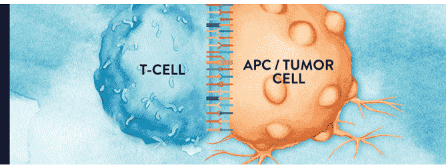
Ultra-pure antibodies for *in vivo* research, targeting immune checkpoints and more.
EXPLORE
Bio Cell



Mechanisms of the Natural Reactivity of Lymphocytes from Noninfected Individuals to Membrane-Associated *Leishmania infantum* Antigens

This information is current as of March 2, 2021.

Atfa Sassi, Beya Larguèche-Darwaz, Alexis Collette, Adrien Six, Dhafer Laouini, Pierre André Cazenave and Koussay Dellagi

J Immunol 2005; 174:3598-3607; ;
doi: 10.4049/jimmunol.174.6.3598
http://www.jimmunol.org/content/174/6/3598

References This article **cites 48 articles**, 15 of which you can access for free at:
http://www.jimmunol.org/content/174/6/3598.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2005 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

Mechanisms of the Natural Reactivity of Lymphocytes from Noninfected Individuals to Membrane-Associated *Leishmania infantum* Antigens[1]

Atfa Sassi,* Beya Larguèche-Darwaz,* Alexis Collette,† Adrien Six,† Dhafer Laouini,* Pierre André Cazenave,† and Koussay Dellagi[2]*

Membrane-associated *Leishmania* Ags (MLA) or soluble *Leishmania* Ags were used in vitro to stimulate cord blood or PBMC from healthy donors noninfected by *Leishmania* parasites. MLA, but not soluble *Leishmania* Ags, constantly induce strong proliferation of cord blood mononuclear cells and PBMC from noninfected individuals. Responding cells are CD3$^+$, CD4$^+$, TCR$\alpha\beta^+$, CD45RO$^+$, and CD45RA$^+$ and secrete IFN-γ and IL-10, but not IL-4. MLA do not activate NK cells nor NKT cells. Membrane Ags also induce purified macrophages from noninfected individuals to secrete IL-10 and TNF-α, but have no effect on IL-1α or IL-12 secretion. The effects of MLA are proteinase K-sensitive and resistant to lipid extraction. The lymphoproliferative responses are inhibited by anti-HLA-DR Abs and require Ag processing by APCs, excluding that the biological effect of MLA could be attributed to a superantigen. Finally, TCR repertoire analysis shows that the T cell expansion induced by MLA uses TCR with various variable β segment rearrangements and CDR3 lengths, features much more characteristic to those observed with a polyclonal activator than with a conventional Ag. These results suggest a particular mechanism developed during the host's natural response to *Leishmania* parasites that allows direct activation of naive CD4 lymphocytes by parasite membrane-associated Ags. *The Journal of Immunology,* 2005, 174: 3598–3607.

Human leishmaniasis are endemic diseases caused by protozoa of the genus *Leishmania*, which are obligate intracellular parasites that selectively infect macrophages and dendritic cells (DC).[3] Human infection by *Leishmania infantum* often runs a subclinical and self-healing course (1), but is also featured by the fatal syndrome of infantile visceral leishmaniasis (VL) (2).

The host immune responses against intracellular pathogens are primarily T cell mediated (3). The outcome of infection in humans is determined by the balance between the contrasting effects of protective (IL-2 and IFN-γ) and nonprotective (IL-3, IL-4, and IL-10) cytokines produced during the initial phase of infection (4). Human leishmaniasis does not actually reproduce the strict dichotomy between the two Th subsets Th1 and Th2, which characterize the murine model of disease, as both IFN-γ and IL-10 are coexpressed in visceral and mucocutaneous diseases and the role of IL-4 is less obvious than in mice (5, 6). It has been suggested that the immune depression, which characterizes VL, is most likely the consequence of the potent inhibitory effects of IL-10 (7). Factors that skew the immune response toward Th1 or Th2 dominance are partially understood. It is considered that direct interaction between parasite Ags and cells of the host innate immunity most likely induces a set of cytokines that participate to shape the subsequent adaptative immune response. For instance, *Leishmania*-infected DC secrete IL-12, which directly activates NK cells to produce IFN-γ, which in turn activates the macrophage leishmanicidal activities and favors differentiation of Th1 cells (8). Several studies have reported that *Leishmania* promastigotes and *Leishmania* extracts induce in vitro proliferative responses of PBMC from nonexposed individuals. Thus, *Leishmania aethiopica* promastigotes activate NK cells (9), while soluble and nonsoluble Ags from *Leishmania major*, *Leishmania donovani*, and *Leishmania guyanensis* induce T cell proliferation (10) and cytokine secretion (11, 12). These effects could indicate the presence in *Leishmania* preparations of mitogenic or superantigenic molecules or expression of T epitopes of *Leishmania* Ags cross-reactive with other microorganisms (10, 13).

Analysis of the innate response of naive individuals to *Leishmania* Ags may provide, better than the use of murine models, important insights in the early cellular events triggered by parasite infection. In addition, it may facilitate discrimination between protective and nonprotective immune responses for better vaccine design and development. In this perspective, we investigated the mechanisms of cellular responses to *Leishmania* Ags expressed by PBMC from noninfected individuals living in endemic or in nonendemic regions. We show that the biological effects characterizing this natural reactivity are mainly triggered by membrane-associated Ags (MLA) and not by soluble *Leishmania* Ags (SLA), are mediated by CD4 T cells secreting IFN-γ and IL-10, and could not be ascribed to any parasite superantigen (SAg). Furthermore,

*Laboratoire d'Immunopathologie, Vaccinologie et Génétique Moléculaire et Laboratoire International Associé Bioingénierie Moléculaire, Institut Pasteur de Tunis, Tunis, Tunisia; and †Unité d'Immunophysiopathologie Infectieuse, Centre National de la Recherche Scientifique Unité de Recherche Associée 1961, Institut Pasteur, Paris, France

Received for publication June 17, 2004. Accepted for publication January 18, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work received financial support from the Tunisian State Secretariate for Research and Technology.

[2] Address correspondence and reprint requests to Dr. Koussay Dellagi, Laboratoire d'Immunopathologie, Vaccinologie et Génétique Moléculaire, Institut Pasteur de Tunis. 13, Place Pasteur B. P 74. 1002, Tunis-Belvédère, Tunisia. E-mail address: koussay.dellagi@pasteur.rns.tn

[3] Abbreviations used in this paper: DC, dendritic cell; BC, constant β segment; BV, variable β segment; CBMC, cord blood mononuclear cell; DBV, difference in BV; LST, leishmanin skin test; MLA, membrane-associated *Leishmania* Ag; PK, proteinase K; PPD, purified protein derivative of tuberculin; SAg, superantigen; SEA, *S. aureus* enterotoxin A; SEB, *S. aureus* enterotoxin B; SI, stimulation index; SLA, soluble *Leishmania* Ag; TT, tetanus toxoid; VL, visceral leishmaniasis.

Copyright © 2005 by The American Association of Immunologists, Inc.

0022-1767/05/$02.00

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

we can demonstrate that they involve both naive and memory T cells and use structurally diverse TCR variable β segment (BV) and CDR3 much as a polyclonal activator does.

Materials and Methods

Donors

Noninfected individuals. In previous experiments, we demonstrated that individuals living in countries endemic for leishmaniasis could be considered noninfected (i.e., naive to *Leishmania* parasites) if they did not have any history of clinically apparent leishmaniasis, are nonreactive to the leishmanin skin test (LST), and are not responsive in in vitro lymphoproliferative assays to SLA (14). Heparinized venous blood samples were collected after informed consent from 22 Tunisian adults fulfilling these criteria.

Nonexposed controls. PBMC from eight healthy Swedish adult blood donors, who had not visited any country endemic for leishmaniasis, were kindly provided by H. Akuffo (Karolinska Institute, Stockholm, Sweden). PBMC were purified in Sweden and deep frozen in 10% DMSO according to standard procedures. Vials were sent to Tunisia in dry ice by rapid delivery. Cell viability was >95% after recovery. Control of viability includes PHA- and purified protein derivative of tuberculin (PPD)-induced proliferative assays, as indicated below.

Cord blood samples. Twenty-four cord blood samples were collected immediately after delivery of full-term Tunisian newborns, who were delivered normally and showed no evidence of infection or congenital anomaly.

Control group. Fifteen children cured of VL (age range 2–11 years, mean 4 ± 2 years) were included in this study. The diagnosis of VL was established by identification of *Leishmania* parasites in bone marrow aspirates and/or in positive culture on NNN culture medium. Blood samples were obtained after parental consent 2 years after disease's cure.

Reagents and Abs

PHA was purchased from Difco Laboratories. PPD and tetanus toxoid (TT) were obtained from Institut Mérieux. Proteinase K (PK), polymixin B, LPS from *Escherichia coli*, SAgs *Staphylococcus aureus* enterotoxin A (SEA) and B (SEB), and the E-Toxate kit (*Limulus* amebocyte lysate test) were purchased from Sigma-Aldrich. TRIzol reagent was obtained from Invitrogen Life Technologies. S. Chouaïb (Institut Gustave Roussy, Villejuif, France) kindly provided the human rIL-2. The Lympho-kwik TH lymphocyte isolation reagents were from One Lambda. Mouse anti-IgG-coated magnetic beads were purchased from Dynal Biotech.

