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Identification and Characterization of T Cell-Stimulating Antigens from \textit{Leishmania} by CD4 T Cell Expression Cloning\textsuperscript{1}

Peter Probst,\textsuperscript{2,*} Erika Stromberg,\textsuperscript{*} Hashim W. Ghalib,\textsuperscript{†} Michelle Mozel,\textsuperscript{‡} Roberto Badaro,\textsuperscript{§} Steven G. Reed,\textsuperscript{*,*} and John R. Webb\textsuperscript{2,*†}

Persistent immunity against \textit{Leishmania} infections in humans is mediated predominantly by CD4\textsuperscript{+} T cells of the Th1 phenotype. Herein we report the expression cloning of eight \textit{Leishmania} Ags using parasite-specific T cell lines derived from an immune donor. The Ags identified by this technique include the flagellar proteins \(\alpha\) and \(\beta\)-tubulin, histone H2b, ribosomal protein S4, malate dehydrogenase, and elongation factor 2, as well as two novel parasite proteins. None of these proteins have been previously reported as T cell-stimulating Ags from \textit{Leishmania}. \(\beta\)-tubulin-specific T cell clones generated against \textit{Leishmania major} amastigotes responded to \textit{Leishmania}-infected macrophages and dendritic cells. IFN-\(\gamma\) enzyme-linked immunospot analysis demonstrated the presence of T cells specific for several of these Ags in PBMC from self-healing cutaneous leishmaniasis patients infected with either \textit{Leishmania tropica} or \textit{L. major}. The responses elicited by \textit{Leishmania} histone H2b were particularly striking in terms of frequency of histone-specific T cells in PBMC (1 T cell of 6000 PBMC) as well as the percentage of responding donors (86%, 6 of 7). Ags identified by T cells from immune donors might constitute potential vaccine candidates for leishmaniasis. \textit{The Journal of Immunology}, 2001, 166: 498–505.

\textit{Leishmania} are protozoan parasites that infect human macrophages and cause a spectrum of clinical diseases including self-healing skin lesions, diffuse cutaneous or mucosal disease, as well as potentially fatal visceral disease. Clinical symptoms result from replication of the parasites in macrophages of the dermis, naso-oropharyngeal mucosa, and the mononuclear phagocyte system, respectively (reviewed in Refs. 1–3). Leishmaniasis is prevalent in four continents and is considered by the World Health Organization to be one of the top six most important human parasitic diseases. In 1990, estimates by the World Health Organization indicated that \(~350\) million people were at risk of acquiring leishmaniasis and that 12 million were currently infected (4). In a recent epidemic of visceral leishmaniasis (VL),\textsuperscript{3} in southern Sudan, Medicins Sans Frontieres estimated that the excess mortality has been about 100,000 deaths among about 300,000 people at risk (5).

\begin{itemize}
  \item[\textsuperscript{1}] This work is supported by National Institutes of Health Grants AI-25038, AI-36810, and TW-00428. J.R.W. is supported by Medical Research Council of Canada Grant MOP-36341. P.P. was supported by Deutsche Forschungsgemeinschaft Grant Pr 4906/1-1.
  \item[\textsuperscript{2}] Address correspondence and reprint requests to Dr. Peter Probst, Corixa Corporation, 1124 Columbia Street, Suite 200, Seattle, WA 98104, E-mail address: probst@corixa.com or Dr. John R. Webb, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada K1H 8 M5. E-mail address: jwebb@uottawa.ca
  \item[\textsuperscript{3}] Abbreviations used in this paper: VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; MoDC, monocyte-derived dendritic cells; LPr, \textit{Leishmania} major promastigote lysate; CFPr, \textit{L. major} promastigote culture filtrate; CFAm, \textit{Leishmania} culture filtrate; SFC, spot-forming cells; LACK, \textit{Leishmania} homologue of the intracellular receptor for activated protein kinase; TCM, RPMI 1640 containing 10% human AB serum, 25 mM l-glutamine, 25 mM HEPES, and 50 \(\mu\)g/ml gentamicin; ORF, open reading frame; ELISPOT, enzyme-linked immunospot; PPD, purified protein derivative; SI, stimulation index.
\end{itemize}
by highly divergent protective immune responses in different forms of leishmaniasis caused by Leishmania tropica or Leishmania donovans with self-healing cutaneous leishmaniasis (CL) due to Leishmania major. T cell clones were obtained from a T cell line generated before the cellular immune responses of T cells against leishmanial Ags were determined. T cell clones were obtained from a T cell line generated from a Montenegro skin test-positive male donor living in a region endemic for VL. T cells from a immune donor residing in a region endemic for VL.

Materials and Methods

Parasites

Promastigotes of L. major (Friedlin strain) were maintained at 26°C in M199 with 10% FCS, 40 mM HEPES pH 7.4, 100 μM adenine, and 0.005% hemin. Lysate of L. major promastigotes (LP) was prepared by sonicating promastigotes in PBS containing protease inhibitors (one complete protease inhibitor tablet (Boehringer Mannheim, Indianapolis, IN) per 25 ml PBS). Culture filtrate of L. major promastigotes (CFPr) was prepared by growing promastigotes (1 x 10^7/ml) overnight in AIMV serum-free medium (Life Sciences, St. Petersburg, FL) followed by removal of parasitoids by centrifugation and filtration through a 0.2-μm sterile filter. Supernatants were concentrated 50-fold by ultrafiltration with a 3-kDa-cutoff filter unit (Amicon, Beverly, MA). Amastigotes of L. major were recovered from the nonulcerative lesions of SCID mice that had received an intradermal inoculation of promastigotes at the base of the tail 6–8 wk earlier. Culture filtrate of L. major amastigotes (CFAm) was prepared as described above for CFPr without concentrateing the supernatants.

