Cellular and Humoral Responses to *Leishmania major* Virulence Factors in Healed Cutaneous Leishmaniasis and Mediterranean Visceral Leishmaniasis Patients

Inès Lakhal-Naouar, Thouraya Boussoffara, Amel Meddeb-Garnonaui, Yosser Ben Achour-Chenik, Hechmi Louzir, and Mehdi Chenik*

Laboratory of Immunopathology, Vaccinology, and Molecular Genetics, Institut Pasteur de Tunis, 13, Place Pasteur, 1002 Tunis-Belvedere, Tunisia

Received 16 January 2009/Returned for modification 17 February 2009/Accepted 23 March 2009

Cellular and humoral immune responses of healed cutaneous leishmaniasis and Mediterranean visceral leishmaniasis patients were evaluated against results for *Leishmania major* virulence proteins *L. major* protein disulfide isomerase (LmPDI) and mitogen-activated protein kinase kinase (MAPKK). Only MAPKK induces significant peripheral blood mononuclear cell proliferation with gamma interferon production as well as antibody responses. Thus, MAPKK may be of interest in *Leishmania* vaccination and serodiagnosis.

The leishmaniases are diseases caused by vector-borne pathogens that represent a major public health problem affecting the lives of millions of people worldwide (http://www.who.int/whr/en). Depending on the parasite species and on the immunological response of the human host, leishmaniasis ranges from an asymptomatic infection to a self-limiting cutaneous lesion(s) or a fatal visceral form.

No anti-*Leishmania* vaccine is available at the moment. Different studies showed that development of Th1- and Leishmania-specific cytotoxic immune responses correlate with healing of patients with cutaneous leishmaniasis (CL) (3, 12). An intense effort is being made to identify antigens that could induce an immune state similar to that developed by individuals who recover from symptomatic infection and are resistant to a subsequent natural challenge. Such antigens may contribute to the development of an anti-Leishmania vaccine.

Diagnostic tools targeting leishmaniasis are available. However, parasite detection is invasive and poorly sensitive. Sero logical diagnosis using various techniques based on detection of Leishmania-specific antibodies that are developed during acute disease are often more sensitive, less time-consuming, and more user friendly (4, 11, 15).

In an attempt to identify new antigens to be used as vaccines or for serodiagnosis, we focused on *Leishmania* virulence factors. Indeed, several studies described these factors as potentially immunogenic in humans, mice, and, more recently, dogs (6, 8, 9, 13). Here, cellular and humoral immune responses of healed CL (hCL) and Mediterranean visceral leishmaniasis (MVL) patients were evaluated against results for *Leishmania major* protein disulfide isomerase (LmPDI) and mitogen-activated protein kinase kinase (MAPKK), which we and others previously described as potential virulence factors (2, 10).

We produced LmPDI (55 kDa) and MAPKK (40 kDa) in the prokaryotic expression pET system (Novagen, Gibbstown, NJ) and then purified them by affinity chromatography (Fig. 1).

Lymphoproliferative responses (Fig. 2) and gamma interferon (IFN-γ) and interleukin-10 (IL-10) production (Fig. 3) induced by recombinant LmPDI and MAPKK (10 μg/ml) were characterized in vitro using peripheral blood mononuclear cells (PBMC) from two groups. The first group consisted of 18 hCL patients (age range, 17 to 42 years; mean ± standard deviation [SD], 28 ± 10.9 years) living in an area of *L. major* infection endemicity (the governorate of Sidi Bouzid, Tunisia). Diagnosis of leishmaniasis was based on the presence of specific scars, leishmanin skin test positivity, and a positive lymphoproliferative response to soluble Leishmania antigen (SLA). The second group consisted of 12 healthy individuals (negative controls) living outside areas of endemicity and having no history of CL. All healed patients showed positive proliferation in response to SLA, with stimulation indices (SI) ranging from 3.04 to 207.76 (mean ± SD, 56.83 ± 58.03) (Fig. 2). While no proliferation was observed with LmPDI, MAPKK induced significant PBMC proliferation levels (SI > 2.5) in two of eight hCL individuals. No proliferative responses against SLA and the two recombinant proteins were observed in PBMC from healthy individuals (SI < 2.5) (Fig. 2). Polymyxin B treatment of PBMC cultures stimulated with recombinant proteins had very little or no effect on proliferative responses, showing that observed stimulation could not be due to lipopolysaccharide contamination (data not shown).