The following Abs were used for immunofluorescence staining: Cyc IgG1, PE IgG1, FITC IgG1, PE IgG2a, FITC CD3, Cyc CD4, Cyc CD8, FITC CD14, FITC CD19, FITC CD45RO, and PE CD161/NKR P1A were from BD Pharmingen; and FITC CD3/PE (CD16 CD56), FITC CD20, PE Vβ11, FITC Vα24, PE CD45RO, FITC CD45RA, anti-TCR$\alpha\beta$, anti-TCR$\gamma\delta$, and FITC goat anti-mouse IgG were from Beckman Coulter. Abs Cyc IgG1, PE IgG1, FITC IgG1, and PE IgG2a were used as isotype controls. Culture supernatants containing murine mAbs to human CD3 (clone CD3.12.37, IgG2a), anti-CD4 (IgG1), anti-CD8 (clone CD8.1.1, IgG2b), and isotypic controls (IgG1 and IgG2b) were from Institut Pasteur de Tunis. Anti-CD45RA (ALB11) and anti-CD45RO (UCHL-1) mAbs used for the depletion of the T $CD45RA^+$ or T $CD45RO^+$ subsets were purchased from Beckman Coulter.

mAbs and recombinant human cytokines for cytokine assays were purchased from BD Pharmingen. The following mAbs were used: IL-1α 364-3B3-114, IL-4 8D4-8, IL-10 JES3-19F1, IL-12 20C2, TNF-α mAb1, and IFN-γ NIB42 for capture, and IL-1α 28.9, IL-4 MP4-25D2, IL-10 JES3-12G8, IL-12 C8.6, TNF-α mAb11, and IFN-γ 4S.B3 for detection. G. Sterkers (Hôpital Robert Debré, Paris, France) kindly provided mAbs directed against MHC class I (clone W6. 32) and MHC class II determinants (HLA-DP clone B7.21.1, HLA-DQB, anti-DQ broad, clone 33.1, and HLA-DR clone L243).

Preparation of Leishmania Ags

MLA were extracted from promastigotes of *L. infantum* (strain MHOM/TN/80/IPT-1), as previously described, with few modifications (15). Briefly, promastigotes were grown at 26°C in RPMI 1640 (Invitrogen Life Technologies) culture medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% of heat-inactivated FCS. All purification steps were performed in sterile conditions on ice or at +4°C. Parasites were washed in 10 mM Tris, 150 mM NaCl, pH 7.5, resuspended in hypotonic lysis buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.0), and disrupted on ice with a Dounce homogenizer. After centrifugation for 30 min at 10,000 × *g*, pellets were resuspended in TM buffer (20 mM Tris-HCl, 3 mM $MgCl_2$, pH 8.0) containing 5% sucrose and centrifuged for 30 min at 10,000 × *g* on a 20% sucrose layer using a swinging rotor (L7 Ultracentrifuge; Beckman Instruments). The pellets were then resuspended in TM buffer 52% sucrose and centrifuged for 2 h 30 min at 100,000 × *g* on a discontinuous gradient consisting of three layers of 42, 52, and 60% sucrose in TM buffer. Membrane extracts, which collect as sediments at the 42–52% sucrose interface, were recovered and washed twice at 10,000 × *g* for 30 min at +4°C with TM buffer and then with sterile 0.01 M PBS, pH 7.4.

SLA were prepared from the same strain, as previously described (16). Briefly, promastigotes were harvested from culture and washed four times in cold PBS. They were then resuspended at 1.2×10^9 parasites/ml in 100 mM Tris-HC1, 1 mM EDTA (pH 8.0) with 50 pg/ml leupeptin, 50 pg/ml α_2-macroglobulin, and 1.6 mM PMSF (all components are from Sigma-Aldrich), and were incubated at 4°C for 10 min. The parasites were then subjected to sonication at 4°C and were viewed microscopically to ensure that all of the parasites had been disrupted. The parasite suspension was then centrifuged at 27,000 × *g* for 20 min, and supernatant was collected and was recentrifuged at 100,000 × *g* for 4 h. The supernatant was then harvested, dialyzed against PBS, sterilized by passage through a 0.22-μm filter, and stored at −80°C until use. The bicinchoninic acid protein assay kit (Pierce) was used to determine protein concentrations. The endotoxin levels in SLA and MLA were <0.06 U/ml, as determined with the *Limulus* amebocyte lysate assay. Aliquots of SLA and MLA were stored at −80°C until use.

To determine whether the membrane Ag(s) that triggers cell proliferation are proteins, MLA were digested with PK (17). Membrane Ags were incubated overnight at 42°C with PK (10 U/mg Ag) and then precipitated with methanol at −20°C for 2 h. The precipitate was collected after centrifugation at 10,000 × *g* for 30 min at +4°C and then washed with 70% methanol. Pellets were air dried and resuspended in sterile PBS (0.01 M, pH 7.4). PPD was treated similarly and used as control.

Lipid extraction from MLA was performed with chloroform/methanol (2:1 v/v). Twenty volumes of chloroform/methanol were added to 1 vol of membrane Ags. After homogenization, the mixture was incubated for 30 min at room temperature and centrifuged at 10,000 × *g* for 30 min, and the supernatant containing extracted lipids was discarded. The pellets were subjected to three successive extractions with chloroform/methanol. The insoluble material was washed with cold 70% methanol, air-dried, and resuspended in sterile PBS (0.01 M).

Cell purification

PBMC and cord blood mononuclear cells (CBMC) were isolated from heparinized blood samples using Ficoll-Paque (Pharmacia) density gradient centrifugation. Cells were washed and resuspended at 10^6 cells/ml in complete medium (RPMI 1640, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated normal human AB serum). Monocytes were purified, as follows: PBMC at $1–2 \times 10^6$/ml were allowed to adhere to 24-well tissue culture plates (Costar) for 2 h at 37°C, under 5% CO_2 in medium containing 2% human AB serum. Nonadherent cells were removed by several washes with prewarmed medium. The adherent fraction consisted on >98% monocytes after staining with anti-CD14, anti-CD19, and anti-CD3 mAbs, and subsequent FACS analysis. In addition, we checked that no IFN-γ was detectable in culture supernatant after incubation of purified monocytes with PHA. T cells depleted in the $CD45RA^+$ or $CD45RO^+$ subsets were obtained, as follows: purified T cells were incubated with anti-CD45RA or anti-CD45RO mAbs for 30 min at 4°C, washed twice in PBS 0.1% BSA, and then mixed with magnetic beads coated with anti-mouse IgG for 30 min at 4°C. Adherent cells on magnetic beads were removed with a magnet (Dynal Biotech). T cell-purified fractions contained >98% $CD45RA^+$ or $CD45RO^+$ cells. Depletion of $CD3^+$ T cells was performed by incubation of PBMC (5×10^6/ml) for 45 min on ice with anti-CD3 mAbs (clone CD3.12.35, IgG2a). After three washes, cells were incubated for 30 min at 37°C with complement from rabbit (1/5 dilution) and washed extensively. Viable cells were adjusted to 10^6/ml in complete medium. Control cell preparations were prepared in parallel, without addition of anti-CD3 mAbs, to check for toxicity due to rabbit complement.

Analysis of Ag processing and presentation

T cells were purified from PBMC by subjecting them to two rounds of adherent cell depletion on polystyrene dishes and by treating the nonadherent cells twice with the Lympho-Kwik TH lymphocyte isolation kit to remove any residual APCs, i.e., $CD19^+$ (B lymphocytes) and $CD14^+$ (monocytes) cells. $CD14^+$ and $CD19^+$ cells within the purified T cell fraction were <0.1 and <0.3%, respectively, as assessed by FACS analysis. Purified T cells did not proliferate to PHA, PPD, or SEB.

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

For Ag-processing experiments (18): PBMC were incubated with 0.14% paraformaldehyde (Sigma-Aldrich) in PBS for 1 min at 37°C and then washed extensively with cold 0.15 M glycine in PBS. These cells were used at 5×10^4 cells/well as APCs to autologous purified T lymphocytes (10^5 cells/well) and cultured for 6 days. Cultures were pulsed with [^{3}H]thymidine, and incorporation was measured in a liquid scintillation counter (model 1900 TR; Packard Instrument).

Proliferation assays

PBMC and CBMC at 10^5 cells/well in 100 μl of complete medium were plated in triplicate in 96-well round-bottom microtiter plates (Nunc) and stimulated with mitogen, Ags, or SAgs. Control cells were left nonstimulated. Plates were incubated for 3 days for PHA and 5–6 days for Ags or SAgs at 37°C in 95% humidified air with 5% CO_2. Mitogen and Ags were used at final concentrations of 10 μg/ml for PHA, 250 U/ml for PPD, and 5–10 μg/ml for SLA and MLA. These concentrations were found in preliminary experiments to be optimal for causing stimulation. SEA and SEB were used at 5 ng/ml. The kinetics of cell proliferation was studied by incubating PBMC and CBMC at 10^6 cells/ml for 2–7 days in the presence of mitogen or Ags. Cells were pulsed with 1 μCi/well [^{3}H]thymidine (sp. act., 5 Ci/mM; Amersham) for the last 6 h of incubation, and incorporated radioactivity was counted in a liquid scintillation counter.

Data are expressed as Δ cpm (cpm in stimulated cultures − cpm in nonstimulated cultures) or as stimulation indexes (SI); Δ cpm of stimulated wells/cpm in control wells). Cell proliferation was considered as significant when Δ cpm and SI were $\geq$5000 and $\geq$3, respectively.

