with C18 reversed-phase mini-column filled in ZipTip C18 (Millipore, Bedford, USA) and eluted with 5 μl of 0.1% TFA in 50% ACN. The purified peptide solution was then mixed with double volume of matrix containing 10 mg/ml Alpha CHCA (ABI, Farmingham, USA) and spotted onto a MALDI sample plate.

MS and MS/MS spectrum were acquired in the positive ion mode on MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Farmingham, USA). Close external mass calibration for MS was performed with 4700 Cal Mix (Applied Biosystems). Only baseline corrections were applied to the raw data.

For protein identifications, database search was carried out using Global Proteome Server v 3.5 software (Applied Biosystems) equipped with MASCOT (Matrix Science) search engine. Search parameters allowed a maximum of one missed cleavage, the modification of cysteine, the possible oxidation of methionine, peptide tolerance of 100 ppm and MS/MS tolerance of 0.1 Da. Spectra were searched against Brugia coding sequence database composed of both genomic (http://www.tigr.org/tdb/e2k1/bma1/intro.shtml) and EST (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gireport.pl?gudb=b malayi) sequences. The significance threshold was set at $P < 0.05$, and identification required that each protein contained at least one peptide with an expected value <0.05. For those spots that did not get a significant match in Brugia database, the search was performed in NCBInr, SwissProt or MSDB (Table 1).

2.7. NO determination

NO determination was carried out according to Dixit et al. [14]. Briefly, before peritoneal macrophage collection abdominal portion of the animals (Groups S and IF6) were cleaned with 70% ethyl alcohol. Aseptically 4–5 ml Dulbecco's modified Eagle's medium (DMEM) containing EDTA (0.1%) and antibiotics (penicillin: 100 U/ml; streptomycin: 100 μg/ml) was injected into peritoneal cavity of the animals and collected the lavage. Peritoneal cells thus obtained, were washed thoroughly with the medium, suspended in the medium containing 10% fetal bovine serum at a conc. of 2×10^6 cells/ml, and dispensed into sterile 48-well tissue culture plates (Nunc-rosklide, Denmark). After overnight incubation at 37 °C in 5% CO_2 atmosphere adherent cells were replenished with fresh medium and stimulants added (F6: 1 μg protein/ml; LPS: 1 μg/ml) followed by incubation at the same atmosphere. The presence of nitrite in culture supernatants of 48 h post-stimulation (PS) was quantified [24].

2.8. Lymphocyte transformation test

To assess CMI response lymphocyte transformation test (LTT) was carried out broadly following the method of Klei et al. [25] with some modifications to suit our condition [26]. Briefly, the animals (Groups S and IF6) were killed by an overdose of anaesthetic ether and spleen was collected in RPMI-1640 medium containing 100 U penicillin and 100 μg streptavidin/ml, splenocyte suspension was prepared, viability of the cells checked by 0.1% trypan blue dye exclusion method, and final conc. of the cells adjusted to 2×10^6 cells/ml [26]. The cells at 4×10^5 in 200 μl medium/well in 96-well plate (Nunc-rosklide, Denmark) were charged with F6 (1 μg protein/ml) or Con A (10 μg/ml) and incubated at 37 °C in 5% CO_2 atmosphere; cells incubated in medium only served as control. At 72 h PS, ^{3}H-Thymidine (1 μCi/well) was added and 16–18 h later the cells were harvested, suspended in scintillation fluid and β-emission was quantified in a scintillation counter (LS Analyzer, Beckman Inc.). The results were expressed as count per minute (cpm).

2.9. Cytokine assay

We have assayed six cytokines TNF-α, IL-1β, IL-6, IL-10, IFN-γ and IL-2 in the supernatants of splenocytes of animals immunized with F6. The reasons for this are (1) the fraction of interest stimulated the release of TNF-α, IL-1β, IL-6 from THP-1 cells and (2) IL-10, IFN-γ and IL-2 are reported to be associated with filarial parasite survival in the host [27].

Splenocytes isolated as above were plated in sterile 24-well plates (Nunc-rosklide, Denmark) at 2×10^6 ml^{-1} conc. and stimulated with F6 (1 μg protein/ml) or LPS (1 μg/ml) for 48 h under the same incubation condition. The cytokines were determined in the 48 h PS culture supernatants.

