Immune Responses of Iranian Patients with Visceral Leishmaniasis and Recovered Individuals to LCR1 of *Leishmania infantum*

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Visceral leishmaniasis is a serious public health problem. *Leishmania infantum* is one of its causative agents. LCR1 is an immunogen from *L. infantum*. Antibodies against this protein have been detected in visceral leishmaniasis patients. The aim of this study was to define the antibody and cellular immune responses against LCR1 in Iranian visceral leishmaniasis patients and recovered individuals. The LCR1 protein was produced in recombinant form. Antibody responses against this protein were studied in Iranian individuals with a recent history of visceral leishmaniasis. Responses of peripheral blood mononuclear cells to this protein were studied in Iranian individuals who had recovered from visceral leishmaniasis. Our data show that (i) there was an antibody response to LCR1 in each individual with a recent history of visceral leishmaniasis studied, (ii) there was neither a proliferative response nor production of gamma interferon (IFN-γ) or interleukin 10 in response to LCR1 by mononuclear cells from individuals who had recovered from visceral leishmaniasis, and (iii) individuals who have recovered from visceral leishmaniasis show ongoing immune responses long after recovery from the disease. These data show that there are no detectable cellular memory responses to LCR1 in Iranian individuals who have recovered from visceral leishmaniasis, while there are detectable antibody responses in patients with this disease. Our data suggest that LCR1 has potential applications for the diagnosis of leishmaniasis through antibody detection, while the application of LCR1 alone for induction of IFN-γ in individuals who recovered from this disease is not supported. The presence of long-lasting immune reactivities in individuals who recovered from the disease may show the necessity of extended medical surveillance for these individuals.

Leishmaniasis is a neglected disease resulting in a global mortality rate of approximately 60,000 per year. Visceral leishmaniasis (VL) is almost always fatal if left untreated. VL accounts for the majority of mortalities from leishmaniasis, represents a serious public health problem in regions where leishmaniasis is endemic, and is rapidly emerging as an opportunistic infection in HIV patients (1). Patients who recover from VL are resistant to reinfection (2). The fact that recovery from VL protects individuals from disease indicates that it should be possible to develop a vaccine against VL. However, there is no vaccine available for VL. There is great interest in finding immunogenic molecules from *Leishmania infantum* with potential applications as a vaccine candidate or a diagnostic molecule. Many immunogenic molecules from *Leishmania infantum* have been reported (1).

Antibody responses are parts of the immune response against *Leishmania infantum* in humans. The presence of anti-*Leishmania* antibodies has been documented in VL (3, 4). The roles of these antibodies in immunity against VL in humans are not clear (2). However, the regulatory (5) and exacerbating (6) roles of antibodies in murine visceral leishmaniasis have been reported. Anti-*Leishmania* antibodies have been used as diagnostic tools for this disease (7, 8).

Lymphocyte proliferation against *Leishmania* antigens is associated with protective immunity to VL (9). Gamma interferon (IFN-γ) is necessary for resistance against VL (10). Interleukin 10 (IL-10) is a counterprotective cytokine in VL (10).

LCR1 is an immunogenic molecule discovered through screening a cDNA library of *Leishmania infantum* chagasi (11). This antigen has reacted with sera from Brazilian VL patients, showing the presence of anti-LCR1 in VL patients (11). Vaccination with LCR1 in a murine model of VL has shown some degree of protection against the disease (11). We were interested in whether LCR1 has potential uses as a vaccine or a diagnostic molecule in Iranian individuals. To approach these goals, we studied antibody responses against LCR1 in Iranian VL patients. We also evaluated lymphoproliferative responses as well as production of IFN-γ and IL-10 in response to LCR1 in individuals who recovered from VL in Iran.