Patients

T cell lines and clones were generated from a Montenegro skin test-positive male donor living in an endemic area for VL due to infection by Leishmania chagasi in Northeastern Brazil. The donor had no clinical history of leishmanial disease. PBMC from CL patients were obtained after spontaneous cure of CL from seven untreated patients living in Abha, Saudi Arabia. Patients were diagnosed with self-resolved CL due to L. tropica (six cases) or L. major (one case) infections. Lesions due to CL healed spontaneously in a period of 2 (five cases), 6 (one case), or 12 mo (one case), respectively.

Generation of T cell lines and clones

PBMC were obtained from the apheresis product of the Montenegro skin test-positive male donor living in an endemic area for VL due to infection by Leishmania chagasi. T cell lines were generated by stimulating PBMC (5 x 10^6) with either LP or CFPr (10 ng/ml each) in TCM (RPNI 1640 containing 10% human AB serum, 25 mM L-glutamine, 25 mM HEPES, and 50 μg/ml gentamicin). After 3 days IL-2 (10 ng/ml) was added to the cultures. Cultures were split every 3–5 days and restimulated with autologous irradiated or HLA-matched PBMC and the respective Ag every 13–16 days. Before restimulation, T cell lines were cultured for at least 3 days in resting medium (TCM containing 10 ng/ml IL-7 and 0.5 ng/ml IL-2). After three rounds of Ag-specific stimulation T cells were expanded with soluble anti-CD3 Ab and irradiated feeder cells. Short-term T cell lines were cultured for 3 days in resting medium before the cellular immune responses of T cells against leishmanial Ags were detected. T cell clones were obtained from a T cell line generated against amastigote culture filtrate by limiting dilution on Terasaki microwell plates (Nunc, Naperville, IL) using a 1:10 dilution of amastigote culture filtrate. IL-2-supplemented TCM, and autologous irradiated feeder cells.

Library screening

A L. major amastigote cDNA expression library (17, 22) prepared in the λ-ZAP vector (Stratagene, La Jolla, CA) was chosen as the initial library to be screened. For screening purposes, this phage library was transferred to a plasmid-based library using the mass excision protocol supplied by the manufacturer (Stratagene). The plasmid library was transferred to 96-well plates at a density of 40–50 clones per well, grown to mid-log phase, and frozen at ~80°C as a glycerol stock. For screening, small aliquots of the frozen libraries were removed to fresh 96-well plates and grown to mid-log phase. Cultures were then induced with isopropyl β-D-thiogalactoside (β-TG) for 3 h, after which time the bacteria were pelleted in the 96-well plates. Bacteria were resuspended in 200 μl of antibiotic-free RPMI 1640 containing 10% FBS, and 10 μl from each well was transferred to new well of a 96-well plate containing 1 x 10^6 irradiated (3000 rad) HLA-DR-, -DP- and -DQ-matched monocyte-derived dendritic cells (MoDC) (23). After a 90-min incubation at 37°C, the adherent MoDC were washed twice with TCM. Parasite-specific T cells (2.5 x 10^6/well) were then added and cultured for 4 days. Proliferative response of the T cells was measured using a standard [3H]thymidine incorporation assay. IFN-γ secretion by T cells was determined by ELISA analysis of supernatants taken after 3 days as described (24).

Recombinant proteins

All proteins were expressed in Escherichia coli as amino-terminal histidine-tagged proteins to facilitate purification by nickel-chelate affinity chromatography. Full-length cDNA clones of Ags 1G6, 4A5, and 1B11 were obtained during T cell screening, and the corresponding full-length proteins were expressed in a recombinant form. A truncated 3’-clone, then, was initially obtained for clone 8G3, and the full-length sequence was determined by 5’ rapid amplification of cDNA end PCR using a spliced leader primer and a sequence-specific 3’ primer. Similarly, the clone 4H6 was initially isolated as a truncated cDNA clone, and the full-length open reading frame (ORF) was obtained by PCR using primers that were based upon the previously detected L. major sequence (accession number X78156). The full-length ORFs were amplified by PCR using primers for the addition of N-terminal 6x histidine tags and 5’ and 3’ restriction sites. Sequences of oligonucleotide primers are as follows: 1G6, 5’-CAATTC ATATGCATCCACCATCACCATGCTTTCTCCCGAAAGCT 3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’; 4A5, 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’; 1B11, 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’; 8G3, 5’-ATGGTCTAGCCCATCACCATCACCATACATGGTACGCTGTTGTTG-3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’; 4H6, 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’.

