Although, drugs are available for chemotherapy of leishmaniasis but they are limited in number and are associated with various side effects. Pentavalent antimony, that has long been the cornerstone of chemotherapy for more than 60 years and is still playing a driving role in treatment, has become resistant in several parts of the world (particularly in North East India) and is quickly becoming venerable (Sundar et al., 1998b). Second-line drugs (pentamidine and amphotericin B) are also related to toxicity and high cost. In recent years four new potential therapies namely liposomized amphotericin B (Meyerhoff, 1999), oral miltefosine (Sundar et al., 2002), paromomycin (Thakur et al., 2000) and oral sitamaquine (Wasunna et al., 2005) have been introduced which are also not without side effects. In view of these facts, only an effective vaccine can control the disease.

During the past several decades, extensive efforts have been made to search for an effective Leishmania vaccine. Vaccine formulations including killed, live attenuated parasites, recombinant Leishmania proteins or DNA encoding leishmanial proteins, as well as immunomodulators from sandfly saliva have been examined (Kedzierski et al., 2006). Although to date, there is no vaccine against Leishmania, several of the vaccine preparations are at advanced stages of clinical testing. In the search of new vaccine candidates, we evaluated the vaccine efficacy of two important heat shock proteins along with ALD and MPLA as adjuvants.

Hsps are evolutionarily ancient and highly conserved intracellular molecular chaperones constituting several multigenic superfamilies. They are expressed at low levels under normal physiological conditions but during stress they are up regulated to a significant level (Lindquist and Craig, 1988). The heat shock response is a general homeostatic mechanism that protects cells or organisms from damaging effects of stressful stimuli. Hsps are known to play crucial roles in protein folding and unfolding, protein translocation and assembly and disassembly of protein complexes. There is also an increasing evidence for the involvement of Hsps in antigen processing and presentation and in other immunological processes. For example, the formation of multimeric molecules such as T-cell receptors (TCR), immunoglobulins (Ig) and major histocompatibility complex (MHC) requires functional Hsps (Srivastava et al., 1998; Srivastava, 2002a; Srivastava, 2002b; Nicchitta, 1998; Rammensee and Schild, 2000).
During infection pathogens are faced with an array of stressful conditions such as changes in temperature, pH and immune responses of the host. To survive inside the host pathogens must activate several immune evasion strategies including synthesis of Hsps (Zugel and Kaufmann, 1999). They play a role during infection by directly activating macrophages to secrete cytokines, stimulate proliferation of NK cells and induce maturation of dendritic cells in mammals (Retzlaff et al., 1994; Galdiero et al., 1997; Asea et al., 2000; Kuppner et al., 2001; Buchmeier and Heffron, 1990). They can also act independent of chaperoned peptides to directly stimulate innate immune responses, however the mechanism is less understood (Tamura et al., 1997; Multhoff et al., 1997; Basu et al., 2000). The Hsps have important role as virulence factors in human protozoan infections and are considered as targets for therapeutic interventions (Przepiorka and Srivastava, 1998). They are able to chaperone antigenic peptides, interact with antigen presenting cells (APC) through a receptor, stimulate APC to secrete inflammatory cytokines, and mediate maturation of dendritic cells (DC). These properties also permit the utilization of heat shock proteins for the development of a new generation of prophylactic vaccines against cancer and infectious diseases (Binder et al., 2000a; Binder et al., 2000b; Singh-Jasuja et al., 2000; Suto and Srivastava, 1995; Castellino et al., 2000; Basu et al., 2000; Srivastava and Amato, 2001).

