Summary
6. Summary

Lymphatic filariosis, caused by *Wuchereria bancrofti* and *Brugia malayi* is not a fatal disease, it is responsible for considerable morbidity causing social stigma. It is the disease of poor people of many tropical and subtropical countries in both urban and rural areas. Globally around 1100 million people are at risk and 78.6 million showing mf or overt disease. In India, more than 400 million people are at risk of and 48.5 million people are affected with lymphatic filariosis (27 million carry mf in the circulation and nearly 22 million suffer from disease manifestations). The acute disease manifestations which affect more than 50% of the exposed subjects in endemic areas are characterized by periodic and self limiting episodes of adenolymphangitis, fever and associated constitutional symptoms, while the chronic disease includes long lasting manifestations such as elephantiasis, lymphoedema and/or hydrocoele. The recurrent episodic bouts of acute manifestations occurring over a period of time lead to the development of chronic disease manifestations.

Our current understanding of the pathogenesis of filarial infection is rather poor. It is generally believed that inflammatory and immunological processes might be involved in the initiation of acute episodes (WHO, 1992). Available information on tissue pathology suggests that though clinical manifestations have their own range of severity, complications and sequelae, they share abnormal lymphatic function. However, the precise mechanism underlying lymphatic pathology is poorly understood. It is believed, almost to certainty, that the clinical manifestations, which are largely due to lymphatic dysfunction, are the result of overt immunological reactions to the continuous release of parasite product rather than simple mechanical obstruction by the dead worms and/or the dying worm products.

During the past decade several important conceptual advances have been made towards understanding the pathogenesis of lymphatic lesions in bancroftian and brugian filariosis. These concepts are based primarily on the observation in experimental animal models of *Brugia* sp. in ferrets, dogs, cats and immunodeficient mice. These models mimic some of the aspects of filarial disease in human beings and provided clear evidence that much of the pathology results from the host immune response to the parasites. There is evidence that a variety of inflammatory mediators such as arachidonic acid metabolites,
prostaglandins, kinins, complement, eosinophilic granules and cytokines like interleukins (IL-1β & 6) are involved in the inflammatory reactions in filarial patients. Although the immunological basis of clinical manifestations is well known, the identity of antigen(s) evoking inflammatory reaction and the profile of the inflammatory mediators responsible have not been clearly delineated. Recent studies in primates with lymphatic filariid, *B. malayi*-infected Indian leaf monkey (*Presbytis entellus*) / *Macaca mulata* have shown some evidences that the parasite antigens may be responsible for the episodic acute inflammatory (edematous) reaction in the limbs. The precise identity of the antigens and how the antigens participate in the inflammatory cascade remain to be investigated. The recent studies showed that inflammatory cytokines play a central role in the inflammatory reactions in parasitic infections. Tumor necrosis factor-alpha (TNF-α) has been shown to be an important inflammatory mediator capable of producing symptoms such as fever, chills and myalgia in acute filarial disease episode development. These findings suggest that certain parasite antigen(s) may play an important role in the pathogenesis of clinical manifestations and emphasize the urgent need to identify the (se) antigens in an animal model that develops the manifestations. Taken together, the literature clearly indicates that the development of filarial disease manifestations is mediated by inflammatory cytokines released by the host's cellular responses against the adult filarial parasite. However the precise triggering agents involve in such reaction is not clearly known. *Mastomys coucha* and jird (*Meriones unguiculatus*) are the two rodent hosts highly susceptible to *B. malayi* infection. These models have been utilized for various studies in this laboratory and elsewhere. These have shown some resemblances with humans infected with *W. bancrofti* or *B. malayi* with regards to parasitological, immunological and pathological. The models appear to be suitable for identifying proinflammatory parasite antigen(s) with respect to their role in the development of disease manifestations. The present study was therefore, undertaken to identify and characterize the antigens of pathogenic potential using *B. malayi-M. coucha* as a model. The adult parasite was chosen for this purpose because: (a) dead adults (and the consequent accessibility of all the parasite molecules) are believed to contribute to filarial pathological manifestations (b) adults provide a large variety of antigens including those of mf; and (c) the large size of this stage facilitated obtaining adequate quantities of extracts.
Objectives:
1. Fractionation and purification of *B. malayi* antigens.
2. Identification of pro-inflammatory antigen(s) of *B. malayi* using mouse/human macrophage cell line assay system.
3. To investigate the effect of identified pro-inflammatory antigen(s) on the course of the infection in the susceptible rodent host, *M. coucha*.
4. To investigate the humoral and cell mediated immune responses of the host exposed to the pro-inflammatory antigen(s).
5. To investigate the *in vitro/in vivo* expression of inflammatory and anti-inflammatory cytokines/mediators in response to the identified antigen(s).