For the assay mouse monoclonal antibodies of TNF-α, IL-6, IL-10, IL-2 (Pharmingen) IFN-γ and IL-1β (Pierce Endogen, Rockford, IL, USA) were used in a paired antibody sandwich ELISA method following the manufacturer's instructions with some modifications to suit our conditions [28]. Triplicates of each sample were run separately. The concentration of the cytokines was calculated using OD readings for standards (suitable for the paired antibodies obtained from the above source).

2.10. Antibody determination

Filaria specific IgG and its subtypes were detected in sera of animals [29]. Briefly, Maxisorp ELISA strips (Nunc) were coated with the fractions (0.1 μg protein/ml) prepared in carbonate buffer (0.06 M; pH 9.6). Optimally diluted sera (diluent: 1% BSA in PBS + 0.05% T_{20}) were added (IgG: 1:250; IgG subclasses: 1:25) to the wells. As IgG2a levels were found very low at this dilution, the sera were titrated at 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 dilutions and compared with non-immune sera. After incubation with the sera the wells were washed and probed with HRP-conjugated rabbit anti-mouse-IgG and its subtypes (Sigma Chem. Co, USA) at 1:1000 dilution. Orthophenylenediamine (OPD) was used as substrate and absorbance was read at 492 nm in an ELISA reader (PowerWave$_X$, BioTek, USA).

2.11. Statistical analysis

Results were presented as mean ± S.D. of two experiments using 6–8 animals per group and the data were analyzed in GraphPad Prism 3.03 using Tukeys multiple comparison or student's '*t*'-tests. Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. Effect of immunization of animals with the fractions on parasite burden

The effect of immunization of *M. coucha* with different fractions on the survival of subsequently intraperitoneally implanted adult worms and establishment of infection after s.c. injection of L_3 were investigated. Adult worm recovery from p.c. was significantly reduced (Fig. 1A) in F6-immunized (IF6-A; 72%; $P < 0.01$) and F14-immunized (F14-A; 66%; $P < 0.05$) animals as compared to immunization controls (S-A). Mf population in the peritoneal

Table 1
MALDI analysis of proteins in 2DE spots of four selected fractions of soluble *Brugia malayi* adult worm extract. % Confidence interval is automatically calculated by the GPS software. Critical score (individual ion scores greater than this indicate identity or extensive homology; $P < 0.05$) was taken from Mascot searches performed online directly.

Fraction	Spot no.	kDa/p*I*	Matched protein (species)	Acc. no.	Database	Peptide matches/Mascot score	% Confidence interval/Critical score	Species	Function
F5	18	73.7/5.4	Probable vacuolar proton translocating ATPase-subunit a	P30628 (VPP1_CAEEL)	Swiss_Prot	1/46	Not available/39	*Caenorhabditis elegans*	Assembly and activity of the vacuolar ATPase.
	20	75.9/5.5	Troponin t protein 2	TC7854	Bmalayi CDS	2/44	Not available/33	*Brugia malayi*	Regulation of muscle contraction [73]
F6	28	62.1/5.5	Hypothetical protein CBG00623	gi 39587170	NCBInr	9/61	91.8/not available	*Caenorhabditis briggsae*	
	29	62.1/5.5	Chaperonin homolog HSP60, mitochondrial precursor, putative	13718.m00044	Bmalayi CDS	1/39	Not available/34	*Brugia malayi*	Immunostimulatory [61]
	31	62.9/6.0	dTDP-D-glucose 4,6-dehydratase	Q8VDR7	Swiss_Prot	10/71	99.6/not available	*Mus musculus*	Similar to CDP-D-glucose 4,6-dehydratase and involved in producing immunologically active sugars [74]
	32	62.6/6.2	Cytoplasmic intermediate filament protein, putative	15463.m00018	Bmalayi CDS	3/42	Not available/34	*Brugia malayi*	Cross-reactive antigen [75]
	33	63.4/6.5	Elongation factor 2	gi 34597234	NCBInr	15/75	99.6/not available	*Scolopocryptops sexspinosus*	Th1 response [56]
F10	50	40.0/5.3	Disorganized muscle protein 1	14972.m07771	Bmalayi CDS	5/135	Not available/33	*Brugia malayi*	Similarity to As37 antigen, role in protective immunity [76]
F14	54	23.0/4.6	Unknown	gi 55233390	NCBInr	7/70	98.8/not available	*Anopheles gambiae*	
	55	22.1/4.5	Unknown	gi 55238336	NCBInr	14/71	99.0/not available	*Anopheles gambiae*	
	58	21.4/6.3	P27 (a small HSP homolog of nematodes)	TC7940	Bmalayi CDS	6/144	Not available/33	*Brugia malayi*	L_3 and L_4 specific antigen in *D. immitis* and *B malayi* [72]

