IgG3 and IL10 are effective biomarkers for monitoring therapeutic effectiveness in Post Kala-Azar Dermal Leishmaniasis

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Abstract

Background

The assessment of chemotherapeutic responses in Post Kala-azar Dermal Leishmaniasis (PKDL), especially its macular form is challenging, emphasizing the necessity for ‘test of cure’ tools. This study explored the diagnostic and prognostic potential of IgG subclasses and associated cytokines for monitoring the effectiveness of chemotherapy in PKDL.

Methods

Participants included PKDL cases at (a) disease presentation, (b) immediately at the end of treatment (12 weeks for Miltefosine or 3 weeks for Liposomal Amphotericin B, LAmB) and (c) at any time point 6 months later, for estimating anti-leishmanial immunoglobulin (Ig, IgG, IgM, IgG1, IgG2 and IgG3) and cytokines (IL-10, IL-6).

Results

In PKDL, Ig levels were elevated, with IgG3 and IL-10 being the major contributors. Miltefosine decreased both markers substantially and this decrease was sustained for at least six months. In contrast, LAmB failed to decrease IgG3 and IL-10, as even after six months, their levels remained unchanged or even increased.

Conclusions

In PKDL, IgG3 and IL-10 proved to be effective predictors of responsiveness to chemotherapy and may be considered as a non invasive alternative for longitudinal monitoring.

Author summary

Post Kala-azar Dermal Leishmaniasis (PKDL) is a dermal condition that occurs in East Africa and South Asia, the latter in 5–10% of patients after apparent cure from Visceral Leishmaniasis (VL). Till date, conventional knowledge in South Asia was that the
polymorphic form of PKDL comprising of macules, papules and nodules was the predominant disease form, constituting 85–90%. However, since 2014, implementation of active-case surveillance led to unearthing of a large number of macular, hypopigmented cases, and was reported to contribute to nearly 50% of the disease burden. In particular, the macular form poses a diagnostic dilemma as microscopically parasites are difficult to identify in their lesions, and repigmentation occurs months after parasite clearance, emphasizing the need for developing non-invasive approaches for measurement of parasite burden. Till date, no formal clinical trial for treatment of PKDL has been undertaken where the parasite load was quantified and treatment remains empirical. This is primarily due to PKDL cases being unwilling to provide a repeat skin biopsy once their lesions have declined. Therefore, in cases where treatment failure occurs, it cannot be precisely identified, and could potentially lead to these cases becoming mobile disease reservoirs, thereby adversely impacting on the ongoing VL elimination programme. This study addressed this critical lacuna, where it was established that in both clinical types of PKDL, circulating levels of IgG3 and IL-10 can be considered as effective markers for monitoring treatment outcome. At disease presentation, the raised levels of IgG subclasses and associated cytokines (IL-10 and IL-6) declined following therapy with Miltefosine, the maximum decrease being with IgG3 along with IL-10; importantly, this decrease was sustained for at least six months. In contrast, LAmB failed to decrease the levels of immunoglobulins and associated cytokines even six months after completion of treatment; in fact the antibody levels either increased or remained unchanged. Taken together, this study has established the potential of IgG3 and IL10 as a non-invasive alternative for monitoring of chemotherapeutic responses in PKDL.

Introduction

Leishmaniasis, a vector-borne parasitic disease, caused by at least 20 species of the genus Leishmania accounts for a clinical pleomorphism that ranges from cutaneous/mucocutaneous lesions to a life threatening visceral involvement, as determined by an interplay of parasite characteristics, vector biology and host immune responses [1]. Possibly the most challenging disease form is Post Kala-azar Dermal Leishmaniasis (PKDL), a dermal sequel that occurs in patients with apparently cured Visceral Leishmaniasis (VL) [2]. Periodic peaks of VL in South Asia are considered as the norm [3], and a consensus is that during the inter-epidemic periods, PKDL cases fuel the transmission [4–6]. Owing to the poor treatment seeking behavior of patients with PKDL, they innocously harbor parasites in their skin and serve as ‘mobile disease reservoirs’. This ensures parasite transmission in the community, making PKDL a major impediment to the ongoing South-East Asia Region Kala-azar Elimination Programme [7–9].

With implementation of active case surveillance in West Bengal, India from 2014, a huge number of macular PKDL cases were unearthed, and translated into the conventional ratio of polymorphic: macular change from 9:1 to 1:1 [10]. In particular, the macular type poses a diagnostic dilemma owing to the minimal presence of parasites, and its clinical features being indistinguishable from other hypopigmentary disorders like vitiligo, pityriasis versicolor or leprosy. Accordingly, quantification of parasite load is the sole objective parameter of efficacy, but is logistically difficult to implement as upon completion of treatment, patients are either lost to follow up or are reluctant to provide a repeat skin biopsy especially if the lesions have resolved. Therefore, as the parasite burden cannot be quantified, conducting clinical trials at least in South Asia (that includes India, Bangladesh and Nepal) is difficult, resulting in important gaps
in our knowledge. Therefore, there is a need for developing non-invasive approaches for monitoring of treatment efficacy.

Miltefosine was empirically recommended for 12 weeks for PKDL, and is associated with a 15–20% relapse rate emphasizing the need for monitoring, preferably with molecular tools [11,12], while the treatment regimen for LAmB in PKDL was empirically set at 30 mg/kg b.w. for 3 weeks [11,12]. The chemotherapeutic efficacy of Miltefosine vs. LAmB was assessed by quantifying the parasite load in skin biopsies [13] wherein irrespective of the lesional type, patients following treatment with Miltefosine showed an absence of parasite DNA that was sustained up to at least 6 months; however, with LAmB there was parasite persistence suggesting treatment inadequacy [13,14]. In resource limited settings, quantification of parasite load using nucleic acid based detection methods is not always feasible, endorsing the need to validate alternatives e.g. serological approaches for developing a ‘test of cure’ [15]. Accordingly this study explored the prognostic potential of measuring IgG subclass responses and cytokine levels for monitoring responses.

**Material and methods**

**Ethics statement**

The study was approved by the Institutional Ethics committees of School of Tropical Medicine, Kolkata and IPGME&R, Kolkata, India; written informed consent was obtained from the individual or their legal representative to publish photographic images of the lesions.

**Study population**

This study included PKDL cases (n = 101) sourced from passive surveillance (n = 24), where patients presented at the Dermatology Outpatient Department of School of Tropical Medicine, Calcutta Medical College and Institute of Post Graduate Medical Education & Research, IPGME&R, Kolkata, India (n = 24, 2009–2015), or following active surveillance (n = 77) in VL hyper-endemic districts of West Bengal, e.g. Malda, Dakshin Dinajpur, Murshidabad and Birbhum from 2015–2020 (Fig 1) [10]. Using standard case definitions and defined risk factors e.g. living in an endemic area and having an epidemiological link (past history of VL), they were sub grouped into polymorphic and macular PKDL, with an initial diagnosis based on clinical features, a prior history of VL, rK-39 positivity and were confirmed by ITS-1 PCR and/or Giemsa staining for the presence of Leishman Donovan bodies [16]. None of the patients had any co-infections or pre-existing disease. As controls, 15 healthy volunteers were recruited from endemic and non-endemic areas; their ages were comparable and were seronegative for anti-leishmanial antibodies. However, as the ratio of male: female varied depending on the type of surveillance, a gender-match for healthy controls was not done. After confirmation by ITS-1 PCR, patients were randomly allocated to receive Miltefosine or LAmB and samples collected at disease presentation, immediately on completion of treatment with Miltefosine (for 12 weeks) or LAmB (for 3 weeks), and at the end of six months post-treatment (Fig 1). Among them, serial monitoring was achievable in 18 and 36 cases who received Miltefosine and LAmB respectively (Fig 1). On a lesional basis, serial monitoring was done in polymorphic (n = 34) or macular (n = 20) cases.

