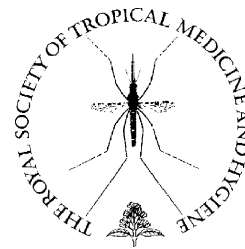available at www.sciencedirect.com

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Leishmania infantum-induced primary and challenge infections in rhesus monkeys (*Macaca mulatta*): a primate model for visceral leishmaniasis

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Summary   Visceral leishmaniasis (VL) was experimentally induced in rhesus macaques (*Macaca mulatta*) by intravenously inoculating 2×10^7 amastigotes/kg of body weight of *Leishmania infantum*. The macaques developed a systemic disease showing characteristic features of human VL such as fever, diarrhoea, body weight loss, anaemia, hypergammaglobulinaemia and transient lymphocytosis, as well as lymph node, liver and/or spleen enlargement. Nine weeks after infection, one primate showed pronounced weight loss, became moribund and was euthanized. The necropsy findings included granulomas composed of parasite-containing macrophages, lymphocytes and plasma cells in the liver, spleen and lymph nodes. The remaining macaques had a sustained course of infection but developed a mild-to-moderate illness that subsequently showed evidence of self-cure. Of note, pathological findings included a typical cell-mediated immunity-induced granulomatous reaction that had an effect on the control of parasite replication. All infected monkeys responded with increased production of anti-*Leishmania*-specific IgG antibodies. Despite the fact that clinical resistance to *L. infantum* was not consistently associated with a parasite-specific cell-mediated immune response, drug-cured macaques from the primary infection acquired immunity to homologous re-infection. These findings point to the feasibility of using the *L. infantum* macaque model for pre-clinical evaluation of novel chemotherapeutics or vaccine candidates for human VL.

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1. Introduction

Leishmaniasis is one of the major infectious diseases affecting mainly the poorest regions of the world. Among the two million new human infections annually in the 88 countries where the disease is endemic, 500 000 cases are clinical visceral leishmaniasis (VL); excluding epidemics, there are 57 000 deaths annually worldwide (www.who.int/tdr/diseases). Concerns about treatment failure for VL are exacerbated by geographical variation in antimonial treatment regimens, severity of disease and sensitivity of *Leishmania* species, and no proven successful vaccine for controlling human leishmaniasis is in routine use (Davies et al., 2003).

The protozoa *Leishmania donovani* and *L. infantum* (syn. *L. chagasi*) are the causative agents of VL. The incidence is rising with urbanisation and HIV co-infection (Desjeux, 2001). *Leishmania infantum* VL is a zoonotic disease found in Latin America, Europe, Asia and Africa. In the Neotropics the parasite is usually transmitted by the sandfly *Lutzomyia longipalpis*, and domestic dogs are the principal reservoir hosts. Control programmes in Brazil focus on residual insecticide spraying and mass elimination of seropositive dogs, but these conventional measures are costly and fail in developing countries (Courtenay et al., 2002).

The artificial induction of protective immunity using second-generation vaccines against leishmaniasis (Coler and Reed, 2005) would be essential for ultimate control of the disease. Nevertheless, there is still much to be done in assessing the effectiveness of vaccination in the absence of a natural challenge. Clinical development of the vaccine comprising the polyprotein Leish-111f in the adjuvant MPL-SE (Coler and Reed, 2005) may not be fully protective across *Leishmania* species. Another vaccine candidate of specific interest is HASPB1, a recombinant stage-specific hydrophilic acylated surface protein that protects against experimental *L. donovani* LV9 in mice (Stager et al., 2000).

Non-human primates are physiologically close to humans (Kennedy et al., 1997) and will probably mimic human response to *L. donovani* complex parasites. Indeed, different Old World monkeys and Neotropical simian species have become useful in studying the biology of infection and in dissecting the host response to these parasites. Those reported as being highly susceptible to this group of pathogens include New World primates such as the owl monkey *Aotus trivirgatus* (Broderson et al., 1986; Chapman et al., 1981), the squirrel monkey *Saimiri sciureus* (Chapman and Hanson, 1981; Dennis et al., 1985, 1986) and the marmoset *Callithrix jacchus jacchus* (Marsden et al., 1981). All of these species have since been used as non-human primate models of VL for antileishmanial chemotherapy studies (Berman et al., 1986; Madindou et al., 1985). East African primate species such as Sykes monkeys (*Cercopithecus mitis*) and baboons (*Papio cynocephalus*) all supported low-grade *L. donovani* infections for periods ranging between 4 and 8 months and subsequently showed evidence of self-cure (Githure et al., 1986). Furthermore, disease mimicking human VL was established in Indian langur monkeys *Presbytis entellus* (Dube et al., 1999) as well as in vervet monkeys *Cercopithecus aethiops* (Binhazim et al., 1993; Gicheru et al., 1995). The *L. donovani*—langur monkey model was also explored to assess different vaccine formulations against VL (Dube et al., 1998; Misra et al., 2001).

In our studies, *L. amazonensis* (Amaral et al., 1996), *L. major* (Amaral et al., 2001) and *L. braziliensis* (Teva et al., 2003) infections in outbred rhesus macaques produced cutaneous leishmaniasis (CL) of variable severity (ranging from self-healing granulomatous cutaneous lesions to non-healing mucocutaneous disease) and the immunological findings were similar to those observed in human CL. The homology between the *Macaca mulatta* immune system (Giavedoni, 2005; Pahar et al., 2003) and that of humans has led to the belief that the efficacy of those antigens selected as being candidates for an anti-*Leishmania* vaccine in this animal model (Campos-Neto et al., 2001; Kenney et al., 1999) may represent suitable vaccine candidates against this parasite in humans. The present studies on infections with *L. infantum* in macaques were performed to develop an animal model for human VL that would optimise progression of vaccine candidates to phase I trials. The parallels found between humans and this primate species with regard to susceptibility, clinicopathological changes and specific immunological responses to *L. infantum* visceral infections are not surprising given their close phylogenetic relationship (Kennedy et al., 1997), thus providing an acceptable model for the purpose of vaccine evaluation.

2. Material and methods

2.1. Animals

Colony outbred (Andrade et al., 2004a), young adult (weighing 6—10 kg) male rhesus macaques (*M. mulatta*) from the Fiocruz Primate Research Centre (Rio de Janeiro, Brazil) were used in this study. Primates were housed individually in stainless-steel squeeze-back cages and fed daily with a commercially available primate diet supplemented with fresh fruits and vegetables. Water was provided ad libitum. Syrian golden hamsters (*Mesocricetus auratus*) were also used in this study as the donor source of the inoculums. The experimental protocols involving monkeys and hamsters were reviewed and approved by the Institutional Animal Care and Use Committee (CEUA-Fiocruz, resolution # P0048-00). Primates were acclimatised to the laboratory conditions for at least 2 weeks before the experimental procedures began; they were monitored daily by non-human primate care specialists and evaluated by a veterinarian. When necessary (before infection and prior to each sampling or testing procedure), the animals were anaesthetised with 10—20 mg ketamine hydrochloride (Vetalar™)/kg of body weight injected intramuscularly.