PCR products were digested with NdeI/EcoRI (1G6), Ndel (4A5), NdeI/XhoI (1B11), NheI/EcoRI (8G3), or NdeI/EcoRI (4H6) and were cloned into pET17b (Novagen, Madison, WI) vector digested with the same enzymes. Clones 4G2, 1E6, and 2A10 were expressed as truncated recombinant proteins. The initial 4G2 expression construct (encoding the carboxy-terminal 595 aa) was prepared by PCR amplification using the primers 5’-CAATGCATCCACCATCACCATCACCATACATGGTACGCTGTTGTTG-3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’ followed by cloning of the PCR product into pET17b using NdeI/XhoI. The recombinant protein encoded by this construct expressed poorly in E. coli; therefore, a smaller construct called 2F11 (encoding the carboxy-terminal 475 aa) was subsequently prepared, also by PCR amplification using the primers 5’-CAATGCATCCACCATCACCATCACCATACATGGTACGCTGTTGTTG-3’ and 5’-CAATGGAATTCCTAGGCGGCGCTGACAC-3’ followed by cloning of the PCR product into pET17b using NdeI/XhoI. Clones 1E6 and 2A10 were subcloned directly from the initial pBluescript plasmids into the vectors pQE30 and pQE32, respectively, (Qiagen, Chatsworth, CA) using BamH1 (5’) and Kpn1 (3’) restriction sites. Recombinant proteins containing N-terminal 6x histidine tags were purified on Ni-NTA agarose (Qiagen) according to previously described protocols (22, 25). All recombinant proteins were routinely assayed for endotoxin contamination using the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) and were uniformly found to contain <100 E.U./mg protein. Purified bovine tubulin was obtained from Molecular Probes (Eugene, OR) and calf thymus-derived histone was obtained from Calbiochem (La Jolla, CA).

Infection of APCs with L. major

To infect human MoDC and macrophages with metacyclic L. major promastigotes, parasites were centrifuged at 1400 x g on 1 x 10^7/ml MoDC or macrophages in a 96-well plate for 30 min at room temperature. After centrifugation, plates were washed twice and then cultured for 7 days in 150 μl TCM before T cells were added. MoDC and macrophages were generated from adherent PBMC as described (23).
Proliferation and cytokine assays

PBMC responses to leishmanial Ags were tested by culturing PBMC in the presence of Ag for 6 days using 3 x 10^6 PBMC/well in 200 μl TCM in 96-well round-bottom plates with a [3H]thymidine pulse (0.5 μCi/well) for the last 18 h. IL-5 and IFN-γ secretion was determined in supernatants taken after 3 days by ELISA. IL-5 and IFN-γ ELISA were performed as described (24). Fine specificities of T cell lines were determined by testing their response to leishmanial Ags using 2.5 x 10^4/well T cells in the presence of either 2.5 x 10^4 T cell-depleted PBMC or 1 x 10^4 HLA-DR-A, -DQ-matched allogenic MoDC in a total volume of 150 μl TCM. Cells were cultured for 3 days with a [3H]thymidine pulse (0.5 μCi/well) for the last 18 h. IL-5 and IFN-γ secretion was determined in supernatants taken after 2 days. Data are presented as the mean of triplicate cultures.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ ELISPOT assays were performed in triplicate following instructions provided by MABTECH (Stockholm, Sweden). Briefly, 96-well Multiscreen-HA plates (Millipore, Bedford, MA) were coated overnight with 10 μg/well Ab to human IFN-γ (1-1DK; MABTECH) at 4°C. After washing the plates three times with PBS, plates were blocked with 100 μg/ml TCM for 1 h at 37°C. Numbers of IFN-γ spot-forming cells (SFC) were determined from wells containing either 3 x 10^4 or 1 x 10^4 PBMC/well and 1.5 x 10^5 irradiated autologous PBMC. PBMC were cultured in the presence of leishmanial Ag at 37°C 5% CO2 in TCM. After 48 h cells were removed and ELISPOT plates were washed three times with PBS containing 0.1% Tween 20 before adding a secondary biotinylated Ab to IFN-γ (1 μg/ml, 7-B6-1; MABTECH). Plates were incubated for 2 h at room temperature, then washed with PBS containing 0.1% Tween 20 before adding 100 μl of avidin-peroxidase-complex (Vectastain). After 1 h plates were washed and developed with Van AEC substrate kit (Vector Laboratories). A computer-assisted video image analysis system (KS Elipsot, Zeiss, Oberkochen, Germany) was used to quantify IFN-γ-producing cells. The cut-off value was set as the mean of triplicates at 4.5 SFC/well, setting an arbitrary lower detection limit of 1 in 70,000 PBMC.

Results

Generation of Leishmania-specific T cells

To facilitate the identification of T cell-stimulating Ags from Leishmania, T cell lines were generated from PBMC of a Montenegro skin test-positive donor living in an area of Northeastern Brazil that is endemic for VL due to infection by L. chagasi. Although the donor had no clinical history of leishmaniasis, the positive Montenegro skin test (DTH) indicates that the donor was previously exposed to Leishmania and developed a protective immune response against the infection. Three different T cell lines were generated by stimulating PBMC with 10 μg/ml LPr, 10 μg/ml CFPr, or a 1:10 dilution of CFAm, respectively. T cell lines were expanded by repeated stimulation with HLA-DR-, -DQ-matched APC and the respective L. major Ag preparation. The established T cell lines were shown to be clonally distinct with the exception of the LPr line. The estimated complexity of the initial T cell line may have been underestimated due to the generation of clonally distinct T cell lines.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>硫</th>
<th>T cell lines against L. major Ag preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPr</td>
<td>56</td>
<td>79</td>
</tr>
<tr>
<td>CFPr</td>
<td>38</td>
<td>74</td>
</tr>
<tr>
<td>CFAm</td>
<td>13</td>
<td>19</td>
</tr>
</tbody>
</table>

