A Leishmania Homologue of Receptors for Activated C-Kinase (LACK) Induces Both Interferon-γ and Interleukin-10 in Natural Killer Cells of Healthy Blood Donors

Kerima Maasho, Iman Satti, Susanne Nylén, Gabriel Guzman, Frits Koning, and Hannah Akuffo

Natural killer (NK) cells from individuals unexposed to Leishmania organisms proliferate with high interferon (IFN)-γ secretion in response to crude Leishmania antigen preparations. In an attempt to identify the molecules that induce blood cells to proliferate and to secrete cytokines, we tested the effect of a 36-kDa Leishmania homologue of receptors for activated C-kinase (LACK) on peripheral blood mononuclear cells from unexposed individuals. Mainly CD8+ and NK cells proliferated in response to LACK. At both the mRNA and soluble protein level, the main sources for LACK-induced IFN-γ and interleukin (IL)-10 were T and NK cells. Furthermore, in the presence of anti-major histocompatibility complex (MHC) class II antibody, there was inhibition of LACK responses in both CD4+ and CD16/56+ cells, with a marked decrease in IFN-γ but with an increase in IL-10 production. We conclude that the response to LACK is part of the response to Leishmania organisms in unexposed donors described elsewhere. That this NK-dominated response is MHC class II sensitive, whether through a direct or indirect effect, is discussed.

Protection against Leishmania major in mice is incurred by the proliferation of Th1 cells producing interferon (IFN)-γ and interleukin (IL)-2, and susceptibility is associated with the proliferation of Th2 cell types and the production of IL-4, IL-5, and IL-10 [1, 2]. Natural healing of human leishmaniasis infection, marked by the development of IFN-γ-producing T cell clones, is believed to render the individual resistant to reinfection [3]. Leishmania-reactive IFN-γ-producing CD8+ T cells have also been associated with cure of cutaneous leishmaniasis [4]. Furthermore, mononuclear cells from a proportion of healthy individuals with no previous exposure to Leishmania have been shown to proliferate in response to L. aethiopica promastigote antigens with high IFN-γ and IL-6 responses [5], coupled with proliferation of CD3+ and CD16/56+ NK cells [6]. Furthermore, subsequent field studies indicate that CD16/56+ cells and CD8+ cells play a role in protection from and healing of Ethiopian cutaneous leishmaniasis [7].

The expansion of CD4+ T cells reactive to a 36-kDa Leishmania homologue of receptors for activated C-kinase (LACK), which is derived from L. major, has been shown to be the focus of the early response in mouse strains susceptible to L. major infection [8]. This early response is associated with a burst of IL-4 that rapidly renders parasite-specific CD4+ T cell precursors unresponsive to the IFN-γ–inducing cytokine IL-12 [9]. Transgenic mice made tolerant to LACK in the thymus resolve infection with L. major [8]; hence, speculation arose that the prevention of such LACK-reactive cells may enhance resistance in this model. This is supported by experiments showing that the administration of LACK, together with IL-12, confers protection in mice [10]. This protein has been shown to be highly conserved in Leishmania species examined to date.

Because the response to LACK is a very early event in a Leishmania infection, it could also include innate elements of immunity. We investigated the reactivity to LACK on the part of human cells not previously exposed to Leishmania organisms and compared the reactivity with the response to whole-cell antigen reported elsewhere [5]. We also evaluated the major histocompatibility complex (MHC) class II restriction of the response.

Materials and Methods

Donors. Mononuclear cells from 10 donors were tested. Six were healthy Swedish blood donors (from the blood bank of Karolinska Hospital, Stockholm) who had no evidence of previous Leishmania exposure, and 4 were healthy laboratory workers. Cells from 12 healthy Sudanese blood donors were also studied.