**Reagents**

All reagents were obtained from Sigma (St. Louis, MO, USA) except anti-human IgG3-HRP from Invitrogen, (Waltham, MA, USA), ELISA kits for IL-10 and IL6 from Immunotools (Friesoythe, Germany), Protease inhibitors and 2,2’-azino-bis[3-ethylbenzthiazoline-
6-sulfonic acid (ABTS) from Roche Applied Sciences (Penzberg, Germany), bovine serum albumin (BSA) from Himedia (Mumbai, India) and polystyrene coated maxisorp strips (Nunc Immunomodules, Roskilde, Denmark).

Preparation of crude *Leishmania* lysate

Crude antigen (LDA) lysate was prepared from a *L. donovani* strain MHOM/IN/1983/AG83 using log phase promastigotes as previously described [17]. Lysates were stored at -20°C until use, and just prior to use diluted in phosphate buffer (0.02 mol/L, pH 7.8) which served as the coating antigen.

ELISA for total anti-leishmanial Ig, IgG and IgM

LDA was added to polystyrene coated wells (1μg/well/100 μl), incubated overnight at 4°C, and following three washings with PBS supplemented with 0.05% Tween-20 (PBS-T); the nonspecific binding sites were blocked by PBS supplemented with 2% BSA (PBS-BSA). After an overnight incubation at 4°C with plasma (1:500 in PBS-BSA; 100 μl/well), binding was detected using horse radish peroxidase (HRP) conjugated protein-A (1:6000 in PBS-BSA, 100 μl/well) or anti human HRP-IgG (1:15,000 in PBS-BSA, 100 μl/well) or HRP-IgM (1:10,000 in PBS-BSA, 100 μl/well) and incubated at 37°C for 30 min. Binding was detected using ABTS and OD \(405\) nm measured on an ELISA reader (Merilyzer EIAQuant, Meril Life Sciences, India).

Measurement of antileishmanial IgG subclasses

For determination of IgG subclasses, wells coated with LDA were incubated overnight with plasma (1:50 in PBS-BSA), followed by incubation with biotinylated anti human IgG1 (1:1000 in
PBS-BSA) or biotinylated anti human IgG2 (1:15,000 in PBS-BSA) for 30 minutes at 37°C, and binding detected using streptavidin-HRP (1:15,000 in PBS-BSA). For IgG3, plasma (1:10 in PBS-BSA) was incubated overnight at 4°C with anti-human IgG3-HRP (1:500 in PBS-BSA), and binding detected using ABTS (100 μl) as the substrate and absorbances measured at 405 nm.

**Cytokine ELISA**

Levels of cytokines (IL-10, IL-6) were measured using commercially available kits (Immuno-tools Friesoythe, Germany). Briefly, the individual cytokine antibodies were coated and after blocking of nonspecific sites with PBS-BSA, individual cytokine standards/plasma was added followed by biotinylated anti-cytokine antibodies, and binding detected using streptavidin-HRP (1:1000 in PBS-BSA) and TMB (100 μl) as the substrate, followed by stop solution (1M H₂SO₄) and absorbances measured at O.D.405nm. The sensitivity of detection of IL-10 and IL-6 was 9.4 and 6.1 pg/ml respectively.

**Statistical analysis**

Results were expressed as median (Interquartile range, IQR), and data analyzed between groups by Kruskal Wallis test followed by Dunn's multiple comparison test for non-parametric data using GraphPad Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA, USA); p<0.05 was considered as significant.

**Results**

**Study population**

Presently, the distribution of polymorphic and macular PKDL is around 1:1 [10], and was reflected in the study population as at disease presentation included 101 polymorphic and macular cases, (Table 1 and Fig 1). Amongst the polymorphic cases, 20 were from passive surveillance and 34 following active surveillance, whereas in the macular type, majority were by active case detection (n = 43), and only 4 by passive surveillance. The disease duration was comparable between the two clinical forms, being 3[1.1–6] and 2 [1–3] years for polymorphic and macular PKDL respectively. There was a male preponderance in the passive surveillance group, and their lesions were predominantly polymorphic (Table 1).

Following ITS-1 PCR positivity, patients were randomly allocated to receive Miltefosine or LAmB (Fig 1), and as a quantifiable parameter of efficacy, the parasite load was measured. Among the 54 polymorphic cases, 34 were serially monitored, and received Miltefosine

| Clinical Features                  | Patients with PKDL (n = 101) | Healthy controls (n = 15) |
|-----------------------------------|------------------------------|---------------------------|
|                                   | Passive Surveillance (n = 24) | Active Surveillance (n = 77) |                         |
| Age (in years)                    | "29(18–40)                  | "20(14–30)                | "27 (25–35)             |
| Gender (male: female)             | 5:1                         | 4:3                       | 1:1                     |
| History of VL (%)                 | 95                          | 92                        | NA                      |
| Polymorphic: macular              | 20:4                        | 34:43                     | NA                      |
| Interval between cure of VL and onset of PKDL (in years) | "5 (3–9) | "4 (3–8) | NA | |
| Duration of PKDL/ patient delay (in years) | "3(2–8.5) | "2 (1–4) | NA |

*Values are expressed as median (IQR); NA = Not applicable; PKDL = Post Kala-azar Dermal Leishmaniasis; VL = Visceral Leishmaniasis

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The parasite burden at disease presentation was high and declined following treatment with Miltefosine, while LAmB failed to reduce the parasite burden (Fig 1). Cases who returned at any time point six months later demonstrated sustained parasite clearance with Miltefosine (n = 2), whereas with LAmB, (n = 5) the parasite load increased (Fig 1). The remaining 20 polymorphic cases included naïve PKDL cases (n = 4), immediately at end of treatment with Miltefosine (n = 4) or LAmB (n = 1), and >6 months completion of treatment with Miltefosine or LAmB (n = 11, Fig 1). Overall, Miltefosine caused a sharp reduction in parasite load whereas with LAmB, the parasite burden persisted (Fig 1).