*Table I. Proliferative response of established L. major-specific T cell lines against different L. major Ag preparations*

To identify Ags that are expressed in both the promastigote and amastigote life stages we screened an amastigote cDNA expression library with T cell lines generated against LPr or CFPr, respectively. The library was a L. major amastigote λ-ZAP phage library (described previously; Refs. 17, 22) that was converted to a plasmid-based library using the excision protocol supplied by the manufacturer (Stratagene). The titer of the plasmid library was determined by serial dilution, and pools were prepared in 96-well format at a density of ~40–50 clones per well. The library was screened as previously described (21) except that HLA-DR-, -DQ-matched MoDC rather than autologous MoDC were used as APC. A total of ten 96-well plates of the cDNA library (38,400–48,000 individual cDNA clones) were screened using both the LPr and CFPr T cell lines. Pools that elicited positive responses from the T cell lines were sequentially broken down until individual immuno-reactive clones were obtained. As an example, Table II shows the identification of two positive library pools and the subsequent breakdown of these pools to the clonal level using a subpooling approach. The pool 1G6 induced a specific proliferative response from the CFPr T cell line, whereas the pool 4H6 induced a specific response from the LPr line. The estimated complexity of the initial library pools was corroborated by the fact that only 1% of the colonies isolated from pool 1G6 and 0.5% of the colonies from pool 4H6 were positive during the second round of screening (data not shown). However, 100% of the colonies identified as positive during the second round of screening remained positive on subsequent assays. This finding, together with a general lack of proliferation in response to control E. coli, confirms that the library screening protocol is both sensitive and specific for the detection of E. coli clones that are expressing leishmanial Ags recognized by T cells.

Immunoreactive pools from all 10 library plates were enriched in the same manner until single immunoreactive cDNA clones were obtained. In summary, a total of 39 independent, positive cDNA clones were identified. Results of genetic database searches using the sequences of positive clones are summarized in Table III. The 39 clones comprised overlapping derivatives of eight distinct genes, three of which were identified with the CFPr T cell line (1G6, 1E6, and 4A5) and five with the LPr T cell line (1B11, 2A10, 2F11, 4H6, and 8G3). Interestingly, the LPr and CFPr T cell lines recognized distinct sets of pools with the exception of the pool 8G3, which was strongly reactive for the LPr T cell line and weakly reactive in screens using the CFPr T cell line. Clones 1G6, 2A10, 4H6, and 8G3 were found to comprise previously characterized Leishmania sequences (histone H2b, α-tubulin, β-tubulin, and malate dehydrogenase, respectively). The sequence of the Leishmania histone H2b was markedly divergent from the human homolog, particularly in the amino terminal half of the protein where there is a large gap in the Leishmania sequence when compared with the H2b proteins from higher eukaryotes (data not shown). Interestingly, two distinct cDNA clones for Leishmania histone H2b were detected by the CFPr line; these clones were divergent between themselves in the amino terminal region. Similarly, the α- and β-tubulin sequences of Leishmania contained blocks of sequence divergence when aligned to the homologous sequences of higher eukaryotes (data not shown). The clone originally obtained for malate dehydrogenase (8G3) contained a severely truncated cDNA sequence that encoded only the carboxyl-terminal 69 aa of the ORF. A peptide comprising the carboxyl-terminal 14 aa of this protein was recognized by the LPr T cell line as demonstrated by epitope-mapping experiments using overlapping synthetic peptides (J.
Table II. Proliferative response of T cell lines against whole intact E. coli comprising pools, subpools, and clones of the L. major cDNA library

<table>
<thead>
<tr>
<th>Pool</th>
<th>Row</th>
<th>Column</th>
<th>Single-positive E. coli clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCL-CFP</td>
<td>1G6</td>
<td>3219 ± 138</td>
<td>2668 ± 296</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>742 ± 63</td>
<td>709 ± 70</td>
</tr>
<tr>
<td>TCL-LPr</td>
<td>4H6</td>
<td>1512 ± 305</td>
<td>7885 ± 1199</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>53 ± 2</td>
<td>93 ± 3</td>
</tr>
</tbody>
</table>

* T cell lines TCL-CFP and TCL-LPr were generated against L. major promastigote culture filtrate and L. major promastigote lysate, respectively. Pools of E. coli-expressing leishmanial proteins were incubated with 1 × 10^6 MoDC. After 2 h, MoDC were washed and 2.5 × 10^5 T cells were added. Proliferation was determined after 4 days with a [3H]thymidine pulse for the last 18 h. Mean ± SD was calculated from duplicate cultures. Single-positive clones were isolated from a 10 × 10 grid array. Rows and columns refer to subpools (each subpool comprised of 10 clones arranged in the 10 × 10 grid array) to facilitate isolation of positive cDNA clones.

* Controls represent the response of the respective T cell lines against control E. coli cultures.