In the present study, we have focused on two heat shock proteins (Hsp70 and Hsp83) which have previously been reported to induce a strong cell mediated and humoral immune responses in various infectious diseases. The Hsp70 has been found to be the most inducible by stress (Moore et al., 1998) and is a major polypeptide antigen in many autoimmune diseases (Engman et al., 1990). It is a potent activator of innate immunity and aberrant expression of it in certain organs promotes immunopathology. The functions of the Hsp70 have recently been extended to their participation in the immunological defense against infectious diseases and cancers and it is suggested that it might have immunotherapeutic potential as Hsp70 purified from malignant and virally infected cells can transfer and deliver antigenic peptides to antigen-presenting cells to elicit peptide specific immunity (Srivastava et al., 1998). A Th1-type of response was potentiated with immunizations of Hsp70 of *L. infantum* (Mora et al., 1999). Hsp70 consists of an NH2-terminal nucleotide (ATPase) domain of 44 kDa and a COOH-terminal 25-kDa domain that binds peptide or polypeptide substrate (Becker et al., 2002). In its ATP-bound state, Hsp70 binds and releases peptide rapidly, whereas after
hydrolysis, in the ADP state, bound peptide is held tightly (Flynn et al., 1989). The B and T cell epitopes of Hsp70 and the Th1-like expression pattern elicited in vitro by this molecule in canine PBMC suggests that Hsp70 is immunogenic (Carrillo et al., 2008). The conservation of epitopes among Leishmania species and the capacity of Hsp70 to generate a specific response against Leishmania in distinct hosts (human patients and dogs) and different clinical manifestations (cutaneous, muco-cutaneous and visceral) show the potential of Hsp70 as a candidate for vaccine trials (Rafati et al., 2007). The immunization of mice with Hsp70 produced a Th1-type of immune response (Rico et al., 1999; Morell et al., 2006). Recently, Hsp70 has been found to enhance the immunogenicity of gp63 based protein and DNA vaccines against L. donovani in mice (Kaur et al., 2011; Sachdeva et al., 2009). The Hsp83 in trypanosomatid parasites (trypanosomes and Leishmania) share high identity to mammalian Hsp90 (Moore et al., 1989). Hsp83 also has been shown to be an immunodominant antigen recognized by sera from diffuse cutaneous leishmaniasis patients (Skeiky et al., 1997). In an earlier study, Hsp83 of L. infantum has been found to be useful for serodiagnostic assays for canine leishmaniasis (Angel et al., 1996). Hsp83 of L. infantum when physically linked to the Maltose Binding Protein (MBP) causes an immunostimulatory effect (Mora et al., 1999). It elicits an increased secretion of IgG2a and IgG1 isotype (Echverria et al., 2006).

Keeping in view the above studies, the immunoprophylactic potential of two heat shock proteins, that is, Hsp70 and Hsp83 of L. donovani in combination with different adjuvants (MPLA and ALD) was evaluated in BALB/c mice in the present study.

The hepatic and splenic parasite load in the immunized and control animals was calculated in terms of LDU (Bradley and Kirkley, 1977) after 1, 2 and 3 months post infection/challenge. Maximum parasite load was observed in the infected groups on 30 days post infection. Thereafter, the parasite load declined on 60 and 90 days post infection days. This is in accordance with the earlier studies of L. donovani infection in BALB/c mice (Engwerda et al., 1998; Gorak et al., 1998; Smelt et al., 1997). The cocktail vaccine comprising of Hsp70 and Hsp83 imparted significant protection against the L. donovani infection in BALB/c mice. In our previous study (Kaur et al., 2011) Hsp70 alone reduced the parasite burden in BALB/c mice by 41.67% to 46.81% from 30 to 90 d.p.c., However the addition of Hsp83 to Hsp70 in the present study further raised the level of protection by 56.27% on 30 d.p.c., 74.05% on 60 d.p.c. and 86.06% on 90 d.p.c. However, the level of protection was raised to a significant extent when both the
heat shock proteins were used in combination with adjuvants. Following vaccination with both the Hsps adjuvanted with ALD, the parasite burden was reduced by 66.91%, 80.61% and 91.90% on 30, 60 and 90 d.p.c. respectively. Maximum protective response was conferred by the vaccine formulation comprising of the two antigens in combination with adjuvant MPLA. The parasite reduction in liver was 75.34%, 85.79 % and 94.78% on 30, 60 and 90 days post challenge. These results are in correspondence with our previous studies where addition of MPLA enhanced the level of protection of 78 kDa antigen in BALB/c mice by 56.5 to 92% in comparison to 51.3-71% imparted by ALD (Nagill and Kaur, 2010). Similarly, Leish-111f + MPL-SE induced significant protection against *Leishmania* infection in mice and hamsters with a reduction of 99.6% in parasite load (Skeiky *et al.*, 2002; Coler *et al.*, 2002; Coler *et al.*, 2007). Recently a subunit vaccine Leish-111f+MPL-SE was shown to have potential immunotherapeutic efficacy in canine visceral leishmaniasis (Trigo *et al.*, 2010).