Antigens and mitogens. Freeze-thawed whole L. aethiopica pro-
mastigote antigen (Ft-Leish Ag) was prepared as described elsewhere [11] and used at a final, optimal concentration of 1.25 × 10^4 parasites/mL. Recombinant LACK protein (a kind gift from Nicolas Papon, l’Institut de Pharmacologie Moleculaire et Cellulaire, University of Nice, Valbonne, France) was checked for purity on gels stained with Coomassie blue. Furthermore, the limulus test was used to test for the presence of endotoxin. LACK was pre-treated with polymyxin B and used at a final concentration of 12.5, 25.0, and 50.0 µg/mL. Unless otherwise stated, the results from the 25-µg/mL concentrations are presented throughout this report. Polymyxin B treatment of the protein was used to neutralize the possible presence of endotoxins [12] that may have been introduced during the preparation of LACK. In initial experiments, polymyxin B was shown not to have any effect on cell viability. Polymyxin B (final 25 µg/mL) treatment of LACK was carried out on ice 2 h before stimulation of cells. Tuberculin purified protein derivative (PPD; Statens Serum Institute, Copenhagen) and the T cell mitogen phytohemagglutinin (PHA; Murex, Harterd, UK) were each added at a final concentration of 12.5 µg/mL.

Recombinant gp63, which is an abundant molecule in the Leishmania parasite and is being considered as a vaccine candidate, was the kind gift of Robert McMaster (University of British Columbia, Vancouver). It was tested at a concentration of 8 µg/mL and 100 µg/mL.

Anti–MHC class II monoclonal antibody. A hybridoma-producing monoclonal IgG1 with broad class II specificity, PDV52, was used [13]. This antibody has been shown elsewhere not to cross-react with MHC class I molecules [14]. Mouse IgG1 monoclonal antibody of irrelevant specificity was used as control.

Preparation and stimulation of mononuclear cells. Peripheral blood mononuclear cells (PBMC) separated fromuffy coats or defibrinated peripheral blood on ficoll gradient [15] were washed 3 times and eventually resuspended to the appropriate concentration in RPMI (Gibco BRL, Paisley, UK) containing 2 mM L-glutamine, 100 U penicillin (Gibco), and 100 µg/mL streptomycin (Gibco) supplemented with 10% heat-inactivated normal Swedish AB serum. Cells were plated at 2 × 10^4 cells/well in 96-well flat-bottom tissue culture plates (Nunclon, Roskilde, Denmark), and each antigen or mitogen was tested in triplicate in the absence or presence of anti-MHC II monoclonal antibodies. Negative control cultures contained RPMI medium with or without anti-MHC II antibodies. Cells were cultured in 5% CO₂ in air at 37°C for 6 days. During the last 18 h of culture, 1 µCi/well tritiated thymidine (Amersham, Aylesbey, UK) was added. Cells were harvested onto a glass fiber filter mat (Wallac, Turku, Finland), and radioactivity was measured in a 1450-microbeta counter (Wallac). Proliferation was measured as counts per minute and calculated as stimulation indices (SI; defined as counts per minute of cells in stimulated cultures/counts per minute of unstimulated cultures).

The cells from donors in Sweden were used fresh, whereas the PBMC from Sudanese donors were stored in liquid nitrogen until stimulated.

Determination of IFN-γ and IL-10 levels in supernatants. Supernatants were harvested from cells incubated for 72 h, as described above, for IFN-γ and IL-10 analysis, using commercial ELISAs (MABTECH, Stockholm), following the manufacturer’s instructions. The sensitivity of the cytokine assays for IFN-γ and IL-10 were 27 pg/mL and 9.8 U/mL, respectively.

Phenotype analysis of proliferating cells. PBMC cultured for 6 days, as above, were prepared for surface marker staining, as described elsewhere [7]. Phenotypic analysis was performed for antibody combinations of CD4 and CD8 (helper and cytotoxic), CD3 and CD16/56 (pan-T and NK cells), or control IgG1 and IgG2a, double-stained with fluorescein isothiocyanate (FITC) and phyco-erythrin (PE), respectively. Cells were assayed on a FACScan (Becton Dickinson, Mountain View, CA). All antibodies were obtained from Becton Dickinson.

Forward and light scatter flow cytometry. In responder PBMC, an enrichment of large blast cells was observed after culture for 6 days in response to antigenic stimulation. Determined by the forward light scatter, smaller cells with the least light scatter, as seen in most unstimulated cultures, were gated in R1, and the area outside this region was gated R2. In response to antigen stimulation, blast cells undergoing activation were scattered in the most forward region, away from the R1 cell population into the R2 region.

