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T-Cell Reactivity to Purified Lipophosphoglycan from *Leishmania major*: A Model for Analysis of the Cellular Immune Response to Microbial Carbohydrates

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Summary

The major macromolecule on the surface of Leishmania major *promastigotes is a lipophosphoglycan (LPG). This glycoconjugate plays a key role in determining infectivity and survival of parasites in the mammalian host cell. In addition,* L. major *LPG is able to induce a host-protective immune response. In this article, we summarise the evidence for recognition of highly purified LPG by T cells and we discuss the potential mechanisms of T-cell stimulation by this non-protein antigen.*

Key-Words: Leishmania, T lymphocytes, glycosyl phosphatidyl-inostitols.

Introduction

Protozoan parasites of the genus *Leishmania* alternate between the promastigote form in the sandfly vector and the obligatory intracellular amastigote form that multiplies in the phagolysosomes of mammalian macrophages. The spectrum of disease patterns seen in humans can be reproduced experimentally in mice. Upon infection with *Leishmania major*, the cause of cutaneous leishmaniasis in the Old World, mice of genetically resistant inbred strains (e.g., C57BL/6) are able to contain the lesions, whereas genetically susceptible mice (e.g., BALB/c) develop progressive disease. The T-cell-dependent immunity is crucial for both resistance and susceptibility to disease[1–3].

Various *L. major* parasite antigen preparations have been shown to induce host protection in mice[4–8], and there is considerable effort to develop a molecularly defined vaccine for immunoprophylaxis or immunotherapy of cutaneous leishmaniasis in humans. The best-characterised vaccine candidates are a glycoprotein (gp63;[9]) and a lipophosphoglycan (LPG;[10]). Both parasite molecules have a similar mode of anchoring into the plasma membrane via glycosyl inositol phospholipids[9,11,12] and are involved in the parasites' invasion of host macrophages. In this process, gp63 binds directly to the complement receptor type 3 (CR3;[13]) and LPG interacts with CR3 as well as another member of the CD18 family of leucocyte integrins, p150,95, on the surface of phagocytes[14]. However, the two ligands presumably bind to CR3 at physically distinct sites[14].

L. major LPG consists of a heterogeneous phosphoglycan (M_r 5,000–40,000), a variably phosphorylated hexasaccharide glycan core and a *lyso* alkyl-phosphatidylinositol lipid anchor[15]. According to estimates of the cellular copy number (approximately 5×10^6 molecules/cell), it is the major macromolecule on the promastigote's cell surface. LPG probably forms a capsular network on the surface of *L. major* promastigotes, as suggested by ultrastructural studies showing that the cell surface is coated by a glycocalyx and that this layer increases in metacyclic promastigotes, coincident with an increase in the average molecular weight of LPG[16]. LPG seems to be the major attachment site for complement, thus facilitating phagocytosis of promastigotes by macrophages via the CR1[17,18] in addition to its direct interaction with CR3[14].

Furthermore, LPG may protect the parasite by impairing the oxidative burst of host macrophages, either by inhibiting the protein kinase C or by acting as a scavenger of oxidative metabolites[19–21].

In addition to its importance for both infectivity and survival of parasites in the mammalian host, *L. major* LPG serves as a parasite antigen for recognition by the host's immune system. Administration of the purified antigen with *Corynebacterium parvum* as an adjuvant vaccinates genetically susceptible mice against cutaneous leishmaniasis[4,11]. The presence of the lipid moiety seems to be essential for the induction of protection, since the lipid-free phosphoglycan is non-protective and, when given with Freund's complete adjuvant, can exacerbate lesion development after subsequent challenge with living parasites[22]. A large body of experimental evidence demonstrates that resistance to leishmaniasis is mediated by T cells and, consequently, the protection achieved by immunisation of mice with *L. major* LPG is likely to be caused by specific activation of host-protective T cells.