Ab inhibition experiments

To investigate whether MLA presentation by APCs requires MHC class I and/or class II Ags, PBMC from noninfected donors and CBMC were incubated with MLA in the presence of mAbs directed against MHC class I or MHC class II determinants. mAbs were used at a 1/400 dilution for MHC class I and a 1/200 dilution for MHC class II. These dilutions did not induce any significant inhibition of PHA-induced cell proliferation. mAbs to CD4 (IgG1) or CD8 (IgG2b) molecules were also used in inhibition experiments. CBMC and PBMC from Tunisian noninfected donors were incubated with mAbs for 1 h at 37°C in 5% CO_2, before the addition of the stimuli. Irrelevant isotype control Abs IgG1 and IgG2b were tested in parallel. Plates were incubated for 5 days, and each well received 1 μCi of [^{3}H]thymidine before harvesting. Results are expressed as percentages of inhibition of the proliferative response.

Phenotype of cells proliferating to MLA

PBMC and CBMC were harvested after 6 days of culture and washed twice in ice-cold PBS supplemented with 1% BSA and 0.1% sodium azide. Cells were labeled for 30 min on ice with the appropriate mAbs and then were washed twice and fixed with 0.3% paraformaldehyde in PBS. Ten thousand events for each sample were analyzed on a FACSVantage cytofluorograph (BD Biosciences) using LYSIS II software.

Analysis of in vitro cytokine production by ELISA

PBMC were cultured at 10^6/ml in complete medium and stimulated as for proliferative assays with optimal concentrations of PHA, PPD, SLA, and MLA. Supernatants from stimulated and nonstimulated cultures were collected after 2 days for IL-10 and IL-4, and after 3 days for IFN-γ. Aliquots were stored at −80°C until analysis. Purified monocytes were cultured at 2–4 $\times 10^5$/ml complete medium (RPMI 1640 containing 2% heat-inactivated human AB serum) in 24-well plates. Cells were incubated in the absence or the presence of 10 μg/ml MLA, 10 ng/ml LPS, or MLA plus LPS. For IL-12 induction, cells were activated with LPS plus human rIFN-γ (100 U/ml). Cytokine secretion was monitored in the supernatant after 24, 48, and 72 h of culture. Cytokines were quantified by an in-house designed ELISA using commercial Abs and recombinant human cytokines purchased from BD Pharmingen (14). Cytokine levels were determined by interpolation from standard curves based on recombinant cytokines. The detection limits were 10 pg/ml for IL-1α, IL-4, IL-10, IL-12, and TNF-α, and 50 pg/ml for IFN-γ. Cytokine concentrations were calculated as the difference between control and stimulated cultures.

TCR BV repertoire and CDR3 size spectratyping analysis

PBMC from three noninfected adults were cultured at 2×10^6/ml for 3, 5, or 7 days with different stimuli (PHA, anti-CD3, PPD, MLA, and SEB) or incubated without stimulation. Cultures were subjected to Ficoll-Paque density centrifugation to enrich for blastic cells and incubated for 36 h in the presence of human rIL-2 (19). Cells were harvested and stored in TRIzol reagent at −80°C until extraction of RNAs. Aliquots of PBMC from each donor were conserved at day 0 in TRIzol reagent at −80°C and used as controls for the repertoire analysis.

Total RNA was extracted from 66 samples using the TRIzol kit. TCR BV gene segment usage was investigated using a semiquantitative adaptation of the Immunoscope approach (20) after standardization of T cell-specific material between samples by quantification of the number of copies of CD3δ transcripts by a competitive PCR (21). Briefly, 30 cycles of PCR were performed with each one of the 24 BV-specific primers and a fluorescent (FAM)-labeled constant β segment (BC)-specific primer using 25 or 50 $\times 10^9$ copies of CD3δ-specific cDNA, as determined in preliminary experiments. Diluted PCR products were loaded on a 6% sequencing gel and run on a 36-well ABI 373 automated sequencer (Applied Biosystems). The Immunoscope software was used to obtain peak areas, nucleotide lengths, and CDR3 profile displays from sequencer raw data. The ISEApeaks software package (22) was used to analyze Immunoscope raw data. ISEApeaks retrieves and smooths the peak data of each sample, consisting of peak areas and nucleotide lengths of the CDR3 region. Relative percentages of use for each CDR3 length and each BV segment were automatically computed. To compare the BV-BC repertoires of each activation condition, data from each sample were gathered in a peak database to study the magnitude of perturbation (DBV-BC) or skewing of the CDR3 spectratypes from a theoretical nonperturbed repertoire exhibiting a Gaussian distribution (23). In our case, perturbation was assessed by comparing the CDR3 length distributions with those exhibited at day 0. The mean of TCR repertoire perturbation (μDBV-BC) of each sample was determined in each stimulation condition by the ISEApeaks software.

Statistical analysis

SD indicates in this study the variance of the whole group. Statistical significance was assessed by paired or unpaired Student's *t* test. Values of $p <$ 0.05 were considered statistically significant.

Results

MLA induce proliferation of CBMC and PBMC from noninfected individuals

In previous epidemiological studies, we have shown that healthy individuals living in Tunisia, a country endemic for leishmaniasis, are exposed to sandfly bites and may be asymptomatically infected with *Leishmania* parasites. Asymptomatic infection leads to the development of an immune memory of the delayed type to *L. major* parasites attested by a positive LST and by in vitro induced lymphoproliferative responses to *Leishmania* soluble extracts. A strong correlation exists between LST reactivity and in vitro lymphoproliferative responses to *L. major* SLA (14). Essentially, similar results were obtained whether *L. major* or *L. infantum* extracts were used, in areas endemic either for *L. major* or *L. infantum* infections (14) (our unpublished data) (young adults). Individuals whose lymphocytes are nonreactive to *L. infantum* SLA are regularly LST negative. In the absence of any history of leishmaniasis, these individuals could be considered as noninfected (i.e., naive to *Leishmania* parasites).

In contrast to the high specificity and sensitivity of the in vitro responses induced by SLA, which accurately identify LST-positive immune individuals including children cured of VL, we found quite unexpectedly that PBMC from all tested individuals, whether LST positive or LST negative, proliferate vigorously when incubated with membranes extracted from *L. infantum* parasites (Fig. 1*A*). Similar results were obtained with MLA extracted from *L. major* parasites (data not shown). These results suggest that human lymphocytes may express a natural reactivity to some components of *Leishmania* membranes.

To confirm these observations, we selected two groups of individuals not exposed to *Leishmania* parasites, namely newborns and blood donors living in a nonendemic country (Sweden). PBMC from both groups constantly proliferate to MLA (SI $\geq$3 and Δ cpm $\geq$5000), but not to SLA (Fig. 1, *C* and *D*). The optimal concentration of MLA was 5–10 μg/ml. Cell proliferation to MLA peaked at days 5–6 as with the classic Ag PPD (data not shown). CBMC did not proliferate to PPD (SI <3), indicating that they were not

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

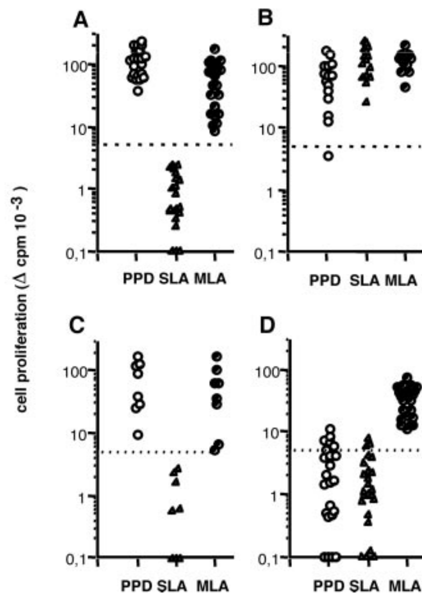


FIGURE 1. In vitro lymphoproliferative responses to MLA, SLA, and PPD Ags. PBMC were obtained form 22 noninfected Tunisian adults (*A*), 15 cured VL children (*B*), and 8 nonexposed Swedish blood donors (*D*), and CBMC were obtained from 24 Tunisian newborns (*C*). Cells were stimulated with 10 μg/ml SLA or MLA and 250 U/ml PPD for 5–6 days. Cell proliferation was considered positive when Δ cpm and SI were ≥5000 and ≥3, respectively. Results are represented in Δ cpm. Each point is representative of one individual.

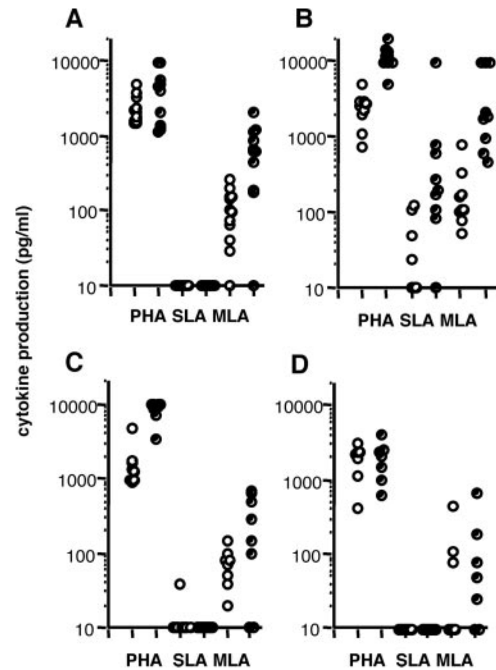


FIGURE 2. IFN-γ (●) and IL-10 (○) secreted by in vitro stimulated blood mononuclear cells. PBMC from noninfected Tunisian adults (*A*), cured VL children (*B*), nonexposed Swedish individuals (*C*), and CBMC (*D*) were stimulated with SLA, MLA, or the mitogen PHA. Cytokine concentration was measured in culture supernatants harvested after 48 or 72 h for IL-10 or IFN-γ, respectively.

contaminated by maternal PBMC (Fig. 1*D*). MLA preparations were LPS/endotoxin-free, as checked by the *Limulus* amebocyte lysate test. In addition, polymixin B treatment of MLA did not abrogate cell proliferation and cytokine secretion (data not shown).