*BmAS* (*B. malayi* soluble extract) was fractionated by Sephadex G-200. proteins of the three main peaks obtained was estimated and for the present study. These three fractions were designated as BmAFI, BmAFII and BmAFIII. Three different batches of BmAS extract were fractionated and all the fractions were tested separately in the THP-1 cell, RAW-264.7 and mouse peritoneal macrophage system for their proinflammatory property.

**Selection and grouping of animals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Group description</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr-1</td>
<td>Normal+FCA/FIA</td>
<td>8</td>
</tr>
<tr>
<td>Gr-2</td>
<td>Normal + Ad</td>
<td>8</td>
</tr>
<tr>
<td>Gr-3</td>
<td>Normal + Mf</td>
<td>8</td>
</tr>
<tr>
<td>Gr-4</td>
<td>Normal + L3</td>
<td>8</td>
</tr>
<tr>
<td>Gr-5</td>
<td>BmAFI Immunized</td>
<td>8</td>
</tr>
<tr>
<td>Gr-6</td>
<td>BmAFI Immunized + Ad</td>
<td>8</td>
</tr>
<tr>
<td>Gr-7</td>
<td>BmAFI Immunized + Mf</td>
<td>8</td>
</tr>
<tr>
<td>Gr-8</td>
<td>BmAFI Immunized + L3</td>
<td>8</td>
</tr>
<tr>
<td>Gr-9</td>
<td>BmAFII Immunized</td>
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</tr>
<tr>
<td>Gr-10</td>
<td>BmAFII Immunized + Ad</td>
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</tr>
<tr>
<td>Gr-11</td>
<td>BmAFII Immunized + Mf</td>
<td>5</td>
</tr>
<tr>
<td>Gr-12</td>
<td>BmAFII Immunized + L3</td>
<td>5</td>
</tr>
</tbody>
</table>
The effect of immunization of *M. coucha* with BmAFI and BmAFII on the different life stages of parasite was studied. Various parameters like parasitological, macrophage population and immunological (humoral, cell proliferation and cytokines) responses, histopathology and mast cell count of the host before immunization, 21-24 days after first dose of immunization, 28-30 days post-adult transplantation (intraperitoneal) or mf-transfusion (intravenous) and 111-118 post-larval (L₃) inoculation (subcutaneous) were carried out. Sera from animals of all the groups at these time points were collected and specific IgG estimated.

**The salient findings of the present study are as follows:**

1. **TNF-α, IL-1β, IL-6 and IL-10 production in THP-1 cells stimulated with live parasites and parasite preparations (BmAS, BmAFI, BmAFII)**

Both live parasite stages and somatic extract of adult parasite were tested *in vitro* for their pro-inflammatory and anti-inflammatory cytokine release stimulating potential in THP-1 cells (human monocyte/macrophage-derived cell line). As the cells were not pre-sensitized, their response reflected the true potential of the stimulant. The results of the present study revealed that both pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokine production is stimulated by the parasite life-stage extracts in the order: Bmmf>BmL₃>BmA and that live mf stimulated the pro-inflammatory cytokines predominantly (the order being: TNF-α > IL-6 > IL-1β). Further, of these TNF-α stimulation by mf was as efficient as that by LPS. Live L₃ and adults were less efficient stimulators of pro-inflammatory cytokines. These observations indicate that mf predominantly induces an inflammatory type 1 response and that L₃ and adults induce primarily type 2 responses and this agrees with the finding of Lawrence *et al.* (1994) who showed this in a mouse model bearing various life stages of the parasite.