Evidence for T-Cell Recognition of *L. Major* LPG

In order to estimate the role of T cells in LPG-induced protection to cutaneous leishmaniasis, we studied both humoral and cellular immune responses in mice immunised with highly purified LPG from *L. major*. The biochemical purification of LPG from stationary-phase promastigotes was performed as described in detail by *McConville* et al.[11], and two sequential cycles of chromatography on separate columns of octyl-Sepharose were included. Each preparation was carefully checked for residual protein, both colorimetrically by commercially available protein assays and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis[11]. However, contamination with protein could not be detected and, as judged by amino acid analysis after hydrolysis, the purity of the preparation is estimated to be greater than 99.95%.

In the serum of mice immunised with purified *L. major* LPG in liposomes and *C. parvum*, we detected high titers of immunoglobulin G (IgG) antibodies binding to LPG[23]. The generation of a specific IgG antibody response indicates that LPG can activate T cells *in vivo* to provide helper signals for the maturation of IgG-secreting B cells.

Using a limiting dilution system for frequency analysis of sensitised T cells, we demonstrated that immunisation of susceptible BALB/c mice with *L. major* LPG and *C. parvum* induces the activation and expansion of *L. major*-reactive T cells[23]. The data in Table 1 show that the frequency of lymphokine-producing T cells or selected $CD4^+$ T cells was significantly higher in mice treated with LPG than in control mice injected with the adjuvant alone. The increase was observed for all the lymphokine activities tested; i.e. Interleukin (IL) 2, IL-3, IL-4 and Interferon (IFN) gamma. On the other hand, lymph node cells from mice immunised with biochemically purified LPG and Freund's adjuvant did not recognise immunogenic peptides of *L. major* gp63 (*F. Y. Liew*, personal communication). This finding confirmed the biochemical evidence that the *L. major LPG* used for the present studies is not contaminated with detectable amounts of peptides.

Pooled spleen and inguinal lymph node cells were collected from LPG-treated BALB/c mice that had resolved the subsequent infection with *L. major*. The lymphocytes were selected for $CD4^+$ cells by treatment with anti-CD8 antibodies and complement and were transferred into irradiated syngeneic recipients prior to infection with *L. major* promastigotes. The course of lesion development in these mice (Fig. 1) shows that protection was mediated by $CD4^+$ cells from LPG-immunised mice but not by $CD4^+$ cells from adjuvant-treated or untreated donors. These

Table 1 Frequency of *L. major*-reactive T cells in mice immunized with *L. major* LPG[a]

Treatment of mice	Reciprocal of frequency of lymphokine-producing cells (95% confidence limits)[b]			
	IL-2+IL-4[c]	IL-4[c]	IFN-γ[c]	IL-3[d]
LPG / liposomes + *C. parvum*	258 (198–370)	1197 (919–1712)	1364 (1010–2083)	10314 (7716–15576)
liposomes + *C. parvum*[e]	6274 (3473–32124)	>100000	3390 (2380–5882)	27100 (18348–51813)

[a] Parts of the data are from ref. 23

[b] All p-values >0.15

[c] After a cycle of *in vitro* stimulation with *L. major* antigen followed by an interval of culture in the absence of antigen, limiting numbers of selected $CD4^+$ T cells from BALB/c mice were cultured with syngeneic accessory cells in the presence or absence of *L. major* antigen. Cultures were restimulated 7 days later and, 24 h thereafter, supernatants from individual cultures were tested for lymphokine activity on HT-2 cells in the absence (IL-2 + IL-4) or presence (IL-4) of anti-IL-2 antibodies. An ELISA was used to test for IFN-γ activity.

[d] Limiting numbers of T cells from BALB/c.H-2^k mice were cultured with syngeneic accessory cells in the presence or absence of *L. major* antigen and were restimulated 7 days later. At 24 h thereafter, supernatants from individual cultures were tested for IL-3 activity on 32D cells.

[e] In the limiting dilution assay for determination of IL-3, T cells had been obtained from control mice treated with *C. parvum* only.