MLA induce cytokine secretion by PBMC and purified monocytes from noninfected individuals

Cytokine production was measured by ELISA in the supernatants of in vitro MLA- or SLA-stimulated CBMC and PBMC from Tunisian and Swedish adults. High levels of IFN-γ and IL-10 were regularly induced by MLA, but not SLA (Fig. 2, *A*, *C*, and *D*). PBMC from individuals with cured VL, used as a positive control, produced high levels of IFN-γ and IL-10 when stimulated with MLA or SLA. However, the highest levels were obtained with MLA (Fig. 2*B*). In these experiments, IL-4 was never detected, except after PHA stimulation (data not shown).

IL-10 is a ubiquitous Th2 cytokine that is produced by either T or B lymphocytes or by monocytes. We therefore investigated whether MLA can directly activate purified monocytes to secrete IL-10 and other monokines. Monocytes from noninfected individuals were purified from PBMC (purity >98%) and incubated with SLA or MLA. IL-1α, IL-12, IL-10, and TNF-α concentrations in the culture supernatants were then measured by ELISA. We confirmed the absence of any contaminating $CD3^+$ T cells or $CD19^+$ B cells using flow cytometry and demonstrating that PHA failed to induce any IFN-γ secretion by the purified cells (data not shown). SLA had no effect on monocytes. In contrast, MLA induced significant secretion of IL-10 and TNF-α, but not of IL-1α or IL-12 (Fig. 3). MLA synergize with suboptimal concentrations of LPS for TNF-α (Fig. 3, *C* and *D*; $p < 0.05$) and particularly for IL-10 secretion (Fig. 3, *A* and *B*; $p < 0.002$). Purified monocytes produce high levels of IL-1α (Fig. 3*E*) or IL-12 (Fig. 3*F*) when incubated with suboptimal doses of LPS or with LPS plus IFN-γ, respectively. Interestingly, addition of MLA to these reagents inhibits IL-1α and IL-12 secretion ($p < 0.02$ and $p < 0.0001$, respectively) (Fig. 3, *E* and *F*).

Altogether, these data suggest that MLA exert a proliferative effect on lymphocytes from noninfected individuals, inducing mainly the Th1 cytokine, IFN-γ. MLA also activate monocytes to secrete the inflammatory cytokine TNF-α, but not the immunoregulatory IL-1α or IL-12. MLA-induced IL-10 was produced by both monocytes and lymphocytes. To ascertain that T lymphocytes from noninfected individuals can secrete IL-10 when activated by MLA, we established a continuous cell line from one donor by iterative stimulation of PBMC with MLA. This cell line, after continuous expansion during 12 wk, consisted only of $CD4^+$ T cells and still proliferated to MLA and secreted IL-10 and IFN-γ, but not IL-4 upon stimulation (data not shown).

Leishmania *Ags, which induce proliferative responses and cytokine secretion in PBMC from noninfected individuals, are proteins*

Considering the high content of glycolipids, especially lipophosphoglycan, in membrane preparations extracted from *Leishmania* parasites, we tested whether component(s)-inducing proliferative response and/or cytokine secretion in noninfected individuals were glycolipids and/or proteins. For that purpose, MLA preparations were delipidated by three rounds of chloroform/methanol extraction, and the capacity of the delipidated fraction to induce lymphoproliferation and cytokine induction was tested. Cell proliferation and IFN-γ secretion were not altered by lipid extraction, whereas production of IL-10 was significantly decreased ($p < 0.01$), although not totally abrogated (Fig. 4*A*). In contrast, when MLA proteins were fully digested by an excess of PK, the in vitro lymphoproliferative responses and cytokine secretion of IFN-γ and IL-10 in culture supernatants were totally abrogated (Fig. 4*B*). These data indicate that the biological effects induced by MLA in

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

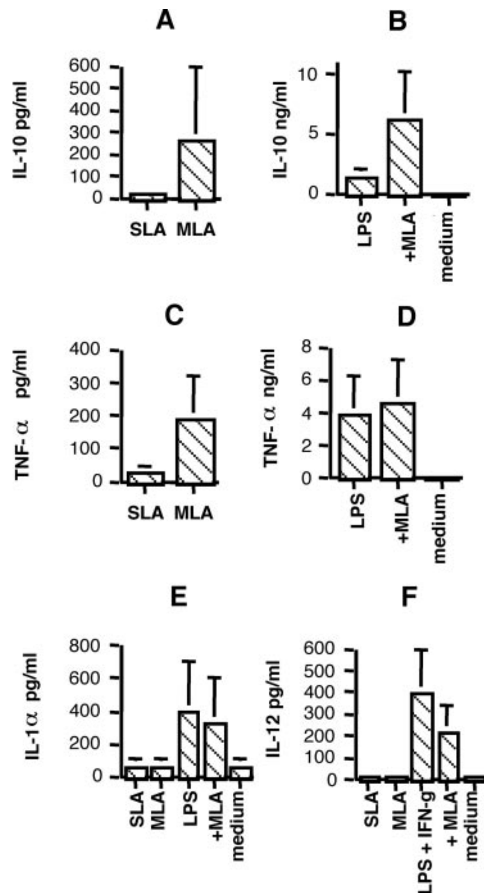


FIGURE 3. Cytokine production by purified human monocytes stimulated in vitro. Adherent cells were purified from PBMC of noninfected Tunisian donors ($n = 6$) and stimulated with MLA (10 μg/ml). + MLA, Indicates stimulation with MLA and LPS (10 ng/ml) (for IL-10, TNF-α, and IL-1α induction) and with MLA, LPS, and IFN-γ (100 U/ml) (for IL-12 induction). The concentrations of IL-10 (*A* and *B*), TNF-α (*C* and *D*), IL-1α (*E*), and IL-12 (*F*) in the culture supernatants, harvested after 24 h of incubation, were determined by ELISA. Results are expressed as mean amounts of cytokine + 1 SD.

PBMC from noninfected individuals are largely mediated by proteins, although some lipidic or proteolipidic component(s) may participate in the induction of IL-10 secretion by monocytes.

MHC class II molecules are required for MLA-induced lymphoproliferative responses in noninfected individuals

To check whether MHC molecules are required for the induction by MLA of proliferative responses in noninfected individuals, we performed inhibition experiments using a panel of mAbs to MHC class I or class II Ags. PBMC or CBMC were incubated with MLA, PPD, or PHA in the presence of MHC-specific mAbs. Cell proliferation to MLA was completely abrogated by mAbs to DR Ags. mAbs to class I and to DP or DQ class II Ags, as well as irrelevant mAbs expressing the same isotypes, were only weakly inhibitory (Fig. 5*A*). DR-specific mAbs also strongly inhibited PPD-induced proliferation, but, as expected, had a minimal effect on PHA-induced cell proliferation. Similar results were obtained with MLA-stimulated CBMC. In the latter experiments, PHA was used as a positive control instead of PPD because CBMC do not proliferate to PPD (Fig. 5*B*). These data indicate that membrane Ags inducing proliferative responses in noninfected individuals are presented by APCs to lymphocytes in the context of DR molecules and exclude the possibility that they merely act as mitogens.

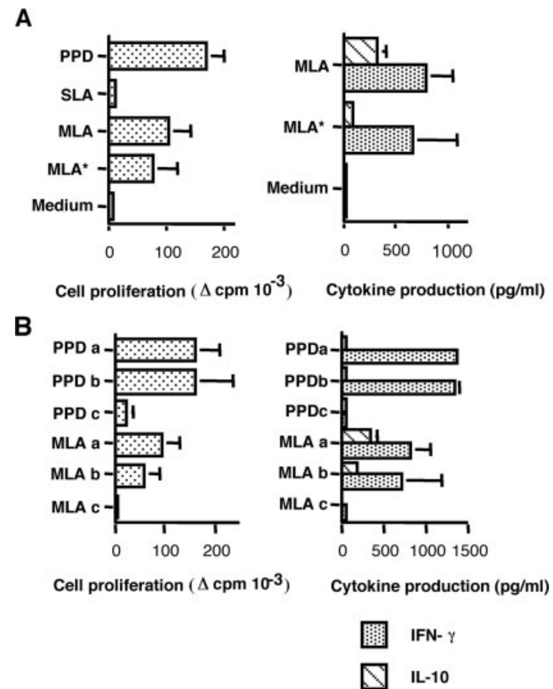


FIGURE 4. Effect of lipid extraction (*A*) or PK digestion (*B*) of MLA on cell proliferation and production of cytokines IFN-γ and IL-10. *A*, PBMC from noninfected donors were cultured in the presence of MLA (5–10 μg/ml) or chloroform/methanol-extracted MLA (MLA*). PPD and SLA were used as controls. *B*, PPDa and MLAa, Ags that were not treated with PK. PPDb and MLAb, Ags that were subjected to the same incubation and washing steps as PPDc and MLAc without the addition of PK. PPDc and MLAc, Ags that were digested by PK, as described in *Materials and Methods*.