Among the 47 macular cases, 20 could be serially monitored following treatment with Miltefosine (n = 4) or LAmB (n = 16). At six months, the parasite load decreased dramatically after treatment with Miltefosine and was sustained. However, LAmB failed to decrease the parasite load and instead, increased or remained unchanged at six months (Fig 1). The remaining 27 macular cases included naïve cases (n = 6), immediately at end of treatment with Miltefosine (n = 6) or LAmB (n = 3), or at any time point 6 months after completion of treatment (n = 12, Fig 1) and the scenario was similar wherein Miltefosine showed complete parasite clearance, whereas LAmB failed to eliminate the parasites (Fig 1).

**Levels of antileishmanial immunoglobulin in PKDL**

The spectrum of anti-leishmanial immunoglobulins included Ig, IgG (and its subclasses, IgG1, IgG2 and IgG3) along with IgM, and levels in healthy non endemic individuals represented the baseline, whose levels of Ig, IgG and IgM were 0.37[0.24–0.39], 0.11[0.09–0.18] and 0.20 [0.14–0.30] respectively (Fig 2A–2Ci); the IgG1, IgG2 and IgG3 levels were 0.20[0.12–0.29], 0.24[0.18–0.33] and 0.17[0.11–0.21] respectively (Fig 2D–2Fi).

PKDL cases with polymorphic features had a higher proportion of anti-leishmanial Ig in polymorphic (n = 38, 0.65[0.42–1.18], p<0.001) than the macular forms (n = 26, 0.59[0.43–0.88], p<0.01, Fig 2Ai) in comparison to healthy controls. Following random allocation to receive Miltefosine or LAmB, polymorphic cases who received Miltefosine showed a significant 2.8 fold decrease in total Ig from 1.07[0.63–1.48] to 0.38[0.26–0.73], p<0.01, whereas in the macular form the decrease was marginal from 0.64[0.57–0.94] to 0.49[0.28–0.78] (Fig 2Aii); with LAmB irrespective of clinical forms were unchanged (Fig 2Aiii).

To delineate the major contributor, IgG and IgM levels were measured, wherein polymorphic and macular cases showed a significant 5.7 fold and 4.3 fold increase in IgG compared to healthy controls, being 0.63[0.42–1.01], p<0.001 and 0.48[0.33–0.82], p<0.001 respectively (Fig 2Bi). Miltefosine decreased IgG in both forms, being 1.00[0.82–1.13] to 0.55[0.31–0.71], p<0.01 and, 0.49 [0.39–1.12] to 0.27 [0.22–0.75] in polymorphic and macular cases respectively (Fig 2Bi). However, in cases who received LAmB, their IgG levels remained unchanged (Fig 2Bii).

With regard to anti-leishmanial IgM, it was significantly increased in polymorphic 0.41 [0.24–0.59], p<0.01 and macular PKDL, 0.34[0.25–0.56], p<0.05, (Fig 2Ci). Akin to IgG, Miltefosine caused a 2.2 fold decrease in total IgM from 1.07[0.63–1.48] to 0.38[0.26–0.73], p<0.01 respectively (Fig 2Bi). Miltefosine decreased IgG in both forms, being 0.81[0.68–1.05] to 0.55[0.31–0.71], p<0.01 and, 0.49 [0.39–1.12] to 0.27 [0.22–0.75] in polymorphic and macular cases respectively (Fig 2Bi). However, in cases who received LAmB, their IgG levels remained unchanged (Fig 2Bii).

In view of the considerable increase in IgG, measurement of IgG subclasses namely IgG1, IgG2 and IgG3 was measured. In polymorphic and macular PKDL, there was a significant elevation of IgG1, being 0.54[0.33–0.75] p<0.001 and 0.44[0.35–0.77], p<0.001 respectively (Fig 2Di). Miltefosine significantly decreased IgG1 in polymorphic cases from 0.51[0.31–0.81] to 0.24[0.17–0.29], p<0.01 and in macular cases from 0.41[0.34–0.60] to 0.22[0.15–0.34], p<0.05 (Fig 2Dii). However, with LAmB, the levels remained unchanged (Fig 2Diii).
**Fig 2.** Impact of chemotherapy upon levels of anti-leishmanial immunoglobulins in patients with PKDL.  

**A:** Effect of treatment upon levels of total antileishmanial Ig in PKDL.

- **i** Scatter plots indicating the median (IQR) of total antileishmanial Ig in patients with polymorphic PKDL (n = 38, ■), macular PKDL (n = 26, □) and healthy controls, HC (n = 15, ●).
- **ii** Bar graphs showing the median (IQR) of total antileishmanial Ig before (pre) and after treatment (t/t) with 12 weeks Miltefosine in patients with polymorphic (Poly, n = 18, □) and macular (Mac, n = 10, ■) PKDL.
- **iii** Bar graphs showing the median (IQR) of total antileishmanial Ig at disease presentation (pre) in patients with polymorphic (Poly, n = 24, □) and macular (Mac, n = 22, ■) PKDL, and after 3 weeks of treatment (t/t) with LamB (Poly, n = 21, □) and (Mac, n = 19, ■).

**B:** Levels of total anti-leishmanial IgG following treatment in PKDL.

- **i** Scatter plots indicating the median (IQR) of anti-leishmanial IgG in patients with polymorphic PKDL (Poly, n = 38, ■), macular PKDL (Mac, n = 26, □) and healthy controls, HC (n = 15, ●).
- **ii** Bar graphs showing the median (IQR) of anti-leishmanial IgG before (pre) and after treatment (t/t) with Miltefosine in patients with polymorphic (Poly, n = 18, □) and macular (Mac, n = 10, ■) PKDL.
- **iii** Bar graphs showing the median (IQR) of anti-leishmanial IgG at disease presentation (pre) in patients with polymorphic (Poly, n = 24, □) and macular (Mac, n = 22, ■) PKDL, and after 3 weeks of treatment (t/t) with LamB (Poly, n = 21, □) and (Mac, n = 19, ■).
Similarly with regard to IgG2, the polymorphic and macular cases demonstrated a significant increase, being 0.54[0.34–0.67], p<0.001 and 0.45[0.31–0.59], p<0.001 (Fig 2Eii) respectively. With Miltefosine, IgG2 decreased significantly in the polymorphic cases from 0.57[0.37–0.65] to 0.29[0.21–0.42], p<0.05, but only marginally in the macular group 0.42[0.31–0.57] to 0.31[0.15–0.38], (Fig 2Eii), while LAmB failed to impact on IgG2 (Fig 2Eiii).

With regard to IgG3, there was a significant elevation in polymorphic, 0.57[0.34–0.78], p<0.001 and macular forms, 0.65[0.40–1.06], p<0.001 (Fig 2Fii). Importantly, Miltefosine significantly curtailed IgG3 in polymorphic, 0.62[0.39–0.86] to 0.20[0.14–0.36], p<0.001 and macular cases, 0.70[0.51–1.13] to 0.34[0.23–0.43], p<0.05, (Fig 2Fii). This was not evident in cases treated with LAmB as IgG3 remained unchanged (Fig 2Fiii).