Webb, manuscript in preparation). Interestingly, this protein contains an SHL tripeptide at its carboxy terminus, indicating that it represents an isoform of malate dehydrogenase that is targeted to the glycosome, an organelle that is unique to kinetoplastid organisms (26). Two other genes isolated herein had significant homology to known sequences (2F11-elongation factor 2 and 1B11-ribosomal protein S4) that have not been previously cloned from Leishmania. The two remaining Ags exhibited either no similarity to existing database entries (4A5) or weak similarity to a family of cell cycle-regulated proteins from higher eukaryotes (1E6). To our knowledge, none of the clones identified in this study have been previously characterized as Ags capable of eliciting responses from human Leishmania-specific T cells.

T cell lines respond to recombinant leishmanial Ags identified by CD4 expression cloning

All eight of the Ags identified by direct T cell screening were expressed in E. coli as recombinant fusion proteins containing N-terminal histidine tags and were purified to homogeneity using nickel affinity chromatography. All eight purified recombinant proteins elicited strong proliferative responses from the respective LPr- or CFPr-specific T cell lines (Fig. 1) as expected from the data of the library screening. Purified recombinant proteins and the respective intact E. coli clones elicited a similar level of response from the respective T cell lines (data not shown). The T cell responses to leishmanial tubulin and histone H2b did not evoke an autoreactive response to the mammalian homologs because both T cell lines did not respond to bovine tubulin or calf thymus-derived histone, which are 100% homologous to the respective human proteins. The responses of short-term T cell lines (PBMC stimulated for one round with parasite lysate or culture filtrate) confirmed that this donor has strong cellular immune responses against the Ags identified by CD4 T cell expression cloning (data not shown). The immune response elicited by these Ags may contribute to a state of protective immunity in this donor.

T cells generated against amastigote leishmanial parasites recognized leishmanial histone H2b, β-tubulin, and malate dehydrogenase as well as infected macrophages and dendritic cells

Because promastigote proteins were used to generate the T cell lines used for expression screening, we next generated a T cell line against CFAm and evaluated the ability of this line to recognize the eight recombinant proteins described above. As shown in Fig. 2, the CFAm T cell line recognized three of the eight Ags identified using the LPr- and CFPr-specific T cell lines. Recombinant Ag 1G6 (histone H2b), 4H6 (β-tubulin), as well as 8G3 (malate dehydrogenase) induced a strong proliferative response in CFAm T cells. This result indicates that these Ags are expressed in both the promastigote and amastigote life stages at levels that are sufficient to evoke strong cellular immune responses. Furthermore, Ags 4H6 and 8G3 were also recognized by Leishmania-specific T cell clones that had been generated against amastigote culture filtrate (Fig. 3). These T cell clones were derived from a CFAm-specific T cell line by limiting dilution in the presence of CFAm and autologous APC. In addition, MoDC and macrophages infected with L. major were capable of evoking proliferative responses (Fig. 3) and IFN-γ production (data not shown) from the 4H6-specific T cell clone CFAm15. Interestingly, no such response was elicited from the 8G3-specific T cell clone CFAm21, suggesting that there are Ag-dependent differences in Ag presentation during natural infection of APC with L. major.

PBMC and short-term T cell lines from CL patients responded to recombinant leishmanial Ags identified by CD4 expression cloning

To further evaluate the human immune response to the eight Ags identified by direct T cell screening, the responses of PBMC from patients with self-resolved CL were characterized. CL patients acquire persistent protective immune responses against Leishmania. Therefore, Ags recognized by PBMC from these patients might be involved in the control of a leishmanial infection. PBMC responses of six CL patients with L. tropica infection and one CL patient with L. major infection were characterized by proliferation and IFN-γ ELISPOT. In addition to the eight Ags identified by expression cloning, two previously described leishmanial proteins (surface protease gp63, reviewed in Ref. 2; Leishmania homolog of the intracellular receptor for activated protein kinase (LACK); Ref. 16), leishmanial promastigote lysate, bovine histone, bovine tubulin, and purified protein derivative (PPD) from M. tuberculosis were tested. Data are shown in Fig. 4. All seven donors had a high frequency of parasite-specific T cells in their PBMC with a mean of one in 1700 ± 430 cells producing IFN-γ in response to leishmanial lysate (as determined by IFN-γ ELISPOT). Furthermore, six of seven CL donors had a strong proliferative response to leishmanial lysate (mean stimulation index (SI) of responders was 29 ± 5.2 with an arbitrary cut-off SI of 4). In general, all CL donors responded to at least one of the eight identified Ags. Most notable were the responses to 1G6 (histone H2b) and 4H6 (β-tubulin). 1G6 and 4H6 elicited responses from six of seven and seven of seven CL patients, respectively, as measured by IFN-γ

* Mean of responders. Mean ± SEM was calculated from responding PBMC. Responders were defined by arbitrary cutoff value of 4.5 SFC for IFN-γ ELISPOT assay or a SI >4 for proliferation, respectively.
ELISPOT and from five of seven and five of seven, respectively, as measured by in vitro proliferative responses. Compared with the other recombinant Ags tested, IFN-γ-producing 1G6-specific T cells had the highest frequency in PBMC from CL patients (1 in 5900 ± 2400, mean of responders; Ref. 4) and elicited the strongest proliferative response (SI of 21 ± 4.3, mean of responders). In addition, 1G6 was not recognized by PBMC from uninfected North American controls, indicating a Leishmania-specific response to 1G6 in PBMC from CL patients. None of the CL patients responded to bovine tubulin or bovine histone, excluding the possibility of cross-reactivity against the human homologs. Ags 1B11 (ribosomal S4 protein) and 2F11 (elongation initiation factor 2A) elicited responses in approximately half of the patients. Interestingly, although 1E6 and 8G3 (malate dehydrogenase) induced a proliferative response in four of seven and two of seven CL patients, respectively, IFN-γ-secreting cells were not detectable by ELISPOT. As shown in Fig. 4, a limited number of normal North American control donors responded to leishmanial lysate or to the recombinant Ags 1E6, 2F11, 4H6, or 8G3 with weak proliferation and/or IFN-γ production, indicating the potential for cross-reactivity to other microbial organisms common in North America. However, to fully evaluate the response of normal donors, a greater pool of donors needs be investigated.