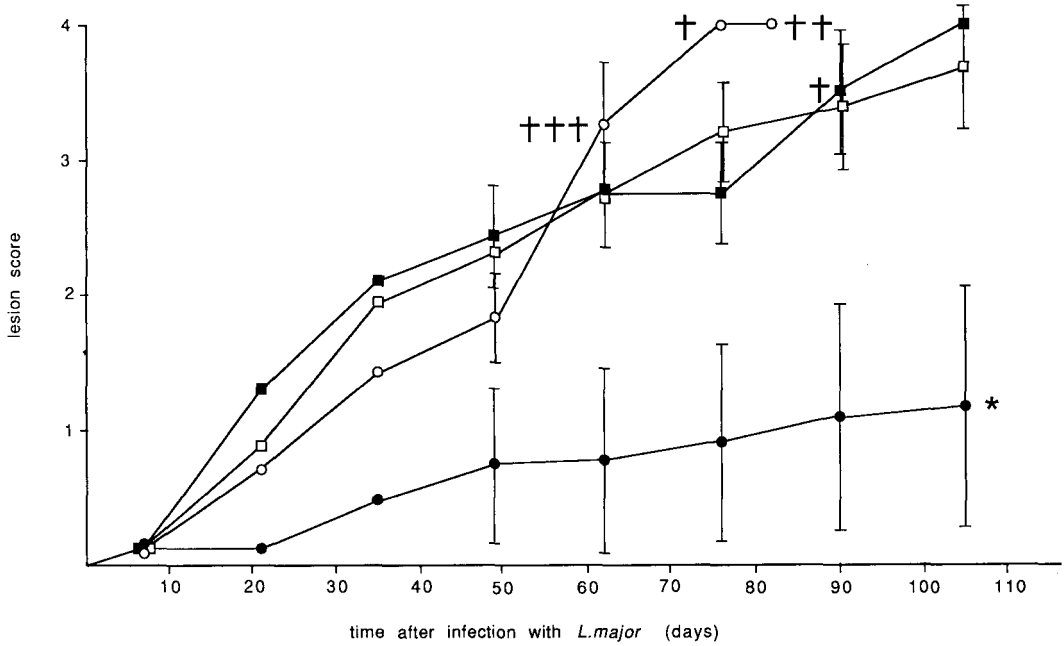

Fig. 1: Reconstitution of BALB/c mice with $CD4^+$ cells from LPG-immunized donors. Groups of 6 irradiated (200R) BALB/c mice were injected intravenously with 3×10^7 $CD4^+$ cells from lymph nodes and spleens of uninfected BALB/c mice (□) or of BALB/c mice that had been immunised with LPG in liposomes and *C. parvum* (●) or with liposomes and *C. parvum* alone (○) prior to infection with *L. major*. On the following day, the recipients were infected intradermally with 2×10^5 *L. major* promastigotes. Irradiated control mice received promastigotes alone (■). Arithmetic means of the cuteanous lesion scores are indicated and the standard errors are given for certain time points. Recipients of lymphocytes from the adjuvant-control mice died between days 60 and 85 (+) and 4 of 6 mice in the group treated with lymphocytes from LPG-immunised mice were unequivocally negative at day 105 when the experiment was terminated (*).

data confirm that LPG-induced resistance to cutaneous leishmaniasis is mediated by T cells of the $CD4^+$ subpopulation.

Mice infected with *L. major* parasites develop an antigen-specific delayed-type hypersensitivity (DTH) response to a challenge with purified LPG in the ear[23]. Resistant C57BL/6 mice were able to respond to LPG throughout the course of disease and after healing, whereas susceptible BALB/c mice reacted only at the onset of infection. Thus, the DTH response to purified *L. major* LPG correlated well with resistance to cutaneous disease. The phosphoglycan moiety of LPG failed to induce a significant swelling in either strain of mice.

In a very recent study, peripheral blood mononuclear cells (PBMC) from cured Kenyan Kala-Azar patients, but not PBMC from controls, were shown to proliferate and produce IFN-gamma *in vitro* in response to purified *L. major* LPG[24]. On the other hand, gp63 failed to activate PBMC from any of the donors tested. The proliferative response to LPG was mainly based on $CD2^+$ T cells. Similar results have been obtained with PBMC from patients with cutaneous leishmaniasis[25]. These data suggest that human T cells recognise LPG as a result of the donor's previous infection with *Leishmania*.