To investigate the pro-inflammatory and anti-inflammatory potential of parasite molecules that were inaccessible in the live parasite, the adult parasite extract (BmAS) and its fractions (BmAFI and BmAFII) were used because: (a) the large size of this stage facilitated obtaining adequate quantities of extracts; (b) adults provide a large number of antigen molecules, including those of mf; and (c) dead adults are believed to be responsible
for the filarial pathological manifestations. Of the 3 fractions BmAFII stimulated the pro-inflammatory cytokine release in the order: TNF-α > IL-1β = IL-6, whereas the anti-inflammatory cytokine IL-10 was barely stimulated. In contrast, BmAFI stimulated IL-10 several times more efficiently than BmAFII, although IL-1β and IL-6 production was also stimulated considerably. Notably, stimulation of TNF-α by BmAFI was minimal. BmAS and BmAFIII were found to be weak stimulators of pro-inflammatory cytokine production, and therefore were not followed up for detailed study. Before, going for characterization in vivo somatic fractions of the parasite were tested for release of pro-inflammatory mediator (nitric oxide: NO) by mouse peritoneal macrophages (M. coucha) and RAW-264.7 cells in culture supernatant. The results revealed that BmAFII stimulated to produce NO significantly than unstimulated macrophages in RAW cell line and peritoneal macrophages; level of NO was in order of BmAFII>LPS>BmAFI>BmAS showing stronger stimulator of NO production. This was proved by using aminoguanidine (inhibitor of NO).

2. **Effect of immunization with BmAFI and BmAFII on the various parasite life stages-induced infection (adult, mf and L3), immune responses and inflammatory mediators (transplanted adult worm recovery, immune responses and inflammatory mediators).**

   Animals immunized with BmAFII and subsequently intraperitoneally instilled with adult worms showed no adult worms or mf recovery from peritoneal cavity. In contrast, BmAFI immunized animals showed worm recovery comparable to control (FCA/FIA only-injected) animals. Further, the worms recovered from BmAFI-immunized animals were all live whereas the control animals showed several dead and calcified masses of worms. Mf were also found in the peritoneal cavity of all the BmAFI-immunized and control animals. Immunized with BmAFI, but not BmAFII, permitted worm survival in the animals.

   BmAFII, but not BmAFI was marginally capable of eliminating peripheral mf count marginally.

   BmAFII imparted protection showing more than 80% lesser establishment of L3 whereas BmAFI promoted establishment of infection in *M. coucha.*
Immunization with BmAFI or BmAFII significantly increased the peritoneal macrophage population with BmAFII; the increase was greater for BmAFII. However, after mf or adult transplantation in BmAFII-immunized animals the macrophage population decreased significantly, mf or adult transplantation in BmAFI-immunized animals could not alter the cell population. In contrast, L3 inoculation substantially increased the macrophage population of BmAFII-immunized animals.

After exposure to adult worm, mf or L3 in BmAFII-immunized animals the NO release increased significantly. In contrast, NO release from BmAFI-immunized remained unaltered after exposure to the parasite life stages indicating that BmAFII has adult specific NO stimulating molecules.

Immunization of animals with BmAFII and subsequent intraperitoneal implantation of adult worms or exposure to L3 enhanced the CMI response, whereas BmAFI+adult/L3 inoculated animals showed either comparable or very low response in CMI response. Mf inoculation did not alter the proliferative response in both BmAFI and BmAFII-immunized animals. BmAFII+L3 inoculated animals showed larger increase to Con A stimulation and to some extend BmAFI-immunized+L3 inoculated ones.

TGF-β release was observed to be increased (P<0.001) in animals of both BmAFI- and BmAFII-immunized animals, the higher level being shown by BmAFI-immunized group. Parasite life stages altered the release more intensely in BmAFI-immunized animals than BmAFII-immunized animals indicating BmAFI has stronger TGF-β release stimulating potential.

BmAFI and BmAFII are able to suppress the IL-10 production significantly but mf or adult inoculation in these animals did not alter the release of cytokine. In contrast, L3 inoculation to BmAFI-immunized animals upregulated the release whereas L3 failed to upregulate the release in BmAFII-immunized. The results show that BmAFI has strong IL-10 release stimulating molecules.

The pattern of TNF-α, release from cells of animals immunized with both the fractions alone or immunized animals exposed to adult worms was almost same as that in
case of IL-10 release. Mf or L3 inoculation increased the release of TNF-α from BmAFI-immunized animals but not from BmAFII-immunized ones indicating that BmAFI fraction has mf and L3 specific TNF-α stimulating molecules; Moreover BmAFI has stronger TNF-α release stimulating molecule than BmAFII.

Immunization of animals with BmAFI or BmAFII resulted suppression in IFN-γ release but implantation of adult worms or L3 inoculation upregulated the cytokine release, indicating that both the fractions have similar type of IFN-γ-inducing molecules of adult and L3 specific. While BmAFI-immunized animals bearing mf showed enhanced IFN-γ response mf exposure in BmAFII-immunized animals could not alter the level.