2.2. Experimental infections

The strain (MHOM/BR/2000/1669) of *L. infantum* originally isolated from the bone marrow of a Brazilian patient with active VL was maintained by hamster-to-hamster passage. The parasite was typed by multilocus enzyme electrophoresis (Cupolillo et al., 1994) in our laboratory before being used for infection. For experimental infections, amastigotes harvested from heavily infected hamster spleens were prepared

according to the procedure as previously described (Amaral et al., 1996).

A study was first designed to provide information regarding the virulence of the infecting parasite. Six of the seven naïve macaques were each inoculated via the saphenous vein with 2×10^7 amastigotes/kg of body weight. One monkey, designated as the control, was injected with uninfected hamster spleen homogenate. An additional study was designed to evaluate the level of clinical resistance to the homologous challenge. These same macaques that had recovered from VL following treatment with meglumine antimoniate (at a dosage of 20 mg of Sb^v/kg/day, given intramuscularly for 28 days; therapy start at week 37 after infection) were each subsequently challenged via the saphenous vein with 2×10^8 amastigotes/kg at week 60 post inoculation (PI).

2.3. Assessment of infection and disease development

Once every 2 weeks, all macaques underwent gross physical examination and body weight, blood samples and rectal temperature were taken from each monkey through the duration of the experiment. Monkeys were examined for six typical signs of human VL (fever, diarrhoea, body weight loss, lymphadenopathy, hepatomegaly and splenomegaly). Following the procedure described by Courtenay et al. (2002), each sign was scored on a semiquantitative scale from 0 (absent) to 3 (severe) and these scores were added together to give an overall clinical score. Monkeys with a total score of 0—2 were arbitrarily classed as asymptomatic, those with a score of 3—6 were classified as oligosymptomatic and those with a score of 7—18 were classified as polysymptomatic.

Heparin- or EDTA-anticoagulated blood samples were collected by venipuncture for haematological and immunological studies. Blood collected into tubes containing EDTA as an anticoagulant was used for a complete haemogram. The following blood components were measured with a computer-directed analyser, using commercially available kits (CELM™ Cia Equipadora de Laboratórios Modernos, Barueri, SP, Brazil): cholesterol, urea nitrogen, total protein and albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Total erythrocyte, leukocyte and haemoglobin counts were carried out with the cellular counter 530/550 (CELM™ Cia Equipadora de Laboratórios Modernos). Haematocrit values were performed according to standard procedures, including Wright's coloration, which was used for blood films. Commercial assays were conducted in accordance with the manufacturer's instructions. Total plasma proteins were determined with a refract meter (American Optical T/C, Scientific Instrument Division, Buffalo, NY, USA). The plasma protein patterns of each monkey were determined by cellulose acetate electrophoresis using a Modular Electrophoresis System (Roche Laboratories, Basel, Switzerland).

For assessment of parasites, biopsy specimens were removed from bone marrow, liver and spleen at distinct stages of infection and processed for DNA isolation and culture or histological examination. For PCR analysis, liver and spleen specimens from the uninfected monkey were included as negative controls. Biopsy samples were cultured using NNN blood agar medium overlaid with Schneider's *Drosophila* insect medium (Sigma, St Louis, MO, USA) supplemented with 20% (v/v) heat-inactivated fetal calf serum (HI-FCS) and 100 μg/ml gentamicin (complete Schneider's medium). One macaque that developed very severe disease was euthanized with sodium pentobarbitone (Euthanol®). Gross and light microscopic examinations of the major organs were performed at necropsy. Paraffin sections from biopsy and necropsy tissues samples (fixed in 10% neutral buffered formalin) were stained with haematoxylin—eosin.

2.4. Diagnosis by DNA analysis

The PCR methods followed for detection of *Leishmania* DNA in infected monkeys have been described previously (Volpini et al., 2004). Briefly, DNA purification from 10—20 mg biopsy fragments of liver, spleen or bone marrow was done using the Wizard® Genomic DNA Purification System (Promega, Madison, WI, USA) following the manufacturer's instructions. Primers were designed to amplify the conserved region of the minicircle of *Leishmania* kDNA producing genus-specific fragments of 120 bp (Degrave et al., 1994). The reaction was carried out using 1 μM of each primer (150: 5′ GGG(G/T)AGGGGCGTTCT(C/G)CGAA 3′; and 152: 5′ (C/G)(C/G)(C/G)(A/T)CTAT(A/T)TTACACCAACCCC 3′) together with 200 μM of dNTPs, 0.8 U of *Taq* DNA polymerase (Invitrogen, São Paulo, Brazil), buffer (10 mM Tris—HCl pH 8.6, 50 mM KCl, 1.5 mM MgCl$_2$) and 2 μl of DNA template in a final volume of 25 μl. Amplification was carried out in an MJ Research PTC-200 machine (MJ Research Inc., Watertown, MA, USA) using an initial denaturing step at 95 °C for 5 min, followed by 29 cycles at 95 °C for 1 min, 55 °C for 30 s, 72 °C for 10 s and a final extension step of 5 min. Each experiment included a positive control (100 fg of *Leishmania* kDNA) and a negative control with no DNA. The expected amplification products of 120 bp were analysed by 8% polyacrylamide gel in a Mini-Protean II apparatus (Bio-Rad, Hercules, CA, USA) and stained using a DNA Silver Staining Kit (Amersham Biosciences, Buckinghamshire, UK) to visualise the bands. All PCR-negative results were tested for reaction inhibitors by adding 100 pg of total *L. infantum* DNA in a new amplification procedure, which then yielded a positive signal.

2.5. Antigens

Promastigotes of *L. infantum* (MHOM/BR/2000/1669) provided the source of the antigens. A preparation of soluble leishmanial antigens (SLA) was made as described previously (Amaral et al., 2001). The SLAs were used at different concentrations for ELISA and in vitro blast transformation assays.

2.6. ELISA for antiparasitic antibodies

Serum from the monkeys was analysed by adapting a standard ELISA technique (Amaral et al., 2001) to detect parasite-specific antibodies (using a peroxidase conjugate rabbit anti-monkey immunoglobulin G; Sigma, St Louis, MO, USA). The reaction was revealed with biotin—avidin peroxidase system. The substrate consisted of 0.04%

o-phenylenediamine dihydrochloride and 0.012% hydrogen peroxidase in phosphate-citrate buffer, pH 5. A group of sera with previously known titres as control values, as well as naïve rhesus controls, were included in each test.