### Longitudinal monitoring of total Ig, IgG, IgM and IgG subclasses

Following treatment with Miltefosine (n = 18), serial monitoring was performed at three time points (i) presentation (ii) on completion of treatment, and (iii) six months later (wherever possible). On completion of 12 weeks treatment, the total Ig decreased significantly and six months later, there was an additional 1.5 fold decrease (Fig 3Ai and Table A in S1 Table). However, in 36 patients who received LAmB, Ig levels failed to decrease at the end of treatment and importantly, at the end of 6 months, remained high in the majority of cases (Fig 3Aii and Table B in S1 Table).

With regard to IgG, Miltefosine demonstrated a significant 1.6 fold decrease, which six months later decreased further by 3.7 fold (Fig 3Bi and Table A in S1 Table), whereas with LAmB, IgG levels remained unchanged even six months later (Fig 3Bii and Table B in S1 Table). Although Miltefosine caused a significant 3.0 fold decrement in IgM, which by the end of six months decreased a further 1.4 fold (Fig 3Ci and Table A in S1 Table), LAmB failed to alter IgM, even at the end of six months (Fig 3Cii and Table B in S1 Table).
Fig 3. Longitudinal monitoring of anti-leishmanial Ig, IgG and IgM in patients with PKDL treated with Miltefosine or LAmB. A: Serial monitoring of anti-leishmanial Ig. i Before after plots indicating the plasma levels of total Ig at disease presentation (Pre, n = 18, ●), end of treatment with Miltefosine (End of t/t, n = 18, ○) and six months later (6 mo.post t/t, n = 4, △). ii Before after plots indicating the plasma levels of total Ig at disease presentation (pre, n = 36, ●), end of treatment with LAmB (End of t/t, n = 36, ○) and six months later (6 mo.post t/t, n = 9, △). B: Serial monitoring of anti-leishmanial IgG. i Before after plots showing the plasma levels of IgG at disease presentation (Pre, n = 18, ●), end of treatment with Miltefosine (End of t/t, n = 18, ○) and six months later (6 mo.post t/t, n = 4, △). ii Before after plots showing plasma levels of IgG at disease presentation (Pre, n = 36, ●), end of treatment with LAmB (End of t/t, n = 36, ○) and six months later (6 mo.post t/t, n = 9, △). C: Serial monitoring of anti-leishmanial IgM. i Before after plots showing the plasma levels of IgM at disease presentation (Pre, n = 18, ●), end of treatment with Miltefosine (End of t/t, n = 18, ○) and six months later (6 mo.post t/t, n = 4, △). ii Before after plots showing plasma levels of IgM at disease presentation (Pre, n = 36, ●), end of treatment with LAmB (End of t/t, n = 36, ○) and six months later (6 mo.post t/t, n = 9, △). D: Serial monitoring of anti-leishmanial IgG1 i Before after plots indicating the plasma levels of IgG1 at disease presentation (Pre, n = 18, ●), end of treatment with Miltefosine (End of t/t, n = 18, ○)
and six months later (6 mo.post t/t, n = 4, Δ). i) Before after plots indicating the plasma levels of IgG1 at disease presentation (Pre, n = 36, ●), end of treatment with LAmB (End of t/t, n = 36, ○) and six months later (6 mo.post t/t, n = 9, Δ). E: Serial monitoring of anti-leishmanial IgG2. i) Before after plots indicating the plasma levels of IgG2 at disease presentation (Pre, n = 18, ●), end of treatment with Miltefosine (End of t/t, n = 18, ○) and six months later (6 mo.post t/t, n = 4, Δ). ii) Before after plots indicating the plasma levels of IgG2 at disease presentation (Pre, n = 36, ●), end of treatment with LAmB (End of t/t, n = 36, ○) and six months later (6 mo.post t/t, n = 9, Δ).

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Serial monitoring of IgG subclasses was done in patients treated with miltefosine (n = 18) or LAmB (n = 36) wherein IgG1 decreased by 1.5 fold with Miltefosine, and in four patients who were monitored six months later, it decreased further by 2.8 fold (Fig 3Di and Table A in S2 Table). With LAmB, at the end of 3 weeks treatment, the IgG1 levels remained unchanged, whereas at six months post-treatment (n = 9), a significant 1.8 fold decrease was evident (Fig 3Dii and Table B in S2 Table) and was attributed to 6/9 (66%) cases. However in 3/9 (33%) cases, there was an increase in IgG1 levels (Fig 3Dii). Similarly, with regard to IgG2, Miltefosine caused a sustained decrease (Fig 3Ei and Table A in S2 Table), whereas LAmB failed to decrease IgG2 levels, and even six months later, decreased marginally (Fig 3Eii and Table B in S2 Table). With regard to IgG3, Miltefosine caused a significant 3.0 fold decrement, which six months later, decreased an additional 1.4 fold (Fig 3Fi and Table A in S2 Table). However, with LAmB there was no decrease in IgG3 at the end of 3 weeks treatment; importantly, six months later IgG3 increased in 8/9 cases (Fig 3Fii and Table B in S2 Table).

Longitudinal monitoring of IL-10 and IL-6 in PKDL

Cytokines can mediate immunoglobulin class switching with IL-10 mediating the switch to IgG1 and IgG3, whereas IFN-γ with IL-6 can drive production of IgG2 [18]. Accordingly, as IgG1, IgG2 and IgG3 were increased (Fig 2D–2F), levels of IL-10 were measured in patients treated with Miltefosine (n = 12) or LAmB (n = 26). At disease presentation, the IL-10 levels were significantly elevated by 2.7 fold vis-a-vis healthy controls, being 481.5 [251.7–862.4] vs. 174.3 [80.0–205.0] pg/ml, p < 0.001 (Fig 4Ai). In 12 serially monitored cases who received Miltefosine, there was an 11 fold decrease from 875.0 [389.8–1271.0] to 80.0 [0.0–283.6] pg/ml, p < 0.001 (Fig 4Aii), whereas with LAmB, the levels of IL-10 increased from 345.0 [211.0–538.0] to 524.0 [279.0–1104.0] pg/ml. In fact, their levels increased in (15/28, 53%) cases, remained unchanged (2/28, 8%) or decreased (11/28, 39%, Fig 4Aiii).

With regard to IL-6, there was a 3.2 fold elevation in patients with PKDL vs. healthy controls 32.0 [18.1–113.8] vs. 10.0 [8.9–82] pg/ml (Fig 4Bi); with Miltefosine (n = 14), there was a significant 9.4 fold decrease from 94.5 [23.1–158.7] to 10.4 [0.0–92.5] pg/ml, p < 0.05, (Fig 4Bii), whereas with LAmB (n = 28), the IL-6 levels remained unchanged being 28.1 [15.3–57.3] and 38.6 [8.0–103.2] pg/ml. In fact, their levels increased in (15/28, 53%) cases, remained unchanged (2/28, 8%) or decreased (11/28, 39%, Fig 4Biii).

Additionally, the efficacy of IL-10 was confirmed by monitoring levels in randomly selected PKDL cases (n = 38), immediately after treatment with LAmB (n = 26) or Miltefosine (n = 12), and at any time point six months later. In the miltefosine treated group, at time points greater than 6 months t (n = 16), IL-10 was non-detectable in the majority of cases (Fig 5), and correlated with total disappearance of dermal lesions (Fig 6A and 6B) and absence of parasite load (Fig 1). However, the levels of IL-10 in patients who received LAmB when measured at or after six months (n = 15), did not demonstrate a reduction, being 410 [0–876] pg/ml (Fig 5),
and importantly, correlated with clinical features as their dermal lesions persisted (Fig 6C and 6D), and corroborated with a persisting parasite burden (Fig 1).