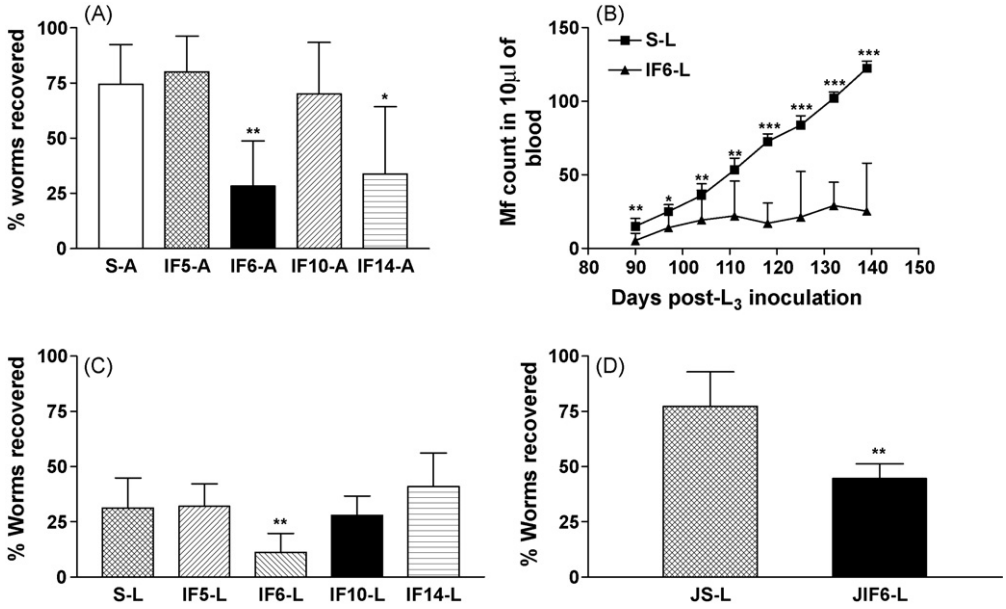

Fig. 1. (A) Worm recovery on day 28/30 post-intraperitoneal instillation of *Brugia malayi* adult worms in *Mastomys coucha* pre-immunized with F5 (IF5-A), F6 (IF6-A), F10 (IF10-A) and F14 (IF14-A). Controls (S-A) were given PBS in place of fractions. Values are mean ± S.D. of data from 6 to 8 animals in two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, (vs. S-A), Student's *t*-test. (B) Microfilaraemia (starting from 90 days post-larval inoculation) in *M. coucha* immunized with F6 and subsequently inoculated with L_3 7 days after last immunization dose (IF6-L) and in non-immunized L_3 inoculated control (S-L). $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, (vs. S-L) Student's *t* test. (C) Percent worm recovery 150 days after *Brugia malayi* L_3 inoculation in *M. coucha* pre-immunized with the fractions (IF5-L, IF6-L, IF10-L and IF14-L). Controls (S-L) were given PBS in place of fractions. $^{**}P < 0.01$ (vs. S-L), Student's *t*-test. (D) Percent worm recovery 90 days after *Brugia malayi* L_3 instillation into peritoneal cavity of jirds pre-immunized with F6 (JIF6-L). Controls (JS-L) received PBS in place of the fraction. $^{**}P < 0.01$ (vs. JS-L), unpaired *t* test.

fluid of IF6-A was significantly less than that found in S-A (data not shown). IF5-A and IF10-A animals showed worm recovery comparable to S-A (Fig. 1A); mf population in IF5-A, IF10-A and IF14-A was also comparable to S-A (data not shown).