IFN-γ and IL-10 levels in culture supernatants of PBMC stimulated with either SLA (10 μg/ml), MAPKK (10 μg/ml), or LmPDI (5 μg/ml) were determined using an enzyme-linked immunosorbent assay (ELISA) (Fig. 3A and B). As expected, SLA induced significantly high levels of IFN-γ but no IL-10 in hCL individuals (mean IFN-γ production levels ± SD of 1,695 ± 1,983 pg/ml and 39.4 ± 49.7 pg/ml, respectively; P = 0.03) (Fig. 3A and B). Interestingly, MAPKK induced high IFN-γ and IL-10 levels in three of eight hCL individuals tested (mean IFN-γ production levels ± SD of 559 ± 642.5 pg/ml and 26.29 ± 25.33 pg/ml and mean IL-10 production levels ± SD of 2,486 ± 3,394 pg/ml and 316.3 ± 174.8 pg/ml in hCL and...
healthy individuals, respectively) (Fig. 3A and B). Interestingly, the difference in production between the two groups was highly significant only for IFN-γ/H9253 (P/H11005 0.007) (Fig. 3A). How-

never, no IFN-γ or IL-10 productions were observed after stim-

ulation of PBMC with LmPDI (Fig. 3A and B). A positive

correlation was found between IFN-γ and IL-10 levels (Spear-

man rank correlation r/H11005 0.594) and between PBMC prolifer-

ation and IFN-γ or IL-10 production (Spearman rank corre-

lation r values of 0.6 [P/H11005 0.03] and 0.583 [P/H11005 0.036] for IFN-γ and IL-10, respectively) in response to MAPKK.

These results show that MAPKK could constitute a potential vaccine candidate. It is well established that IFN-γ is a key cytokine in resistance against CL and is implicated in parasite killing by activated macrophages (14). However, IL-10 is the main downregulating cytokine of the Th1 immune response and exhibits macrophage-deactivating properties (5, 7). Inter-

estingly, the MAPKK IL-10-inducing capacity was not suffi-

cient for suppression of significant proliferation and high, sig-

nificant levels of IFN-γ in hCL patients. The IL-10 production

by PBMC from hCL as well as healthy individuals might be due
to the high level of conservation of MAPKK proteins in eu-

karyotic species. Although MAPKK stimulates high IL-10 lev-
els, it could constitute a potential vaccine candidate since it was
recently reported that the human immune response to crude
and defined Leishmania antigens generated during immuniza-
tion can differ from that induced by natural infection (1).

Similarly, MAPKK used as a vaccine might induce a dominant
Th1 response compatible with protection.

For the second part of the study, we chose to analyze humoral
responses to LmPDI and MAPKK in MVL patients rather than
CL patients for the following reasons: (i) human sera from CL
patients generally show low-level reactivity against Leish-

mania antigens, compared to sera from MVL patients; (ii) diagnosis of MVL is more problematic than that of CL; and (iii) LmPDI and MAPKK are highly conserved with their Leishmania infantum homologue, with 92% and 97% identities, respectively.