Lastly, short-term T cell lines against leishmanial lysate were generated from PBMC of CL patients to further characterize T cell

![image](Insert Image)

**FIGURE 1.** Proliferative responses of T cell lines generated against LPr and CFPr to recombinant Ags identified by T cell expression cloning. Proliferative responses were determined by stimulating 2.5 × 10⁴ T cells for 3 days with either LPr (10 μg/ml), CFPr (10 μg/ml), bovine tubulin (bov-Tubulin, 2 μg/ml), bovine histone (bov-Histone, 2 μg/ml), or the respective recombinant Ag (2 μg/ml) in the presence of 1 × 10⁴ irradiated HLA-DR-, -DQ-matched MoDC. Cultures were pulsed with [³H]thymidine for the last 18 h. Data represent the mean of triplicate cultures.

**FIGURE 2.** Proliferative response of T cell line TCL-CFAm generated against recombinant leishmanial Ags. Proliferative responses were determined by stimulating 2.5 × 10⁴ T cells for 3 days with either LPr (10 μg/ml), CFPr (10 μg/ml), bovine tubulin (bov-Tubulin, 2 μg/ml), bovine histone (bov-Histone, 2 μg/ml), or the respective recombinant Ag (2 μg/ml) in the presence of 1 × 10⁴ irradiated HLA-DR-, -DQ-matched MoDC. Cultures were pulsed with [³H]thymidine for the last 18 h. Data represent the mean of triplicate cultures.

### Table III. Identity of leishmanial Ags identified by CD4 expression cloning

<table>
<thead>
<tr>
<th>Ag</th>
<th>Homology</th>
<th>No. of Times Picked</th>
<th>Recombinant Protein Expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G6 (histone H2b) (accession no. AF099108 and AF099109)</td>
<td>92% sequence identity with histone H2b from <em>L. infantum</em> (accession no. Y13396)</td>
<td>11</td>
<td>Full length (12.7 kDa)</td>
</tr>
<tr>
<td>2F11, 4G2 (elongation factor 2) (accession no. AF199017 and AF199016)</td>
<td>92% sequence identity with elongation factor 2 from <em>Trypanosoma cruzi</em> (accession no. D50806)</td>
<td>10</td>
<td>2F11-carboxyl-terminal 475 aa 4G2-carboxyl-terminal 595 aa</td>
</tr>
<tr>
<td>1E6 (p45) (accession no. AF099106)</td>
<td>Partial sequence identity with cell cycle-regulated proteins from multiple spp. Highest sequence identity was 33% with PrlA from <em>Dictyostelium discoideum</em> (accession no. U61403)</td>
<td>6</td>
<td>Δ20–401 (45.7 kDa)</td>
</tr>
<tr>
<td>2A10 (α-tubulin) (accession no. AF199019)</td>
<td>99% sequence identity with α-tubulin from <em>L. donovani</em> (accession no. U09612)</td>
<td>3</td>
<td>Δ250–451 (25 kDa)</td>
</tr>
<tr>
<td>4H6 (β-tubulin)</td>
<td>100% sequence identity with β-tubulin from <em>L. major</em> (accession no. X93567)</td>
<td>3</td>
<td>Full length (51 kDa)</td>
</tr>
<tr>
<td>4A5 (p21) (accession no. AF099107)</td>
<td>Novel</td>
<td>2</td>
<td>Full length (22.2 kDa)</td>
</tr>
<tr>
<td>1B11 (ribosomal protein S4) (accession no. AF199018)</td>
<td>91% sequence identity with ribosomal protein S4 from <em>Trypanosoma cruzi</em> (accession no. AF005904)</td>
<td>2</td>
<td>Full length (31.6 kDa)</td>
</tr>
<tr>
<td>8G3 (malate dehydrogenase)</td>
<td>100% sequence identity with malate dehydrogenase from <em>L. major</em> (accession no. AL117268)</td>
<td>2</td>
<td>Full length (32 kDa)</td>
</tr>
</tbody>
</table>

* New sequences were submitted to GenBank under the indicated accession numbers. Proteins without obvious sequence identity (p45, p21) are designated according to molecular weight.
* Databank search results showing protein with highest overall sequence identity.
* cDNA clones (out of a total of 48,000) encoding the same protein or truncated derivatives thereof.
* Two reactive histone H2b genes with 82% overall sequence identity were identified during this screen.
* Two reactive elongation factor 2A genes with differing sequence in the 3′-untranslated region were identified during this screen.