The cell mediated immune response to *Leishmania* is usually evaluated *in vivo* by delayed-type hypersensitivity (DTH) skin test reaction using leishmanin as antigen (Mosmann and Fong, 1989). The DTH response can be measured easily and semi-quantitatively (Dannenberg, 1991). It is done for recalling the antigens to which previous exposure has occurred. Memory T cells play an important role in this process. When antigens are administered into the skin, they come in contact with antigen presenting cells (APCs), which engulf the antigens, process and present them on their surface. Memory T cells recognize them and trigger the secretion of several pro-inflammatory cytokines. It brings about the dilation of blood vessels and massive cellular infiltration (predominantly T cells and macrophages) which results into induration and swelling of the affected area (Black, 1998). Since the infection is associated with depressed cell mediated immune responses, the lowest DTH responses were observed in infected controls. In our study, immunization of mice with a cocktail of two antigens brought about a significant protective response. It significantly raised the DTH responses in comparison to the infected controls. The addition of adjuvants further increased the DTH responses significantly. MPLA elicited strong and maximum DTH response followed by heat shock proteins plus ALD antigen. Successful vaccination of humans and animals is often related to antigen induced DTH responses *in vivo* and T-cell stimulation with antigen *in vitro* (Howard and Liew, 1984; Bhowmick *et al.*, 2007). Similarly in our previous study, mice immunized with 78 kDa+MPLA showed significantly higher DTH responses in
comparison to those immunized with 78 kDa+ALD (Nagill and Kaur, 2010). Furthermore immunization with Hsp70+63 induced a significantly high DTH response (Kaur et al., 2011). In another study, untreated patients with kala-azar were unable to control their visceral infection with *Leishmania donovani* and it was attributed to a defective cell-mediated immune response to leishmanial antigens. These patients were unable to respond to parasite antigens in delayed-type hypersensitivity skin tests (de Andrade et al., 1982; Manson-Bahr, 1961). Our results are also in accordance with another study in which it was demonstrated that 89.9, 94.9, 96.9, 97.1 kDa proteins stimulate remarkable cellular responses and significant (P < 0.001) DTH responses in cured *Leishmania* infected hamsters (Kumari et al., 2008). Mice immunized with liposome-encapsulated gp63 (Jaafari et al., 2006) induced good DTH response. Furthermore, vaccination of mice with gp63 entrapped in positively charged liposomes induced the highest level of DTH response whereas free gp63 vaccinated groups exhibited lower responses (Afrin et al., 2002; Bhowmick and Ali, et al., 2008).

Antibody isotype switching provides a convenient surrogate marker of Th1 and Th2 CD4⁺ T-cell differentiation (Coffman et al., 1999). In a *L. infantum* model, it has been shown that the levels of IgG1 antibodies and the specific anti-*Leishmania* IgG2a subclass detected in the dog sera could be considered as a predictor of progression and cure of the infection, respectively (Deplazes et al., 1995). It has been reported that, higher levels of anti-*Leishmania* IgG2a antibodies in sera from IL-4 deficient mice were associated with resistance to the infection (Guimaraes et al., 2006). In leishmaniasis, resistance and resolution of the illness have been associated with IgG2a production (Mohammadi et al., 2006) and activation of Th1 cells with secretion of IL-2 and IFN-γ. In mice, IL-4 secreted by Th2 cells induces an immunoglobulin isotype switch on B lymphocytes to IgG1 whereas IFN-γ produced by Th1 clones increased the response of the IgG2a isotype (Stavnezer, 1996). To verify the cytokine responses and to understand the possible mechanism of protection, we measured serum levels of parasite-specific IgG1 and IgG2a isotypes. The low levels of IgG1 antibody detected in sera of the animals immunized with antigens in combination with different adjuvants suggests that the disease in immunized animals is being controlled, while in the control animals the infection progresses. Maximum level of IgG1 was seen in serum samples of mice immunized with Hsp70+Hsp83 followed by the animals immunized with both the antigens in combination with ALD and Hsp70+Hsp83 along with MPLA. Moreover,
higher levels of anti-\emph{L. donovani} IgG2a were detected in serum samples of immunized animals, again pointing towards the control of the infection in these animals. Maximum levels of IgG2a were seen in serum samples of mice immunized with MPLA vaccine followed by ALD vaccine and Hsp’s alone. Indeed, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses. The relative production of these isotypes can thus be used as a marker for the induction of Th1-like and Th2-like immune responses, respectively. Our studies are in accordance with other studies in which immunization with purified P4 nuclease protein and Hsp70 of \emph{L. amazonensis} resulted in significant increase in IFN-\(\gamma\) and reduction in parasite-specific IgG1, suggesting an enhancement in Th1 responses (Campbell et al., 2003). Mice immunized with LACK or LmSTI1 antigen stimulates Th1 or mixed Th1/Th2 responses in lymph node cells of BALB/c mice infected with \emph{L. major}. The sera of these animals contain high titers of IgE, IgG1, and IgG2a antibodies (Webb et al., 1996). In another study, BALB/c mice immunized with rHsp70 of \emph{L. amazonensis} showed enhanced levels of IgG1 and IgG2a pointing towards the generation of a mixed Th1/Th2 responses (Rafati et al., 2007).