**MATERIALS AND METHODS**

**Antigen**. LCR1 recombinant protein was produced and characterized as reported (12). Briefly, the procedure was performed as follows: *Escherichia coli* strain BL21(DE3) pLyS (Invitrogen) was transformed by a pRSETA plasmid, including the lcr1 insert. The transformed bacteria was cultured and precipitated by centrifugation, the supernatants were discarded, and pellets were stored frozen at −20°C until use. The cell pellet was then resuspended in phosphate-buffered saline (PBS) and passed through five cycles of freeze and thaw (liquid nitrogen and 37°C, respectively), then centrifuged. The pellet was discarded and the supernatant which contained LCR1 was aliquoted and stored at −70°C until use. Since the recombinant LCR1 was produced in *Escherichia coli*, lysis of the same bacteria transformed by the same plasmid but without *lcr1* insert was used as negative control in immunoblotting.

Soluble *Leishmania* antigens (SLA) were prepared as follows. *L. infantum* (strain MHOM/04/IR/IPI-UN10) was grown in culture medium (RPMI 1640, 10% fetal bovine serum [FBS], 2 mM t-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin). The stationary-phase parasites were harvested, washed in PBS (2 times), freeze-thawed (6 times),
and centrifuged (16,000 \times g, 20 min, 4°C), and then the supernatant was collected, aliquoted, and stored in −70°C until use. The protein content of SLA was 500 \mu g/ml as determined by the Bradford method (13).

**Study population.** Six patients with a recent history of VL were selected. The disease was diagnosed in these patients 4 to 11 months before sampling for the present study. The patients were 5 females and 1 male, with ages of 3.8 ± 1.9 years, from Bojnord in the North Khorasan province located in northeast Iran. VL was confirmed in all patients by observation of parasites in bone marrow smears. In one case, the titer of a direct agglutination test (DAT) was available, which was 1/102,400. All patients had received meglumine antimoniate (Glucantime) as therapy for 28 days. Parents of all patients gave written informed consent, and the study protocol was approved by the ethics committee of the Pasteur Institute of Iran. Thirteen VL-recovered individuals, 8 females and 5 males, with ages of 4.3 ± 1.7 years, were selected from Ahar in the East Azerbaijan Province in northwest Iran. These individuals had received meglumine antimoniate (for 28 days in 12 patients and 14 days in 1 patient). They had recovered from VL between 3 to 42 months (27.53 ± 12.59 months) before blood draw for our study. VL was confirmed in these individuals by documented compatible clinical presentation in addition to either isolation (or microscopic detection) of parasite from bone marrow aspirate or a positive direct agglutination test (DAT) test (titer, ≥1/6,400). A leishmanin skin test (LST) was carried out in recovered cases and all cases were LST positive (LST inductions were from 7 mm to 15 mm, and the average was 8.95 ± 2.37 mm).

Ten healthy individuals, 8 male and 2 female, with ages of 31.2 ± 9.2 years, and without any history of VL or exposure to Leishmania infection, were selected from Tehran, which is an area where leishmaniasis is not endemic. LST was done, and all results were negative (LST inductions of <5 mm). Three healthy individuals were used as negative controls in immunoblotting. These individuals were male adults with no history of leishmaniasis.

**Sample collection.** Peripheral blood was withdrawn from all subjects of the study population. For use in immunoblotting, serum samples were separated after clotting and stored at −70°C until use. Serum samples were used in immunoblotting, individually. Pooled serum was used in immunoblotting. These individuals were male adults with no history of leishmaniasis.