**Discussion**

Disease progression in human VL has been consistently associated with an abundant production of Th1 (IFN-γ and TNF-α) and Th2 (TGF-β and IL-4) cytokines, with the latter coupled with IL-10 taking the upper hand [19,20]. Similarly in PKDL, a raised mRNA expression of both pro- and anti-inflammatory cytokines has been demonstrated at lesional sites [21–23] along with a raised mRNA expression of counter-regulatory cytokines, TGF-β and IL-10 [21,22,24–26], that collectively supported a pro-parasitic immunosuppressive milieu [27–29].

Although studies have proposed the ability of Th1 and Th2 cells to support B cell responses [30], the latter is generally accepted to be more adept in this respect, as validated by their

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**Fig 4. Levels of circulating cytokines following treatment with Miltefosine or LAmB.**

A: Levels of IL-10. i Scatter plots showing the median (IQR) levels of IL-10 in healthy controls (HC, n = 15, ●) and patients with PKDL (n = 38, ■). ii Before after plots of plasma IL-10 at disease presentation (Pre t/t, n = 12, ●) and end of treatment with Miltefosine (End of t/t, n = 12, o). iii Before after plots of plasma IL-10 at disease presentation (Pre t/t, n = 26, ●), end of treatment with LAmB (End of t/t, n = 26, o).

B: Levels of IL-6. i Scatter plots showing the median (IQR) levels of IL-6 in healthy controls (HC, n = 15, ●) and patients with PKDL (n = 42, ●). ii Before after plots indicating the plasma levels of total IL-6 at disease presentation (Pre t/t, n = 14, ●), end of treatment with Miltefosine (End of t/t, n = 14, o). iii Before after plots indicating the plasma levels of total IL-6 at disease presentation (Pre t/t, n = 28, ●), end of treatment with LAmB (End of t/t, n = 28, o).

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**Fig 5. Kinetics of IL-10 in PKDL.** Scatter plots indicating the median (IQR) of IL-10 in patients with PKDL at disease presentation (Pre, n = 38, ●, 1), end of t/t with LAmB (3 wks post LAmB, n = 26, ■) or Miltefosine (12 wks post Miltefosine, n = 12, ●), six months post t/t with LAmB (>6 mo. post LAmB, n = 15, Δ) or Miltefosine (>6 mo. post Miltefosine, n = 16, □).

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cytokines being key contributors towards B cell proliferation and differentiation [31,32]. Accordingly, in Leishmaniasis, the Th2 predominant microenvironment stimulates B cell activation leading to secretion of IgG and enhanced isotype switching [17,33]. In VL, this paved the way for development of serological assays, with the rk39 ‘rapid diagnostic test’ revolutionizing diagnosis [34]. However, analogous tests for PKDL till date remain limited.

Ideally, monitoring the chemotherapeutic effectiveness of PKDL requires direct detection of parasite or its DNA, the latter being the first choice. However, there are logistic limitations, as once the lesions decline patients are reluctant to provide a repeat skin biopsy. Furthermore,
although PCR is a clear winner [35,36], the associated expenses, availability of sophisticated facilities and trained personnel precludes its applicability in resource limited settings. An excellent alternative would be detection of parasite antigens and has been implemented in VL using urine-based assays. However, its moderate sensitivity [15 and references therein] necessitates that efforts be aimed at developing a non invasive ‘test of cure’, especially for PKDL.

Studies have alluded to the presence of a high proportion of anti-leishmanial antibodies in PKDL [33,37], but their potential in monitoring their treatment efficacy has not been investigated, and was the focus of this study. Anti-leishmanial antibody levels of total Ig, IgG, IgM, IgG1, IgG2 and IgG3 were monitored in polymorphic and macular PKDL, and their raised levels (Fig 2) corroborated with previous studies [33,38,39]. Miltefosine impacted substantially upon all antibody subclasses especially in the polymorphic form, and accounted for the decrease in IgG (Fig 2Aii) as also IgG1, IgG2 and IgG3 (Fig 2D–2F). This was in agreement with the parasite load wherein Miltefosine caused a dramatic decline in parasite load, more so in the polymorphic form [13,14]. Miltefosine mediates its leishmanicidal activity directly via parasite apoptosis [40] and indirectly by its immunomodulatory ability to skew macrophages towards a M2 phenotype, accompanied by a decrease in circulating levels of IL-10, TGF-β and IL-4 [24] which in this study translated into a decrease in the levels of Immunoglobulins (Fig 3). However, it remains an open ended yet pertinent question as to why the macular type vis-a-vis the polymorphic form failed to demonstrate a similar decrease in antibody levels, notable exceptions being IgG1 and IgG3 (Fig 2).

IL-10 is a key cytokine that drives Leishmania infection [41] and based on its propensity to induce naïve slgD⁺ B cells to secrete IgG1 and IgG3 [42], is responsible for the increased levels of IgG1 and IgG3 in PKDL (Fig 1). As Miltefosine is known to decrease IL-10 [24], it translated into lowering of levels of IgG1 and IgG3 (Figs 2–4). A similar scenario was reported in serially monitored patients with CL [43] and American VL [44], endorsing its applicability in monitoring chemotherapeutic responses in PKDL (Fig 3). This study also corroborated the chemotherapeutic superiority of Miltefosine vis-à-vis LAmB in PKDL [13], as antibody levels failed to decrease with LAmB (Fig 3). However, the study by Moulik et al., (2018) [13], had a limited number of samples especially at the time point of 6 months post treatment, owing to an inability to collect skin biopsies following completion of treatment. It is anticipated that a non-invasive approach as applied in this study can circumvent this problem. It can be justifiably argued that following three weeks of LAmB, antibody levels are unlikely to decrease, and measurement at a later time point would be more relevant. Accordingly, antibody levels in patients treated with LAmB were measured at 6 months post-treatment in 9 cases, wherein the IgG3 levels increased in 8/9 cases (Fig 3). Therefore, it is imperative such cases of treatment failure be identified, as they may require additional or alternative treatment, or else will become mobile disease reservoirs, and derail the progress of the ongoing VL elimination programme.

The superiority of Miltefosine over LAmB in PKDL has been reported [45], but the underlying reasons remain to be elucidated. As monocytes/macrophages at lesional sites are critical for delivering LAmB, the degree of infiltration could impact on its lesional concentration as validated in a murine model of CL [46]. Therefore, it may be suggested that in macular cases having a lower proportion of macrophage infiltration [47], translated into a lowered accumulation of LAmB, and contributed towards its reduced therapeutic response.