2.7. Assessment of delayed-type hypersensitivity (DTH) responses

Animals were assessed for DTH reactions according to the method used for humans (leishmanin skin test (LST)). The antigen (leishmanin), provided by the Fiocruz, Biomanguinhos Unit (Rio de Janeiro, Brazil), consisted of pooled heat-killed cross-species promastigotes suspended in phosphate-buffered saline (PBS) with 0.5% phenol. For the LST, a volume of 0.1 ml containing macromolecules from 5×10^6 parasites was inoculated intradermally into the forearm and the induration produced was measured 72 h later. Induration diameters of ≥ 5 mm were considered positive.

2.8. Assessment of the in vitro blastogenic and cytokine responses

The antigen-specific recall proliferative and cytokine responses were measured in peripheral blood leukocytes (PBL) using phytohaemagglutinin (PHA-P) (Sigma, St Louis, MO, USA) stimulation as a positive control. Basic experimental protocols have been reported elsewhere (Amaral et al., 1996). Briefly, purified PBL concentration was adjusted to 5×10^6 cells/ml in RPMI-1640 supplemented with 20% HI-FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5×10^{-5} M β-mercaptoethanol (Merck, Darmstadt, Germany). Aliquots (0.1 ml) of the cell suspension were distributed in triplicate cultures in 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark). PHA-P and SLA were added in 0.1 ml of the same media to a final concentration of 12.5 µg/ml and 10 µg/well, respectively. Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 3 days in the case of PHA-P or for 4 days in the case of SLA. The cells were pulsed with 1 µCi of ^{3}H-thymidine (specific activity 74 GBq/nmol; Amersham International, Buckinghamshire, UK) for the last 24 h before cell harvest. Radioactivity uptake was measured by liquid scintillation counting. The stimulation index (SI) was determined as the ratio of the mean counts per minute (cpm) of cultures stimulated with PHA-P or SLA to the mean cpm of cultures with RPMI alone (control cultures). A SI ≥ 3 was considered significant based on the 95th percentile of pre-infection values of seven macaques.

Concentrations of IFN-γ and IL-5 in culture supernatants of stimulated cells were measured by ELISA. Cell-free culture supernatants were harvested by centrifugation at $200 \times g$ for 10 min and cytokine levels were estimated using either a rhesus monkey IFN-γ (Biosource, Camarillo, CA, USA) or human IL-5 (R&D Systems Inc., Minneapolis, MN, USA) commercial ELISA kit. The procedure was carried out following the manufacturer's instructions. In brief, plates were coated with an optimum concentration (2 µg/ml) of mouse anti-rhesus IFN-γ or anti-human IL-5 monoclonal antibody (mAb). Then, 100 µl of cell culture supernatants and multiple dilutions of the standard (0–1400 pg/ml) were added in duplicate. Detection was performed by the addition

of horseradish peroxidase—streptavidin conjugate at a concentration of 1:2000 in PBS. Reaction was developed using tetramethylbenzidine as the substrate. Cytokine concentrations for unknown samples and controls were read from the plotted standard curve.

2.9. Statistical analysis

Student's t-test was used in comparative analysis and a P-value of <0.05 was considered statistically significant. Concordance between cellular immune responses was assessed as reported elsewhere (Amaral et al., 2001).

3. Results

3.1. Establishment of infection and disease development

Leishmania infantum primary infection in outbred macaques was assessed through time by parasitological examination or PCR of the liver, spleen and bone marrow. All of the monkeys had established infection by week 2 PI and parasites persisted in lymphoid organs as long as 25 weeks PI, except for the monkey (O45) that recovered spontaneously from VL (Table 1). Overall, attempts to detect parasite-positive specimens by culture (61%; 14/23) were more successful than PCR (53%; 17/32) and direct microscopic examination (50%; 9/18). However, there was variation in the sensitivity of each diagnostic test following the time course of infection. Considering only macaques positive for Leishmania DNA by PCR, the sensitivity of the test was high (90.9%; 10/11) during active infection, but lower (20%; 2/10 samples) in the self-healing phase and after antimony therapy.

Table 2 shows that a similar picture to human VL, characterised by systemic disease of varying severity, develops in L. infantum-infected monkeys. According to their clinical condition, two macaques (N13 and O59) with a total score of 10 and 7, respectively, and four others (O45, O21, F14, O43) with scores of 4—6 were classed as polysymptomatic and oligosymptomatic, respectively. The most consistent clinical parameters were an intermittent rise in body temperature by 2—4 °C, diarrhoea and body weight loss. These changes were evident by week 2 PI and became more pronounced during the first 4 months of infection. Although visceral organ enlargement was seen as a result of the experimental infection, no animal showed a syndrome similar to human VL with progressive hepatosplenomegaly.

Nine weeks after infection, one primate (N13) developed a severe illness after losing 42% of his body weight. At this time he was anorectic, listless and became weak and dehydrated, and was euthanized. Gross necropsy findings included enlarged spleen (five-fold), mesenteric lymph nodes and fatty liver. The surviving five chronically infected macaques lost between 7% and 28% of their body weight. These primates clinically recovered from infection following antimony-based therapy and regained normal weight by week 45 PI. At this time, the weight of the uninfected control monkey (O15) increased by 5%. Of note, in the second experiment we found that the same macaques were refractory to the disease (as indicated by complete clinical resistance)

Table 1 Comparative results of conventional diagnostic procedures and PCR of *Leishmania infantum* visceral infection in rhesus macaques (*Macaca mulatta*)

Monkey	Time PI (weeks)	Clinical status (stage)	Demonstration of the parasite (biopsy specimen)		
			Histological sections[a]	In vitro culture	PCR[b]
O15	0	Uninfected control	n.d.	n.d.	− (L); − (S)
N13	2	Oligosymptomatic (active)	+ (L)	+ (S)	+ (L)
	5	Polysymptomatic (active)	+ (L)	n.d.	+ (BM); + (S)
	9	Polysymptomatic (active)	+ (L)	+ (S)	+ (L); + (S)
O59	2	Oligosymptomatic (active)	+ (L)	+ (L)	+ (S)
	5	Polysymptomatic (active)	+ (L)	+ (BM); + (L)	+ (BM); + (S)
	25	Oligosymptomatic (healing)	− (BM); − (L)	− (L)	+ (BM); + (L)
	45	Asymptomatic (drug-cured)	n.d.	− (L)	− (L)
F14	2	Oligosymptomatic (active)	+ (L)	+ (L)	+ (S)
	5	Oligosymptomatic (active)	− (L)	− (L)	− (BM); − (S)
	25	Oligosymptomatic (healing)	− (L)	+ (BM)	− (BM); −(S)
	45	Asymptomatic (drug-cured)	n.d.	− (L)	− (L)
O43	2	Oligosymptomatic (active)	+ (L)	+ (L)	n.d.
	5	Oligosymptomatic (active)	− (L)	+ (BM)	n.d.
	25	Oligosymptomatic (healing)	− (L)	+ (L)	− (BM); − (S)
	45	Asymptomatic (drug-cured)	n.d.	− (L)	− (L)
O21	2	Oligosymptomatic (active)	+ (L)	+ (L)	+ (S)
	5	Oligosymptomatic (active)	n.d.	− (BM)	+ (L); + (S)
	25	Oligosymptomatic (active)	− (L)	+ (BM); + (L)	+ (BM); − (S)
	45	Asymptomatic (drug-cured)	n.d.	− (L)	− (L)
O45	2	Oligosymptomatic (active)	+ (L)	+ (L)	+ (S)
	5	Oligosymptomatic (active)	− (L)	n.d.	+ (BM); − (S)
	25	Asymptomatic (healing)	− (L)	− (BM)	− (BM); − (S)
	45	Asymptomatic (drug-cured)	n.d.	− (L)	− (L)

PI: post inoculation; BM: bone marrow; L: liver; S: spleen; +: positive; −: negative; n.d.: not done.
[a] Haematoxylin—eosin stain.
[b] Each experiment included positive and negative controls (see Section 2.4).

when re-challenged by homologous leishmanial parasites at 60 weeks after the primary infection.