In *M. coucha* immunized with F6 and subsequently inoculated with L_3 (IF6-L) and in non-immunized control (S-L), mf appeared in the peripheral blood between day 90 and 118 p.l.i. (mean ± SD: 99.33 ± 16.17). While the microfilaraemia increased progressively in S-L animals, there was no significant increase in microfilaraemia in IF6-L animals (Fig. 1B) till the day of sacrifice (150 p.l.i). Further, IF6-L animals showed 64% ($P < 0.01$) lesser adult worm burden (Fig. 1C). No significant difference was found in adult worm yield between immunized animals receiving L_3 (IF5-L, IF10-L, IF14-L) and controls (S-L groups) (Fig. 1C). Microfilaraemia in these animals was comparable to control animal (data not shown). In summary, immunization with F6 suppressed the establishment of L_3-initiated infection in *M. coucha* as evident by suppressed microfilaraemia and low adult worm yield.

Jirds immunized with F6 and subsequently inoculated with L_3 (JF6-L) showed 42% lesser yield ($P < 0.01$) of adult parasites than in control animals (Fig. 1D). However, number of mf present in this group was not as high as its counterpart JS-L (data not shown).

Taken together, these findings demonstrate that immunization with F6 (i) imparted protection against establishment of L_3-initiated infection both in terms of mf load (*M. coucha*) and adult worm burden (*M. coucha* and jirds) and (ii) reduced the survival of i.p. instilled adult worms (*M. coucha*). Immunization with F14 also reduced the peritoneally instilled adult worm yield in *M. coucha* but to a lesser extent.

3.2. 2DE and MALDI analysis

Eleven well resolved protein spots in CBB stained gels are shown in the four fractions of interest (Fig. 2). The details of the protein spots, their molecular weight and *p*I identified by MALDI are shown in Table 1. F5 has two protein spots identified to be vacuolar proton

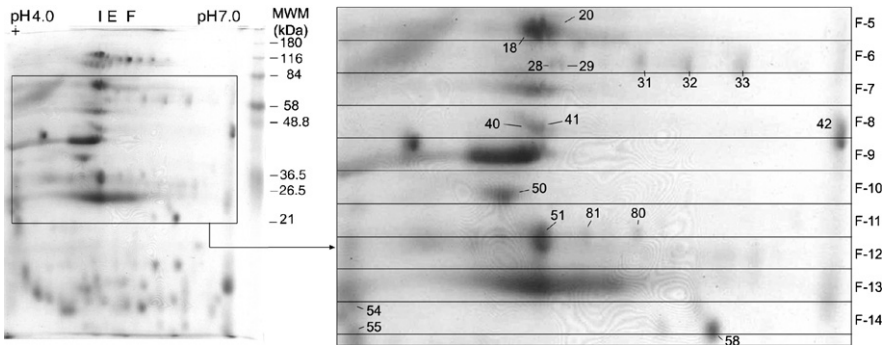

Fig. 2. Soluble *Brugia malayi* adult worm extract (70 µg protein) separated by 2DE and stained with CBB. First dimension isoelectric focusing was performed on an Ettan IPGPhor (GE Healthcare, Bio-sciences Ltd.) with a pH 4–7 linear Immobiline DryStrip of 7 cm (GE Healthcare, Bio-sciences Ltd.). Second dimension separation was on a 12.5% SDS-PAGE. Boxed area is enlarged to show details. Numbers indicate protein spots that were processed for MALDI.

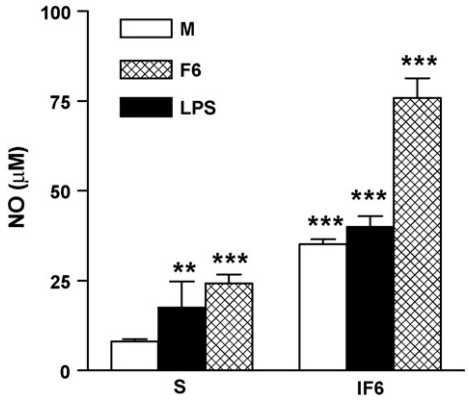

Fig. 3. Nitric oxide release from peritoneal macrophages of *M. coucha* immunized with F6 (IF6) in response to *in vitro* challenge with the F6, LPS or medium only (M). NO in the cell-free supernatants was quantified using Griess reagent. The assay was performed 7 days after the last immunizing dose. Values are mean ± S.D. of data from 6 to 8 animals in two experiments. $^{**}P < 0.01$ (S-M vs. S-LPS); $^{***}P < 0.001$ (S-M vs. S-F6; IF6-M vs. IF6-F6; S-M vs. IF6-M; S-LPS vs. IF6-LPS; S-F6 vs. IF6-F6). Tukeys multiple comparison test/Student's *t*-test.