**Lymphocyte proliferation assay.** The lymphocyte proliferation assay was performed according to the reported procedure (14). PBMC samples of each individual were cultured in triplicates in the presence of phytomagglutinin (PHA), SLA, and LCR1, with final concentrations of 5, 5, and 7.5 \mu g/ml, respectively. A wide range of LCR1 concentrations (5 to 40 \mu g/ml final concentrations) were used in cell culture of selected VL-recovered individuals. After 4 days of culture, about half of the culture supernatant was harvested for cytokine assay, then \(^3\)H-thymidine (0.5 \mu Ci/well) (Amersham Pharmacia Biotech, Buckinghamshire, England) was added to each well, and after 16 to 18 h cells were harvested on filter paper suitable for cell harvesting. Filter papers containing the cells were dried overnight at room temperature or for a few hours at 37°C. Filter papers corresponding to each well were transferred into separate scintillation vials. Two milliliters of scintillation fluid (Ready Safe; Beckman Coulter, Fullerton, CA) was then added to tubes and the tubes were counted in liquid scintillation counter (Wallac 1410, Turku, Finland). The lymphoproliferative results are presented in counts per minute (cpm) or stimulation index (SI). SI was calculated by dividing the cpm of the stimulated well by the cpm of the unstimulated well of the same individual.

**Cytokine assay.** PBMCs from recovered individuals were cultured in the presence of PHA, SLA, and LCR1 as described above. Supernatant were collected after 4 days and cytokines were assayed in the supernatants. IFN-γ and IL-10 were assayed by kits from e-Bioscience (human IFN-γ enzyme-linked immunosorbent assay [ELISA] Ready-SET-Go, and human IL-10 ELISA Ready-SET-Go, respectively). The assay procedure is briefly described as follows. ELISA plates (Corning, Lowell, MA) were coated with pretitrated capture antibody in ELISA coating buffer, sealed, and incubated overnight at 4°C. The plates were washed and any residual buffer was removed. Wells were blocked with assay diluent. The wells were aspired and washed. Six consecutive 2-fold serial dilutions of standard for IFN-γ or IL-10 were prepared in assay diluent. Standards and samples from cell culture supernatants were added to the appropriate wells and incubated. The detection antibodies (diluted in assay diluent) were added to wells and incubated. Plates were washed and Avidin–horseradish peroxidase (HRP) (diluted in assay diluent) was added and incubated. Tetramethylbenzidine (TMB) substrate solution was added to wells and incubated. Stop solution (2 N H\textsubscript{2}SO\textsubscript{4}) was added to wells and the plates were read by a microplate reader (Anthos 2020; Eugendorf, Austria) at 450 nm and 620 nm. The values at 620 nm were subtracted from the values at 450 nm and the data were analyzed.

**Bacterial sample preparation.** Escherichia coli strain BL21(DE3) pLysS (Invitrogen), which was transformed by the prSETA plasmid lacking the lcr1 insert (12), was used as the negative control. The bacteria were grown in Luria Bertani (LB) medium supplemented with 50 \mu g/ml ampicillin and 35 \mu g/ml chloramphenicol at 37°C overnight. The cultures were then subcultured in fresh LB medium (1:50, old/new medium) without antibiotics at 37°C with vigorous shaking for 5 h. The bacteria from 25 ml culture media were divided to 1-ml aliquots and precipitated by centrifugation (3,000 \times g, 10 min, 4°C), and then the supernatants were discarded. The pellets were stored frozen at −20°C prior to use. The cell pellet of each aliquot was then resuspended in 100 \mu l of phosphate-buffered saline (PBS) and passed through five cycles of freeze and thaw (liquid nitrogen and 37°C, respectively), then centrifuged at 16,000 \times g, for 10 min at 4°C. Since LCR1 is soluble (12), the pellet was discarded and the supernatant was kept at 4°C until use.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** SDS-PAGE was performed using a 10% concentration of acrylamide (CinnaGen, Tehran, Iran) in the resolving gel. Samples were prepared by adding a protein loading buffer (Fermentas) (1:6, loading buffer/protein suspension) and boiling for 5 min before loading onto an SDS-PAGE gel. The running buffer consisted of 1g SDS (CinnaGen, Tehran, Iran), 3.03 g Tris base (Sigma, St. Louis, MO) and 14.0 g glycine (AppliChem, Darmstadt, Germany) in 1,000 ml double-distilled water (ddH\textsubscript{2}O). The samples were subjected to electrophoresis at 15 mA and 10 mA in stacking and resolving gel, respectively. Finally, the gel was stained with Coomassie brilliant blue R250 (Acros Organics, New Jersey).