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**![Image](Insert Image)**

**![Image](Insert Image)**
response against the eight identified Ags (Fig. 5). IFN-γ production by the short-term T cell lines revealed a similar pattern of Ag reactivity as was observed with the PBMC of CL patients. The short-term T cell lines did not produce IFN-γ in response to malate dehydrogenase, protein 1E6, or protein 4A5. Again, 1G6 (histone H2b) and 4H6 (β-tubulin) induced the strongest response in these short-term lines. In contrast, only one of the short-term CL T cell lines produced significant amounts of the Th2-associated cytokine IL-5. Interestingly, this particular line produced both IL-5 and IFN-γ in the presence of either *Leishmania* lysate or recombinant 1G6, indicating a Th0-like response. However, all other T cell lines produced a Th1-like pattern with high levels of IFN-γ and low to undetectable amounts IL-5 in response to the recognized Ags.

**Discussion**

The aim of this study was to identify dominant T cell-stimulating Ags from *Leishmania* using human T cells from an immune donor. Previously, T cell responses to *Leishmania* were characterized by testing biochemically purified proteins or recombinant Ags identified by serological expression cloning. The use of these techniques to define T cell responses to individual *Leishmania* Ags are problematic because they rely on non-T cell components to identify the Ag. In a different approach, Moungneou et al. (16) used the response of a murine-protective T cell clone to screen pools of purified recombinant Ags from a *L. major* promastigote cDNA library to identify a LACK. Herein we used a recently described direct T cell-screening approach (21) to identify Ags recognized by *Leishmania*-specific human T cell lines. To identify T cell epitopes that are shared by multiple *Leishmania* species, a cDNA library of amastigote *L. major* (endemic in the Old World) was screened with T cells from a donor controlling a *L. chagasi* infection (a *Leishmania* species endemic in Latin America). By using this approach we identified eight leishmanial T cell-stimulating Ags. To our knowledge none of

**FIGURE 3.** A β-tubulin specific T cell clone generated against amastigote culture filtrate responds to macrophages and MoDC infected with *L. major*. MoDC (1 × 10⁴) and 5-day-old macrophages (1 × 10⁴) were infected with *L. major* at the indicated multiplicity of infection (MOI). After 7 days, 2.5 × 10⁴ cells of the indicated T cell clone TCC-CFAm21 (8G3/malate dehydrogenase-specific) or T cell clone TCC-CFAm15 (4H6/β-tubulin-specific) were added to infected APC, uninfected APC (medium), or uninfected APC in the presence of the respective recombinant Ag (2 µg/ml). Proliferative responses were determined by incubating cultures for 3 more days. Cultures were pulsed with [³H]thymidine for the last 18 h. Cultures were performed in triplicate and the figure is representative of three separate experiments.

**FIGURE 4.** Cellular immune response of PBMC from CL donors and healthy North American control donors to recombinant leishmanial Ags. Cellular immune responses were determined by stimulating 3 × 10⁵ PBMC with recombinant leishmanial proteins (2 µg/ml) identified by CD4 expression cloning, recombinant Ags LACK (2 µg/ml) and Gp63 (2 µg/ml), purified bovine tubulin (bovTubulin), purified bovine histone (bovHistone), PPD from *M. tuberculosis* (5 µg/ml), and LPr (10 µg/ml), respectively. PBMC were obtained from Saudi Arabian donors with self-healed CL (●) or healthy North American individuals (control group, ○). Proliferation was measured after 6 days with [³H]thymidine pulse for the last 18 h. Counts of unstimulated PBMC cultures were in the range of 200-1500 cpm. Number of IFN-γ SFC was determined after 48 h. Data represent the mean of triplicate cultures.
These Ags have been previously shown to induce T cell responses in leishmaniasis patients, although histone H2b, α- and β-tubulin, as well as malate dehydrogenase have been previously characterized in terms of biochemistry or molecular biology (27–30).

Our rationale for screening an amastigote cDNA library with T cell lines raised against promastigote lysate and culture filtrate was to define Ags that are shared by both life stages. Indeed, at least five of the T cell-stimulating Leishmania Ags reported herein (α- and β-tubulin, histone H2b, elongation factor 2α, and ribosomal protein S4) represent cytoplasmic or nuclear proteins that are abundantly expressed in the promastigote as well as amastigote life stage of the parasite (31, 32). Furthermore, α- and β-tubulin are also major components of the leishmanial flagella. The isoform of malate dehydrogenase identified herein is located within the glycosome, a membrane-bound organelle unique to kinetoplastid organisms.

Interestingly, the T cell lines generated against promastigote lysate and culture filtrate exhibited distinct differences in terms of the pattern of Ags recognized. Of the Ags studied herein, only malate dehydrogenase (8G3) was recognized by both long-term T cell lines. Nonetheless, both lines react strongly with preparations of total lysate or total culture filtrate. This result suggests that these lines may recognize additional Ags, which have not been cloned during this screen possibly due to either underrepresentation in the cDNA expression library or poor expression and/or processing during the screening procedure.

Interestingly, T cell reactivity to histone H2b, β-tubulin, and malate dehydrogenase was observed in T cell lines generated against promastigote Ag preparations as well as in the T cell line generated against amastigote culture filtrate. Although the presence of these proteins in culture filtrate is more likely to be the result of parasite autolysis rather than active protein secretion, a combination of high abundance and (apparent) high solubility may favor the MHC class II-mediated presentation of these proteins in Leishmania-infected APC. Indeed, β-tubulin-specific T cell clones strongly recognized both L. major-infected macrophages as well as L. major-infected dendritic cells, indicating that β-tubulin is presented by infected cells at levels sufficient to elicit strong class II-restricted T cell responses. In contrast, Leishmania-infected monocytes or dendritic cells did not stimulate a malate dehydrogenase-specific T cell clone despite the fact that these same infected cells were capable of processing and presenting exogenous recombinant malate dehydrogenase (data not shown). Interestingly, our data with malate dehydrogenase resemble recently published studies showing that in the murine system Leishmania-infected APC were not able to stimulate LACK- or cysteine proteinase-specific T cells (33, 34).

