CHAPTER-5

4 DISCUSSION

Active modulation of the host’s immune response is part of the parasites’ strategies for long-term survival which is characterized by a marked cellular hyporesponsiveness and a shift of the cytokine balance toward a Th2/Th3 response (Doetze et al., 2000; Plier et al., 1996; Satoguina et al., 2002). How the parasite achieves this is not clear but some of secreted products of the parasites were shown to down-regulate inflammatory reactions in the host (Allen & MacDonald, 1998; Hewitson et al., 2009; Whelan et al., 2000). However, it is also known that the dying and dead parasites elicit intense inflammatory responses (either during normal course of infection or in response to chemotherapy) that are involved in or responsible for the granuloma formation, lymphadenitis, lymphangitis and other immunopathological lesions. Therefore, it is evident that some of the parasite products or cellular/tissue constituents (possibly other than those secreted) are proinflammatory. Nevertheless, identifying these proinflammatory molecules of the parasite and whether using them to jeopardize the development and survival of the parasite in the host would provide opportunity to search protective molecules, were not known for many years.

*Brugia malayi*, a lymphatic filariid, is a well-studied pathogen. Some proteins of the parasite were reported to be immunogenic (Yenbutr & Scott, 1995; Zvelebil et al., 1993) or prophylactic (Dixit et al., 2004; Gnanasekar et al., 2005; Kazura et al., 1990; Pokharel et al., 2006; Vasu et al., 2000) or some are immunogenic but not prophylactic (Peralta et al., 1999; Probst et al., 2001). It has been hypothesized that in the host the soluble filarial proteins are degraded into peptides, which, upon association with major histocompatibility complex class II molecules and presentation to CD4 T cells, trigger host cellular immune responses. Murthy and her group (Dixit et al., 2004) while working with BmAFII fraction (Sephadex G-200 eluted fraction of *B. malayi* adult worm) have shown that proteins between 21 and 84 kDa of the fraction was protective against *B. malayi* in *M. coucha* and suggested that it might have functioned in the same way in protecting rodent host against *B. malayi* challenge. Further, MALDI-TOF analysis of the fraction within this molecular weight range showed 11
proteins, of which 6 proteins were identified to be immunostimulatory. These are: Heat Shock Protein 60 (HSP60), Cytoplasmic intermediate filament (CIF), Elongation factor 2 (EF2), dTDP-D-glucose 4,6-dehydratase, Disorganize muscle protein 1 (DIM-1), small HSP (p27) (Sahoo et al., 2009). However, the role of these proteins in protection or pathogenesis of lymphatic filariasis is not clear. Recently (Verma et al., 2013) have shown that immunization with recombinant mitochondrial HSP60 of B. malayi modulated and balanced the host’s immune responses to favour parasite survival without inducing any pathology. Continuous immune stimulation with the helminth infection is known to generate strong regulatory anti-inflammatory network which provides favourable environment to parasites strategies for long-term survival, typically characterized by a marked cellular specific hyporesponsiveness (Harnett & Harnett, 2008) and a shift of cytokine balance toward a Th2/Th3 response (Doetze et al., 2000; Plier et al., 1996). The hyporesponsiveness caused by regulatory networks are likely to play a role in the development of infection. With these background in the present study we used three different immunization schedules [3-dose (standard immunization protocol), 3- and 6-week (non-standard repeated administration or repeated administration for prolonged period)] for vaccination of animals with rDIM-1bm in order to understand the role of a stimulatory protein ‘DIM-1’ molecule in host-parasite interactions and their effects on subsequent invasion/attack on the parasites. The animals were immunized with rDIM-1bm by using standard and non-standard immunization protocols and subsequently exposed to L3 of B. malayi and assessed the effects on the various responses of the host.