3.2. Haematology and blood chemistry

The results for the haematological parameters evaluated in the rhesus macaques prior to and at different time points after *L. infantum* challenge infections are given in Table 3. The pre-infection mean blood counts were within the normal range compared with the baseline haematological data previously found for clinically healthy rhesus macaques from the Fiocruz colony (Andrade et al., 2004b). The most consistent haematological finding was a normchromic, normocytic anaemia. An appreciable fall in the red blood cell (RBC) count occurred from week 2 PI onward, and the count was minimal at 46 weeks PI. Packed cell volumes and haemoglobin levels paralleled the RBC values, but exhibited a wide variation. White blood cell counts also varied during infection. Whilst the haemogram revealed neutrophilia and lymphopenia by 9 weeks PI, neutropenia, eosinophilia and lymphocytosis were detected at week 15 PI. Alterations in serum chemistries of infected macaques included increases in blood urea nitrogen, total protein and globulin. Of note, in

at least three of these macaques the plasma concentration of gamma globulins increased from a pre-inoculation mean value of 0.3 g/dl to 0.8 g/dl at week 9 PI (Table 2). Typical changes are also illustrated in Figure 1.

One monkey (N13) that had severe disease showed significantly altered haematological values. The RBC values decreased from a pre-infection value of 5.695×10^6/ml to 3.300×10^6/ml by 6 weeks PI. Packed cell volumes and haemoglobin levels decreased from 38% and 13.3 g/dl at pre-infection to 20% and 7.4 g/dl, respectively, at 6 weeks PI. The leukocyte count reduced from an initial value of 6.20×10^3/ml to 1.90×10^3/ml. At this time, the levels of AST (72 IU/l) or ALT (88 IU/l) were elevated, but the cholesterol level declined (108 mg/dl) in this animal.

3.3. Histopathological findings

Hepatic granulomas were found in all infected macaques. Granulomas were formed as early as 2 weeks PI and persisted as active in some monkeys through at least 33 weeks PI. The poorly differentiated granulomas of the initial infection consisted primarily of widespread macrophages (some of which contained amastigotes) and variable numbers of

Table 2 Clinical outcome of experimental *Leishmania infantum* visceral leishmaniasis in rhesus macaques (*Macaca mulatta*)

Clinical and laboratory features of sick macaques[a]	Monkey					
	N13[*]	O59	F14	O43	O21	O45
Overall clinical score[b]	10	7	6	5	5	4
Fever	1	1	1	1	1	1
Diarrhoea	2	1	1	1	1	1
Decrease in body weight (% change)	3 (42)	2 (28)	1 (18)	1 (16)	1 (13)	1 (7)
Enlarged lymph nodes	1	1	1	1	0	0
Splenomegaly	2	1	1	0	1	0
Hepatomegaly	1	1	1	1	1	1
Leishmania in biopsy specimens[c]	+ (2 to 9)	+ (2 to 25)	+ (2 to 25)	+ (2 to 25)	+ (2 to 25)	+ (2 to 5)
Plasma concentration of γ-globulins (g/dl)[d]	0.2 (0.6—0.8)	0.3 (0.6—0.7)	0.3 (0.6—0.9)	0.2 (0.2—0.3)	0.3 (0.2—0.3)	0.2 (0.2—0.3)
Leishmania-specific serum IgG (OD value)[d,e]	0.02 (0.26—0.52)	0.01 (0.33—0.57)	0.04 (0.08—0.37)	0.13 (0.36—1.10)	0.15 (0.21—0.54)	0.07 (0.31—0.83)
Leishmanin skin test (mm)[f]	<2 (2 to 9)	9 (21)	10 (21)	10 (21)	<3 (2 to 21)	9 (21)
Specific lymphoproliferative response (SI)[d,g]	1 (0.7—1)	1 (1—23)	1 (2—27)	2 (1—19)	2 (1—3)	1 (1—12)
IFN-γ levels in culture supernatants (pg/ml)[d,h]	0 (452—580)	0 (133—1587)	0 (124—941)	0 (152—464)	0 (0—60)	0 (141—1489)
IL-5 levels in culture supernatants (pg/ml)[d,h]	0 (17—20)	0 (0—0)	0 (0—0)	0 (0—20)	0 (0—25)	0 (0—25)

[a] Each macaque was inoculated intravenously with a single dose of 2×10^7 amastigotes/kg of body weight. [*]This monkey developed fulminating disease and was therefore euthanized at week 9 post infection. At necropsy, the spleen was enlarged five-fold.

[b] Monkeys were scored for six typical signs of human visceral leishmaniasis as described in Section 2.3.

[c] For assessment of parasites, biopsy specimens were removed from bone morrow, liver and spleen at distinct stages of infection and then processed for *Leishmania* DNA, culture and histological examinations (see Table 1). Figures in parentheses are the number of weeks following infection.

[d] Data presented as pre-infection value, followed by the minimum and maximum values obtained during the time course of infection in parentheses.

[e] Antigen-specific antibody concentrations were determined by ELISA and results are expressed as the optical density (OD) at 492 nm (see Figure 3).

[f] Delayed-type hypersensitivity reactions to *Leishmania* antigens are expressed as the mean diameter of skin induration and were considered positive if $\geq$5 mm. Figures in parentheses are the number of weeks after infection.

[g] Lymphocyte proliferation in response to parasite antigen was expressed as stimulation index (SI) and was considered positive if $\geq$3 (see Section 2.8).

[h] Measurement of cytokine secretion in the supernatants of antigen-stimulated cultures was assayed by ELISA (see Section 2.8).