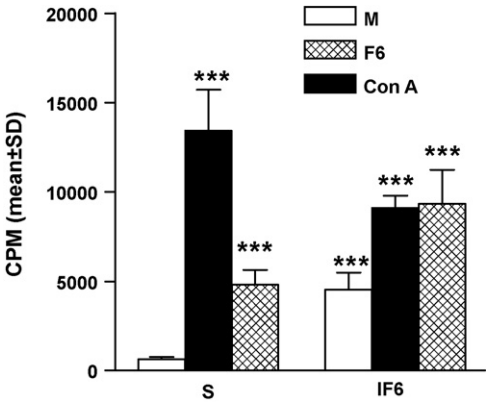

Fig. 4. Proliferative response of spleen cells from *M. coucha* immunized with F6 (IF6) or PBS in place of fractions (S), to *in vitro* challenge with F6, Con A or medium only (M). The assay was performed 7 days after the last immunizing dose. Values (CPM of ^{3}H-Thymidine incorporated into cells) are mean ± S.D. of data from 6 to 8 animals in two experiments. $^{***}P < 0.001$ (S vs. IF6; S-M vs. S-ConA/S-F6; IF6-M vs. IF6-ConA/IF6-F6; S-M vs. IF6-M, S-F6 vs. IF6-F6), Tukeys multiple comparison/Student's *t*-test.

translocating ATPase-subunit 'a' of *Caenorhabditis elegans*, and Troponin t protein 2 of *B. malayi*. Five proteins were identified in F6: chaperonin homolog HSP60, cytoplasmic intermediate filament, elongation factor 2; one protein matched with dTDP-D-glucose 4,6-dehydratase of *Mus musculus* and a hypothetical protein. One protein spot identified in F10 was disorganized muscle protein 1 which has similarity with *Ascaris* antigen As37. Out of three spots in F14 one was identified to be P27, a small HSP of *B. malayi*; the other two protein spots could not be identified.

3.3. NO response

NO release from macrophages of the animals (Groups S and IF6) was increased by exposure to F6 or LPS in vitro as compared to cells of S animals ($P < 0.05$-0.001; Fig. 3). In summary, F6 was able to induce greater NO production.

3.4. Cellular proliferative response

Experiments were carried out to see the effect of immunization with F6 on the proliferative responses of splenocytes of the animals to in vitro stimulation with the fraction and to correlate these effects with the survival of adult worms in the peritoneal cavity and establishment of L_3-induced infection. As expected cells from S animals proliferated several folds by Con A compared to unexposed cells. However, proliferation of cells of the F6-immunized animals in response to Con A was comparable to response of cells from S animals. Response of cells to F6 of F6-immunized animals was significantly high ($P < 0.001$) as compared to cells of their counterpart (S animals) (Fig. 4). In summary, molecules of F6 upregulated cellular proliferation.

3.5. Cytokine response

In this study we have investigated the effect of immunization with F6 on the cytokine release by cells from the immunized animals when challenged with the same fraction in vitro. TNF-α (Fig. 5A), IL-1β (Fig. 5B), IL-6 (Fig. 5C) and IL-10 (Fig. 5D) release was upregulated ($P < 0.05$–0.001) in cells of IF6 animals compared to cells from S group animals. There was upregulation in all the above cytokines when the cells of immunized groups were exposed to LPS or F6 in vitro. Unstimulated and F6 stimulated cells from IF6 animals showed enhanced ($P < 0.001$) IL-6 response but not LPS stimulated in vitro compared to cells from S group animals (Fig. 5C). Cells from IF6 animals showed increased IL-10 release only when challenged with F6 ($P < 0.001$) but not with LPS (Fig. 5D) as compared to cells of their counter parts (S Group animals). Interestingly, in vitro exposure of F6 downregulated the IL-10 release from cells of control animals.