**Immunoblot analysis.** The reactivities of sera from each VL patient against LCR1 and SLA were studied through immunoblotting according to established methods (15). Briefly, proteins were transferred from unstained SDS-PAGE gel to nitrocellulose membranes (Sigma, St. Louis) by a semidry apparatus (Multiphore II electrophoresis system; Pharmacia, Sweden) at 14 V for 60 min. The transfer was confirmed by observing prestained protein ladder (Vivantis, Malaysia) bands on the membranes. The membranes were incubated in 50 ml of 3% bovine serum albumin (BSA) (Merck, Darmstadt, Germany) in Tris-buffered saline (TBS) (Tris-HCl 100 mM [pH 7.5] and NaCl 150 mM) overnight. The membranes were then washed three times (15 min each time) in 200 ml of Tween-20-buffered saline (TTBS) washing buffer (TBS and 1% Tween 20). Each diluted serum was then added to a membrane containing the following proteins: LCR1, SLA, BSA, and lysozyme of bacteria lacking the lcr1 insert (negative control). Sera were used at 1:500 in TBS containing 1% BSA. The membranes were incubated for 2 h and were then washed and incubated for 2 h in HRP-conjugated anti-human IgG (Bethyl) (diluted at 1/8,000 in TBS containing 1% BSA) as secondary antibody (10 ml for each membrane). The membranes were washed again and 3,3’-diaminobenzidine (DAB) (Sigma, St. Louis) solution (15 ml of TBS, 9 mg DAB powder, and 27 ml 30% H\textsubscript{2}O\textsubscript{2}) was added to each membrane. The membranes were washed with ddH\textsubscript{2}O after signal development, and results were recorded.
Statistical analysis. A t-test was used for comparison between different groups, and differences were considered significant at P values of ≤0.05.

RESULTS

No proliferative response to LCR1 in VL-recovered individuals. Lymphocyte proliferation assays were performed with PBMCs from VL-recovered individuals as well as naive ones as controls. The results of lymphoproliferative responses of naive and VL-recovered individuals are presented in Fig. 1 (upper panel).

The baseline lymphoproliferative responses are seen in cultures of PBMCs without any in vitro stimulants (i.e., without PHA, SLA, or LCR), which is actually a negative control of the assay. Immune individuals showed significantly (P < 0.001) higher baseline responses than naive ones (counts per minute of 3,691 ± 1,627 and 1,442 ± 684, respectively) (Fig. 1, upper panel). This baseline responsiveness is not an artifact of the assay, because the proliferative responses to PHA and LCR, in the same assay, were not significantly different between naive and immune individuals (P > 0.94 and P > 0.40, respectively) (Fig. 1). This higher baseline responsiveness of immune individuals may show that there is an ongoing response in these individuals due to the remaining activity of VL.

The SI results of naive and VL-immune individuals are shown in Fig. 1 (lower panel). The SI values for PHA, as an antigen-nonspecific positive control, were 19.79 ± 20.97 and 5.61 ± 2.22 in naive and VL-immune individuals, respectively. These results show the assay is sufficiently optimized and can be reliable. The proliferative responses to SLA, as an antigen-specific positive control, were significantly (P < 0.01) higher in VL-immune individuals than in naive individuals (SIs, 4.53 ± 2.85 and 1.86 ± 1.16, respectively) (Fig. 1). These results show the overall correct selection of naive and VL-immune individuals, because there is immune memory against SLA in VL-immune individuals, while the naive ones do not show such memory.

The lymphoproliferative responses of VL-immune individuals against LCR1 were lower than those of negative controls (no in vitro stimulation) (SI, 0.90 ± 0.15), which shows that there is no memory response against LCR1 in VL-immune individuals. The lymphoproliferative response against LCR1 was slightly but significantly (P < 0.001) higher in naive individuals than in immune ones (SI, 2.87 ± 2.06 and 0.90 ± 0.15, respectively). The reason for the responses of naive individuals to LCR1 is not clear and needs further study.