The reactivities of MVL patients to LmPDI (10 μg/ml), MAPKK (5 μg/ml), and SLA (2 μg/ml) were assayed by ELISAs with two groups. The first group consisted of 12 MVL children (age range, 1 to 5 years; mean age ± SD, 35 ± 21 months) living in the governorate of Kairouan (a region of MVL endemivity), blood sampled before treatment. Diagnosis of MVL was established on clinical criteria (fever, anemia, splenomegaly, and weight loss) and on demonstration of Leish-

FIG. 1. Expression of recombinant proteins in Escherichia coli. Rec-

ombinant LmPDI (lane 1) and MAPKK (lane 2) were synthesized in

BL21, purified by affinity chromatography over Ni-nitrotriacetic acid

resin, and analyzed on 14% sodium dodecyl sulfate-polyacrylamide gel
electrophoresis gel, followed by Coomassie blue staining. MW, molec-

ular mass markers (kDa).

FIG. 2. Lymphoproliferative response induced by MAPKK or LmPDI. Levels of lymphocyte proliferation in response to either SLA (10 μg/ml) or recombinant proteins MAPKK (10 μg/ml) and LmPDI (5 μg/ml) incubated for 5 days at 37°C and 5% CO₂ are expressed as SI. The cutoff value (SI = 2.5) is indicated by a horizontal bar.

FIG. 3. Immune response induced by MAPKK or LmPDI: IFN-γ (A) and IL-10 (B) induction. PBMC from hCL or healthy subjects were stimulated with either SLA (10 μg/ml) or recombinant proteins MAPKK (10 μg/ml) and LmPDI (5 μg/ml). Supernatants were harvested at 48 h and 72 h and were used for quantification of IL-10 and IFN-γ. The dashes indicate the mean cytokine values obtained for the different groups of individuals. NS, nonstimulated cultures.
healthy controls (C) with SLA (2 μg/mL) and recombinant Leishmania proteins MAPKK (5 μg/mL) and LmPDI (10 μg/mL). Bars show the cutoff value for each ELISA, which is defined as the mean OD + 2 SDs for the values obtained with sera from healthy controls. The significance of differences between P and C groups was evaluated by the Mann-Whitney test. P values of <0.05 were considered significant. Arrows indicate mean OD values.

FIG. 4. ELISA reactivity of sera from patients with MVL (P) and healthy controls (C) with SLA (2 μg/mL) and recombinant Leishmania proteins MAPKK (5 μg/mL) and LmPDI (10 μg/mL). The best sensitivities, with 100% and 91.7%, respectively. Interestingly, the best specificities and positive predictive values were obtained with MAPKK (100% for MAPKK versus 92.3% for SLA). However, SLA gave the best results for negative predictive values (100% versus 92.9% for MAPKK). The ELISA using LmPDI showed only 66.7% sensitivity, with a specificity of 100%. For all proteins, significant differences in measured OD were observed between MVL patients and healthy controls, with P values of <0.05. Taken together, these results indicate that only MAPKK constitutes a major target of humoral response during MVL.

This is the first step in the determination of MAPKK as an interesting antigen for MVL serodiagnosis. Indeed, recruitment of individuals must be improved by (i) increasing the size of MVL patient and control groups and (ii) increasing the number of control groups with healthy individuals living outside the area of endemity, healthy individuals living inside the area of endemity, and patients infected by other pathogens and showing similar symptoms.

To our knowledge, this is the first description of MAPKK from *Leishmania* as a potential vaccine candidate and an MVL serodiagnostic antigen.

### REFERENCES

1. Azeredo-Coutinho, R. B., D. C. Matos, G. G. Armoa, R. M. Maia, A. Schubach, W. Mayrink, and S. C. Mendonca. 2008. Contrast personality cytokine responses to promastigote whole-cell extract and the Leishmania analogue receptor for activated C kinase antigen of *L. amazonensis* in natural infection versus immunization. Clin. Exp. Immunol. 153:369–375.

2. Ben Aouch, Y., M. Chenik, H. Louiz, and K. Delliagi. 2002. Identification of a disulfide isomerase protein of *Leishmania* major as a putative virulence factor. Infect. Immun. 70:3576–3585.