The proportional increase of responding cells of a particular cell type was computed taking the total large cells in R2 into consideration using the following formula: [(percent large cells of total × percent large CD1 of stimulated culture) + 1]×[(percent large cells of total × percent large CD1 of unstimulated culture) + 1]. The mathematical correction factor of +1/1 was necessary, because, in some instances, the percentage of large cells of a particular phenotype in the unstimulated cultures was 0.

Separation of cells. Magnetic beads (Dynal, Oslo) coated with specific cell surface marker antibodies were used according to the manufacturer’s instructions. Plastic-adherent cells were prepared by allowing cells to adhere to the plastic.

Identification of cell types in which cytokines are induced. In order to evaluate which cell types were involved in the cytokine induction in the bulk cultures, we used 2 approaches. In the first, cells cultured as above were harvested after 72 h stimulation and were positively selected for CD16/56- NK cells or CD4+ or CD8+ T cells. Positively selected cells, as well as plastic-adherent cells, were then treated with lysis buffer, and mRNA was isolated using Dynabeads mRNA DIRECT kit (Dynal), according to the manufacturer’s protocol. Complementary DNA (cDNA) was prepared from the RNA using reverse transcriptase (RT) polymerase chain reaction (PCR) analysis. The total mRNA was assessed by performing a PCR analysis with the primers for β₂-microglobulin [16], which is structurally expressed by all cells. The mRNA for a given cytokine in the individual cell type cDNA preparation was determined in the PCR using primers designed by Pisa et al. for IFN-γ [17] and IL-12 (Pharmacia Biotech, Uppsala, Sweden) and IL-10 (Scandinavia Gene Synthesis, Köping, Sweden). Primers of IL-12 and IL-10 were custom made (IL-10 sense: GCCTAACATGGTTC; IL-12 sense: CAGCAGTTGGTCATCTCTTG; IL-12 GCTTCGAGATC; IL-10 antisense: TGATGTCGTGGTCTT TTGATGTCGTGGTCTT GGTT; IL-12 antisense: CCAGCA GTGGTCA ACTCTT; IL-12 antisense: CCGACAC CGTAAACGTC). The reaction was run in a GeneAmp thermocycler 2400 or 9600 (Perkin-Elmer, Alameda, CA). The PCR analysis was programmed for 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C, and 60 s extension at 72°C. On completion of the cycle, the template was allowed a final extension of 7 min at 72°C. The PCR product was analyzed by loading samples onto a 1.5% agarose gel and electrophoresed at 100 V for 20–25 min.
In the second approach for identifying cytokine-producing cells, PBMC prepared as above were taken as a whole or were depleted of CD56+ NK cells or CD4+ or CD8+ T cells. Particular phenotype-depleted cells were then stimulated with antigen and supernatant harvested after 72 h for cytokine analysis. Purity of depletion in the cultures was checked by a fluorescence-activated cell sorter (FACS), using specific cell marker antibodies.

**Semiquantitative evaluation of cytokine mRNA.** Gel pictures were taken with a Polaroid camera (International Biotechnologies, New Haven, CT) and were scanned, and the band intensity was analyzed using Whole Band Analyzer software (BioImage, Ann Arbor, MI). Results are expressed in arbitrary units.

**Statistical analysis.** One-group $\chi^2$ test and correlation coefficient were used to evaluate the magnitude of differences and the relationship of the independent antigen responses, respectively.

**Results**

**Proliferative responses to LACK.** At all concentrations tested (12.5, 25.0, and 50.0 $\mu$g/mL), LACK induced proliferation of PBMC in responder donors. The background counts of unstimulated cultures were 531–2451 cpm. The dose inducing highest response varied among the donors. Data from 25 $\mu$g/mL are presented in this study. Tritiated thymidine incorporation into proliferating cells in response to LACK was evaluated and compared with Ft-Leish Ag and PPD stimulation (figure 1). The results showed that 8 of 9 of the healthy donor cells tested proliferated in response to LACK stimulation (SI, 3), with 1488–41,141 cpm. Of the 9, 6 had SI = 3 in response to Ft-Leish Ag (range, 2714–62,464 cpm). The magnitude of the responses to LACK was much higher than that of the responses to the crude *Leishmania* Ag stimulation ($P < .0001$) in those cells responding to both stimuli. The SI of responses to PHA in these donors were 7–38 (mean ± SD, 21 ± 10).