The major findings obtained in the study were: i) B. malayi DIM-1 was successfully clone, expressed and rDIM-1bm protein (~40 kDa) was purified. rDIM-1bm belongs to immunoglobulin superfamily (IgSF) bearing similarity with DIM-1 of C. elegans, A. sum and L. loa and ‘blastn’ results showed that protein coding sequence (CDS) has almost no homology with human and mouse nucleotide sequences, ii) the protein was located in body wall muscle, iii) sera of rDIM-1bm immunized animals reacted strongly with ~40 kDa (near to 36 kDa) molecule of B. malayi mf, L3, L4 and adult worms in western blots suggested that DIM-1 was present in all life stages of B. malayi, iv) M. coucha immunized with rDIM-1bm caused reduction in adult worm recovery and microfilaremia accompanied with embryostatic
effect. Of the three immunization schedules, 3-dose immunization (standard vaccination schedule) caused better reduction in mf burden (36-63%) in peripheral circulation, adult worm load (52%) with 55% embryostatic effect, whereas immunization for 3 weeks or 6 weeks (non-standard immunization) exerted lesser effect on peripheral mf count (23-58%), adult worm burden (9-12.5%) and embryostatic effect (12.5-27.5%).

v) 3-dose immunization resulted in upregulation of celluar proliferation, macrophages activity, NO release, specific IgG, IgG1, IgG2a, IgG2b, IgE and IgA levels; both Th1 (IFN-γ, TNF-α and IL-2) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) cytokine release whereas repeated administration of rDIM-1bm (3- and 6-week) exerted lesser effect on parasite burden and mixed immunological responses. None of the rDIM-1bm-administration schedules induced any pathology in lymphoid tissues, or alteration in mast cell number and granularity.

In a preliminary vaccine trial Tsuji and his group reported that rAs37 of A. suum, which bore similarity with DIM-1bm, could induce poor protection (20%) against A. suum in BALB/c mice (Tsuji et al., 2002). Later on (Yan et al., 2013) reported that vaccination with DNA vaccine encoding DIM-1 (pVAX1/DIM-1) partially protected goat against Haemonchus contortus with uniform reduction in egg output (45.7%) and abomasal worm burden (51.1%). In our study 3-dose immunization with rDIM-1bm produced better protection against B. malayi L3 induced infection in M. coucha.

IgG is known to be involved in removal of the filarial parasites by antibody-dependent cell-mediated cytotoxicity (ADCC) (Chandrashekar et al., 1990). ADCC is one of the well known immunological mechanisms by which the host kills a large multicellular helminthes including filarial parasites both in vitro and in vivo (Chandrashekar et al., 1990; Dabir et al., 2008; Diagne et al., 1990; Subrahmanyam et al., 1976). 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 (Chandrashekar et al., 1990). IgG1 and IgG2b are Th2 type whereas IgG2a is indicative of Th1 type responses (Stevens et al., 1988). Filarial parasite specific IgG1, IgG2 and IgG3 are shown to be predominant in chronic lymphatic filariasis (Joseph et al., 2012; Rahmah et al., 1994). Previous studies carried out by
Murthy and her group demonstrated that immunization with F6 (a proinflammatory fraction of B. malayi adult worm) intensely upregulated IgG1, IgG2a, IgG2b in mouse (Sahoo et al., 2009). A similarly phenomenon was also observed in other nematodes, such as in A. suum (Tsuji et al., 2002) and in Trichostrongylus colubriformis (Kiel et al., 2007). Although we did not perform ADCC assay in this study high IgG and IgG1, IgG2b and IgG2a antibody responses with high cellular responses might have helped in significant parasite reduction in 3-dose immunized host indicating that rDIM-1bm had ability to stimulate the host immune response. It also provides evidence that the antibody mediated responses during Th1 response might have synergized the cellular components to facilitate more effective clearance of the pathogen. Nevertheless, mouse IgG3 is an early effector molecule of the immune system (Gavin et al., 1998) that appears early in immune responses independently of T helper cell. Briles et al. (1981) reported that mouse IgG3 subclass can protect against bacterial infection. Parasite specific IgE has been implicated in protective responses in helminthes which promote parasite killing (Gurish et al., 2004; Spencer et al., 2001). King et al. (1997) reported that deletion of IgE gene resulted in raised worm burdens. In the present study we observed different immunization schedules produced different status of IgE levels in animals and this situation could have modulated the specific IgE effector mechanisms as evidenced by different status of parasite load in animals receiving different doses of the r-protein. IgA constitutes nearly 15%–20% of the total immunoglobulins in humans (van Egmond et al., 2001). Sahu et al. (2008) reported protective role of IgA in human filariasis as shown by elevated levels of filaria-specific IgA in endemic controls than in subjects with chronic disease and suggested a role for IgA in anti disease immunity. Furthermore, higher IgA levels were significantly correlated with the absence of filarial infection. Similar results were reportedly associated with reduced percentage of L3 for Ostertagia circumcincta in sheep (Stear et al., 1995) and O. ostertagi in calves (Geldhof et al., 2002). However in this study, IgA levels were significantly increased in 3- and 6-week immunized animals but not in 3-dose rDIM-1bm-immunized animals, the reason for this is not clear at present. Macrophages activated by Brugia infection display high levels of IgM on their surface, in comparison to those from naive mice, suggesting an up-regulation of IgM receptor(s) during the activation process (Rajan et al., 2005). Abraham and his colleagues have correlated IgM titers with host protection against the parasitic organisms, Strongyloides stercoralis (Abraham et al., 1989;
Herbert et al., 2002). In the present study we observed elevated level of IgM in both immunized and immunized L₃ infected animals suggesting protective role of anti rDIM-1bm IgM antibodies against B. malayi.