MLA do not contain SAg(s)

The fact that MLA induce strong proliferative responses of PBMC from noninfected individuals and that the responses require presentation by MHC class II molecules raises the possibility that MLA may contain SAg(s). To test this hypothesis, we checked whether MLA processing by APCs is required. T cells were purified from PBMC from noninfected individuals by the double step of adherence to plastic and complement depletion of $CD14^+$ or $CD19^+$ cells by the Lympho-Kwik kit. We assessed the high purity of T cells using flow cytometry (>99%) and by demonstrating that they could no longer proliferate to PPD, MLA, and SEB because of the total depletion of APCs (Fig. 6). Autologous PBMC either untreated or Formalin fixed were used as APCs. We established the optimal conditions for Formalin fixation of APCs in preliminary experiments, by demonstrating that APCs that had already processed MLA or PPD before Formalin treatment were still able to induce proliferative responses of T cells. Using these experimental conditions, we found that PBMC from noninfected individuals once fixed with Formalin and therefore unable to process Ags could no longer present MLA or the conventional Ag PPD to purified T cells. Control experiments using similar experimental conditions showed that SAg SEB was still able to induce cell proliferation (Fig. 6).

Phenotype of blood mononuclear cells responding to MLA

To determine which T cell subsets are targeted by MLA, we treated PBMC from two noninfected adults with anti-CD3 mAbs and complement. Depletion of $CD3^+$ T cells totally abrogated cell proliferation (Fig. 7*A*) and cytokine production (data not shown).

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

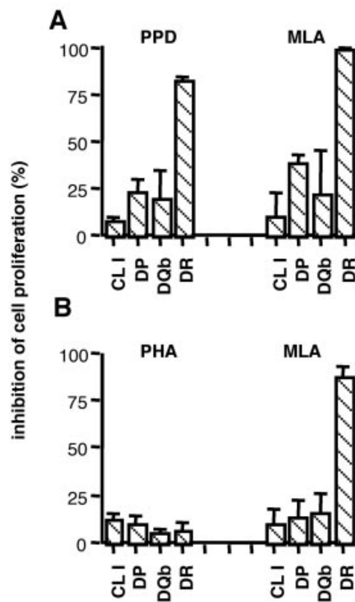


FIGURE 5. Inhibition of the lymphoproliferative responses by anti-MHC mAbs. PBMC from noninfected Tunisian donors ($n = 4$) (*A*) and CBMC ($n = 4$) (*B*) were stimulated with PPD, PHA, or MLA in the presence or absence of anti-class I, anti-DP, anti-DQ, or anti-DR class II mAbs. Results are expressed as mean percentage of inhibition of cell proliferation by anti-MHC mAbs + 1 SD.

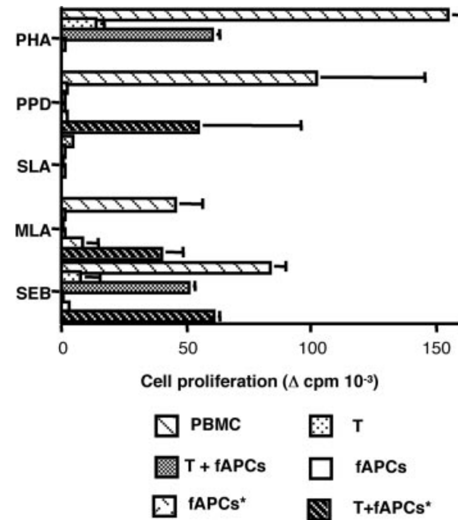


FIGURE 6. Proliferation to MLA requires processing by APCs before presentation to T cells. T cells were purified from PBMC of two noninfected adults and incubated in the absence or presence of autologous paraformaldehyde-fixed PBMC (pfAPCs). Fixed APCs∗ (pfAPCs∗) are autologous PBMC, which were allowed to process Ag before paraformaldehyde fixation. Results are represented as means of Δ cpm + 1 SD.

Similarly, addition of CD4-specific mAbs strongly inhibited MLA-induced cell proliferation, while addition of mAbs specific to CD8 Ags had no effect (Fig. 7*B*). To further determine whether T cells responding to MLA have the phenotype of naive or memory cells, we performed proliferative assays using T cells depleted in either the $CD45RA^+$ (naive) or the $CD45RO^+$ (memory) T subset. Irradiated autologous PBMC used as APCs were added to T cells depleted in either of these subsets. Fig. 8 shows that both fractions, containing residual $CD45RA^+$ or $CD45RO^+$ T cells, respectively, still proliferate to MLA. As expected, proliferation to recall Ags, such as PPD or TT, was maintained in T cells depleted of $CD45RA^+$ naive lymphocytes, but was abrogated in T cell preparations depleted of $CD45RO^+$ memory cells.

Using a panel of mAbs to various cell surface Ags, we characterized the phenotype of lymphocyte subpopulations responding to MLA by gating the flow cytometer on activated cells (R2 gate). As shown in Fig. 9*A*, >80% of R2-gated cells activated by MLA, from adult noninfected donors, express $CD3^+$, $CD4^+$, and $CD45RO^+$ surface markers ($p < 0.005$). This fraction contained very few $CD45RA^+$ cells, $\gamma\delta^+$ T cells, B ($CD20^+$) cells, monocytes ($CD14^+$), or NKT ($V\alpha\ 24^+V\beta11^+$) cells. There was only a marginal increase in the R2 gate of $CD8^+$ T cells, NK ($CD3^-CD16^+CD56^+$) cells, or $CD161^+$ compared with nonstimulated cells. PPD-stimulated cells were also tested as control. In this case, R2-gated cells bear either the $CD4^+CD45RO^+$ or $CD8^+CD45RO^+$ phenotype or the $CD3^-CD16^+CD56^+$ phenotype ($p < 0.05$). In experiments using CBMC, similar results were observed, except that slightly larger numbers of $CD4^+CD45RA^+$ cells ($p < 0.05$), $CD8^+$ cells ($p < 0.01$), and NK ($CD3^-CD16^+CD56^+$) cells ($p < 0.05$) were recorded among the R2-gated cells, although with large individual variations. However, it is interesting to note that even MLA-stimulated CBMC showed a large dominance of $CD4^+CD45RO^+$ over $CD4^+CD45RA^+$ cells (Fig. 9*B*). Finally, cell surface analysis of the T cell line established by MLA + IL-2 iterative stimulation

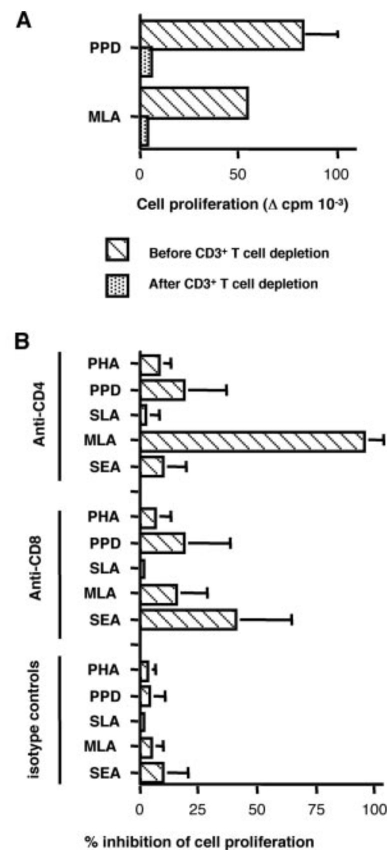


FIGURE 7. Effect of $CD3^+$ T cell depletion on cell proliferation to MLA and blocking assays of the lymphoproliferative response to MLA by mAbs. *A*, PBMC from noninfected Tunisian donors ($n = 2$) were incubated with anti-CD3 mAbs and then with complement to deplete $CD3^+$ T cells. *B*, Inhibition of proliferation of PBMC from noninfected Tunisian adults ($n = 9$) to PHA, PPD, SLA MLA, and SEA by anti-CD4 and anti-CD8 mAbs. Results are expressed as percentage of inhibition of cell proliferation compared with cultures without added mAbs + 1 SD.

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

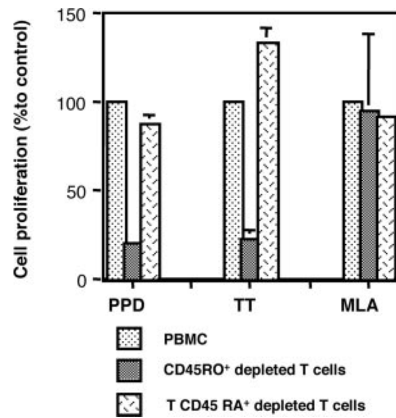


FIGURE 8. Role of CD45RA$^+$ and CD45RO$^+$ T cells in the lymphoproliferative responses induced by MLA. T lymphocytes were purified from PBMC of two noninfected donors and depleted in the CD45RA$^+$ or CD45RO$^+$ subsets. Cell preparations were incubated in the presence of recall Ags (PPD and TT as controls) or MLA and in the presence of autologous irradiated PBMC as APCs. Results are expressed as percentages of proliferative responses to bulk PBMC preparation response before depletion.

