

Phagocytes and the Leishmania Parasite: A Marriage of Convenience

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ABSTRACT

The current drive by the Government of India to eliminate Leishmaniasis has pinpointed post-kala azar dermal leishmaniasis (PKDL) as the strongest contender for the disease reservoir. This emphasizes the necessity to consider the eradication of PKDL as top priority, and hinges on its early diagnosis and management. We undertook this challenge and have provided insights into *Leishmania* biology, by focusing our efforts in (i) delineating the immunopathogenesis of PKDL, a disease unique to South Asia (ii) developing diagnostic/prognostic tools for monitoring antileishmanial treatment in patients with visceral leishmaniasis and PKDL. In order to delineate the immunopathogenesis of PKDL, it was established that the parasite adopts multiple approaches to deviously manipulate host monocytes/macrophages, and thus facilitate parasite survival and disease progression. The parasite adopts a multipronged approach that includes attenuation of the oxidative burst within phagocytes, polarization of monocytes/ macrophages towards alternate activation, enhancement of CD8 T-cell exhaustion and a decreased presence of Langerhans cells. Identification of these immunological changes have allowed for development of biomarkers that have been exploited to develop diagnostic and prognostic markers for monitoring the disease progression, either in terms of antibody based markers, or quantification of the parasite load, the latter being the most definitive approach. Measurement of parasite load has proved to be an effective tool for monitoring the effectiveness of chemotherapy. Taken together, the identification of biomarkers and new chemotherapeutic modalities has helped in improved management and potential elimination of leishmaniasis.

Keywords: Leishmania biology, post-kala azar dermal leishmaniasis (PKDL), visceral leishmaniasis, Th-associated cytokines, Toll-like receptors, antimonials, miltefosine.

Leishmaniasis caused by the digenetic parasite *Leishmania* is a diverse group of neglected tropical diseases of poverty, that ranges from a self limiting cutaneous lesion to a fatal visceral form called kala-azar, and has a dermal sequel called post-kala azar dermal leishmaniasis (PKDL) (1). Its relevance to public health in India lies in the fact that over

60% of the world's cases of visceral leishmaniasis (VL) are reported from three countries, namely India, Nepal and Bangladesh, with an estimated 150 million people being at risk of VL in 109 districts (2). In India, the states of Bihar and adjoining areas of West Bengal, Jharkhand and Uttar Pradesh account for India's burden of VL.

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In PKDL, the dermal sequel of VL, where *Leishmania* parasites remain restricted to the skin, manifest with nodular, papular, hypopigmented macular lesions, erythematous plaques and/or a mixed phenotype, termed as polymorphic (1-3) or a diffuse hypopigmentation considered as the macular variant. The etiopathogenesis of PKDL is still unclear and there is yet to emerge a consensus regarding possible causes for the generally viscerotropic *L. donovani* parasite to become dermatropic (4). An important limitation in PKDL is the absence of an animal model and therefore, information is derived solely from human studies, and understandably remains limited. In PKDL, similar to other leishmaniasis, *Leishmania* have developed several strategies to outmanoeuvre host immunity by subverting and/or suppressing macrophage microbicidal activities, thereby sustaining chronic infection (5).

A pivotal pathogenic event in Leishmaniasis is harboring of the causative *Leishmania* parasite within phagolysosomes of macrophages. To achieve this, the parasite deviously initiates mechanisms to modulate the macrophage microbicidal machinery (5). Additionally, as macrophages are sentinels of the immune system, establishment of infection critically hinges on the parasite's ability to modulate the hosts signaling systems, the end point being immunosuppression. Accordingly, the therapeutic armamentarium against *Leishmania* albeit limited, includes compounds that are directly parasitocidal and/or indirectly immunomodulatory with notable examples being antimonials, miltefosine, amphotericin B, and paromomycin (6).