Table 3 Comparative haematological data in rhesus macaques (Macaca mulatta) prior to and after primary and challenge Leishmania infantum infections[a,b]

Weeks post infection[c]	RBC (×10^6/ml)	Hct (%)	Hb (g/dl)	TLC (×10^3/ml)	Bas. (%)	Eos. (%)	Neu. (%)	Mon. (%)	Lym. (%)	AST (IU/l)	ALT (IU/l)	Urea nitrogen (mg/dl)	Cholest. (mg/dl)	Total protein (mg/dl)	Albumin (mg/dl)
0 (7)	5.667 ± 0.18	38.8 ± 1.9	13.3 ± 0.63	5.51 ± 2.63	0.0 ± 0.0	0.7 ± 0.9	61.0 ± 2.5	3.5 ± 1.5	29.3 ± 3.8	27.0 ± 6.5	42.1 ± 2.2	17.8 ± 3.8	176.0 ± 38.1	5.37 ± 0.35	3.38 ± 0.26
2 (5)	4.334** ± 0.24	38.2 ± 2.8	12.8 ± 0.5	9.36 ± 4.07	0.0 ± 0.0	4.2 ± 2.4	53.4 ± 12.5	5.6* ± 1.7	29.2 ± 4.4	36.4 ± 12.3	35.6 ± 11.3	27.8** ± 6.3	190.0 ± 24.0	5.72 ± 0.35	3.64 ± 0.33
6 (5)	4.596** ± 0.55	36.2 ± 2.4	11.9** ± 0.4	5.78 ± 1.37	0.0 ± 0.0	4.0 ± 4.0	60.6 ± 2.6	3.4 ± 1.7	30.0 ± 3.8	34.0 ± 8.6	42.0 ± 10.6	28.0 ± 11.6	201.2 ± 17.9	5.54 ± 0.44	3.80 ± 0.40
9 (5)	5.082** ± 0.35	35.6 ± 2.5	12.1* ± 0.9	4.58 ± 1.37	0.0 ± 0.0	3.0 ± 1.9	69.4** ± 3.5	4.2 ± 1.9	23.4* ± 5.2	28.8 ± 12.4	36.4 ± 14.3	18.0 ± 5.2	183.4 ± 24.0	5.38 ± 0.55	3.64 ± 0.41
15 (5)	5.080** ± 0.19	36.0 ± 1.4	12.2* ± 0.4	6.42 ± 1.66	0.4 ± 0.5	8.4* ± 5.7	43.4** ± 8.3	5.4 ± 2.7	41.6* ± 7.2	33.2 ± 16.4	39.6 ± 18.9	25.8 ± 11.7	208.0 ± 23.2	5.36 ± 0.65	3.78 ± 0.38
33 (5)	4.754** ± 0.47	34.8 ± 2.9	11.9* ± 0.8	6.86 ± 1.15	0.0 ± 0.0	0.8 ± 0.8	55.8 ± 11.3	4.2 ± 0.4	38.6 ± 11.6	46.0 ± 23.7	52.2 ± 28.9	35.4** ± 12.6	159.0 ± 51.7	5.56 ± 0.41	3.48 ± 0.40
46 (5)	4.240** ± 0.36	36.0 ± 2.2	12.2* ± 0.7	10.44* ± 1.54	0.0 ± 0.0	0.8 ± 0.8	69.8* ± 10.1	2.8 ± 1.6	26.0 ± 8.9	22.8 ± 11.4	38.8 ± 27.7	35.4** ± 16.6	108.0* ± 20.7	7.08** ± 1.15	3.50 ± 0.63
60 (5)	5.164* ± 0.44	37.6 ± 1.8	12.7 ± 0.7	5.20 ± 0.87	0.2 ± 0.4	1.6 ± 0.9	52.6 ± 10.5	5.2 ± 2.8	40.4* ± 11.7	28.6 ± 4.2	32.6* ± 8.7	36.0** ± 12.7	106.0* ± 27.0	4.92 ± 1.00	2.96 ± 0.51
65 (5)	4.954* ± 0.39	36.8 ± 2.9	12.5 ± 1.0	5.80 ± 1.47	0.0 ± 0.0	0.4 ± 0.9	62.4 ± 14.2	3.5 ± 1.5	34.4 ± 14.8	18.8* ± 3.6	25.2** ± 3.2	26.6* ± 7.1	111.0** ± 16.1	6.22* ± 0.40	3.96* ± 0.50
76 (5)	7.330* ± 0.93	36.4 ± 2.5	12.9 ± 2.0	8.44 ± 6.29	0.0 ± 0.0	0.8 ± 0.8	61.6 ± 2.9	5.6* ± 1.1	32.0 ± 2.9	18.4* ± 1.6	25.2** ± 2.2	21.8 ± 4.6	120.0* ± 16.1	6.38** ± 0.60	3.84* ± 0.28

RBC: total red blood cell count; Hct: haematocrit; Hb: haemoglobin; TLC: total leukocyte count; Bas.: basophils; Eos.: eosinophils; Neu.: neutrophils; Mon.: monocytes; Lym.: lymphocytes; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Cholest.: cholesterol.
*,**Statistically significant differences between pre- and post-infection mean values (except for macaque N13, which was a complete outlier from the rest of the group) are indicated as *(P ≤ 0.05−0.01) or **(P < 0.01−0.001) (Student's unpaired t-test).
[a] Each macaque was inoculated intravenously with a single dose of 2 × 10^7 amastigotes/kg of body weight. Cured primates (following treatment with meglumine antimoniate at 20 mg Sb[V]/kg, given intramuscularly for 30 days) were re-challenged with 2 × 10^8 amastigotes/kg at week 60 post infection.
[b] Values are expressed as the mean ± SD.
[c] Figures in parentheses are the number of analysed samples.

lymphocytes, plasma cells and occasional neutrophils. This inflammatory process evolved to form well circumscribed, well differentiated granulomas, with epithelioid cells fusing to form Langhans-type multinucleated giant cells, surrounded by lymphocytes and occasionally plasma cells (Figure 2A and B). These granulomas contained less marked parasitised macrophages at week 9 PI compared with those of the initial infection. Additional histological findings in the liver included proliferation and hyperplasia of parasite-laden Kupffer cells, associated with diffuse fatty changes in the stellate cells (Figure 2C and D). Amastigote-containing macrophages were also seen in the spleen, lymph nodes and bone marrow (data not shown). Sections from the spleen revealed that lymphoid follicles increased in number and size, as well as congested red pulp, with reticular cells proliferating and macrophages (some of which contained haemosiderin) aggregating in the cords and walls of dilated sinuses (Figure 2E and F). Following therapy, older hepatic granulomas observed in portal zones by week 45 PI (the latest time that biopsy samples were taken) developed an enclosing rim of fibroblasts and connective tissue (data not shown). In addition, parasites were not found in sections at this time.

3.4. Humoral responses

As shown in Figure 3, the antibody responses detected by ELISA in experimental macaques varied following infection. The serum levels of L. infantum-specific IgG antibodies reached a plateau by 15 weeks PI and subsequently declined slightly after antimonial therapy. Following re-challenge, these animals had heightened levels of antigen-specific antibodies, continuing up to 76 weeks PI. Interestingly, the antibody titres produced by polysymptomatic macaques (N13 and O59) were not consistently higher compared with oligosymptomatic animals.