Human visceral leishmaniasis is characterised by a depression of cell-mediated immunity to \emph{Leishmania} spp. and a marked humoral response. Patients have negative delayed-type hypersensitivity skin test (Manson-Bahr, 1961), no response in lymphocyte proliferation assay \textit{in vitro} and decreased IL-2 and IFN-\(\gamma\) production when cultured with parasite antigens. Restoration of cell-mediated immunity to the parasite is necessary for an effective pentavalent antimonial therapy (Carvalho et al., 1985). In murine visceral leishmaniasis, susceptibility to \emph{L. donovani} infection is associated with the loss of capacity of spleen cells to produce IFN-\(\gamma\) \textit{in vitro} (Kaye et al., 1991). Posterior development of parasite-specific cell-mediated response requires both, CD4\(^+\) and CD8\(^+\) T-cells (Stern et al., 1988), and reduces the parasite burden by the production of endogenous IFN-\(\gamma\) and TNF-\(\alpha\), macrophages activation and the formation of hepatic granulomas (Kaye et al., 1991; Murray et al., 1989; Tumang et al., 1994).

The course of leishmaniasis has been associated with different cytokine patterns, which parallel cure or non-cure of disease in mice. Contrasting signals are induced by the \emph{Leishmania} when it enters into the human macrophage. It may cause the secretion of IFN-\(\gamma\) and TNF-\(\alpha\), which promotes macrophage activation, or to the secretion of IL-10 or TGF-\(\beta\) associated with macrophage deactivation and inhibition of IFN-\(\gamma\) (Barral et al., 1995; Barral-Netto et al., 1991). Initial survival of \emph{Leishmania} inside the macrophage
probably depends on which of these or similar cytokines predominate in the microenvironment of infection. The polarized responses do not alter easily \textit{in vivo} after 2 to 3 weeks of \textit{Leishmania} infection in mice. A change from a Th1 to a Th2 type of immune response was observed by the use of IL-4 \textit{in vitro} (Mocci and Coffman, 1995) or \textit{in vivo} (Coffman \textit{et al}., 1995). Treatment with antimony (Nabors and Farrell, 1994; Nabors \textit{et al}., 1995) in combination with anti-IL-4 or IL-12 promoted a change from a Th2 to a Th1 type of response in \textit{L. major} model of mice. Cytokine pattern during the very early stages of \textit{Leishmania} infection probably influences Th1/Th2 dichotomy. The Th1 type of immune response is assessed in terms of IFN-\(\gamma\) and IL-2 levels. IFN-\(\gamma\) produced by NK cells (Solbach and Laskay, 2000) activates macrophages to clear the parasites (Afonso \textit{et al}., 1994; Mattner \textit{et al}., 1996). IFN-\(\gamma\) plays an important role in limiting the growth of \textit{Leishmania} in murine and human macrophages and limiting leishmaniasis progression (Basu \textit{et al}., 2005). The production of IFN-\(\gamma\) is mediated by IL-12. However, in mice infected with \textit{L. donovani}, the differential production of Th1 and Th2 cytokines does not control the cure rate, although Th1 response correlates with resistance to infection, the Th2 response does not determine susceptibility (Kaye \textit{et al}., 1991). Although IL-10 responses are typically generated to balance excessive Th1 and CD8\(^+\) T-cell responses and prevent immunopathology, overproduction of IL-10 has been shown to inhibit proinflammatory responses leading to susceptibility to infectious pathogens such as \textit{Leishmania} (Anderson \textit{et al}., 2005; Belkaid \textit{et al}., 2001). There is also a trend that Th1 and Th2 responses coexist in humans who have been cured of visceral leishmaniasis (Karp \textit{et al}., 1993; Kemp \textit{et al}., 1993). Thus, the Th1/Th2 paradigm of resistance/susceptibility is an oversimplification of a far more complicated network of regulatory/counter-regulatory interactions (Alexander and Bryson, 2005) and a successful protective response in VL need not necessarily follow the Th1/Th2 paradigm, although a Th1 response is essential for imparting immunity (Ravindran and Ali, 2004). Immunization with heat shock proteins along with different adjuvants induced substantial production of both Th1 and Th2-specific cytokines. Though the concentrations of IFN-\(\gamma\) and IL-2 were greater in the animals immunized with the two heat shock proteins in combination with adjuvants, the levels of IL-4 and IL-10 were also significant. Maximum levels of IFN-\(\gamma\) and IL-2 were seen in cultures of splenocytes from animals immunized with vaccine formulation with MPLA followed by those immunized with vaccine formulation with ALD. Minimum level was seen in cultures of splenocytes from animals immunized with Hsps alone. In contrast, IL-4 and IL-10 levels were high in control
animals but decreased significantly in immunized groups. The minimum levels of IL-4 and IL-10 were found in cultures of splenocytes obtained from mice immunized with MPLA vaccine followed by those immunized with ALD vaccine. These results correlate with our previous study where MPLA and ALD increased the level of Th1-specific cytokines and decreased the concentration of Th2-specific cytokines generated by 78kDa antigen and the generation of protective immune response was significantly greater in the animals immunized with 78 kDa+MPLA in comparison to those immunized with 78 kDa+ALD (Nagill and Kaur, 2010).