T-Cell Responses to Carbohydrate Antigens from Bacteria

Antigenic structures with the ability to induce cell-mediated immune responses are commonly believed to be peptides and it is still controversial whether T cells are capable of recognising polysaccharide antigens. However, a large body of literature provides evidence for DTH reactivity to carbohydrates isolated from bacteria (e.g., references 26–29; for a review, see reference 30). Furthermore, it is well-established that bacterial carbohydrates or glycolipids have the potential of modulating T-cell responses both *in vivo* and *in vitro*[31–33]. This effect has been studied particularly well for the mycobacterial cell wall glycolipids. The phosphorylated lipoarabinomannan found in most mycobacteria is a potent inhibitor of the *in vitro* response of PBMC to mycobacterial antigens[34] as well as other antigens like influenza virus[35]. Similar results have been obtained with the glycopeptidolipids from the *M. avium-M. intracellulare* complex and the studies indicate that both antigens exert their effect at the level of antigen processing by macrophages, thus interfering with T-cell activation. In contrast, the target of the phenolic glycolipids from *M. leprae*, *M. bovis* BCG and *M. kansasii* appears to be the $CD4^+$ T cell[36]. The phenolic glycolipids exert a nonspecific inhibitory effect on T-cell proliferation *in vitro*[37]. However, de facto specificity has been suggested to occur *in vivo* because large amounts of the glycolipid are present within the lesions of leprosy. *M. leprae*-induced suppression of T-cell proliferation may also be mediated by $CD8^+$ suppressor cells[34] that can be isolated from the lesions of lepromatous patients[38].

The T-cell-mediated immune response to a capsular polysaccharide of *Bacteroides fragilis* can be induced not only in euthymic mice but also in athmyic nude mice[39]. Since nude mice are able to generate T cells bearing the γδ T-cell antigen receptor, in addition to some T cells with αβ receptors in older animals, it has been suggested that γδ T cells may respond to carbohydrate antigens[40]. In accordance with this hypothesis, *Pfeffer* et al.[41] recently showed that human T cells expressing the γδ receptor respond to mycobacterial antigens that are resistant to protease digestion. The biochemical nature of this antigenic component, presumably a non-peptide molecule, has not yet been elucidated. In the same experimental system, using T cells from healthy individuals, a proliferative response to purified *L. major* LPG could not be detected (*Pfeffer* and *Moll*, unpublished results). The possibility exists that antigen priming *in vivo* is a pre-

requisite for anti-LPG reactivity of γδ T cells, as it has been observed for unselected human T cells. Only PBMC from donors with a history of leishmaniasis, but not those from healthy controls were able to respond to the purified parasite antigen *in vitro*[24,25].

Potential Mechanisms of T-Cell Stimulation by *L. major* LPG

The immune system has evolved two pathways for presentation of antigens and their recognition by T cells. Recent data favour the idea that antigens present in extracellular body fluids are processed by the endosomal pathway and are presented by molecules of the major histocompatibility complex (MHC) class II, whereas intracellular antigens are processed by the cytosolic pathway and are presented by MHC class I molecules. Thus, $CD4^+$ T cells are stimulated by antigen-presenting cells that have encountered exogenous antigen and $CD8^+$ T cells respond to antigen-presenting cells bearing endogenous antigen[42,43].