Cells from another group of 10 donors were tested with recombinant gp63. The responses to gp63 in cells from these individuals were 0–2603 cpm (mean ± SD, 976.7 ± 952.7 cpm), and their responses to Ft-Leish were 665–13,751 cpm (mean ± SD, 7654 ± 4496 cpm).

**Phenotype of proliferating cells.** The percentage of large blast cells in the R2 gated region was compared in unstimulated and stimulated cultures (table 1). Compared with cells in unstimulated cultures, the percentage of LACK-induced CD4+ cells was lower in the cells of all but 1 donor tested. The percentage of CD8+ large cells was, however, enhanced in LACK-stimulated cultures in 8 of 10 donors. The percentage of large CD4+ cells in Ft-Leish–stimulated cultures was unchanged or increased as compared with the unstimulated cultures. The percentage of CD8+ cells in such cultures was appreciably increased in 3 of 10 donors’ cells. The percentage of CD16/56+ cells tended to be higher after stimulation with both LACK and Ft-Leish.

Data were analyzed in terms of proportional increase in cell types, taking into consideration the total cell number in the R2 population. It is important to note that the number of cells in R2 after stimulation, which indicates whether there is a response, is larger than the number of cells in R2 of unstimulated cells. Thus, there is a need to calculate the proportional in-

**Figure 1.** Proliferative responses of peripheral blood mononuclear cells (PBMC) to the *Leishmania* homologue of receptors for activated C-kinase (LACK) in relation to freeze-thawed whole *Leishmania aethiopica* promastigote antigen (Ft-Leish) stimulations in 6-day cultures in healthy Swedish blood donors. RPMI represents unstimulated cultures. The bars represent SDs of triplicate cultures.

<table>
<thead>
<tr>
<th>Donor</th>
<th>% CD4+/CD8-</th>
<th>% CD8+/CD4+</th>
<th>CD16/56+/CD3+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPMI</td>
<td>LACK</td>
<td>Ft-Leish</td>
</tr>
<tr>
<td>D1</td>
<td>43.59</td>
<td>38.64</td>
<td>77.76</td>
</tr>
<tr>
<td>D2</td>
<td>33.04</td>
<td>25.13</td>
<td>58.00</td>
</tr>
<tr>
<td>D3</td>
<td>36.13</td>
<td>25.97</td>
<td>50.38</td>
</tr>
<tr>
<td>D4</td>
<td>32.39</td>
<td>25.53</td>
<td>58.00</td>
</tr>
<tr>
<td>D5</td>
<td>22.38</td>
<td>26.32</td>
<td>29.73</td>
</tr>
<tr>
<td>D6</td>
<td>43.10</td>
<td>16.15</td>
<td>57.37</td>
</tr>
<tr>
<td>D7</td>
<td>30.65</td>
<td>12.68</td>
<td>52.95</td>
</tr>
<tr>
<td>D8</td>
<td>46.07</td>
<td>12.68</td>
<td>52.95</td>
</tr>
<tr>
<td>D10</td>
<td>41.25</td>
<td>22.70</td>
<td>77.53</td>
</tr>
</tbody>
</table>

**Table 1.** Percentage of cell phenotypes in large cell populations.

NOTE. RPMI, unstimulated cultures; LACK, *Leishmania* homologue of receptors for activated C-kinase; Ft-Leish, freeze-thawed *Leishmania aethiopica* promastigote antigen.
increases, because they will not be obvious from the raw percentage data alone.

LACK stimulation induced the highest proportional increase in proliferating cells in the CD16/56$^+$ and CD8$^+$ populations (figure 2). Cells from 2 donors, D2 and D11, had a preferential proportional increase of CD8$^+$ cells, whereas the other donor cells had higher proportional increases of CD16/56$^+$ cells. Cells from donor D3 did not show any change in the cell phenotype after stimulation with LACK or Ft-Leish, although the cells from this donor induced thymidine incorporation.