There are many challenges in the diagnosis of the leishmaniasis. Early diagnosis of the disease is difficult because the clinical and epidemiological findings in various forms of leishmaniasis resemble several other diseases. Untreated VL patients also act as a reservoir for parasites and therefore contribute to disease transmission in anthropoontic VL areas. Therefore, early case-finding is considered an essential component of VL management. However, there is a lack of gold standard technique to detect VL infections. The visualization of the amastigote form of the parasite by microscopic examination of aspirates from lymph nodes, bone marrow or spleen is the classical confirmatory test for VL. However, splenic aspiration can be complicated by life-threatening hemorrhages in approximately 0.1% of individuals and therefore requires considerable technical expertise (Kager and Rees, 1983) as well as facilities for nursing surveillance, blood transfusion and surgery. Moreover, the accuracy of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents used. The detection of parasites in the blood or organs by culture or by using molecular techniques such as PCR is more sensitive than microscopic examination but these techniques remain restricted to referral hospitals and research centres, despite efforts to simplify them (Reithinger and Dujardin, 2007). As a result, many serological tests such as DAT, IFAT and ELISA have been developed to diagnose visceral leishmaniasis. DAT has been extensively validated in most endemic areas. The performance of the DAT was influenced by neither the region nor by *Leishmania* species. The fast agglutination screening test (FAST) is a simplified and more rapid version of the DAT. The IFAT has been the most common serological test used for the diagnosis of CL, MCL, PKDL and canine leishmaniasis, but it is not readily adaptable for large-scale seroepidemiological studies. Conversely, ELISA has proven to be at least as sensitive and specific as the IFAT and is suitable for large scale studies (Barbosa-de-Deus *et al*., 2002).
Discussion

Serological tests are very sensitive and specific, but they are also limited by antibody detection limits and cross-reactivity (Ferrer et al., 1995).