IF6 animal cells showed enhanced specific IFN-γ ($P < 0.05$; Fig. 5E) and IL-2 release ($P < 0.01$; Fig. 5F). In these cells, LPS was a better stimulant for IFN-γ release ($P < 0.001$; Fig. 5F) but a poor stimulant of IL-2 (Fig. 5F). In summary, the findings indicate that F6 upregulated pro-inflammatory cytokine release from cells of unimmunized or immunized animals.

3.6. Serum IgG responses

Sera of immunized animals had significantly increased levels of IgG ($P < 0.001$; Fig. 6A) as compared to control animals. Analysis of IgG subclasses revealed that all the subclasses (1:25 dilution) increased several folds over the controls with IgG2a showing the greatest increase (5.5-fold; $P < 0.001$) followed by IgG1 (3.4-fold; $P < 0.001$) and IgG2b (2.2-fold; $P < 0.001$). In IF6 animals IgG2a titre was high ($P < 0.01$–0.001) up to 1:128 dilution (Fig. 6B).

4. Discussion

Parasites elicit a broad spectrum of immune and inflammatory responses in their hosts. Majority of these responses are irrelevant in terms of protection of host and only few contribute directly to resistance to infection. However, the identity of the protective responses and their target protein antigens remained elusive. In the present study rodent hosts were immunized with four cytokine-stimulating fractions (F5, F6, F10 and F14) and were subsequently exposed to adult worms or L_3 of *B. malayi* to determine which of these fractions has the potential to influence the survival and establishment of infection.

When splenocytes of F6 immunized animals were challenged in vitro with the fraction, release of IFN-γ, TNF-α, IL-2, IL-6 and IL-1β increased. Th1-type immunity defined by IL-2 and IFN-γ production by T cells and IL-12 by NK cells and monocytes can cross-regulate the Th2-type immunity at the T cell level by enhanced blocking of antibody production [27,30–32]. Although F6 produced largely Th1 response, it also activated Th2-like immune response. It was shown that individual cytokines can have cru-

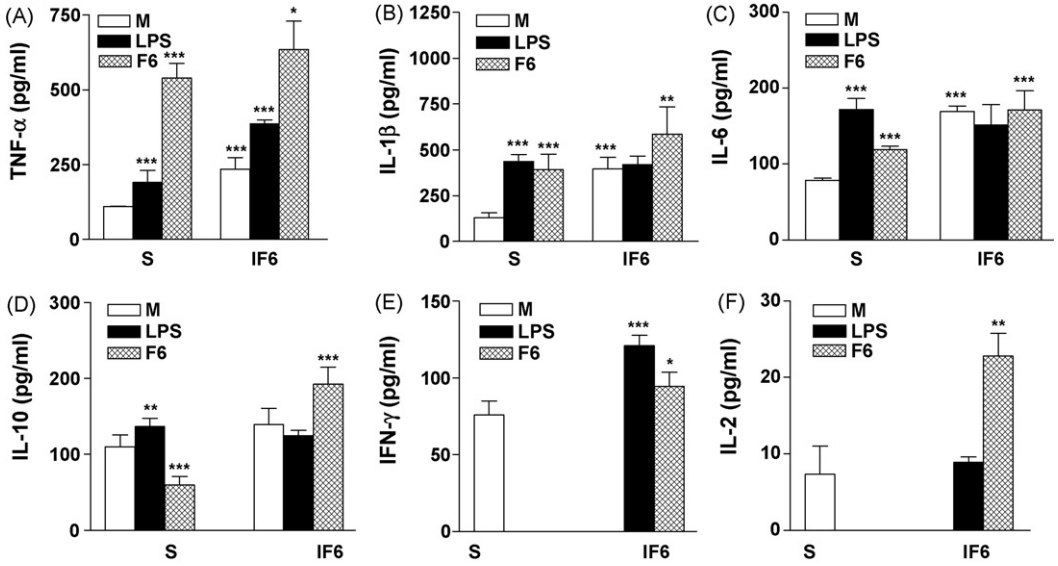

Fig. 5. TNF-α (A), IL-1β (B), IL-6 (C), IL-10 (D), IFN-γ (E) and IL-2 (F) release from spleen cells of *M. coucha* immunized with F6 (IF6) or PBS in place of fractions (S), in response to *in vitro* challenge with F6, LPS or medium only (M). Cytokines in the cell free supernatants were quantified by antibody capture ELISA. The assay was performed 7 days after the last immunizing dose. Values are mean ± S.D. of data from 6 to 8 animals in two experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ (S-M vs. S-LPS/F6 in A, B, C, D); $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (S-M/LPS/F6 vs. IF6-M/LPS/F6 in A-F). Student's *t*-test.