Cellular immune responses to the eight Ags identified by CD4 expression cloning were further evaluated with PBMC as well as short-term T cell lines generated from patients with self-resolved CL due to L. tropica or L. major infections. These patients control infection and acquire persistent immunity against Leishmania. T cells from CL donors responded to histone H2b (1G6), β-tubulin (4H6), ribosomal protein S4 (1B11), elongation factor 2 (2F11), and α-tubulin (2A10). However, most notable were the dominant T cell responses to histone H2b and β-tubulin. Histone H2b-reactive IFN-γ-producing T cells were present at a very high frequency in PBMC from CL patients. In addition, histone H2b elicited strong proliferative responses in a broad range of CL patients. Interestingly, humoral immune responses against histone H2b were recently reported in dogs with canine VL. Abs to histone H2b as well as other members of the histone family were detected after the dogs developed VL due to a Leishmania infantum infection (35, 36). These data suggest that histone H2b is available for immune recognition not only in situations where the immune system has controlled the infection but also in situations where the parasite has evaded protective immune mechanisms. Notably, neither H2b-reactive T cells (this study) nor histone H2b-reactive dog sera recognized their mammalian homologs, indicating that histone H2b reactivity is not indicative of an autoreactive immune response.

Like histone H2b, β-tubulin is one of the most abundant proteins in leishmanial promastigotes and amastigotes (31). However, unlike the histone H2b protein, which is relatively divergent from its mammalian homolog (50% identity to human histone H2b), β-tubulin exhibits 86% identity to the human counterpart. Despite this level of homology, the tubulin-specific T cell reactivity observed in seven of seven tested CL patients was completely specific for parasite tubulin as no response could be elicited by purified mammalian tubulin. This was also indicated by preliminary epitope-mapping experiments showing that T cell reactivity was restricted to a small block of nonconservative amino acid substitutions (data not shown). This finding demonstrates the exquisite sensitivity of T cells to detect even subtle sequence differences between the proteins of an invading pathogen and the corresponding host homologs and that recognition of such differences may have profound consequences on the course of infection.

Interestingly, although malate dehydrogenase (8G3) and 1E6 elicited strong T cell responses in short-term T cell lines from the Brazilian donor (data not shown), they did not evoke a specific T cell response in the CL patients. Because all these Ags were initially derived from a L. major library, the nonresponsiveness of CL donors against these three Ags may be indicative of HLA class II restriction differences. Alternatively, differences in the disease profile of these disparate types of patients may result in distinct patterns of Ag recognition.

There is currently little information available regarding the response of human T cells to LACK. In this study LACK did not
induce a T cell response in PBMC from CL patients. A 24-kDa portion of LACK protected susceptible BALB/c mice when administered as a vaccine with IL-12 in the experimental L. major mouse model. Interestingly, the susceptibility of BALB/c to L. major may be mediated by an early IL-4 T cell response to LACK because mice made tolerant to LACK are ablated of early IL-4 production and exhibit a healing phenotype (reviewed in Ref. 37). However, cellular immune responses to Leishmania in humans are probably more complex and less polarized than they are in mice.

In this data study support a recently reviewed theory about the association of Ag presentation by Leishmania-infected macrophages and the control of infection and its implications for the design of subunit vaccines (38). Amastigote Leishmania replicate in resting macrophages. Upon activation with INF-γ the macrophages kill the parasite and during the subsequent degradation of amastigote present an extremely heterogeneous peptide repertoire. Under these circumstances, the abundance of a particular Ag might be an important factor in eliciting a strong immune response. Herein, we have demonstrated that β-tubulin-specific T cells have the capacity to recognize infected macrophages and dendritic cells. It is envisioned that, in vivo, cells such as these β-tubulin-specific T cells mediate the initial activation of infected macrophages to kill intracellular parasites and, consequently, drive the presentation of a more diverse repertoire of parasite Ags in the class II pathway. The T cell response to abundant cytoplasmic proteins like histone H2b, tubulins, and ribosomal protein S4 might then be triggered by presentation of these killed organisms by activated APC. In an intercellular chain reaction these T cell responses would then activate more macrophages in the vicinity. Eventually, this combination of parasite-specific T cell responses would be expected to reach a point at which the parasite is forced into either a low level subclinical latent infection or is eliminated entirely.

In summary, this report describes the identification of T cell-stimulating Ags from Leishmania, recognized by T cells from donors who developed a protective immune response against leishmanial infections. Although it is unclear at this point whether T cell responses to leishmanial histone H2b and β-tubulin either alone or in combination participate in the control of the initial infection, the Th1-type immune responses elicited against these Ags may contribute to a state of protective immunity in these donors. Thus it is likely that Ags like histone H2b and β-tubulin identified by a direct T cell-screening approach might constitute potential candidates for inclusion in a subunit vaccine against Leishmania.

References