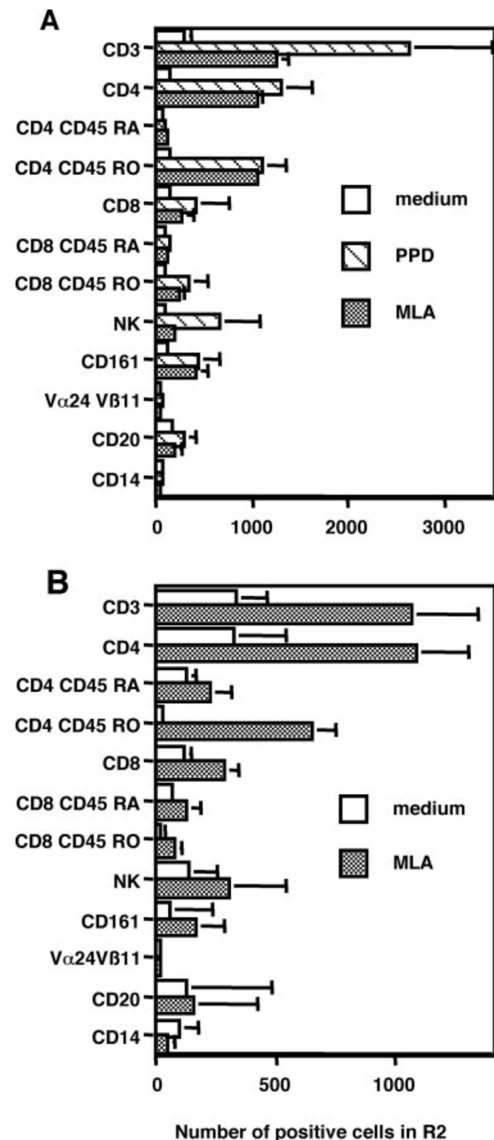


FIGURE 9. Phenotypic characterization of PBMC activated by MLA. PBMC (n = 4) and CBMC (n = 5) were cultured with MLA (10 μg/ml) for 6 days and analyzed by immunofluorescence staining and flow cytometry after gating on activated cells (R2 gate); 10,000 total events were counted in each experiment. PPD was used as a positive control for PBMC stimulation. Results are illustrated as absolute numbers of positive cells expressing the cell surface markers among PBMC from noninfected adults (*A*) and among CBMC (*B*).

showed that 100% of cells present after 12 wk of expansion had the phenotype of CD3$^+$CD4$^+$CD45RO$^+$ cells (data not shown).

TCRB repertoire analysis of MLA-stimulated PBMC of naive individuals

We checked whether the MLA-induced T cell expansion expressed a narrow or a broad range of BV genes and whether it alters the TCR β-chain CDR3 length distributions. For this purpose, we analyzed the Vβ repertoire of cells from three noninfected donors using the semiquantitative Immunoscope technique. As expected, the lymphoproliferations induced at days 3, 5, and 7 by polyclonal T cell activators such as PHA and anti-CD3 mAbs or by a complex Ag mixture such as PPD did not induce the expansion of specific Vβ-bearing T cells compared with ex vivo PBMC (day 0) or with control nonstimulated cultures (Fig. 10*A*; day 7 stimulation results are represented for one donor, and data for PHA and PPD are not shown). In contrast, the SAg SEB induced, as expected, a selective proliferation of Vβ3, Vβ12, Vβ14, Vβ15, Vβ17, and Vβ20 T cells (24, 25). T cell activation by MLA was associated with some expansion of Vβ17 and Vβ18 T cells; however, this association was modest and not consistent from donor to donor (Fig. 10*A*). These results further support that MLA-TCR interaction is different from the one that is established between SAg and TCR.

Finally, we investigated the diversity of CDR3 usage in BV-amplified transcripts using a sensitive method based on estimation of the mean perturbation of TCR repertoire (μDBV-BC). Analysis showed that ex vivo PBMC (day 0), as well as PHA and anti-CD3- or SEB-stimulated cells tested at days 3, 5, and 7 were characterized by a Gaussian distribution of CDR3 lengths (data not shown). μDBV-BC at various times of incubation were remarkably similar between the three individuals tested, confirming that TCR used CDR3-expressing random length sequences. In contrast, the conventional Ag PPD induced a perturbation in the TCR BV repertoire featured by a deviation in the CDR3 length usage (Fig. 10*B*). In addition, analysis of CDR3 spectratypes demonstrated the presence of monoclonal and oligoclonal expansions (data not shown). Finally, when PBMC were incubated with MLA, we found that the mean perturbation induced by the latter stimulator was closer to that induced by polyclonal activators than to that induced by the conventional Ag PPD.

Discussion

The present study characterizes the mechanisms underlying a remarkable biological property of membrane Ags of *L. infantum*, namely their capacity to induce in vitro lymphoproliferative responses and cytokine secretion by PBMC from noninfected individuals (i.e., naive to *Leishmania* parasites). The latter were represented by newborns, healthy noninfected Tunisian adults, or Swedish blood donors nonexposed to *Leishmania* infection. Similar results were obtained with MLA extracted from *L. major* parasites (data not shown). In contrast to soluble Ags extracted from *L. infantum*, which react only with PBMC from individuals immune to *Leishmania* parasites, MLA extracted from the same parasite strain constantly stimulate PBMC from all individuals. These results confirm that only SLA should be used as *Leishmania* Ags in in vitro studies to discriminate accurately between immune and

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

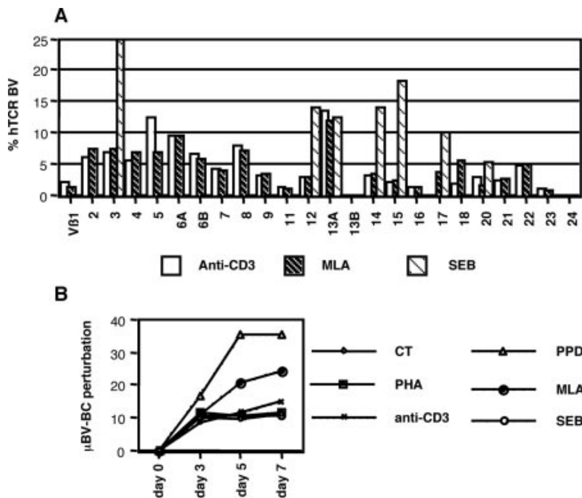


FIGURE 10. BV gene segment usage and mean of TCR BV-BC repertoire perturbation in T cells stimulated with MLA, anti-CD3, PHA, PPD, or SEB. *A*, TCR BV-BC at day 7 from anti-CD3-, SEB-, and MLA-stimulated PBMC of three noninfected donors are depicted as the relative percentages of use compared with control cultures. Results from one representative individual are shown. *B*, Alteration of TCR β-chain CDR3 length distributions. The mean of TCR BV-BC repertoire perturbation (μDBV-BC) was analyzed at days 3, 5, and 7 in PPD-, CD3-, SEB-, PHA-, and MLA-stimulated cells. Data from ex vivo PBMC (day 0) were used as controls (CT). Results from one representative individual are shown.

nonimmune individuals (14). MLA-induced proliferation of lymphocytes from noninfected donors is not trivial or due to an artifact: MLA preparations were LPS/endotoxin free and polymixin B treatment did not affect either cell proliferation or cytokine production. In addition, the extraction procedure of MLA is unlikely to have generated these properties because whole parasites of *L. infantum* are also able to induce cell proliferation of PBMC from naive adult individuals (data not shown). The effect is to some extent species restricted because splenocytes from BALB/c mice did not proliferate to MLA (data not shown). The latter result indicates, à contrario, that MLA reactivity with human PBMC does not merely reflect the presence in the antigenic preparation of DNA or RNA acting through TLR9, as this receptor can be expressed by murine B and T lymphocytes (26, 27), but not by human T cells (28).

The natural reactivity of noninfected individuals has been previously reported: studies using Ags from protozoan parasites, including several *Leishmania* species (9–13), *Trypanosoma cruzi* (29), *Toxoplasma gondii* (30), and *Plasmodium sp.* (31). Our study, however, is the first to our knowledge to specifically ascribe these effects to membrane-associated Ags.

The property of PBMC of almost all individuals reacting with membrane Ags raises the possibility that MLA may contain (a) mitogenic component(s). We could exclude this hypothesis by showing that cell proliferation to MLA peaks at day 5–6, that it requires macrophage processing, and that Ag presentation to T cells occurs in the context of MHC class II molecules. The latter characteristic prompted us to test whether responding cells are actually activated by a putative parasite SAg. Several parasites have previously been reported to contain a SAg that can induce proliferation of T cells bearing particular Vβ TCR. Thus, *T. gondii* and *Plasmodium yoelii* trigger, in nonimmune mice, selective expansions of $CD8^+$ $V\beta5^+$ T cells and of $CD4^+$ and $CD8^+$ $V\beta9^+$ T cells, respectively (32, 33). However, our analysis of the T cell repertoire did not detect any significant expansion of $CD4^+$ T cells bearing a particular Vβ TCR after MLA stimulation. In addition, MLA need to be processed by APCs before association with MHC DR molecules and presentation to T cells, a further indication that the biological effects of MLA are most likely not due to a SAg. A further confirmation of this crucial fact was recently obtained using PBMC from a patient with the Bare lymphocyte syndrome (a congenital immune deficiency due to a genetic defect leading to nonexpression of class II Ags) whose PBMC were not able to proliferate in response to MLA, although they effectively proliferate in response to PHA (A. Sassi and K. Dellagi, unpublished results).