3.5. Cell-mediated immune responses

A very high degree of variability was observed in the parasite-specific cell-mediated immune responses between individuals over time following infection with L. infantum. Marked DTH responses were demonstrated in four macaques by 21 weeks PI (Table 2). In contrast, the remaining infected (N13 and O21) and uninfected (O15) monkeys injected with the same leishmanial antigen developed either minimal skin indurations (mean score, <3 mm) or no reaction.

Mitogen (PHA)-induced proliferation of PBLs from primates was comparable prior to and throughout infection (data not shown). The parasite-specific recall proliferative and cytokine responses of T-lymphocytes from individual macaques through the course of the experiment are shown in Figure 4. Whereas the specific responses were negative in primates before the challenge, four animals (O59, F14, O43 and O45) converted to a positive proliferative response (maximum SI values ranging from 12 to 27) after infection (Figure 4A). Of note, one oligosymptomatic monkey (O21) demonstrated a specific proliferative response (SI value of 27.5) only by week 76 PI.
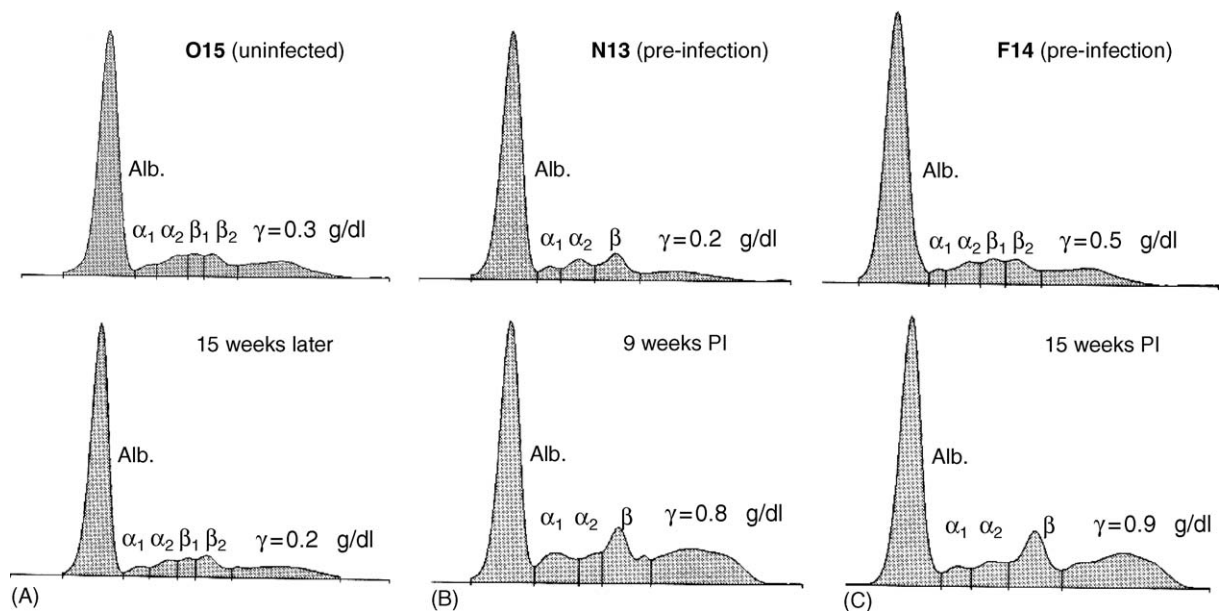
Figure 1 Typical changes in cellulose acetate electrophoretic patterns of plasma components in two representative *Leishmania infantum*-infected macaques that developed either acute (N13) or chronic (F14) disease, compared with data obtained from the control monkey (O15). Similar changes were observed in other infected macaque (see Table 2). PI: post inoculation.

Significant but varying levels of IFN-γ were secreted in PBL cultures from all the infected macaques when stimulated with either PHA (data not shown) or SLA (Table 2; Figure 4B). In the uninfected control monkey (O15), IFN-γ was only detected when PBLs were stimulated with PHA. Noteworthy, a strong *Leishmania*-specific IFN-γ response was elicited in the rhesus monkey (N13) that had the most protracted fatal course of infection. In contrast, either PHA-(data not shown) or SLA-induced IL-5 secretion in supernatant fluids was at low or undetectable levels (Table 2; Figure 4C). Comparison of immune responses in terms of antigen-specific IFN-γ production with the blastogenic response of PBLs from infected macaques showed that there was no positive correlation between the results of the two assays ($r^2 = 0.038$; $P = 0.1453$).

4. Discussion

Typically, *L. infantum* VL affects young children and immunocompromised adults. The disease can have a long incubation period and has been reported to present as a smouldering subclinical illness characterised by weight loss, fever and diarrhoea that can evolve either into a self-healing or full-blown disease (Evans et al., 1995). The clinical features of VL in humans include intermittent fever, progressive emaciation, anaemia, lymphadenopathy and hepatosplenomegaly. Although malnutrition is a risk factor for the development of human VL, our understanding of the pathogenesis of the disease is still rudimentary.

Non-human primates have varying degrees of susceptibility to *L. donovani* complex parasites. The results of this study revalidate original reports (Manson-Bahr and Wilson, 1976; Shortt, 1923) identifying *M. mulatta* as a moderately susceptible host to *L. donovani* complex parasites. In the original model description (Shortt, 1923), induced fulminat-

ing infections were regarded as exceptions rather than the rule. Likely, macaques in this study had a sustained course of *L. infantum* visceral infection, ranging from mild self-curing to severe disease. The macaques lost between 7% and 42% of their body weight and developed progressive liver lesions as revealed by the granulomatous inflammatory reaction studied chronologically after infection. Hepatomegaly and splenomegaly were difficult to evaluate in monkeys surviving the disease, but the post-mortem findings detected in rhesus N13 included a five-fold enlarged spleen.

The major haematological changes (anaemia associated with haemosiderosis and tissue iron deposition) in rhesus macaques with VL paralleled the haemolysis associated with hypersplenism observed to occur in humans with VL (Cartwright et al., 1948) and other infected monkey species (Binhazim et al., 1993; Broderson et al., 1986; Dennis et al., 1985). Moreover, one monkey (N13) that had severe disease showed a significant increase in the levels of AST and ALT, indicating liver disorders. The hepatic lesions resolved following drug-induced recovery from the disease, confirming the antileishmanial efficacy of the reference drug *N*-methylglucamine antimoniate (Glucantime®) in the rhesus monkey model (Teva et al., 2005).