Levels of plasma IL-10 are raised in human VL [48,49] as also PKDL [25,26,28], and declined following treatment. Additionally, in PKDL, a raised mRNA/protein expression of IL-10 has been demonstrated in skin lesions [26]. Importantly, the significant decline in plasma IL-10 after treatment with Miltefosine indicated its strong association with parasite clearance and resolution of dermal lesions (Figs 4 and 6). Furthermore, the decrease in
antibody levels was most prominent with IgG1 and IgG3, whose switching is mediated by IL-10 [42]. Additionally, in PKDL cases that received LAmB, the unchanged or increased levels of IL-10 (Fig 3A) coincided with their unchanged or increased levels of IgG3 (Fig 3Fii). Studies have reported a positive correlation between IL-10 and parasite load in human VL [50] and lesional tissue of PKDL patients [26]; accordingly, this study endorsed the translational potential of IL-10 and/or IgG3 as biomarkers for monitoring PKDL.

In patients with VL, increased levels of circulating IFNγ regularly accompany progressive infections as also cure, and was reflected in raised IgG2 and IgG4 at disease presentation and cure [17,25,39]. A similar scenario was reported in Disseminated Leishmaniasis wherein high levels of IFNγ were accompanied by an increased IgG2, and importantly, was an effective predictor of disease [51]. At disease presentation, the raised IFNγ reported in human VL [21,52,53], CL [54], and PKDL [21,41] indicates a mixed Th1/Th2 milieu, the latter being predominant [20–22]). Accordingly, as IFNγ drives the levels of IL-6, it accounted for the increase in IL-6 (Fig 3B) along with IgG2 which is dependent on IFNγ and IL-6 [18]. With Miltefosine, the resultant parasite elimination led to a decrease in IFNγ [55], and a concomitant decrease in IgG2 (Figs 2E and 4B). Moreover, the lack of efficacy observed with LAmB reflected in the unchanged or even raised levels of IL-6 and IgG2 (Figs 4B and 3Eii).

This study was an extension of a randomized sampling mode study where suspected cases of PKDL reported at a medical camp, and after confirmation of diagnosis were randomly allocated Miltefosine or LAmB depending on drug availability [13]. In 2010, the prevalence of PKDL was reported to range from 4.4 to 7.8 per 10,000 endemic population, and later declined to 1.1 per 10,000 population in a 2017 study [56 and references therein]. Accordingly, taking a real world, pragmatic approach, sample size calculation was not attempted. Another drawback of this study was irrespective of treatment received, there was a high attrition rate, especially at six months post-treatment (Fig 1), and therefore, no statistical analysis in terms of paired data was attempted at the 6 months time point. Although detection of LD bodies was independently performed anonymously by a pathologist, other laboratory diagnostic tests were not carried out in a blinded fashion, and therefore a potential for bias existed.

The overall management, chemotherapy and control of transmission of Leishmaniasis largely depend on the availability of robust diagnostic tools that can provide early and unequivocal results. The success of the ongoing kala-azar elimination programme requires addressing the last mile challenges that include availability of tools for monitoring therapy and epidemiological surveillance to allow for prompt detection of potential outbreaks. It would therefore be prudent to consider a non-invasive approach such as measurement of circulating IgG3 and IL-10 to identify patients with PKDL who fail to respond to chemotherapy.

Supporting information

S1 Table. Impact of Miltefosine (A) or LAmB (B) upon levels of antileishmanial Ig, IgG and IgM in PKDL.

S2 Table. Monitoring of anti-leishmanial IgG subclasses (IgG1, IgG2 and IgG3) in patients with PKDL following treatment with (A) Miltefosine or (B) LAmB.

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References

1. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. Nat Rev Microbiol. 2011; 9:604–15. https://doi.org/10.1038/nrmicro2608 PMID: 21747351

2. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018; 392:951–970. https://doi.org/10.1016/S0140-6736(18)31204-2 PMID: 30126638

3. Singh OP, Hasker E, Boelaert M, Sundar S. Elimination of visceral leishmaniasis on the Indian subcontinent. Lancet Infect Dis. 2016; 16:e304–e309. https://doi.org/10.1016/S1473-3099(16)30140-2 PMID: 27692643

4. Mondal D, Bern C, Ghosh D, Rashid M, Molina R, Chowdhury R, et al. Quantifying the Infectiousness of Post-Kala-Azar Dermal Leishmaniasis Toward Sand Flies. Clin Infect Dis. 2019; 69:251–258. https://doi.org/10.1093/cid/ciy891 PMID: 30357373

5. Molina R, Ghosh D, Carrión E, Monnerat S, Bern C, Mondal D, et al. Infectivity of Post-Kala-Azar Dermal Leishmaniasis Patients to Sand Flies: Revisiting a Proof of Concept in the Context of the Kala-azar Elimination Program in the Indian Subcontinent. Clin Infect Dis. 2017; 65:150–153. https://doi.org/10.1093/cid/cxx245 PMID: 28520851

6. Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM, el-Hassan AM. Post-kala-azar dermal leishmaniasis. Lancet Infect Dis. 2003; 3:87–98. https://doi.org/10.1016/s1473-3099(03)00517-6 PMID: 12560194

7. World Health Organization, Regional Office for South-East Asia. (2005). Regional strategic framework for elimination of kala azar from the South-East Asia Region (2005–2015). Available at: https://apps.who.int/iris/handle/10665/205825.

8. Hirve S, Kroeger A, Matlashewski G, Mondal D, Banjara MR, Das P, et al. Towards elimination of visceral leishmaniasis in the Indian subcontinent-Translating research to practice to public health. PLoS Negl Trop Dis. 2017 Oct 12; 11:e0005889.https://doi.org/10.1371/journal.pntd.0005889 PMID: 29023446

9. Rijal S, Sundar S, Mondal D, Das P, Alvar J, Boelaert M. Eliminating visceral leishmaniasis in South Asia: the road ahead. BMJ. 2019; 364:k5224. https://doi.org/10.1136/bmj.k5224 PMID: 30670453

10. Sengupta R, Chaudhuri SJ, Moulik S, Ghosh MK, Saha B, Das NK, et al. Active surveillance identified a neglected burden of macular cases of Post Kala-azar Dermal Leishmaniasis in West Bengal. PLoS Negl Trop Dis. 2019; 13:e0007249. https://doi.org/10.1371/journal.pntd.0007249 PMID: 30856178

11. Ramesh V, Singh R, Avishek K, Verma A, Deep DK, Verma S, et al. Decline in Clinical Efficacy of Oral Miltefosine in Treatment of Post-Kala-Azar Dermal Leishmaniasis (PKDL) in India. PLoS Negl Trop Dis. 2015; 9:e0004093. https://doi.org/10.1371/journal.pntd.0004093 PMID: 26492039

12. Ghosh S, Das NK, Mukherjee S, Mukhopadhyay D, Barbhuiya JN, Hazra A, et al. Inadequacy of 12-Week Miltefosine Treatment for Indian Post-Kala-Azar Dermal Leishmaniasis. Am J Trop Med Hyg. 2015; 93:767–9. https://doi.org/10.4269/ajtmh.14-0721 PMID: 26175030
13. Moulik S, Chaudhuri SJ, Sardar B, Ghosh M, Saha B, Das NK, et al. Monitoring of Parasite Kinetics in Indian Post-Kala-azar Dermal Leishmaniasis. Clin Infect Dis. 2018; 66:404–410. https://doi.org/10.1093/cid/cix088 PMID: 29020350

14. Moulik S, Sengupta R, Ghosh MK, Das NK, Saha B, Chatterjee M. Differential efficacy of liposomal amphotericin B in polymorphic versus macular post kala-azar dermal leishmaniasis. Indian J Dermatol Venereol Leprol.2020 https://doi.org/10.105259/jidvl_338_20 Epub ahead of print. PMID: 33969651.