In this study the body weights of the rDIM-1bm immunized animals were significantly lower than the body weights of non-immunized animals whereas rectal temperature of immunized and immunized L₃ infected animals was greater than their counterparts. Schutz et al. (2012) reported that non-vaccinated (control) and vaccinated (bovine rhinotracheitis) without dewormed calves had greater rectal temperatures compared with dewormed calves at the time of vaccination. This shows that raise in rectal temperature was due to the intestinal parasites but not due to vaccine. Our immunized and non-immunized animals were free from intestinal parasites indicating the rDIM-1bm did not adversely affect rectal temperature. Interestingly, the relative weights of organs (liver, kidney, testes, lymph nodes) were remained unaltered in immunized animals.

In the present study we found that animals receiving 3 doses of rDIM-1bm showed decreased in Hb level. Animals infected with Hemonchus L₃ showed reduced Hb level (Qamar & Maqbool, 2012). However, in this study there was no obvious relationship could be ascertained between worm burden and some alterations in the haematological parameters in the individual animals. Thus these findings suggest that Hb value may only provide a useful aid to group diagnosis of filariosis, but is not reliable indicators of worm burdens. A similar situation was reported by (Yan et al., 2013) in goats with haemonchosis.

Eosinophils (Granulocytes) play an important part in immune responses against helminth infections and are often associated with the expression of resistance to the parasites (Balic, 2000; Pfeffer et al., 1996). Yan et al. (2013) reported higher numbers of eosinophils in the peripheral blood of DIM-1 vaccinated animals. In this study, we observed increase in granulocytes (eosinophil, neutrophils and basophils) in 3-dose immunized animals and immunized+L₃ infected animals. These findings concurred with the general observations of close association between eosinophils and resistance to helminth parasites (Yacob et al., 2004). Eosinophil recruitment is hypothesized to be dependent on cytokines secreted by CD4+ Th2 cells (Winter et al., 1997). Antigens produced by helminth parasites are thought to
be strong inducers of reaginic and hemocytotropic IgG elements which, together with eosinophils, are responsible for antibody-dependent and cell-mediated cytotoxicity (ADCC) (Butterworth, 1984). Thus, it was assumed that in vivo parasite elimination must have followed this phenomenon.

Modulation of lymphocyte proliferation has been shown to result in alterations in survival of parasites (Dixit et al., 2004; Maizels & Lawrence, 1991; Maizels et al., 2004). In the present study enhanced host’s CMI responses by the rDIM-1bm was considered as one of the modes through which the survival of the parasites could have been influenced suggesting rDIM-1bm is a potent stimulator of cell proliferation and may have implicated in protection against *B. malayi* infection.