ELISA has shown high diagnostic accuracy in several studies (Ho et al., 1983; Iqbal et al., 2002; Sinha and Sehgal, 1994; Sreenivas et al., 2002). In order to develop specific assays for the serodiagnosis of leishmaniasis, several promastigote and amastigote antigens, purified antigens such as FML, defined, synthetic peptides or recombinant antigens have been characterized and evaluated. An rK39 based ELISA showed excellent sensitivity (93–100%) and specificity (97–98%) in many VL-endemic countries (Braz et al., 2002; Ozensoy et al., 1998; Maalej et al., 2003; Kurkjian et al., 2005). This test was then developed into a dipstick format that was more suitable for field use (Chappuis et al., 2006). Among the many antigens used in ELISA for the diagnosis of leishmaniasis are total soluble antigen (Hommel et al., 1978), dp72 (Jaffe and Zalis, 1988), gp 63 (Okong‘o-Odera et al., 1993), a 78-kDa (Ravindran et al., 2004) and rK39, a recombinant and a kinesin-related protein (Burns et al., 1993). Some other antigens from L. infantum such as the acidic ribosomal proteins P2a and P2b, the ribosomal protein P0, the histones H2A (Soto et al., 1995b) and H3 (Soto et al., 1996) were isolated and characterized for diagnosing canine VL. Similarly, the heat shock proteins Hsp70 and Hsp83 have also been shown to have serodiagnostic potential against canine VL (Quijada et al., 1998; Angel et al., 1996).

An antigen detection test would, in principle, provide better means of diagnosis since antigen levels are expected to broadly correlate with the parasite load. Antigen detection systems are also an ideal alternative to the antibody detection systems in immunocompromised patients and more particularly with the growing number of HIV co-infected cases, especially in advanced cases where the immune response is impaired (Rosenthal et al., 1995). A latex agglutination test detecting a heat-stable, low-molecular-weight carbohydrate antigen in the urine of VL patients has shown promising results (Attar et al., 2001; Sarkari et al., 2002). Several studies conducted in East Africa and the Indian subcontinent showed good specificity but only low to moderate (48–87%) sensitivity (Rijal et al., 2004; Sundar et al., 2005; Sundar et al., 2007). Apart from its low sensitivity, there are two practical limitations: the urine must be boiled to avoid false-positive reactions and it is difficult to distinguish weakly positive from negative results, which affects the reproducibility of the test (Chappuis et al., 2006; Rijal et al., 2004). Therefore, no satisfactory antigen detection test is currently commercially available and
Discussion

attempts to develop such tests have been unconvincing (Kohanteb et al., 1987). In the present work, an effort was made to develop an antigen detection test. For this the simultaneous occurrence of two heat shock proteins Hsp70 and Hsp83 was analyzed. A specificity and sensitivity of 100% was observed in *L. donovani* model of experimental murine leishmaniasis. The absorbance value was 8.18 fold on 30 p.i.d in infected controls as compared to normal uninfected controls. However it decreased on 60 (5.85 fold) and 90 (5.23 fold) days post infection. Several other investigators have also reported heat shock proteins (Hsp) from *Leishmania* belonging to the 60, 70, 83, and 90 families that have been tested for the serodiagnosis of leishmaniasis. *L. major* Hsp 60 was tested with cutaneous leishmaniasis sera (Rey-Ladino et al., 1997) and *L. braziliensis* Hsp83 and Hsp70 with cutaneous, mucocutaneous and diffuse cutaneous leishmaniasis sera (Skeiky et al., 1997). In another study, two polypeptide fractions of 72-75 kDa were detected in the urine of 14 of 15 patients with visceral leishmaniasis (VL) and another fraction of 123 kD was found in 10 of the 15 patients with 96% sensitivity and 100% specificity (De Colmenares et al., 1995). This study may be extended for analyzing the diagnostic potential of these two antigens (Hsp70 and Hsp83) in early asymptomatic cases.

Western blotting was used to identify the parasite antigens recognized by serum samples from the experimentally *L. donovani* infected BALB/c mice. The main advantage of western blotting over other serologic techniques that use whole *Leishmania* antigen is its capacity to discriminate early infections. The results showed that bands of both heat shock proteins (Hsp70 Hsp83) were simultaneously present in all infected controls as well as in immunized animals on all post infection/challenge days. Other antigens frequently recognized were those with molecular weights of 36, 46, 54, 58, 78, 100 and 104kDa. Therefore, the results suggest that Hsp70 and Hsp83 in combination may be considered good candidates for diagnosis of visceral leishmaniasis.

To conclude, the cocktail of Hsp70 and Hsp83 in combination with MPL-A provided best protection followed by the cocktail plus ALD and the only cocktail without any adjuvant. The diagnostic potential of both heat shock proteins in combination has also been proved.