Sensitive molecular techniques have allowed the identification of leishmanial parasites directly in scars after clinical cure of *L. (L.) braziliensis* CL in patients (Schubach et al., 1998) and in experimentally infected macaques (Teva et al., 2003). By contrast, whereas persistence of *L. infantum* parasites was documented in all monkeys during active infection, no parasites were found (using either conventional diagnostic procedures or PCR for detecting *Leishmania* DNA) after treatment. Whether exposure to a particular parasite has led to sterile cure could not be ascertained by this study. The ability of *Leishmania* to establish latency in immune individuals is evidence of parasite determinants to escape the host's immune response by clonal
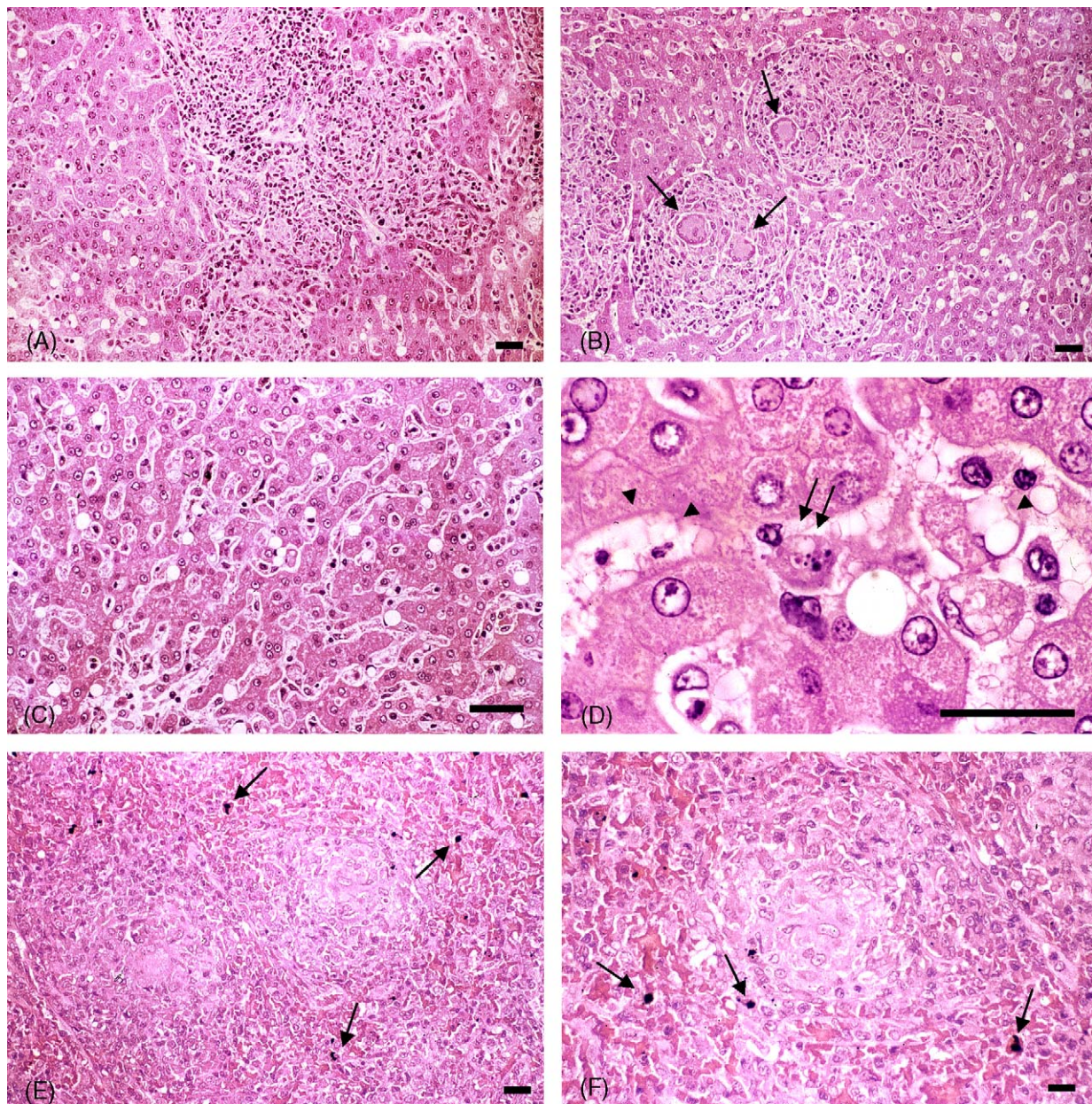
Figure 2 Photomicrographs of the liver and spleen from a rhesus macaque (N13) 9 weeks after inoculation with *Leishmania infantum*. (A,B) Sections showing multifocal coalescing hepatic immune granulomas consisting of an aggregation of activated macrophages (epithelioid cells) and multiple Langhans-type giant cells (arrows), surrounded by lymphocytes, at the portal spaces, which obliterate the sinusoids and protrude the parenchyma. (C,D) Also illustrated are the proliferation and hyperplasia of parasite-laden Kupffer cells (double arrow), associated with fatty changes of the stellate cells (arrowheads). (E,F) Sections from the spleen showing granulomas consisting of differentiated macrophages in the pulp, with widespread macrophages aggregating in the cords and walls of dilated medullary sinuses. Numerous haemosiderin deposits are present (arrows). (H&E stain; bar scale = 10 μm.)

selection or molecular changes resulting from mutational or recombinational processes. Of note, our genotyping analyses showing genetic variations among *L. (L.) braziliensis* isolates from humans and experimentally infected macaques (Teva et al., 2003) indicated that persistent parasites do not retain the characteristics of the parental clones.

It should be noted that our model is artificial in the sense that intravenous injection of a large number of amastigotes initiates the infection, which may account for the parasitic invasion of lymphopoietic organs with the result-

ing chronic granulomatous inflammatory response that was observed. Descriptions of the histopathological lesions in the liver either of patients with VL (Sen Gupta et al., 1956) or of experimentally infected *L. donovani* macaques (Manson-Bahr and Wilson, 1976) resemble the granulomas described here. The hepatic granulomas in this study were most prominent 2–9 weeks PI and had an effect on the control of parasite replication. Of note, the marked regression of immune granulomas and the eventual absence of parasites 25 weeks PI correlated with satisfactory resolution of the disease.
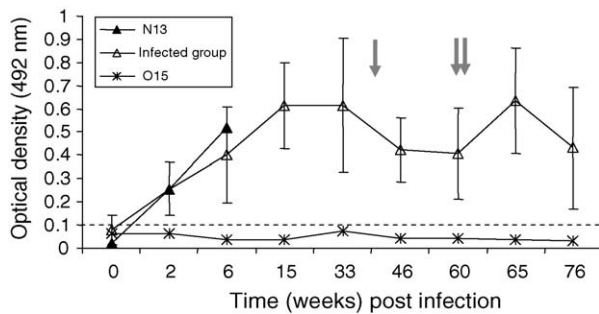
Figure 3 Evolution of serum levels of *Leishmania*-specific antibodies (IgG) in rhesus macaques following infection with *L. infantum*. Experimental animals were infected as indicated (Table 2). Following drug-induced recovery from the primary infection (arrow indicates that start of antimonial therapy at week 37 post infection (PI)), the primates were re-challenged (see Table 3) at week 60 PI as indicated by the double arrow. Each point represents the optical density value (mean ± SD) at 492 nm of sera from five infected monkeys, as determined by ELISA. Individual humoral responses are also shown for the macaques N13 (that developed fulminating disease) and O15 (uninfected control). Broken line indicates the positive cut-off (0.10 ± 0.073) as determined by the mean of negative controls (representing pre-infection values of seven macaque sera) ± 2 SD.