cial effects in both type-1 and type-2 responses. For example, although TNF-α is regarded as a type-1 cytokine, it is required at an early stage of infection to synergize with the Th2-effector mechanism that determines the parasite elimination or persistence [33]. Experimental evidence shows that regulation of Th1- and Th2-responses protects schistosome infected animals against 'overzealous' immune responses of either type 1 or type 2 [34].

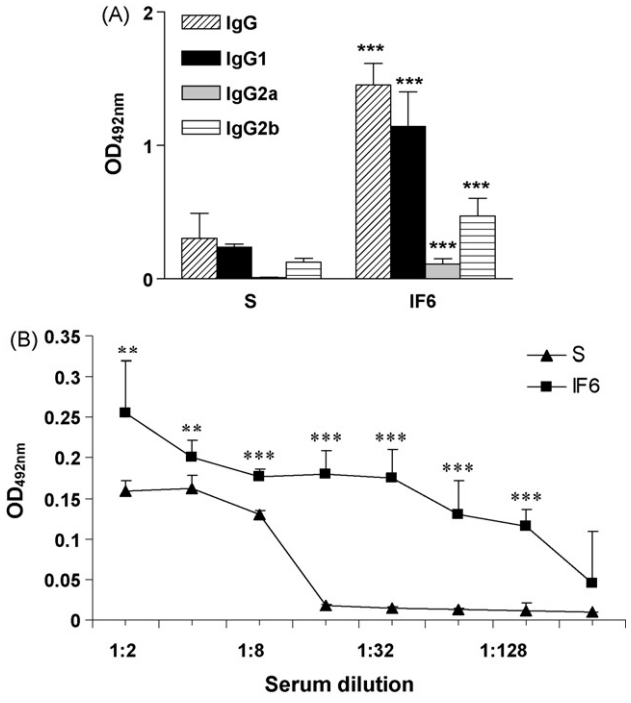

Fig. 6. (A) Specific IgG and its subtypes in sera *M. coucha* immunized with F6 (IF6) or PBS in place of fractions (S). IgG and its subtypes were determined by ELISA using commercially available probes (rabbit anti-mouse-IgG and its subtypes conjugated with horseradish peroxidase). Values are mean ± S.D. of data from 6 to 8 animals in two experiments. $^{***}P < 0.001$ (vs. corresponding parameters of S), Student's *t*-test. (B) Specific IgG2a titre determined by ELISA in sera of *M. coucha* immunized with F6 (IF6) or PBS (S). $^{**}P < 0.01$, $^{***}P < 0.001$ (vs. S), Student's *t*-test.

In filarial endemic areas Th1-like antifilarial immune response predominate in antigen negative subjects and contributes to protection in putatively immune individuals [35] but in chronic lymphatic filarial patients, both antigen specific tolerance and bystander suppression operate [36–38]. Several investigators [39–42] have reported that certain fractions/products of the parasites facilitate parasite survival by immunosuppression of host while other products of the parasite facilitate immunostimulation and inflammatory pathology. In the present study we found that immunization with F6 a strong pro-inflammatory cytokine stimulator largely prevented the survival of intraperitoneally instilled adult parasites and establishment of L_3-induced infection in *M. coucha*. Further, significant inhibition in L_3 establishment in F6-immunized jirds substantiated the protective efficacy of F6. Immunization with F14, a weak anti-inflammatory cytokine (IL-10) stimulator, failed to affect the development of L_3 induced infection though it decreased the survival of intraperitoneally instilled adult worms. The mechanism behind this selective ability of F14 to affect adult worms but not L_3 is not clear. It has recently been shown that IL-10 stimulates pro-inflammatory cytokines probably by feed back mechanisms [43]. Though F5 and F10 also induced the pro-inflammatory cytokine IL-1β and were expected to show protection against the infection, they failed to do so indicating that IL-1β alone was not sufficient to confer protection.