It is universally accepted that monocytes-macrophages play a range of fundamental biological roles being inducers, regulators and effectors of innate and acquired immunity. Upon stimulation with Th1-associated cytokines, notably IFN γ , they acquire a heightened effector function against intracellular pathogens, referred to as a

classically activated or M1 phenotype. Conversely, in the milieu of Th2 associated cytokines e.g. IL-4, IL-13, IL-33, TGF- β and IL-10 or by microbial triggers, M2 polarization or alternative activation occurs (7). As M2 polarization requires a milieu comprising IL-4, IL-10 and IL-13, levels of these cytokines were estimated in patients with PKDL. The levels of IL-4 were significantly raised as compared to healthy individuals as was IL-10 and IL-13. Treatment caused a significant decrease in IL-10 reiterating its importance in leishmaniasis (8).

These M1 and M2 monocytes/macrophages are differentiated by cardinal genes regulated by inducible nitric oxide synthase (*iNOS*), arginase 1 (*ARG1*), mannose receptor (*CD206*) and *Fizz1* (9, 10). M2 macrophages can impede protective immunity to protozoan infection. In general, understanding of the phenotypic and functional complexity of M2 monocytes-macrophages is limited by a conspicuous discordance between data derived from murine vs. human systems (11). Unlike classically activated macrophages, where human and murine cells respond similarly, the molecular phenotype of alternatively activated macrophages in mice and humans have to date shown a limited overlap (12). Accordingly, we delineated the activation status of monocytes in peripheral blood and dermal macrophages of patients with PKDL, thus providing the first characterization of M2 polarized macrophages in human dermal leishmaniasis.

In PKDL, as compared to healthy controls, the frequency of CD14⁺ monocytes expressing TLR-2⁺ and TLR-4⁺ was significantly reduced which was restored following treatment (Fig. 1A & B) (8). As attenuation of the oxidative burst, secondary to reduced phosphorylation of MAPKs occurred through the TLR-2 pathway or the CD40 signalosome, it was proposed that in PKDL, the decreased expression of TLR-2/4 translated into an impaired MAPK signalling, resulting in the intramonocytic redox imbalance tilting towards