The negative memory responses against LCR1 in VL-immune individuals were obtained using one concentration of LCR1 (7.5...
μg/ml) in the lymphocyte proliferation assay. To study whether these results were not due to unoptimized concentrations of LCR1 antigen, different concentrations of LCR1 were used in further lymphocyte proliferation assays. A wide range of concentrations of LCR1 (5, 10, 20, and 40 μg/ml final concentrations) were used in lymphocyte proliferation assays of PBMCs of two recovered individuals. The results were negative for all concentrations studied (SIs of 1.17, 1.41, 1.20, and 1.04 for LCR1 concentrations of 5, 10, 20, and 40 μg/ml, respectively) (Fig. 2).

IFN-γ and IL-10 were produced in response to SLA by VL-immune individuals. PBMCs of naive and VL-immune individuals were cultured in the presence of PHA, SLA, and LCR1, and cytokines of IFN-γ and IL-10 were determined in culture supernatants after 4 days. IFN-γ and IL-10 responses to SLA of VL-immune individuals and healthy controls are presented individually in Fig. 3. These data show that, in response to SLA, each of the VL-immune individuals produced IFN-γ as well as IL-10 more than each of naive controls. IFN-γ was not produced in response to LCR1 by VL-immune individuals. The results of IFN-γ secretion are presented in Fig. 4 (upper panel). These results show that IFN-γ responses to PHA (as antigen-nonspecific controls) in naive individuals are significantly ($P < 0.0001$) higher than those in the same individuals with no in vitro stimulation (negative controls) ($2,477 \pm 1,145$ and $31 \pm 11$, respectively). Responses to SLA in VL-immune individuals (as antigen-specific positive controls) were significantly ($P < 0.00001$) higher than those in the same individuals with no in vitro stimulation (baseline response) ($3,205 \pm 70$ and $1,195 \pm 1,360$, respectively). These results show that the assay is reliable and the selection of recovered individuals was performed correctly (Fig. 4, upper).

The basal levels of IFN-γ responses in negative-control cultures of VL-immune individuals were higher than the same values in naive individuals ($P < 0.02$) (Fig. 4, upper panel), which shows the presence of higher levels of immune responses in immune individuals even in the absence of any in vitro stimulation. This may show that the cells of immune individuals have already been stimulated in vivo by Leishmania parasites due to the remaining activity of visceral leishmaniasis.

In VL-immune individuals, IFN-γ responses against LCR1 did not differ significantly ($P > 0.40$) those of from negative controls ($1,321 \pm 1,366$ and $1,195 \pm 1,360$, respectively). These data show that there are no memory responses against LCR1 resulting in IFN-γ secretion in VL-immune individuals. On the other hand, in naive individuals, IFN-γ responses against LCR1 were slightly but significantly ($P < 0.01$) higher than those in negative controls ($136 \pm 95$ and $31 \pm 11$, respectively) (Fig. 4, upper panel). This finding may show a relatively weak immune response against LCR1 in naive individuals, which needs further study.

IFN-γ responses against PHA were significantly ($P < 0.02$) higher in naive individuals than in recovered ones ($2,477 \pm 1,145$ and $1,111 \pm 1,014$) (Fig. 4, upper panel). This shows that cells of naive individuals have higher capacities for stimulation than VL-immune ones. This may show that the capacity of being stimulated in vitro has been reduced in VL-immune individuals because they are already in a state of stimulation by Leishmania parasites due to remaining activities of VL.