Modulation of lymphocyte proliferation has been shown to result in alterations in survival of parasites [13,44]. In the present study immunization with F6 resulted in about threefold enhancement in cell proliferation in response to F6 challenge *in vitro* compared to control. F6 challenge of cells of even non-immunized animals significantly enhanced the cell proliferation indicating that F6 is a potent stimulator of cell proliferation.

F6 also stimulated the release of NO from cells of immunized animals indicating that enhanced NO release may contribute to the mechanism(s) by which the parasite may be eliminated by F6. NO is a critical mediator in Th1/Th2 balance and also an important self-regulatory molecule that prevents the over expression of Th1 response [45]. In filariasis, NO-mediated mechanisms have been shown to be capable of killing mf *in vitro* and L_3 *in vivo* and protect the host through type 1 responses and IFN-γ stimulated toxic mediators' release [46,47]. Filarial parasites can induce NO produc-

tion from endothelial cells [48] and its release may directly affect parasites' survival in the lymphatics.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is one of the well known immunological mechanisms by which filarial parasites are killed in vitro and in vivo [49–53]. ADCC reaction involves engagement of macrophages, eosinophils and neutrophils with surface receptors FcR that bind to antibody attached to the parasite surface, and release of toxic mediators by these cells onto the parasite surface leading to death of the parasites [52]. In the present study IgG and its subclasses were estimated because IgG is known to be involved in removal of the filarial parasites by ADCC [52]. IgG1 is indicative of antigen specific antibody response whereas IgG2a and IgG2b are indicative of Th1 and Th2 type responses, respectively [54]. We observed inverse correlation between elevated levels of the IgG subclasses (IgG1, IgG2a and IgG2b) and establishment of infection in F6-immunized animals. We also observed adherence of normal *M. coucha* splenocytes to *B. malayi* L_3 when incubated with serum of F6-immunized animals resulting subsequently in paralysis and death of the larvae (authors' unpublished observation). These findings suggest that one of the mechanisms by which F6 might protect host against infection is an ADCC reaction. Further, the findings support the report that classical killing of filarial parasite also depends on type 2 responses in which large amounts of antibody of different isotypes, including IgE are produced [55]. MALDI-TOF analysis revealed five proteins in F6 fraction of which three were immunostimulatory, viz. elongation factor 2 (EF2), heat-shock protein 60 (HSP60) and intermediate filament. EF-2 identified in our study has not been reported earlier in *B. malayi* parasites. This protein was reported to induce Th1 response in cured visceral leishmaniasis patients and in leishmanial parasite-specific T cell lines derived from an immune donor [56]. HSPs are evolutionarily highly conserved molecular chaperones and are known to participate in protein folding [57] antigen-processing pathway [58–60], innate immunity [61], antigen-specific immunity through predominantly Th1 [62] and cytokine pathways [63] in several microbial and parasitic diseases [64–66]. HSP70, was earlier reported to be present in larval and adult *B. malayi* [67]. Recently Suba et al. [68] have shown elevated levels of *B. malayi Wolbachia* HSP60 specific IgG1 in the sera of chronic filarial patients compared to microfilaraemics and endemic normals. The antibody is also known to play a role in autoimmune arthritis, atherosclerosis and idiopathic diseases [69–71]. p27, a small HSP identified in anti-inflammatory F14 fraction, was reported to be expressed constitutively throughout the life stages of *B. malayi* in the host [72]. We presume that protection conferred by F14 against intraperitoneally instilled adult worms in the animals may be due to the presence of p27 protein but anti-inflammatory cytokine release stimulating property of F14 and presence of p27 protein is not clear at present.

In summary, F6 imparted protection against establishment of *B. malayi* L_3-initiated infection or survival of intraperitoneally implanted adult worms; F14 affected intraperitoneally instilled adult worms only whereas F5 and F10 failed to provide protection against L_3-initiated or adult transplanted infection. F6 induced intense upregulation of both Th1 (IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IgG1, IgG2a and lymphoproliferation) and Th2 (IgG2b and IL-10) responses and NO release. These findings suggest that F6 protects the host against the parasite stages via Th1/Th2 type responses. Presence of three immunostimulatory proteins (HSP60, intermediate filament protein, EF-2) in the fraction as revealed by 2DE and MALDI-TOF analysis further substantiated these findings. Studies directed towards cloning, expression and characterization of stimulatory proteins of F6 for development as a possible vaccine candidate are in progress.

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