The basic requirement for direct T-cell responsiveness to carbohydrate antigens is the availability of antigenic determinants bound to MHC molecules on the surface of antigen-presenting cells. The current knowledge of processing and presentation of antigen is based on studies with proteins and suggests that association of antigenic fragments and MHC molecules takes place in intracellular compartments; i.e., the endosome for association of exogenous antigens with MHC class II molecules and the endoplasmic reticulum for binding of endogenous antigens to MHC class I molecules[42,43]. If these mechanisms also hold true for the trafficking of carbohydrate antigens, they have some important consequences for the presentation of amphipathic molecules like the *L. major* LPG. During natural infection, promastigotes gain access to the phagolysosome of the host's macrophages. In this cellular compartment, degradation of at least some parasites may enable the association of LPG, or fragments of it, with MHC molecules. It is conceivable that fragmentation of the phosphodiester-linked oligosaccharide repeat units of LPG, for example, is achieved by esterases and glycosidases present in the phagolysosome. Furthermore, the phosphoglycan moiety of LPG may be released by amastigotes in the same compartment and may also be available for processing and binding to MHC molecules. Association of antigenic fragments with MHC class II molecules may occur either in the phagolysosome itself or in another endosomal compartment for subsequent transport to the cell surface membrane. The possibility exists that those complexes of antigen and MHC account for the LPG determinants detectable on the surface of infected macrophages[44] and for the *L. major*-reactive responses of T cells from LPG-immunised mice[23]. Alternatively, LPG may insert into the phagolysosome membrane or may bind to the same receptor that enabled parasite attachment to the macrophage initially and recycle to the cell surface[45]. The various possibilities are illustrated in Figure 2. Further characterisation of the LPG epitopes displayed on infected macrophages will be of great importance for the understanding of how LPG interacts with the host's immune system.

In contrast to LPG inserted in the parasite membrane, administration of purified LPG in its soluble form may not be suitable for the stimulation of a cellular immune response. Owing to its hydrophobic lipid moiety, purified LPG passively inserts into cell membranes[46]. It is conceivable that LPG captured in the surface membrane of an antigen-presenting cell may not reach an endosomal compartment for processing and association with MHC molecules and, thus, may not be available for recognition by T cells. Coupling of purified LPG to a suitable antigen carrier may be more appropriate for endosomal targeting