IL-10 was not produced in response to LCR1, SLA, and PHA by VL-immune individuals. PBMCs of naive and VL-immune individuals were cultured in the presence of PHA, SLA, and LCR1, and levels of cytokines of IL-10 were determined in culture supernatants after 4 days. Results of IL-10 responses are presented in Fig. 4 (lower panel). In naive individuals, IL-10 responses against PHA were significantly higher ($P < 0.0002$) than those in negative controls, but in these individuals the responses to the other two stimulants (SLA and LCR1), were not (Fig. 4, lower panel). This shows that cells of naive individuals produce IL-10 in response to PHA as a stimulant, while SLA or LCR1 do not have the same capacity to produce responses. On the other hand, in VL-immune individuals as well as negative controls, the IL-10 response was not significantly different from the responses to any of the stimulants (PHA, SLA, and LCR1). This shows that cells from VL-immune individuals do not secrete IL-10 in response to any of the stimulants used, even in response to a powerful stimulant such as PHA. This may show that a basal level of IL-10 is being produced due to remaining disease activity and no further IL-10 production is pos-
It is noteworthy that the levels of IL-10 in no-stimulant controls were significantly higher in immune individuals than in naive ones ($P < 0.01$), confirming higher basal levels of responses in VL-immune individuals than in naive ones (Fig. 4, lower panel).

Sera from all VL patients recognize the recombinant LCR1 protein. LCR1, SLA, and BSA were run in 10% SDS-PAGE. The results showed significant bands for each protein with expected molecular weights (~40 for LCR1, ~90 for BSA, and several protein bands for SLA) (Fig. 5, part (a), right panel). To study the antibody responses of VL patients against the recombinant LCR1 protein, sera from patients were pooled and were used in immunoblotting. The pooled sera reacted with LCR1 and SLA, but did not react with BSA, ruling out any nonspecific reactions between the sera and LCR1 or SLA proteins (Fig. 5a, left panel, and Fig. 5b, right panel). Serum from a healthy control individual did not recognize BSA, LCR1, and SLA, showing the specificities of reactions of LCR1-VL patient sera (Fig. 5b, left panel). Both sera of normal healthy controls and VL patients reacted with bacterial lysate (negative control), showing the presence of anti-*E. coli* antibodies in patients as well as normal healthy individuals (Fig. 5b, both panels). Sera from two additional healthy control individuals were used in immunoblotting against LCR1 and SLA. The results showed that the two sera did not recognize LCR1 and SLA.

Immunoblotting experiments were also carried out with serum of each individual patient. The results (Fig. 5c, panels 1 to 6) show that serum of each VL patient recognizes recombinant LCR1 and SLA (a single band with a molecular weight of ~40, corresponding to LCR1, and several bands corresponding to SLA).
Omission of serum (primary antibody) in this experiment resulted in no visible band in the immunoblot, confirming the absence of any nonspecific reaction in this assay (Fig. 5c, “No serum”).

DISCUSSION
Our data show that there are no proliferative responses against the recombinant LCR1 protein by PBMCs of VL-immune individuals. Lack of proliferative responses in individuals who recovered from VL were not due to improper concentrations of LCR1 in cell cultures, because a wide range of LCR1 concentrations, from 5 to 40 μg/ml, did not result in any LCR1-specific proliferation (IS, ≤1.41). These findings show that there are no proliferative memory immune responses against LCR1 in VL-immune individuals, or at least such memory is not sufficiently high that it could be detected in our assay setting.

The higher SI against LCR1 in naive individuals was quite unexpected, and the reason for such responses cannot be determined without further study. However, these responses may originate from cross-reactivity between LCR1 and other antigens encoun-