Other mechanism of parasite killing is activation of macrophages by IFN-γ that leads to either by antibody dependent cytotoxicity, resulting in the release of reactive oxygen intermediates or directly by generation of reactive nitrogen intermediates (Rajan et al., 1996; Taylor et al., 1996). Macrophages are critical mediators of immune regulation in lymphatic filariasis (Allen & Loke, 2001). NO mediated mechanisms have been shown to be capable of killing mf *in vitro* and L₃ *in vivo* and protect the host through type 1 responses and IFN-γ stimulated toxic mediators’s release. In the present study, macrophage activity, NO and IFN-γ release was increased in cells of all the immunized and immunized L₃ infected animals and reduced worm burdens (both mf and adult) in them reflect the involvement of IFN-γ and NO mediated role in suppressing establishment of infection. This finding is in the line of earlier findings of (Dixit et al., 2006; Sahoo et al., 2009) who have reported that IFN-γ (Th1) and NO were involved in protection of the host against *B. malayi* in *M. coucha*. In human patients, elevated levels of IFN-γ were found in both endemic normals and chronic filarial patients which suggested that type 1 inflammatory responses may be involved in killing of L₃ and this is probably how endemic normals remain infection free (Al-Qaoud et al., 1997; Thomas et al., 1997). In the present study, immunization with the rDIM-1bm significantly protected *M. coucha* against establishment of infection as evidenced by low recovery microfilaraemia and parasite burden. Enhanced activity of macrophages, IFN-γ and NO responses in these animals suggest participation of macrophage mediated mechanism(s) in the immune response.
It is well documented that filarial infection is associated with impairment of T cell responses which persists even after clearance of the parasite (Steel & Ottesen, 2001). When splenocytes of immunized (3-dose, 3- and 6 week schedule) animals and L₃ infected animals were challenged in vitro with rDIM-1bm, showed greater release of Th1 cytokines (IFN-γ, TNF-α, IL-2). 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 blocking of enhanced antibody production (de Almeida et al., 1998; de Boer et al., 1998; Mahanty et al., 1996). It has been reported that IFN-γ involved in protection of the host against B. malayi in M. coucha (Dixit et al., 2006; Sahoo et al., 2009). 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 (Artis et al., 1999). Dixit et al. (2006) demonstrated that BmAFII a pro-inflammatory fraction has strong TNF-α stimulating molecules and eliminated intraperitoneally transplanted adult worms in M. coucha.

IL-4 (Th2 type cytokine) in the elimination of parasites has been implicated in protective responses to Litomosoides sigmodontis in BALB/c mice as IL-4 knockout mice have shown greater worm burdens and circulating mf. Also, infection of IL-4 deficient C57BL/6 mice resulted in full parasite development and patency, demonstrating that in fact a Th2 response is the key determinant of susceptibility and resistance in non-permissive mice (Babu et al., 2000; Volkmann et al., 2003). Other studies have revealed that IL-4 production and IL-4 receptor expression by T cells are both dispensable for T-cell-mediated host protection (Spencer et al., 2003). In the present study it has been observed, that increased IL-4 release was relatable with significant reduction in parasite load in 3-dose immunized animals but not in 3- or 6-week immunized animals where IL-4 release was not elevated indicating non-association of IL-4 in the parasite elimination.

IL-5 is reported to provide protection via recruitment of eosinophils in conjunction with parasite-specific antibodies (Al-Qaoud et al., 1997). In the present study IL-5 responses were upregulated in all the immunized groups as well as immunized animals receiving L₃. On the other hand animals belonging to 3- and 6-week immunization schedule showed increase
in IL-5 on immunization but after L$_3$ exposure the response declined. The findings indicate that enhanced IL-5 following exposure of L$_3$ to 3-dose immunized animals may have caused killing of significant number of parasites. In present study, IL-6 release from cells of animals immunized with 3-dose or 6-week and subsequently given L$_3$ to these animals was observed to be increased but not in animals receiving immunization for 3 weeks. Investigators (Dixit et al., 2004; Sahoo et al., 2009) reported that proinflammatory fractions protected the host against *B. malayi* infection via proinflammatory cytokine release. (Muhsin, 2013) reported increased *L. sigmodontis* worm recovery during early infection in IL-6 deficient mice and suggested protective role of IL-6 against filarial parasites.

Anti-inflammatory cytokine (IL-10 and TGF-β) network plays an important role in modulation of effector mechanism of the host against longstanding parasite survival/invasion in filarial infection (Doetze et al., 2000; Mahanty & Nutman, 1995). The prominence of IL-10 as a crucial mediator of regulation in parasite infections has long been recognized (Sher et al., 1992) particularly for its role in attenuating pathogenesis. Studies with IL-10 deficient mice showed a failure to control pathological reactions, with fatal results in infections with *Trichuris muris* (Schopf et al., 2002) and increased mortality in *Schistosoma mansoni* (Wynn et al., 1998). It was also observed in the present study that immunization of animals with rDIM-1bm for 3- and 6-week schedules resulted in upregulation of IL-10 release but not in animals receiving 3 doses of rDIM-1bm; these findings provided evidence of poor protection against L$_3$ induced infection in repeated immunization for prolonged period.