Cytokine analysis. LACK induced high levels of IFN-γ in cells of all but 1 donor from Sweden, and PPD stimulation induced high levels of IFN-γ in all Swedish donors tested (figure 3). IFN-γ levels were low or undetectable in supernatants from these cells in response to Ft-Leish. All PBMC from the Swedish healthy blood donors secreted substantial levels of IL-10 in response to LACK, whereas PPD induced considerably less IL-10 in all donors. Measurable IL-10 was not induced by the Ft-Leish Ag in any of the donor cells. Levels of IFN-γ and IL-10 in PHA-stimulated cells were 899–13,349 pg/mL and 254–829 U/mL, respectively. We had the opportunity to test cells from another ethnic group, healthy Sudanese donors. LACK-induced IFN-γ was measured in 8 of 12 healthy Sudanese donors' cells tested. The levels in the responders were 221–705 pg/mL (table 2).

Effect of anti–MHC class II antibodies on the proliferative responses of LACK. We wished to evaluate whether the proliferative responses observed to LACK in these healthy donors were MHC class II restricted. Cells from 3 healthy donors were tested in the presence and absence of anti–MHC class II antibodies. The proliferative responses to LACK, as well as to Ft-Leish Ag and PPD, were appreciably inhibited in the presence of anti–MHC class II antibodies (figure 4A). PHA responses were not affected by this antibody (data not shown). The anti–MHC class II antibody alone did not have any effect on the background counts per minute of the cells. Isotype control antibody had no effect on the observed responses.

Effect of anti–MHC class II antibodies on the proliferating cell types. The effect of anti–MHC class II antibodies on the potential of CD4$^+$ and CD16/56$^+$ cells to proliferate in response to LACK, Ft-Leish, and PPD antigens was evaluated. Proliferation and the resulting proportional increase of LACK-reactive CD4$^+$ cells were considerably inhibited in all cultures in the presence of anti–MHC class II antibodies (data not shown). Unexpectedly, however, the proliferation of CD16/56$^+$ cells was also appreciably reduced by this antibody (figure 4B). Control antibodies had no such effect. Similar results were obtained from PBMC of 3 donors on different occasions.

Effect of anti–MHC class II antibodies on the level of measurable IFN-γ and IL-10. The effects of anti–MHC class II antibodies on IFN-γ and IL-10 levels in response to LACK, Ft-Leish, and PPD stimulation were evaluated. IFN-γ production in response to LACK and PPD was appreciably, but not completely, inhibited in the presence of anti–MHC class II antibodies. The Ft-Leish IFN-γ levels in the supernatants were low or not measurable; however, the levels of LACK-induced IL-10 increased in the presence of anti–MHC class II antibodies, whereas this same antibody had an inhibitory effect on the levels of IL-10 produced in response to PPD but had no effect on the Ft-Leish responses (figure 4C).

Phenotype of cytokine inducing cells. To determine which cells were responsible for the secretion of IFN-γ and IL-10 at 72 h, mRNA for IFN-γ, IL-10, and IL-12 in response to LACK and Ft-Leish Ag stimulations were evaluated after 72 h culture in unfractionated PBMC as well as in positively selected CD16/56$^+$, CD4$^+$, and CD8$^+$ cells. Consistent with the ELISA data, LACK was a stronger inducer of IFN-γ mRNA than was Ft-Leish Ag in CD4$^+$, CD8$^+$, and CD16/56$^+$ cells (figure 5A). A background of IL-10 mRNA was detected in the unstimulated cultures; however, the levels induced in response to LACK were higher than Ft-Leish Ag in all cell types, after subtracting the...
Levels of measurable interferon (IFN-γ) and interleukin (IL-10) in 72-h culture supernatants in response to Leishmania homologue of receptors for activated C-kinase (LACK), Leishmania aethiopica promastigote antigen (Ft-Leish), and purified protein derivative (PPD) in peripheral blood mononuclear cells from healthy Swedish individuals. RPMI represents unstimulated cultures.