3. Boussafara, T., H. Louiz, A. Ben Salah, and K. Delliagi. 2004. Analysis of granzyme B activity as a surrogate marker of *Leishmania*-specific cell-mediated cytotoxicity in zoonotic cutaneous leishmaniasis. J. Infect. Dis. 189:1265–1273.

4. Changsuek, F., S. Rijal, L. K. Jha, P. Desjeux, R. M. Karki, S. Koirala, L. Loutan, and M. Bards. 2006. Field validity, reproducibility and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal. Trop. Med. Int. Health 11:31–40.

5. de Waal Malefyt, R. J., A. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174:1290–1220.

6. Fernandes, A. P., M. M. Costa, E. A. Coelho, M. S. Michalick, E. de Freitas, M. N. Melo, W. Luiz Tafuri, M. Resende, V. Hermont, F. Abrantes, and R. T. Gazzinielli. 2008. Protective immunity against challenge with *Leishmania* (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. Vaccine 26:5888–5895.

7. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O’Garra. 1993. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. 147:3815–3822.

8. Jaafari, M. R., A. Gharafian, A. Farrokh-Giour, A. Samiei, M. T. Kherei, F. Mahmoudi, F. Barkhordari, A. Khamesipour, and W. R. McMaster. 2006. Immunoreponse and protection assay of recombinant major surface glyco-protein of *Leishmania* (rg63) reconstituted with liposomes in BALB/c mice. Vaccine 24:5708–5717.

9. Khoshgo, N., F. Zahedifard, H. Azizi, Y. Taslimi, M. J. Alonso, and S. Rafati. 2008. Cystine proteinase type III is protective against *Leishmania* infantum infection in BALB/c mice and highly antigenic in visceral leishmaniasis individuals. Vaccine 26:5822–5829.

10. Kuhn, D., and M. Wiese. 2005. LmxPK4, a mitogen-activated protein kinase homologue of *Leishmania* mexicana with a potential role in parasite differentiation. Mol. Microbiol. 56:1169–1182.

11. Pedras, M. J., L. de Gouvea Viana, E. J. de Oliveira, and A. Rabello. 2008. Comparative evaluation of direct agglutination test, rK39 and soluble antigen ELISA and IFAT for the diagnosis of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 102:172–178.

12. Reithinger, R., J. C. Dujardin, H. Louiz, C. Firmesse, B. Alexander, and S. Brooker. 2007. Cutaneous leishmaniasis. Lancet Infect. Dis. 7:581–596.

13. Resende, D. M., B. C. Caetano, M. S. Dutra, M. L. Penido, C. F. Abrantes, R. M. Verly, J. M. Resende, D. Pilo-Veloso, S. A. Rezende, O. Bruna-Belloso, A. P. Fernandes, and R. T. Gazzinielli. 2008. Epitope mapping and protective immunity elicited by adenovirus expressing the Leishmania amastigate specific A2 antigen: correlation with IFN-gamma and cytokite activity in CD8+ T cells. Vaccine 26:4585–4595.

14. Sacks, D., and N. Noben-Trauth. 2002. The immunology of susceptibility and resistance to *Leishmania* major in mice. Rev. Nat. Rev. Immunol. 2:845–858.

15. Schallig, H. D., G. J. Schoone, C. C. Kroon, A. Hallu, F. Chappuis, and H. Veek. 2001. Development and application of ‘simple’ diagnostic tools for visceral leishmaniasis. Med. Microbiol. Immunol. 190:69–71.

### TABLE 1. Sensitivities and specificities of ELISAs using crude SLAs and recombinant *Leishmania* proteins MAPKK and LmPDI

| Antigen | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---------|----------------|----------------|---------|---------|
| Crude SLA | 100 | 92.3 | 92.3 | 100 |
| MAPKK | 91.7 | 100 | 100 | 92.9 |
| LmPDI | 66.7 | 100 | 100 | 76.5 |

* PPV, positive predictive value; NPV, negative predictive value.