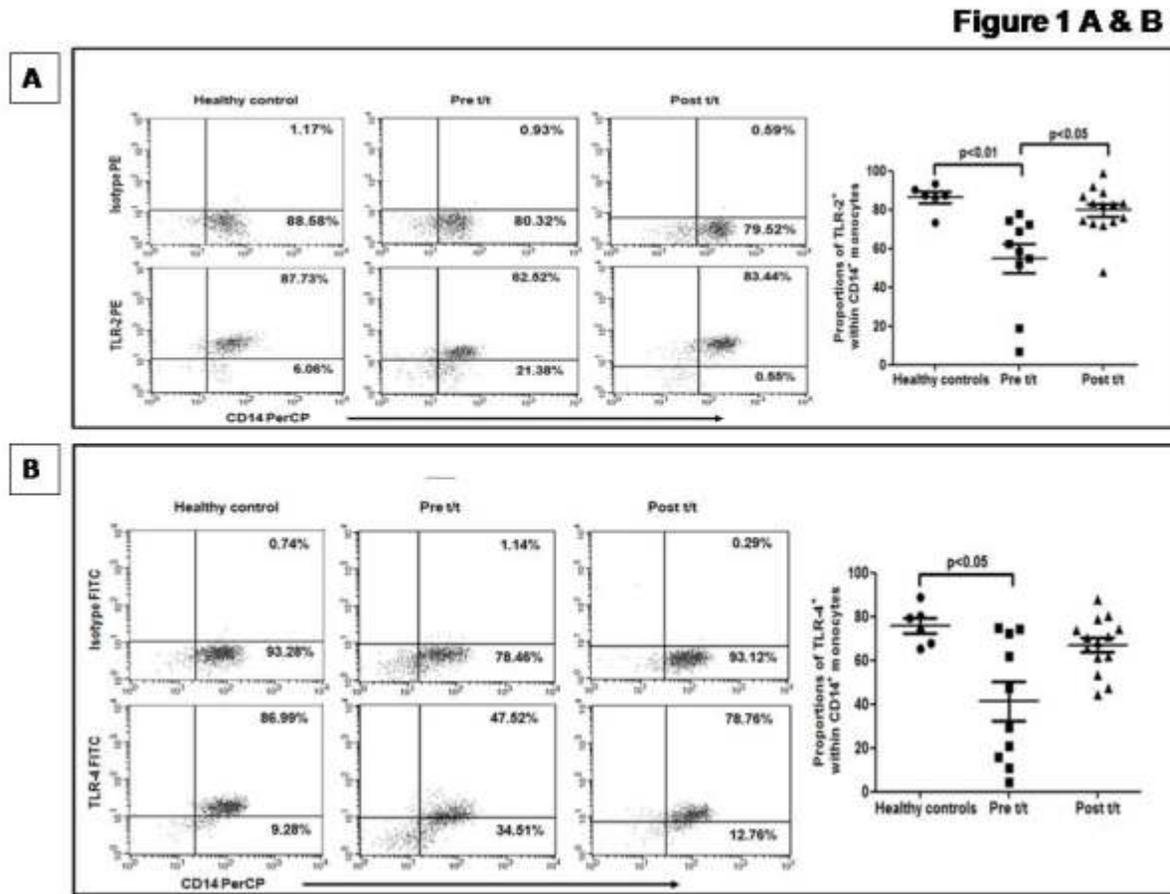


Fig. 1: Decreased expression of TLR-2 and -4 and altered redox status within monocytes
A-B: Representative data showing expression of TLR-2 (A) and TLR-4 (B), within CD14⁺ monocytes in a healthy control, a patient with PKDL pre (Pre t/t) and post treatment (Post t/t). Isotype control staining is also shown. *Scatter plots showing frequency of CD14⁺ monocytes expressing TLR-2 or TLR-4 in healthy controls (●), patients with PKDL (Pre t/t, ■) and post treatment (Post t/t, ▲). *The proportion of CD14⁺TLR-2⁺ and CD14⁺TLR-4⁺ monocytes was calculated by dividing the percentages of upper right quadrant with the sum of upper and lower right quadrant.

an anti-inflammatory milieu (Fig. 2) (13). Alongside, the *ex-vivo* levels of NO in monocytes from PKDL patients was significantly diminished as compared to controls and following treatment, monocytes regained their ability to generate NO (Fig. 3A). Similarly, the generation of ROS was significantly attenuated at presentation *vis-a-vis* controls and treatment increased fluorescence, but remained lower than controls (Fig. 3B). These variations in the anti-oxidant status impacted on the redox

balance and impaired macrophage host defence functions, facilitating parasite survival (8).

Monocytes can differentiate into inflammatory or anti-inflammatory subsets, but their classification in relation to functional phenotypes remains to be precisely defined. Three subsets of blood monocytes, namely classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and non-classical (CD14⁺CD16⁺⁺) have been described with