15. Moulik S, Sengupta S, Chatterjee M. Molecular Tracking of the Leishmania Parasite. Front Cell Infect Microbiol. 2021; 11:62343. https://doi.org/10.3389/fcimb.2021.62343 PMID: 33692966

16. Das NK, Singh SK, Ghosh S, Sarkar A, Mukhopadhyay D, Roy S, et al. Case series of misdiagnosis with rK39 strip test in Indian leishmaniasis. Am J Trop Med Hyg 2011; 84:688–91. https://doi.org/10.4269/ajtmh.2011.10-0590 PMID: 21540376

17. Chatterjee M, Basu K, Basu D, Bannerjee D, Pramanik N, Guha SK, et al. Distribution of IgG subclasses in antimicrobial unresponsive Indian kala-azar patients. Clin Exp Immunol. 1998; 114:408–13. https://doi.org/10.1046/j.1365-2249.1998.00752.x PMID: 9844051

18. Kawano Y, Noma T, Kou K, Yoshizawa I, Yata J. Regulation of human IgG subclass production by cytokines: human IgG subclass production enhanced differentially by interleukin-6. Immunol. 1995; 84:278–84. PMID: 7751005

19. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. Front Immunol. 2012; 3:251. https://doi.org/10.3389/fimmu.2012.00251 PMID: 22912637

20. Kumar R, Bhatia M, Pai K. Role of Cytokines in the Pathogenesis of Visceral Leishmaniasis Clin Lab. 2017; 63:1549–1559. https://doi.org/10.7754/Clin.Lab.2017.170404 PMID: 29035452

21. Ansari NA, Ramesh V, Salotra P. Interferon (IFN)-gamma, tumor necrosis factor-alpha, interleukin-6, and IFN-gamma receptor 1 are the major immunological determinants associated with post-kala-azar dermal leishmaniasis. J Infect Dis. 2006; 194:958–65. https://doi.org/10.1086/506624 PMID: 16960784

22. Ganguly S, Mukhopadhyay D, Das NK, Chaudhula M, Sadhu S, Chatterjee U, et al. Enhanced lesional Foxp3 expression and peripheral anergic lymphocytes indicate a role for regulatory T cells in Indian post-kala-azar dermal leishmaniasis. J Invest Dermatol. 2010; 130:1013–22. https://doi.org/10.1038/jid.2009.393 PMID: 20032994

23. Nylén S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol. 2007; 28:378–84. https://doi.org/10.1016/j.it.2007.07.004 PMID: 17689290

24. Mukhopadhyay D, Das NK, Roy S, Kundu S, Barbhuiya JN, Chatterjee M. Miltefosine effectively modulates the cytokine milieu in Indian post kala-azar dermal leishmaniasis. J Infect Dis. 2011; 204:1427–36. https://doi.org/10.1093/infdis/jir551 PMID: 21933878

25. Ganguly S, Das NK, Panja M, Pal S, Modak D, Rahaman M, et al. Increased levels of interleukin-10 and IgG3 are hallmarks of Indian post-kala-azar dermal leishmaniasis. J Infect Dis. 2008; 197:1762–71. https://doi.org/10.1086/588387 PMID: 18444882

26. Katara GK, Ansari NA, Verma S, Ramesh V, Salotra P, Foxp3 and IL-10 expression correlates with parasite burden in lesional tissues of post kala azar dermal leishmaniasis (PKDL) patients. PLoS Negl Trop Dis. 2011; 5:e1171. https://doi.org/10.1371/journal.pntd.0001171 PMID: 21655313

27. Mukhopadhyay D, Mukherjee S, Roy S, Dalton JE, Kundu S, Sarkar A, et al. M2 Polarization of Monocytes-Macrophages Is a Hallmark of Indian Post-Kala-Azar Dermal Leishmaniasis. PLoS Negl Trop Dis. 2015; 9:e0004145. https://doi.org/10.1371/journal.pntd.0004145 PMID: 26496711

28. Mukherjee S, Sengupta R, Mukhopadhyay D, Braun C, Mitra S, Roy S, et al. Impaired activation of lesional CD8+ T-cells is associated with enhanced expression of Programmed Death-1 in Indian Post Kala-azar Dermal Leishmaniasis. Sci Rep. 2019; 9:762. https://doi.org/10.1038/s41598-018-37144-y PMID: 30679687

29. Chatterjee M, Sengupta R, Mukhopadhyay D, Mukherjee S, Dighal A, Moulik S, et al. Immune Responses in Post-Kala-azar Dermal Leishmaniasis. Indian J Dermatol. 2020; 65:452–460. https://doi.org/10.4103/ijd.IJD_258_20 PMID: 33487699

30. DeKruyff R.H., Ju S.T., Hunt A.J., Mosmann T.R., Umetsu D.T. Induction of antigen-specific antibody responses in primed and unprimed B cells. Functional heterogeneity among Th1 and Th2 T cell clones. J. Immunol. 1989; 142:2575. PMID: 2522962

31. Parker DC. T cell-dependent B cell activation. Annu Rev Immunol. 1993; 11:331–60. https://doi.org/10.1146/annurev.iy.11.040193.001555 PMID: 8476565

32. Oracki SA, Walker JA, Hibbs ML, Corcoran LM, Tarlinton DM. Plasma cell development and survival. Immunol Rev. 2010; 237:140–59. https://doi.org/10.1111/j.1600-065X.2010.00940.x PMID: 20727034

33. Mukhopadhyay D, Das NK, De Sarkar S, Manna A, Ganguly DN, Barbhuiya JN, et al. Evaluation of serological markers to monitor the disease status of Indian post kala-azar dermal leishmaniasis. Trans R Soc Trop Med Hyg. 2012; 106:668–76. https://doi.org/10.1016/j.trstmh.2012.07.005 PMID: 22920931
34. Sundar S, Singh RK, Maurya R, Kumar B, Chhabra A, Singh V, et al. Serological diagnosis of Indian visceral leishmaniasis: direct agglutination test versus rK39 strip test. Trans R Soc Trop Med Hyg. 2006; 100:533–7. https://doi.org/10.1016/j.trstmh.2005.08.018 PMID: 16325874

35. Salotra P, Sreenivas G, Pogue GP, Lee N, Nakhasi HL, Ramesh V, et al. Development of a species-specific PCR assay for detection of Leishmania donovani in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. J Clin Microbiol. 2001; 39:849–54. https://doi.org/10.1128/JCM.39.3.849-854.2001 PMID: 11230394