This type of immune response capable of creating a protective granuloma (Murray, 2001) is firmly linked to the IL-12-dependent IFN-γ production pathway (Lammas et al., 2002).

Whilst the generation of anti-*Leishmania* antibodies apparently plays no role in resistance to leishmaniasis, a strong cell-mediated immune response is essential. The protective immune response in mice against *L. major* is dependent on the ability to mount an IL-12-driven T-helper cytokine type 1 (Th1) response, which activates infected macrophages by production of IFN-γ for the killing of intracellular parasites (Alexander and Bryson, 2005). Vaccine efficacy against *Leishmania* is also thought to be largely associated with the emergence of a specific Th1 response (Coler and Reed, 2005). In humans, whilst there is a good correlation between Th1 responses and resistance to CL, no association between increased IL-4 and kala-azar has been identified (Kharazmi et al., 1999). However, a direct correlation between production of IL-10 and active disease is reported in patients with VL (Ghalib et al., 1993). One could speculate that the exacerbation of disease observed in macaque N13 could be due to the suppressor activity of IL-10-secreting CD4+CD25+ regulatory T-cells (Belkaid et al., 2002).

In the present study, *L. infantum*-specific antibodies in serum were detected by ELISA in all infected animals; levels of IgG antibodies rose during active infection and then declined during treatment, but they increased after re-challenge infection. Positive DTH responses were seen in most of the resistant monkeys with self-limiting disease when skin tested with leishmanin at 21 weeks after infection, whereas the control uninfected monkey and one monkey (N13) with progressive illness did not respond. Skin test reactivity following natural exposure is also thought to
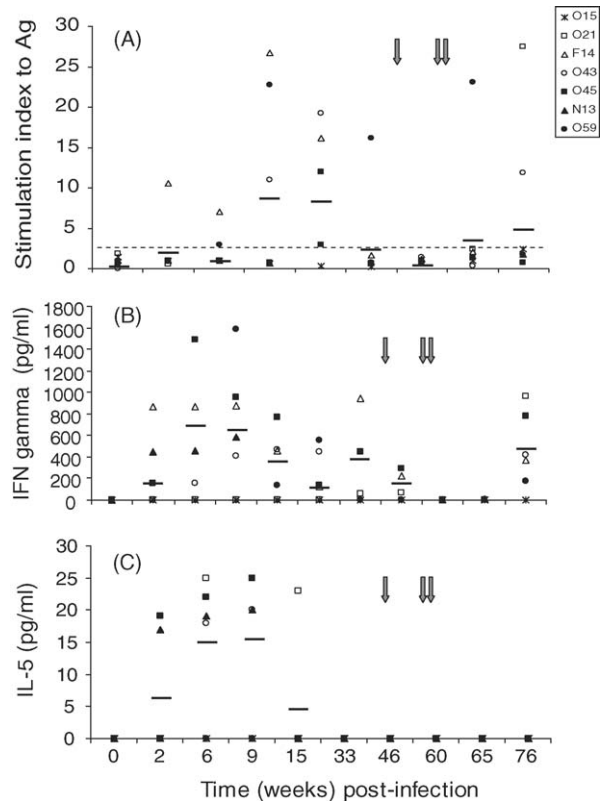

Figure 4 (A) Parasite-specific recall proliferative response and (B,C) cytokine (IFN-γ and IL-5) responses of T-lymphocytes from rhesus macaques prior to and after experimental infection with *Leishmania infantum*. After recovery from the primary infection (arrow indicates the start of antimonial therapy at week 37 post infection (PI)), the primates were subsequently re-challenged at week 60 PI (double arrow). Suspensions of purified peripheral blood leukocytes were re-stimulated in vitro in the presence of soluble antigens (Ags) prepared from *L. infantum*. (A) Cell proliferation was assessed by measuring thymidine incorporation. Results are expressed as the stimulation index (SI; mean counts per minute of stimulated cultures/mean counts per minute of unstimulated cultures). Broken line indicates the positive cut-off value (SI ≥ 3). Determinations were made at minimum in triplicate. (B,C) Cell culture medium samples collected after 72 h of stimulation were used for quantitative determinations of cytokine secretion in the supernatants by ELISA. Bars depict the mean SI, IFN-γ and IL-5 values of infected monkeys at different time points. Individual cellular immune responses are also indicated for the uninfected (control) monkey (O15).

reflecting resistance to infection, with genetic epidemiology studies (Shaw et al., 1995) demonstrating a strong genetic component for mounting a skin test response to leishmanial antigen.

We have also used conventional in vitro assays of cellular immunity to understand some of the mechanisms that underlie resistance to *L. infantum*. The cytokines of interest were primarily selected because of their importance as markers for Th1 or Th2 immune responses or inflammatory responses. The anti-human IL-5 mAb evaluated in this study has been used previously for ELISA detection of

macaque IL-5 (Mäkitalo et al., 2002). In our experiments, the antigen-stimulated cells produced IL-5 at low or undetectable levels, whereas production was slightly enhanced by PHA stimulation. This, and the finding (Mäkitalo et al., 2002) of the poor cross-reactivity displayed by the mAbs to cynomolgus and rhesus macaque IL-5, highlight the need for a thorough investigation of mAb cross-reactivity. Our results confirmed some studies on the basic immunological parameter in *M. mulatta* (Pahar et al., 2003), showing that the magnitude and kinetics of lymphocyte proliferation in response to antigen is not directly related to effector or T-cell functions such as secreted IFN-γ.

Despite the fact that clinical healing was not consistently linked with an abundance of antigen-specific IFN-γ-producing cells, animals that recovered from previous infection acquired immunity to homologous re-infection. This was also the situation in vaccinated monkeys (Campos-Neto et al., 2001; Kenney et al., 1999) in which neither pre-challenge in vitro antigen-specific recall T-cell proliferative and IFN-γ responses nor positive DTH response were predictive of clinical protection. Taken together, this confirms the importance of quantitative assessment of low-frequency, antigen-specific T-cells from rhesus macaques by ELISPOT and cytokine flow cytometry assays (Pahar et al., 2003), as well as using luminex technology (Giavedoni, 2005), to determine correlates of protection against leishmanial infection. Finally, the demonstration of a specific immunological response in *L. infantum*-infected macaques, such as described here, suggests that this primate model of human VL should be of practical importance since it shows several features underlying immunity that could be met by an effective vaccine.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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