Table 2. Amounts of interferon (IFN-γ) induced by Leishmania homologue of receptors for activated C-kinase (LACK) and phytohemagglutinin (PHA) in cells from healthy Sudanese blood donors.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Unstimulated</th>
<th>L. donovani</th>
<th>LACK</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>598</td>
<td>1706</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>221</td>
<td>838</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1127</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>555</td>
<td>1309</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>547</td>
<td>1319</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>456</td>
<td>111</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>705</td>
<td>989</td>
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<td>9</td>
<td>0</td>
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<td>969</td>
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<td>0</td>
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<td>258</td>
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</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>397</td>
<td>598</td>
</tr>
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</table>

NOTE. Data are picograms per milliliter.
Figure 4. Effect of anti-major histocompatibility complex (αMHC) class II antibody, added at the start of the culture, on responses induced by Leishmania homologue of receptors for activated C-kinase (LACK), freeze-thawed whole Leishmania aethiopica promastigote antigen (Ft-Leish), and purified protein derivative (PPD) of tuberculin. Results of 1 of 3 representative donors are shown. A, Proliferative responses to antigen (Ag) and Ag plus αMHC class II antibody; B, proportional increases in CD16/56+ cells; C, interferon (IFN)-γ and interleukin (IL)-10 levels in 72-hour supernatants. RPMI represents unstimulated cultures.

CD8+ cells had higher proportional increases in response to both LACK and Ft-Leish Ag than did CD4+ cells, which started out at high levels in the unstimulated cultures. In most donors whose cells proliferated in response to LACK, the highest proportional increase was in both CD16/56+ and CD8+ cells. In 2 donors, however, the CD8+ response was the most prominent. Thus, both CD16/56+ and CD8+ cells are induced to proliferate in responders, but 1 cell type may be preferentially induced in individual donors.

Although mRNAs for both these cytokines were detected in response to the 2 stimuli, secreted cytokine levels were low or undetectable in the supernatants of Ft-Leish-stimulated cultures, which suggests that the levels of protein translated in these cultures are probably inhibited. The mechanisms of this inhibition are not known and have not been addressed in this study. In the highly susceptible BALB/c mouse, LACK induces an early induction of IL-4–secreting cells [9], which are responsible for subsequently driving the immune response toward the highly susceptible Th2 phenotype seen in these mice [8]. In many forms of human cutaneous leishmaniasis, IL-4 is not induced at levels as high as those in mice. In our studies with L. aethiopica, IL-4 has not been seen as a consistent feature, even in patients with diffuse cutaneous leishmaniasis, whereas an association of IL-10 induction and susceptibility to L. aethiopica has been observed [20]. IL-10 is a cytokine of the Th2 phenotype but also a cytokine secreted by other, non-T cells. It is interesting to note that IL-10 is induced in response to LACK in healthy donors with no known history of leishmaniasis.

As expected, both T cells and NK cells were induced to express mRNA for IFN-γ. mRNA for IL-10 was induced in all cell types isolated after stimulation with LACK or the Ft-Leish Ag, including NK cells. Other investigators have reported that NK cells secrete IL-10 after stimulation with IL-12 and IL-2 [21].

mRNA for IL-12 was, however, not detectable in cells from 72-h cultures, which is not surprising because the induction of IL-12 is an early event occurring 18 h after stimulation [22]. When evaluated at 24 h, however, LACK induced high levels of IL-12 mRNA, whereas the Ft-Leish antigen induced no or
very weak IL-12 mRNA signals (data not shown). In the plastic-adherent cells (presumably monocyte/macrophages), LACK induced more mRNA for IL-10 than did Ft-Leish. This suggests that the IL-10 induction of Ft-Leish may be greater in the nonadherent cells than in the adherent cells.

Because both CD4- and CD16/56-related responses were obtained in our results, we wished to evaluate what contribution MHC class II expression had on the measured in vitro responses to LACK. We showed that the proliferative and the IFN-γ responses, but not the IL-10 responses, are inhibited by the addition of anti–MHC class II antibodies to the cultures. This was reflected in the lower number of not only proliferating CD4+ cells but also CD16/56+ cells, as measured by FACS.

The effect of anti–MHC class II antibodies on NK cell proliferation could be an indirect consequence of their effects on CD4+ cells. We therefore stimulated cells depleted of specific cell types with LACK. In cells from some donors, NK cells were stimulated in the absence of CD4+ cells. In other donors, depletion of NK cells had only a slight effect, whereas removal of CD4+ cells abrogated the induction of IFN-γ. This suggests that there are donors whose response is entirely NK dependent, whereas others have a mixed response. The finding of enhanced MHC class II+ NK cells in LACK-stimulated cells suggests that MHC class II-expressing CD16/56+ cells are also contributing to the response to LACK and Ft-Leish Ag. A population of NK cells expressing MHC class II has been shown to have