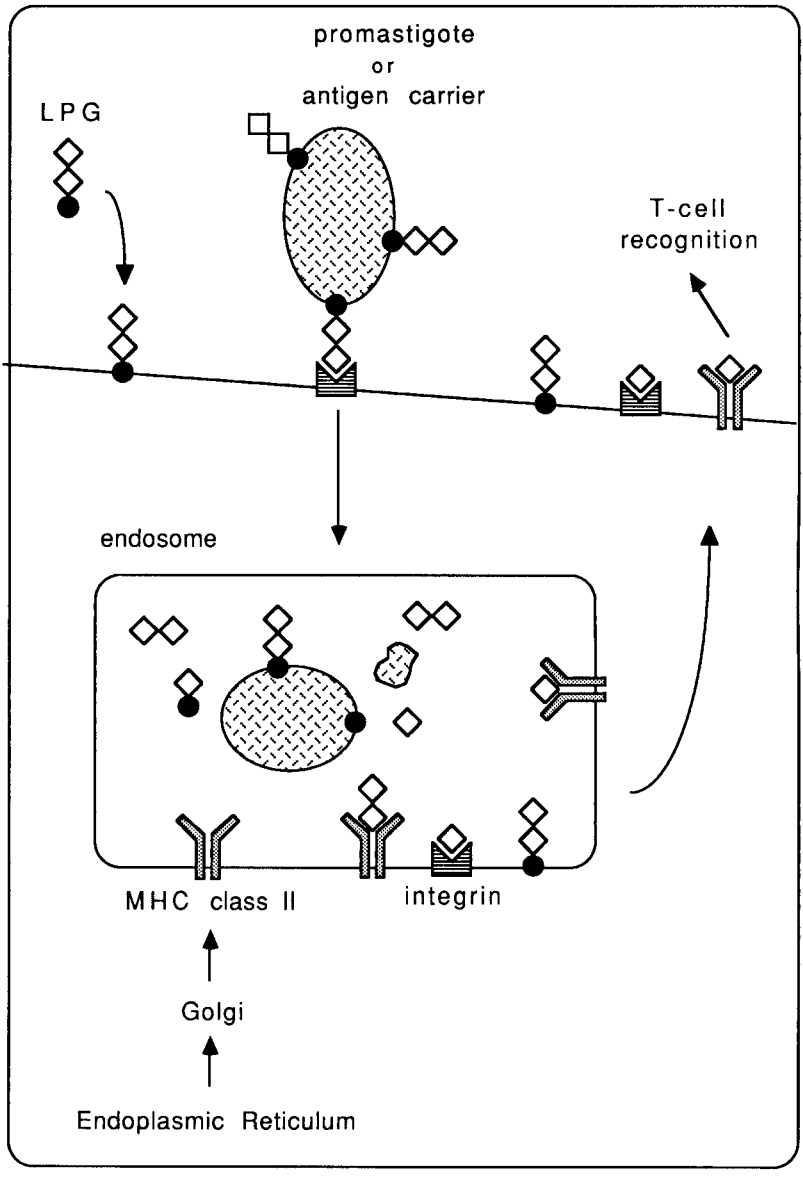

Fig. 2: Potential mechanisms of the uptake, processing and presentation of *L. major* LPG by macrophages. Association of LPG, or fragments thereof, with MHC class II molecules may occur either in the phagolysosome or in another endosomal compartment. All elements are drawn schematically without any implication on their exact molecular structures.

of this protective parasite antigen. This assumption is favoured by the finding that vaccination of mice with purified LPG was more efficient when the antigen had been incorporated into liposomes[11].

Immunisation of mice with purified *L. major* LPG increased the frequency of *L. major*-reactive T cells with the capacity to secrete lymphokines[23]. This T-cell reactivity to *L. major* antigen was significantly decreased by

adding the phosphoglycan moiety of LPG to the limiting dilution cultures. Thus, the phosphoglycan may have competed for T-cell recognition of relevant antigenic structures, a situation that is reminiscent of the ability of competitor peptides to interfere with the binding of antigenic peptides to MHC molecules and their presentation to T cells[47]. However, polysaccharides have been reported not to compete for the binding to MHC class II molecules[48] but to inhibit antigen presentation by interfering with antigen processing (*E. Unanue*, personal communication). It is possible that only glycolipid or phosphoglycan structures are capable of associating with MHC molecules. For LPG, this interaction may be favoured by the helical structure of its phosphoglycan moiety[49] because an alpha-helical propensity has been suggested to be a prominent feature of peptides binding to MHC[50]. Alternatively, LPG may behave in a way similar to staphylococal enterotoxin B, SEB, that induces the proliferation of $CD4^+$ T cells but does not compete with peptide binding to MHC. Thus, SEB does not interact in the same way as antigenic peptides[51].

Stimulation of $CD4^+$ T cells by *L. major* LPG may be caused by direct T-cell recognition of LPG in conjunction with MHC class II molecules on the surface of antigen-presenting cells, as outlined above. Alternatively, activation of T cells may be mediated by other cells of the immune system, or their soluble factors, specifically sensitised by LPG. Such a mechanism has recently been described for activated B cells that were able to deliver helper signals for the generation of antiviral T cells[52]. This B-cell help for T cells was mediated by soluble factors and was therefore not based on the ability of B cells to serve as antigen-presenting cells. Another possibility is the activation of T cells by anti-idiotypic interactions. Anti-idiotypic antibodies may mimic the relevant carbohydrate epitopes and induce protection, as it has been described in various systems[53,54].

Concluding Remarks

There is accumulating evidence for the capacity of carbohydrate antigens to elicit T-cell-mediated immune responses. Several molecules that have been shown to induce T-cell-dependent host protection against microbial infections are not proteins but polysaccharides or glycolipids[4,30,31]. In studies on the effect of purified antigens from *M. tuberculosis*, proteins have often proved to be pathogenic for the host, whereas glycolipids and polysaccharides were frequently found to be protective (reviewed in reference 30). The mechanisms of T-cell recognition of these epitopes are at present unknown, but the recent advances in the knowledge of antigen processing and presentation to T cells may help to clarify this issue in the near future. Obviously, the use of highly purified antigen preparations and selected lymphocyte subsets will be crucial for elaboration of the molecular mechanisms. Furthermore, the effect of different adjuvants or antigen carriers as well as routes of administration has to be considered. These factors are important for appropriate targeting of antigens and induction of an immune response that promotes protection of the host rather than survival of the parasite.

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