36. Ghosh P, Hasnain MG, Hossain F, Khan MAA, Chowdhury R, Faisal K, et al. Evaluation of Real-time PCR for Diagnosis of Post-Kala-azar Dermal Leishmaniasis in Endemic Foci of Bangladesh. Open Forum Infect Dis. 2018; 5:ofy234. https://doi.org/10.1093/ofid/ofy234 PMID: 30320150

37. Datta S, Modak D, Sarker S, Saha B, Mukhopadhyay S. Identification and glycobiological characterization of circulating immune complexes in patients with visceral leishmaniasis and post kala azar dermal leishmaniasis. Indian J Exp Biol. 2015; 53:321–8. PMID: 26155670

38. Jaiswal P, Datta S, Sardar B, Chaudhuri SJ, Maji D, Ghosh M, et al. Glycoproteins in circulating immune complexes are biomarkers of patients with Indian PKDL: A study from endemic districts of West Bengal, India. PLoS One. 2018; 13:e0192302. https://doi.org/10.1371/journal.pone.0192302 PMID: 29420575

39. Bhattacharyya T, Ayandeh A, Falconar AK, Sundar S, El-Safi S, Gripenberg MA, et al. IgG1 as a potential biomarker of post-chemotherapeutic relapse in visceral leishmaniasis, and adaptation to a rapid diagnostic test. PLoS Negl Trop Dis. 2014; 8:e3273. https://doi.org/10.1371/journal.pntd.0003273 PMID: 25340782

40. Paris C, Loiseau PM, Bories C, Bréard J. Miltefosine induces apoptosis-like death in Leishmania donovani promastigotes. Antimicrob Agents Chemother. 2004; 48:852–9. https://doi.org/10.1128/aac.48.3.852-859.2004 PMID: 14982775

41. Dayakar A, Chandrasekaran S, Kuchipudi SV, Kalangi SK. Cytokines: Key Determinants of Resistance or Disease Progression in Visceral Leishmaniasis: Opportunities for Novel Diagnostics and Immunotherapy. Front Immunol. 2019; 10:670. https://doi.org/10.3389/fimmu.2019.00670 PMID: 31024534

42. Brière F, Servet-Delpret C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. J Exp Med. 1994; 179:757–62. https://doi.org/10.1084/jem.179.2.757 PMID: 8294889

43. Fagundes-Silva GA, Vieira-Gonçalves R, Nepomuceno MP, de Souza MA, Favoreto S Jr, Oliveira-Neto MP, et al. Decrease in anti-Leishmania IgG3 and IgG1 after cutaneous leishmaniasis lesion healing is correlated with the time of clinical cure. Parasite Immunol. 2012; 34:486–91. https://doi.org/10.1111/j.1365-3024.2012.01379.x PMID: 22742527

44. Gomes IT, Carvalho SF, Rocha RD, Peruyerre-Magalhães V, Dietze R, Martins-Filho OA, et al. Anti-Leishmania chagasi immunoglobulin G3 detected by flow cytometry for early cure assessment in American visceral leishmaniasis. J Immunol Methods. 2010; 360:75–83. https://doi.org/10.1016/j.jim.2010.06.011 PMID: 20598707

45. Pandey K, Pal B, Siddiqui NA, Lal CS, Ali V, Bimal S, et al. A randomised, open-label study to evaluate the efficacy and safety of liposomal amphotericin B (Ambisome) versus miltefosine in patients with post-kala-azar dermal leishmaniasis. Indian J Dermatol Venereol Leprol. 2021; 87:34–41. https://doi.org/10.25259/IJDVL_410_19 PMID: 33580944

46. Duthie MS, Guderian J, Vallur A, Bhatia A, Lima dos Santos P, Vieira de Melo E, et al. Alteration of the interleukin 10 and transforming growth factor β (TGFβ) kinetics and efficacy in ambisome treatment of murine cutaneous leishmaniasis. Antimicrob Agents Chemother. 2004; 48:852–9. https://doi.org/10.1128/AAC.48.3.852-859.2004 PMID: 11230394

47. Bhattacharya P, Ghosh S, Ejazi SA, Rahaman M, Pandey K, Ravi Das VN, et al. Induction of IL-10 and TGFβ from CD4+CD25+Foxp3+ T Cells Correlates with Parasite Load in Indian Kala-azar Patients Infected with Leishmania donovani. PLoS Negl Trop Dis. 2016; 10:e0004422. https://doi.org/10.1371/journal.pntd.0004422 PMID: 26829554

48. Costas DL, Rocha RL, Carvalho RM, Lima-Neto AS, Harhay MO, Costa CH, et al. Serum cytokines associated with severity and complications of kala-azar. Pathog Glob Health. 2013; 107:78–87. https://doi.org/10.1179/2047773213Y.0000000078 PMID: 23683334

49. Bhattacharyya T, Ayandeh A, Falconar AK, Sundar S, El-Safi S, Gripenberg MA, et al. IgG1 as a potential biomarker of post-chemotherapeutic relapse in visceral leishmaniasis, and adaptation to a rapid diagnostic test. PLoS Negl Trop Dis. 2014; 8:e3273. https://doi.org/10.1371/journal.pntd.0003273 PMID: 25340782

50. Magalhães A, Carvalho LP, Costa R, Pita MS, Cardoso TM, Machado PRL, et al. Anti-Leishmania IgG is a marker of disseminated leishmaniasis caused by Leishmania braziliensis. Int J Infect Dis. 2021; S1201–9712 00102–8. https://doi.org/10.1016/j.ijid.2021.02.016 PMID: 33578011
52. Dos Santos PL, de Oliveira FA, Santos ML, Cunha LC, Lino MT, de Oliveira MF, et al. The Severity of Visceral Leishmaniasis Correlates with Elevated Levels of Serum IL-6, IL-27 and sCD14. PLoS Negl Trop Dis. 2016; 10:e0004375. https://doi.org/10.1371/journal.pntd.0004375 PMID: 26814478

53. Soni B, Saha B, Singh S. Systems cues governing IL6 signaling in leishmaniasis. Cytokine. 2018; 106:169–175. https://doi.org/10.1016/j.cyto.2017.11.001 PMID: 29128405

54. Bahrami F, Harandi AM, Rafati S. Biomarkers of Cutaneous Leishmaniasis. Front Cell Infect Microbiol. 2018; 8:222. https://doi.org/10.3389/fcimb.2018.00222 PMID: 29998089

55. Souza MA, Castro MC, Oliveira AP, Almeida AF, Reis LC, Silva CJ, et al. American tegumentary leishmaniasis: cytokines and nitric oxide in active disease and after clinical cure, with or without chemotherapy. Scand J Immunol. 2012; 76:175–80. https://doi.org/10.1111/j.1365-3083.2012.02717.x PMID: 22537157

56. Ghosh P, Roy P, Chaudhuri SJ, Das NK. Epidemiology of Post-Kala-azar Dermal Leishmaniasis. Indian J Dermatol. 2021; 66:12–23. https://doi.org/10.4103/ijd.IJD_651_20 PMID: 33911289.