FIG 5 Patient sera recognize recombinant LCR1 protein. (a) Identities of proteins were verified by SDS-PAGE and immunoblotting. A single band (~40 kDa) corresponding to LCR1, and several bands corresponding to SLA were detected in both SDS-PAGE (SDS) and immunoblotting analysis (IB). A single band (~90 kDa) corresponding to BSA was observed in SDS-PAGE, while no such band was observed in immunoblotting analysis, showing the specificities of reactions of VL patient sera to Leishmania antigens (LCR1 and SLA). (b) Pooled sera from six patients with a recent history of VL (Positive serum) reacted with both LCR1 and SLA, while a normal healthy control serum sample (Negative serum) reacted with none of them. Both VL and normal healthy control sera recognize bacterial lysate lacking the lcr1 insert (Neg), showing the presence of anti-E. coli antibodies in patients as well as normal healthy individuals. Data shown for “Negative serum” are the results of one representative serum out of three negative healthy control sera with identical results. (c) Each patient’s serum (1 through 6) show the reactivity against recombinant LCR1 and SLA (as a positive control). When no serum was applied on one immunoblot (No serum), it resulted in no visible band on the immunoblot, confirming the absence of a nonspecific reaction in the immunoblot. Abbreviations: SLA, soluble Leishmania antigens; LCR, recombinant protein of LCR1; BSA, bovine serum albumin; Mw, molecular weight in thousands; Neg, E. coli bacteria containing uninserted plasmids; IB, immunoblotting; SDS, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
tered by naive individuals throughout their lives. Alternatively, these responses may be artifacts of the assay due to the possible residual endotoxin (lipopolysaccharide) remaining in the recombinant LCR1 protein. It is noteworthy that we had sufficiently depleted the endotoxin in the recombinant LCR1. The LCR1 used in our assays had less than 0.1 endotoxin IU/ml (12), which cannot induce such in vitro immune responses.

No significant increases of IFN-γ or IL-10 production in response to LCR1 were observed in PBMC cultures of VL-immune individuals. At the same time, all VL-immune individuals produced high levels of IFN-γ in response to SLA, showing the presence of memory immune responses to Leishmania antigens. These results show that there is no IFN-γ-producing memory response against LCR1 in Iranian VL-recovered individuals. These data show that LCR1 may not have the capability of inducing an IFN-γ-protective immune response in Iranian individuals.

An interesting finding of our data is the presence of baseline immune responses in individuals who did not need any in vitro stimulation. These baseline responses were detected by lymphocyte proliferation in the absence of any in vitro stimulation and were confirmed by detection of IFN-γ and IL-10. It is important to consider that these individuals had recovered from clinical VL long before sample collection for the current study (more than 2 years on average). This means that the immune responses against the parasite had lasted for a long time even after clinical recovery from VL. This reactivity may be due to the presence of an ongoing response against Leishmania regardless of clinical recovery from VL. The long-lasting immune memory against Leishmania parasites is a well-accepted phenomenon in Leishmania infections. However, as far as we know, the presence of a long-lasting immune response (and not immune memory) after clinical recovery from VL has not been reported and our findings are novel. This finding may underscore the need for extended medical surveillance for VL-recovered individuals. A weakness of our study was the difference between the average ages of patients and healthy controls. This was due to the difficulty in recruiting children (through their parents’ consent) who agreed to enter our study voluntarily. Our conclusion regarding presence of an ongoing immune response in VL-recovered individuals is based on the comparison of VL-recovered individuals with healthy controls who were not age matched. Does this mismatch invalidate our conclusion? It seems that our conclusion may be valid because it is based on the comparison of baseline immune responses (unstimulated PBMCs), and all of the previous studies considering the effect of age on immune response showed that the proliferative responses of unstimulated PBMCs are not significantly affected by the age of donors, although PHA-stimulated proliferation declines with increasing age (16–19).

Antibody responses against LCR1 were present in all six of the VL patients in the present study, while no antibody responses were detectable in a control individual. Our findings regarding the presence of antibody responses against LCR1 in VL patients are in accordance with those of another reported study (11). These findings show the potential of LCR1 as a diagnostic molecule for VL. More studies are needed for assessment of the diagnostic utilities of this antigen for VL.

In summary, our findings show that LCR1 may have utilities for diagnosis of VL, while the applications of this molecule for vaccine purposes are not supported. Our findings also show that there are long-lasting immune responses in VL-recovered individuals that continue years after clinical recovery. These long-lasting responses may show the necessity of extended medical surveillance of VL-recovered individuals.

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