Figure 2

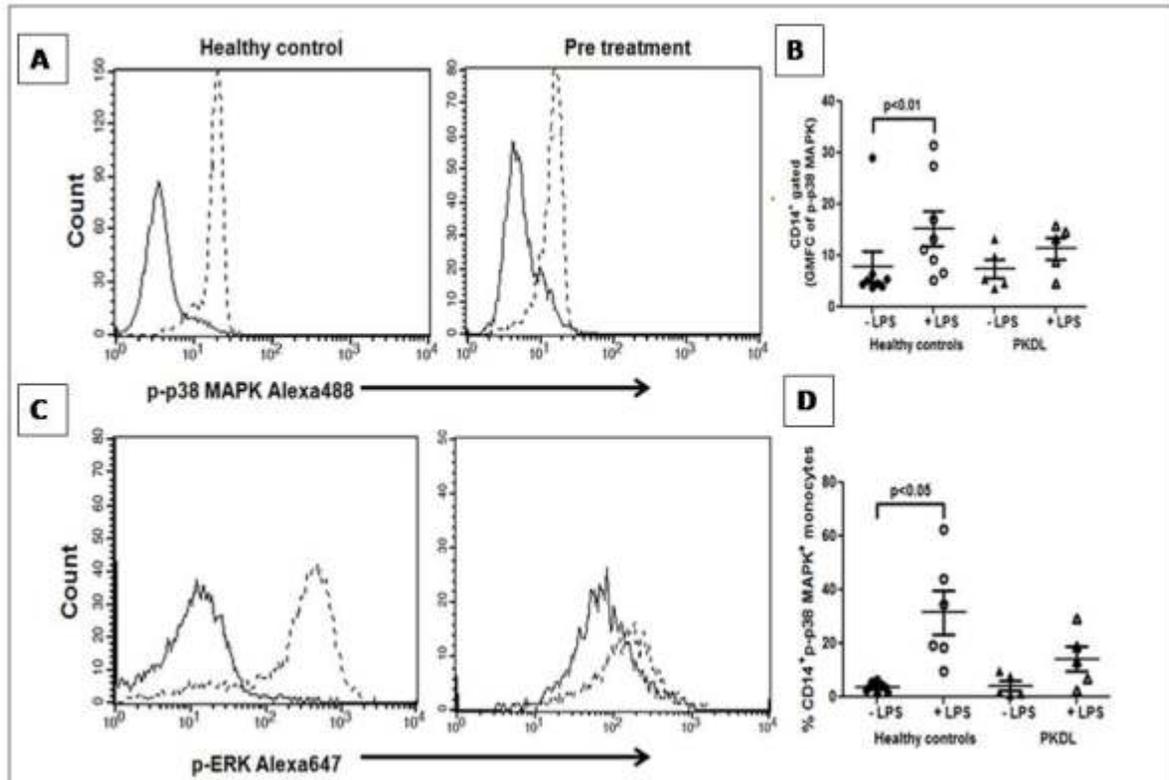


Fig. 2: Intramonocytic phosphorylation status of p38 MAPK and ERK

A. and C. Representative profile showing baseline (—) and LPS-stimulated (---) expression of p-p38 MAPK (A) and p-ERK (C) in monocytes from a healthy control and a patient with PKDL.

B and D. Scatter plots showing baseline and LPS-stimulated expression of p-p38 MAPK (B) and p-ERK (D) in healthy controls (●, ○) and patients with PKDL (▲, △).

discrete functions (14). In PKDL, at disease presentation, there was minimal decrease in classical (CD14⁺⁺CD16⁻) monocytes whereas there was an increase in the intermediate variant (CD14⁺⁺CD16⁺) and non-classical monocytes.

In mouse monocytes/macrophages, the intricate network of signalling molecules, associated transcription factors along with post transcriptional regulators mediating the different forms of activation are well delineated. IL-4 and IL-13 via STAT6 activation are known to skew the macrophage function towards the M2 phenotype leading to transcription of genes typical of M2 polarization, notably Mannose

receptor (*Mrc1*), Arginase (*Arg1*), PPAR and *Fizz1* among others (7). As decreased generation of reactive oxygen and nitrogen radicals suggested alternative activation (9, 10), monocytes from PKDL patients were examined for a M2 phenotype using a panel of robust markers. In circulating monocytes from PKDL cases, the mRNA expression of nuclear receptor *PPARG* which regulates oxidative metabolism in macrophages was increased ~50-fold, as was mRNA expression of *ARG1*. Confocal immunofluorescence confirmed localisation of arginase-1 within CD68⁺ macrophages, which decreased post-treatment (Fig. 4A). The lesional 13.9 fold increase in *CD206* mRNA reinforced