Figure 5. Phenotype of cytokine-inducing cells after 72 h of stimulation in culture. mRNA for interferon (IFN)-γ (A) and interleukin (IL)-10 (B) were evaluated by polymerase chain reaction in positively selected cells stimulated with Leishmania homologue of receptors for activated C-kinase (LACK) and freeze-thawed whole Leishmania aethiopica promastigote antigen (Ft-Leish). Results are expressed in arbitrary units with semiquantitative evaluation of the band intensity described in Materials and Methods. The corresponding measured mRNA gel pictures of the various cell types are shown beneath the graphs. Bands showing the total mRNA in the samples using β2-microglobulin are also displayed (mean arbitrary units, 1.062 ± 0.1).
the capacity to present both conventional antigens and super-
antigens [23, 24]. We have no evidence to show that NK cells
on their own can do this, however; in the presence of antigen-
presenting cells, measurable cytokines could be induced. MHC
class II molecules have been shown to regulate NK cell function
and target recognition in studies looking at NK–target cell
interaction using the NK-sensitive K562 cell line [25]. A model
can be envisaged where in some donors LACK causes early
IL-12 induction in monocyte/macrophages, which in turn stim-
ulate NK cells and T cells for IFN-γ secretion. Once produced,
the IFN-γ may stimulate NK cells to proliferate and produce
more cytokines. In this model, proliferation includes the in-
duction of MHC class II–positive NK cells, which in turn pres-
tent LACK to T cells, amplifying the response further. Inactiva-
tion of MHC class II would result in lowered levels of CD4+ as
well as CD16/56+ cells. This model is consistent with our
finding that CD16/56+ cells alone cannot be activated by Ft-
Leish Ag but require the presence of other cell types to be
preferentially induced to proliferate and secrete cytokines [18].
Our data also show that, in the presence of plastic-adherent
cells and in the absence of CD4+ cells, NK cells can secrete
high levels of IFN-γ in response to LACK stimulation. It would
appear that such NK cells may also play a role in a PPD
response, because our data have shown that the anti–MHC
class II antibody had an effect on PPD-stimulated NK cells as
well. PHA responses were unaltered in the presence of this
antibody. Thus, our data are consistent with this model; how-
ever, the alternative model of anti–MHC class II having an
effect on the CD4+ cells, with the NK cell effect being of a
bystander nature, may occur in some donors. Alternative mech-
anisms in individuals are supported by differential importance
of NK and CD4+ cells observed in different donors.

The LACK preparation used here was expressed in Escher-
ichia coli, and thus it could be argued that the observed re-
sponses may be due to bacterial contamination. This, however,
was a pure preparation, as determined by Coomassie blue stain-
ing of gels. The LACK responses were unlikely to be due to
bacterial contamination with mitogenic activity, because not all
donors responded to LACK. To rule out any possible contam-
ination by other bacterial products, we have used polymyxin
B to treat the LACK in all the experiments to inhibit potential
endotoxin activity. Thus, our accumulated data do not suggest
bacterial contamination of the LACK preparations. Furthe-
more, we have determined the endotoxin level in the prepara-
tion using the limulus amoebocyte lysate test. In comparison
with the accepted levels of 30–50 EU/dose/70 kg (used by bio-
medical companies) and 350 EU/dose/70 kg (used by the US
Food and Drug Administration), the levels we measured in our
antigen were between 24- and 40-fold and 280-fold lower,
respectively.

We have previously indicated that the “innate” response to
Leishmania antigen seen in healthy unexposed donors would
be a protective phenotype against subsequent infection [6]. If
this response is primarily to LACK, however, the protein to
which the initial IL-4 secretion is directed in susceptible mouse
strains, the protective potential of this innate response needs
to be readdressed, because LACK stimulates IL-10 induction
even in uninfected donors. Systematic studies in Leishmania-
infected individuals may clarify whether this response is pro-
tective. Furthermore, the protective role of LACK as a vaccine
in humans is not straightforward; however, driving of the im-
munodominant LACK response toward a Th1 phenotype may
be the key for preventing the ill effects of Th2 responses. Indeed,
mouse studies show that LACK administered together with IL-
12 is able to direct the T cells toward a Th1 response [10].
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References