The effects of MLA on PBMC from noninfected adults could reflect a secondary immune response to some cross-reactive microorganisms or Ag(s) previously encountered by these individuals. Kemp et al. (10) have reported that lymphocytes from Danish nonexposed individuals respond to *Leishmania* Ags by proliferation and IFN-γ secretion. They suggested that the responding cells are memory cells to cross-reactive Ags because depletion of $CD45RO^+$ (memory) lymphocytes abolishes the lymphoproliferative responses to nonsoluble or soluble Ags of *L. donovani* or *L. major*. These cells were $CD2^+CD4^+$ (but not $CD8^+$) and secrete IFN-γ and IL-4. The latter cytokine was detected only when cells were preactivated by PMA and ionomycin (11). Our results differ from those reported by Kemp in several respects: we show that T cells depleted in either the $CD45RA^+$ or the $CD45RO^+$ T cell subsets equally proliferate to MLA. In addition, the biological effects are triggered only by membrane-associated Ags and not by soluble Ags. Finally, the cytokines secreted in response to MLA are mainly IFN-γ and IL-10, but not IL-4. Our results also differ from those reported by Akuffo et al. (9), who stimulated PBMC from naive individuals with *L. aethiopica* Ags or live parasites, and showed that responding cells are mainly NK cells (9, 34). In our results, NK cells are not targets of *L. infantum* Ags, as shown by the phenotypic analysis of proliferating cells and from our demonstration that MLA effects were totally abrogated by depletion of CD3-positive T cells. Finally, it has been reported that $CD8^+CD45RA^+$ clones and $CD4^+CD45RA^-$ clones from nonexposed individuals secrete IFN-γ or IL-10, respectively, upon stimulation with live *L. guyanensis* parasites or with the *Leishmania* homologue of receptors of activated C kinases Ag. Only *Leishmania* homologue of receptors of activated C kinases, but not whole parasites, induces IL-10 secretion by $CD4^+CD45RA^-$ clones (12).

The differences reported above indicate that various *Leishmania* species might react with different lymphocyte subsets in noninfected individuals, which would not be surprising for parasitic protozoa of the genus *Leishmania*, evolutionary diverse and from which significant differences in host-parasite interactions have evolved (35). It is tempting to consider that these slight variations in the innate reactivity of the host to Ags from different *Leishmania* species shape, at least in part, the subsequent immune response and may account for differences in the natural history of the infections induced by these pathogens.

The reactivity of lymphocytes from noninfected individuals with Ags from *Leishmania* parasites or other pathogens (i.e., *T. gondii*, *Plasmodium sp.*, helminths) (30, 31, 36) has been detected to date with PBMC obtained from adult individuals. Therefore, it was considered that they could reflect a mere cross-reactivity of memory cells with environmental Ag(s) previously encountered by these individuals during their lives. In the present study, we used CBMC from human healthy newborns, a source of cells that are unlikely to have previously encountered microbe Ags, and that hence provide strong argument that human CD4 T cells have an innate property to react with parasite Ags. The fact that CBMC did

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

not proliferate to SLA argues against an in utero exposure of newborns to *Leishmania* Ags. In addition, the fact that CBMC did not proliferate to PPD, a recall Ag to which almost all adults in Tunisia are reactive (because of generalized bacillus Calmette-Guérin vaccination), contends against a contamination of CBMC with maternal cells. Phenotypic analysis of ex vivo T cells indicates that they almost exclusively (>99%) express the CD45RA cell surface marker (37), a further indication that naive (nonmemory) T lymphocytes are able to proliferate in response to MLA. The fact that R2-gated cells, which correspond to MLA-activated cells, are mainly $CD45RO^+$ and not $CD45RA^+$ suggests together with CD45RA or RO depletion experiments that once activated by MLA, $CD45RA^+$ cells may express the CD45RO marker either stably or transiently.

Our data corroborate the concept that the biological effects revealed by MLA in naive individuals do not reflect some cross-reactivity with another microorganism, but most likely indicate an innate property of the host immune system of reacting with some membrane-associated *Leishmania* Ag(s). The latter are most likely proteins and not glycolipids, as shown by extraction experiments as well as by the demonstration that proliferating cells are not NKT cells bearing the Vα24 Vβ11 TCR, a T cell subset that recognizes glycolipidic Ags presented by CD1 molecules (38, 39).

Our results indicate that the biological effects of MLA are most likely mediated through the TCR and also involve the CD4 molecule. The ability of anti-CD4 mAbs to strongly inhibit the lymphoproliferation induced by MLA suggests that the interactions between TCR and MLA-DR complexes have a rather weak affinity and could be destabilized by blocking the CD4 coreceptor molecule. Interestingly, our analysis of the CDR3 spectratype elicited by MLA stimulation shows that despite the requirement of Ag processing and MHC presentation, MLA behave more like a polyclonal mitogen than as a classic Ag such as the PPD. Considering the effect of MLA on CBMC, it most likely represents a particular innate mechanism allowing parasite Ags to activate the host immunity by directly targeting naive CD4 T lymphocytes. Obviously, these characteristics are intermediate between those characterizing specific responses induced by conventional Ags (like PPD) and those associated with responses to SAgs. Our results may indicate an original mechanism used by the parasites to directly stimulate some naive T cell subsets. It should be stressed that we cannot exclude at this step the possibility that, in addition to the processed Ag(s) that interacts with TCR, some components of MLA (acting as pathogen-associated microbial patterns) could directly react with other surface molecules on T lymphocytes and deliver costimulatory signals. Dedicated receptors for microbe Ags have been characterized on macrophages and DC. TLRs and DC-specific ICAM-grabbing nonintegrin have been shown to play crucial roles in modulating the innate responses to microorganisms (40, 41) and to discriminate among *Leishmania* species (42). To date, very few data have indicated a direct recognition by lymphocytes of parasite Ags through these specific receptors, except for the recently reported reactivity of *Leishmania* lipophosphoglycan with TLR2 expressed on NK cells (43). In contrast, one should mention that TLRs can be expressed in humans by B and NK, but not by T lymphocytes (28), although it was reported that these receptors could be expressed by murine T lymphocytes, including regulatory T cells (26). Whether other receptors for microbial products are also expressed by T lymphocytes and can transduce activation signals to these cells is presently unknown.

The biological effects reported in this study were induced by a crude extract of parasite membranes. The identification of the exact Ag that triggers these responses is in progress. Preliminary results using electroeluted fractions from membrane-associated Ags resolved by SDS-PAGE indicate that the biological effects of MLA are mainly associated with the low molecular mass (molecular mass ~20 kDa) fractions.

Our results raise the question of the biological significance of the natural reactivity to membrane-associated Ags in the context of host-parasite interactions, and whether this reactivity should be considered as a host-protective or a parasite-protective response. On the one hand, IFN-γ is known to play a central role in *Leishmania* infection control by activating the leishmanicidal activities of macrophages and amplifying the protective Th1 responses (44). In contrast, the concomitant secretion upon MLA stimulation of IL-10, a well-known macrophage-deactivating cytokine (45–47), may indicate that the effect is rather parasite protective. The fact that MLA also antagonize the secretion by macrophages of the LPS-induced proinflammatory cytokines IL-1α and IL-12 argues in favor of this view. It is noteworthy that IL-10 secretion by $CD4^+CD25^+$ regulatory T cells has recently been demonstrated, in murine experimental leishmaniasis, to play a central role in maintaining a residual baseline level of parasite infection in macrophages (i.e., avoiding a sterile cure), a requisite for the maintenance of a long-lasting anti-*Leishmania* immunity (48). One should note that to exert a significant effect in vivo, the Ag responsible for the biologic properties of MLA should also be expressed by MLA of the amastigote stage, which represents the major parasite burden during *Leishmania* infection.

In summary, we present data demonstrating that MLA Ags regularly induce marked T cell proliferation of CBMC and PBMC derived from noninfected individuals. This MHC-restricted proliferation targets both $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$, but not NK, NKT, or $CD8^+$ lymphocytes; it induces IFN-γ and IL-10 secretion and appears to be essentially polyclonal and not provoked by a SAg. Experiments are in progress to unravel the cellular requirements of such natural reactivity.

Acknowledgments

We thank Dr. H. Akuffo (Karolinska Institute) for kindly supplying PBMC from Swedish blood donors, Dr. S. Chouaïb (Institut Gustave Roussy) for the human rIL-2, and Dr. Guislaine Sterkers (Hôpital Robert Debré) for mAbs directed against MHC Ags.

Disclosures

The authors have no financial conflict of interest.