IL-6 response increased in BmAFI or BmAFII-immunized animals but the level being more in BmAFII-immunized animals. Adult exposure in BmAFII-immunized animals upregulated the response for LPS challenge and comparable for BmAFII challenge in vitro whereas converse (P<0.01) was seen with BmAFI-immunized animals indicating that BmAFII has adult specific IL-6 up regulation inducing molecules, but not BmAFI. However after mf exposure in these animals the response decreased; the decrease was more in BmAFI-immunized animals than BmAFII-immunized animals. In contrast L3 exposure downregulates the response in BmAFII-immunized animals. The findings indicate that BmAFII has stronger L3 specific IL-6 inducing molecules as compared to BmAFI.

Filarias specific IgG levels increased after immunization with BmAFI or BmAFII. Adult or L3 exposure upregulated the response in immunized animals; the increase was more in BmAFII-immunized animals. However, mf transfusion in these animals, the IgG level was marginally decreased in BmAFII-immunized ones but not in BmAFI- immunized animals though the level of IgG was much higher in BmAFII-immunized animals bearing mf. In general, specific IgG levels were higher in immunized, immunized +parasite life stages; highest level being shown by BmAFII immunized +adult worms or L3 exposed group.
3. Effect of BmAFI and BmAFII on the weight and cellularity of draining popliteal lymph node (PLN) and mast cells.

Popliteal lymph node weight and cellularity was determined 7-8 days after a single s.c. injection of BmAFI or BmAFII in the right hind paw of *M. coucha* and compared with that of PBS injected left leg PLNs of the same animal. BmAFI induced significant increase in the PLN weight (P<0.01) without increasing its cellularity. This disparity between node weight and node cellularity was due to the fact that 50% of these PLNs were edematous. And this oedematous change appears to be due to stimulation of pro-inflammatory IL-1β and IL-6 production. BmAFII increased (P<0.001) both the weight and cellularity index of the nodes of the animals.

Further in the PBS injected lymph nodes of normal animals, almost all the mast cells showed rich granularity (grade 3 granularity) while BmAFI sensitized nodes showed grade 2 granularity. However, BmAFII sensitized nodes showed more degranulated mast cells (grade 1 granularity).

4. Cytokine responses to NCP-bound BmAS fractions.

Having found indications from above experiments that BmAFI and BmAFII fractions possess pro- and anti-inflammatory potential, it was planned to further fractionate the fractions. But due to paucity of adequate quantity of the fractions another approach was applied to fractionate the crude extract of adult *B. malayi* (BmAS) to short-list the molecular entities with respect to their *in vitro* cytokine-releasing efficacy in THP-1 system. Fifteen fractions resolved by SDS-PAGE were blotted on to NCP and tested their-pro- (TNF-α, IL-1β and IL-6) and anti-inflammatory (IL-10) cytokine responses in THP-1 cells. While B1 to B9 correspond to molecular weight range of BmAFI, B5 to B15 correspond to molecules covered by BmAFII. B5 and B6 are common to BmAFI and BmAFII. The strongest IL-1β response was elicited by B5 (67.8-84.3 kDa) and B6 (54.3-67.8 kDa) and moderate response by B10 (38.4-41.8 kDa). B6 was identified to be a stronger stimulator of IL-6 production too. B1 (>180 kDa) and B2 (169-180 kDa) were strong and B6 was lowest stimulator of TNF-α.
release. While B2 was strong, B1 and B14 (22.5-23.44kDa) was moderate and low stimulators respectively, of IL-10 release.

**Conclusion**

In conclusion, immunization with BmAFII eliminates or suppresses parasite life stages-induced infection particularly adult worms and L3 in *M. coucha* by stimulating predominantly pro-inflammatory responses (TNF-α, IL-6, IL-1β and NO) to the parasites. BmAFII also enhanced CMI response, lymph node weight and cellularity and stimulated lymph node mast cells. In contrast, immunization with BmAFI elicited low CMI responses, moderately stimulated mast cells and facilitated survival of the parasite life stages. BmAFI stimulated predominantly IL-10 and comparatively greater TGF-β release. 50% of naive animals exposed to BmAFI showed edematous lymph nodes and increased the node weight. Further, NCP-bound molecules corresponding to BmAFI and BmAFII showed pro- and anti-inflammatory cytokine stimulating potential *in vitro*. Thus, the study reveals that BmAFII is protective and stimulates proinflammatory cytokines while BmAFI facilitates parasite survival and stimulates several folds higher release of IL-10 and TGF-β indicating that it possesses anti-inflammatory molecules.