In several nematode models of infection, IL-13 has been shown to compensate for the absence of IL-4 in mediating host protection (McKenzie et al., 1998). When IL-4 knock out mice on the 129xC57Bl/6 background were infected with *B. malayi* via the i.p. route, no difference was observed in the recovery of L$_3$ at early time points (i.e. p.l.i.) demonstrating that the survival of a primary infection with L$_3$ is not IL-4-dependent (Lawrence et al., 1995). However, IL-4 KO mice infected through s.c. route with *B. pahangi* L$_3$ showed very high levels of IL-13, a cytokine which shares some biological activities with IL-4 (Zurawski & de Vries, 1994). Two types of receptors have been reported for IL-4. The first is composed of the IL-4R-α-chain dimerized to the common γ-chain, and the second is made up of the IL-4R-
\( \alpha \)-chain and the IL-13R-\( \alpha_1 \)-chain. IL-13 has been shown to mimic many of the effects of IL-4 by binding to the second type receptor. In this study, upregulated IL-13 in animals receiving 3 doses of the antigen seemed effective in parasite removal via this mechanism. On the contrary in 3- and 6-week immunized animals where IL-13 response was not observed and perhaps no significant inhibition in parasite recovery was observed. Nevertheless recently (Joseph et al., 2011) reported that anti-inflammatory BmAFI fraction derived from \( B. \) malayi adult worm extract facilitated parasite development and survival via downregulation of IL-13 response. Thus, in the present study elevated level of IL-13 (Th2) mediated protective responses was due to vaccination of \( M. \) coucha with 3 doses (standard immunization schedule) of rDIM-1bm.

Of the other cell types of defence system, mast cells are important as initiators and effectors of innate immunity. In addition, mast cells that are activated during innate immune responses to pathogens, or in other contexts, can secrete products and have cellular functions with the potential to facilitate the development, amplify the magnitude or regulate the kinetics of adaptive immune responses. Thus, mast cells may influence the development, intensity and duration of adaptive immune responses that contribute to host defence, allergy and autoimmunity, rather than simply functioning as effector cells. In filariasis mast cells play an important role in the protection of the host against the parasite (Dixit et al., 2004). However which of the myriads of the parasite antigen molecules influence or mobilize the mast cell responses is not known. In the present study it was found that none of the rDIM-1bm immunization schedules induced any histological changes, alteration in total number of mast cells and degranulation in mast cells indicating that inhibition in parasite load was not due to the role of mast cells.

In summary, \( B. \) malayi DIM-1 was successfully cloned, expressed and rDIM-1bm protein (~40 kDa) purified. The protein was localized in body wall muscles, and present in all life stages of \( B. \) malayi. DIM-1bm belongs to immunoglobulin superfamily (IgSF) bearing similarity with DIM-1 of \( C. \) elegans, \( A. \) sum and \( L. \) loa and ‘blastn’ results showed that DIM-1 coding sequence (CDS) has almost no homology with human and mouse nucleotide sequences. Of the 3 different immunization schedules, 3-dose immunization schedule was the
most effective in protecting *M. coucha* against establishment of L₃ induced infection as inferred by a low recovery of mf in circulation and parasite burden. The enhanced activity of macrophages, cellular proliferation and NO responses, and elevated levels of specific IgG, IgG1, IgG2a, IgG2b, IgE and IgA, and intense upregulation of both Th1 (IFN-γ, TNF-α and IL-2) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) responses produced by 3-dose rDIM-1bm immunization schedule (standard immunization protocol for vaccination) correlated with parasitological findings. Administration of rDIM-1bm for 3 or 6 weeks (non-standard procedure) provided poor protection against L₃-initiated infection and produced mixed immunological responses which provided poor protection in the host. Thus in conclusion, findings of the present study suggest that rDIM-1bm (3-dose schedule) protects the host against the parasite stages via Th1/Th2 type responses. This is the first report on cloning, expression, structural modeling and purification of rDIM-1bm and its ability to prevent establishment of *B. malayi* infection. DIM-1bm’s almost complete lack of homology with the human counterpart makes it an important protein for exploring its vaccine potential.