References

1. Badaro, R., T. C. Jones, E. M. Carvalho, D. Sampaio, S. G. Reed, A. Barral, R. Teixeira, and W. D. Johnson, Jr. 1986. New perspectives on a subclinical form of visceral leishmaniasis. *J. Infect. Dis. 154:1003*.
2. Ben Salah, A. B., R. Ben Ismail, F. Amri, S. Chlif, F. Ben Rzig, H. Kharrat, H. Hadhri, M. Hassouna, and K. Dellagi. 2000. Investigation of the spread of human visceral leishmaniasis in central Tunisia. *Trans. R. Soc. Trop. Med. Hyg. 94:382*.
3. Liew, F. Y. 1989. Functional heterogeneity of $CD4^+$ T cells in leishmaniasis. *Immunol. Today 10:40*.
4. Kharazmi, A., K. Kemp, A. Ismail, S. Gasim, A. Gaafar, J. A. Kurtzhals, A. M. El Hassan, T. G. Theander, and M. Kemp. 1999. T-cell response in human leishmaniasis. *Immunol. Lett. 65:105*.
5. Carvalho, E. M., R. Badaro, S. G. Reed, T. C. Jones, and W. D. Johnson, Jr. 1985. Absence of γ interferon and interleukin 2 production during active visceral leishmaniasis. *J. Clin. Invest. 76:2066*.
6. Ghalib, H. W., J. A. Whittle, M. Kubin, F. A. Hashim, A. M. el-Hassan, K. H. Grabstein, G. Trinchieri, and S. G. Reed. 1995. IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. *J. Immunol. 154:4623*.
7. Holaday, B. J., M. M. Pompeu, S. Jeronimo, M. J. Texeira, A. Sousa Ade, A. W. Vasconcelos, R. D. Pearson, J. S. Abrams, and R. M. Locksley. 1993. Potential role for interleukin-10 in the immunosuppression associated with kala azar. *J. Clin. Invest. 92:2626*.
8. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol. 13:251*.
9. Akuffo, H., K. Maasho, and R. Howe. 1993. Natural and acquired resistance to *Leishmania*: cellular activation by *Leishmania aethiopica* of mononuclear cells

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021

from unexposed individuals is through the stimulation of natural killer (NK) cells. *Clin. Exp. Immunol. 94:516*.
10. Kemp, M., M. B. Hansen, and T. G. Theander. 1992. Recognition of *Leishmania* antigens by T lymphocytes from nonexposed individuals. *Infect. Immun. 60:2246*.
11. Kurtzhals, J. A., M. Kemp, L. K. Poulsen, M. B. Hansen, A. Kharazmi, and T. G. Theander. 1995. Interleukin-4 and interferon-γ production by *Leishmania* stimulated peripheral blood mononuclear cells from nonexposed individuals. *Scand. J. Immunol. 41:343*.
12. Bourreau, E., M. Collet, G. Prevot, G. Milon, D. Ashimoff, H. Hasagewa, C. Parra-Lopez, and P. Launois. 2002. IFN-γ-producing CD45RA$^+$CD8$^+$ and IL-10-producing CD45RA$^-$CD4$^+$ T cells generated in response to LACK in naive subjects never exposed to *Leishmania*. *Eur. J. Immunol. 32:510*.
13. Jensen, A. T., K. Kemp, T. G. Theander, and E. Handman. 2001. Cloning, expression and antigenicity of the *L. donovani* reductase. *APMIS 109:461*.
14. Sassi, A., H. Louzir, A. Ben Salah, M. Mokni, A. Ben Osman, and K. Dellagi. 1999. Leishmanin skin test lymphoproliferative responses and cytokine production after symptomatic or asymptomatic *Leishmania major* infection in Tunisia. *Clin. Exp. Immunol. 116:127*.
15. Dwyer, D. M. 1980. Isolation and partial characterization of surface membranes from *Leishmania donovani* promastigotes. *J. Protozool. 27:176*.
16. Scott, P., E. Pearce, P. Natovitz, and A. Sher. 1987. Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. *J. Immunol. 139:221*.
17. Mendonca, S. C., D. G. Russell, and S. G. Coutinho. 1991. Analysis of the human T cell responsiveness to purified antigens of *Leishmania*: lipophosphoglycan (LPG) and glycoprotein 63 (gp 63). *Clin. Exp. Immunol. 83:472*.
18. Roska, A. K., and P. E. Lipsky. 1985. Dissection of the functions of antigen-presenting cells in the induction of T cell activation. *J. Immunol. 135:2953*.
19. Ohmen, J. D., P. F. Barnes, C. L. Grisso, B. R. Bloom, and R. L. Modlin. 1994. Evidence for a superantigen in human tuberculosis. *Immunity 1:35*.
20. Pannetier, C., J. Even, and P. Kourilsky. 1995. T-cell repertoire diversity and clonal expansions in normal and clinical samples. *Immunol. Today 16:176*.
21. Pannetier, C., S. Delassus, S. Darche, C. Saucier, and P. Kourilsky. 1993. Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. *Nucleic Acids Res. 21:577*.
22. Collette, A., and A. Six. 2002. ISEApeaks: an Excel platform for GeneScan and Immunoscope data retrieval, management and analysis. *Bioinformatics 18:329*.
23. Gorochov, G., A. U. Neumann, A. Kereveur, C. Parizot, T. Li, C. Katlama, M. Karmochkine, G. Raguin, B. Autran, and P. Debre. 1998. Perturbation of CD4$^+$ and CD8$^+$ T-cell repertoires during progression to AIDS and regulation of the CD4$^+$ repertoire during antiviral therapy. *Nat. Med. 4:215*.
24. Kappler, J., B. Kotzin, L. Herron, E. W. Gelfand, R. D. Bigler, A. Boylston, S. Carrel, D. N. Posnett, Y. Choi, and P. Marrack. 1989. Vβ-specific stimulation of human T cells by staphylococcal toxins. *Science 244:811*.
25. Choi, Y. W., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA 86:8941*.
26. Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express Toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med. 197:403*.
27. Gelman, A. E., J. Zhang, Y. Choi, and L. A. Turka. 2004. Toll-like receptor ligands directly promote activated CD4$^+$ T cell survival. *J. Immunol. 172:6065*.
28. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol. 168:4531*.
29. Piuvezam, M. R., D. M. Russo, J. M. Burns, Jr., Y. A. Skeiky, K. H. Grabstein, and S. G. Reed. 1993. Characterization of responses of normal human T cells to *Trypanosoma cruzi* antigens. *J. Immunol. 150:916*.
30. Subauste, C. S., F. Fuh, R. de Waal Malefyt, and J. S. Remington. 1998. $\alpha\beta$ T cell response to *Toxoplasma gondii* in previously unexposed individuals. *J. Immunol. 160:3403*.
31. Goodier, M. R., and G. A. Targett. 1997. Evidence for CD4$^+$ T cell responses common to *Plasmodium falciparum* and recall antigens. *Int. Immunol. 9:1857*.
32. Denkers, E. Y., P. Caspar, and A. Sher. 1994. *Toxoplasma gondii* possesses a superantigen activity that selectively expands murine T cell receptor Vβ5-bearing CD8$^+$ lymphocytes. *J. Exp. Med. 180:985*.
33. Pied, S., D. Voegtle, M. Marussig, L. Renia, F. Miltgen, D. Mazier, and P. A. Cazenave. 1997. Evidence for superantigenic activity during murine malaria infection. *Int. Immunol. 9:17*.
34. Nylen, S., K. Maasho, K. Soderstrom, T. Ilg, and H. Akuffo. 2003. Live *Leishmania* promastigotes can directly activate primary human natural killer cells to produce interferon-γ. *Clin. Exp. Immunol. 131:457*.
35. McMahon-Pratt, D., and J. Alexander. 2004. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol. Rev. 201:206*.
36. Steel, C., and T. B. Nutman. 1998. Helminth antigens selectively differentiate unsensitized CD45RA$^+$ CD4$^+$ human T cells in vitro. *J. Immunol. 160:351*.
37. Wilson, C. B. 1991. The ontogeny of T lymphocyte maturation and function. *J. Pediatr. 118:S4*.
38. Ulrichs, T., and S. A. Porcelli. 2000. CD1 proteins: targets of T cell recognition in innate and adaptive immunity. *Rev. Immunogenet. 2:416*.
39. Sugita, M., P. J. Peters, and M. B. Brenner. 2000. Pathways for lipid antigen presentation by CD1 molecules: nowhere for intracellular pathogens to hide. *Traffic 1:295*.
40. Beutler, B., K. Hoebe, X. Du, and R. J. Ulevitch. 2003. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J. Leukocyte Biol. 74:479*.
41. van Kooyk, Y., and T. B. Geijtenbeek. 2003. DC-SIGN: escape mechanism for pathogens. *Nat. Rev. Immunol. 3:697*.
42. Colmenares, M., A. L. Corbi, S. J. Turco, and L. Rivas. 2004. The dendritic cell receptor DC-SIGN discriminates among species and life cycle forms of *Leishmania*. *J. Immunol. 172:1186*.
43. Becker, I., N. Salaiza, M. Aguirre, J. Delgado, N. Carrillo-Carrasco, L. G. Kobeh, A. Ruiz, R. Cervantes, A. P. Torres, N. Cabrera, et al. 2003. *Leishmania* lipophosphoglycan (LPG) activates NK cells through Toll-like receptor-2. *Mol. Biochem. Parasitol. 130:65*.
44. Bacellar, O., C. Brodskyn, J. Guerreiro, M. Barral-Netto, C. H. Costa, R. L. Coffman, W. D. Johnson, and E. M. Carvalho. 1996. Interleukin-12 restores interferon-γ production and cytotoxic responses in visceral leishmaniasis. *J. Infect. Dis. 173:1515*.
45. De Waal Malefyt, R., J. 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. 174:1209*.
46. Murphy, M. L., U. Wille, E. N. Villegas, C. A. Hunter, and J. P. Farrell. 2001. IL-10 mediates susceptibility to *Leishmania donovani* infection. *Eur. J. Immunol. 31:2848*.
47. Ghalib, H. W., M. R. Piuvezam, Y. A. Skeiky, M. Siddig, F. A. Hashim, A. M. el-Hassan, D. M. Russo, and S. G. Reed. 1993. Interleukin 10 production correlates with pathology in human *Leishmania donovani* infections. *J. Clin. Invest. 92:324*.
48. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. CD4$^+$CD25$^+$ regulatory T cells control *Leishmania major* persistence and immunity. *Nature 420:502*.

Downloaded from http://www.jimmunol.org/ by guest on March 2, 2021