Figure 3 A & B

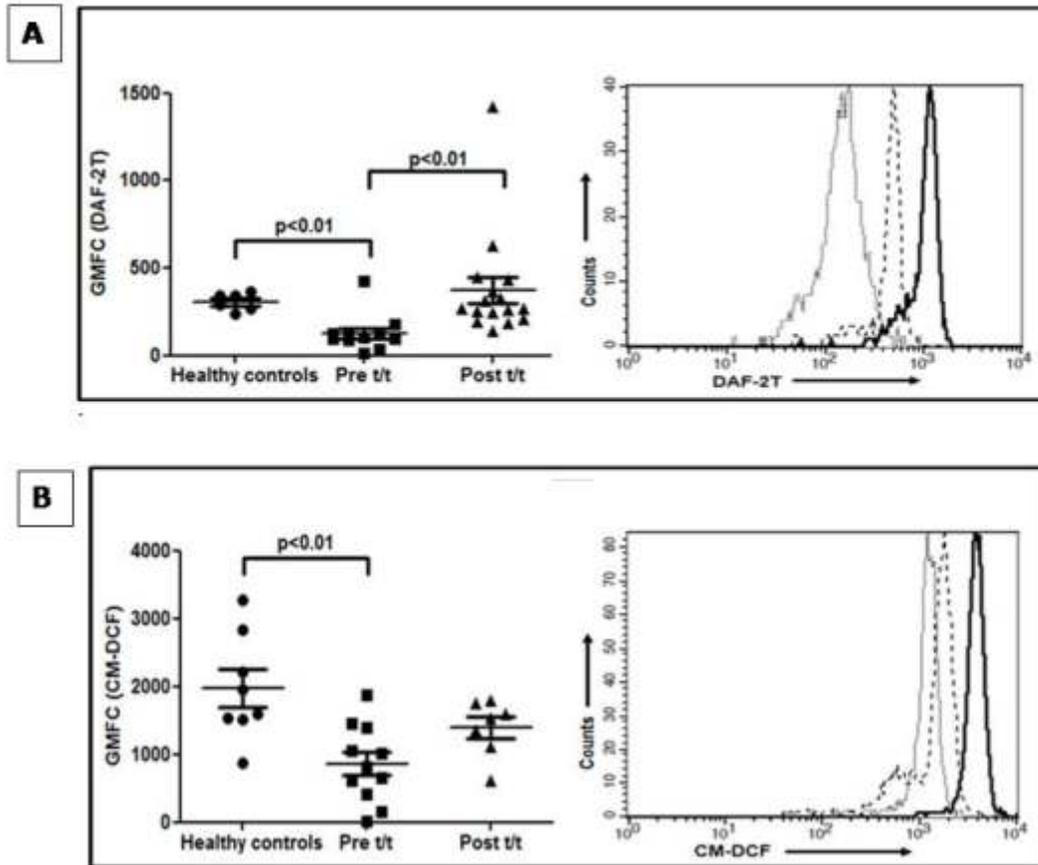


Fig. 3: Production of reactive oxygen-/nitro-free radicals in circulating monocytes in healthy and patients with PKDL

A: Scatter plots showing intracellular NO in circulating monocytes of healthy controls (●), patients with PKDL (Pre t/t, ■) and post treatment (Post t/t, ▲).

Representative histogram overlay showing DAF-2T fluorescence in monocytes from a healthy control (—), patient with PKDL (···) and on completion of treatment (---).

B: Scatter plots of intramonocytic generation of ROS as for C above. A representative histogram overlay showing CM-DCF fluorescence, as for D above.

the M2 polarized status (Fig. 4B) and was mirrored by raised protein expression evident via confocal microscopy (8).

As vitamin D receptor signaling has been linked to M2 polarization and generation of antimicrobial peptides (15), this pathway was examined as it may underlie the systemic and local M2 polarization in PKDL. Plasma $1\alpha,25$ -dihydroxyvitamin D_3 (1,25D3) was significantly raised during PKDL and in keeping with the

elevated plasma levels of 1,25D3, an increase in VDR, *CYP27B1* and LL-37 mRNA accumulation endorsed the upregulation of the vitamin D-related pathways.

In our efforts to delineate mechanisms that promote immunopathology, we evaluated the contribution of T-cells in PKDL by immunophenotyping in peripheral blood for T cells (CD3), T helper cells (CD3/CD4), cytotoxic T cells (CD3/CD8), NK cells (CD56),

Figure 4 A & B

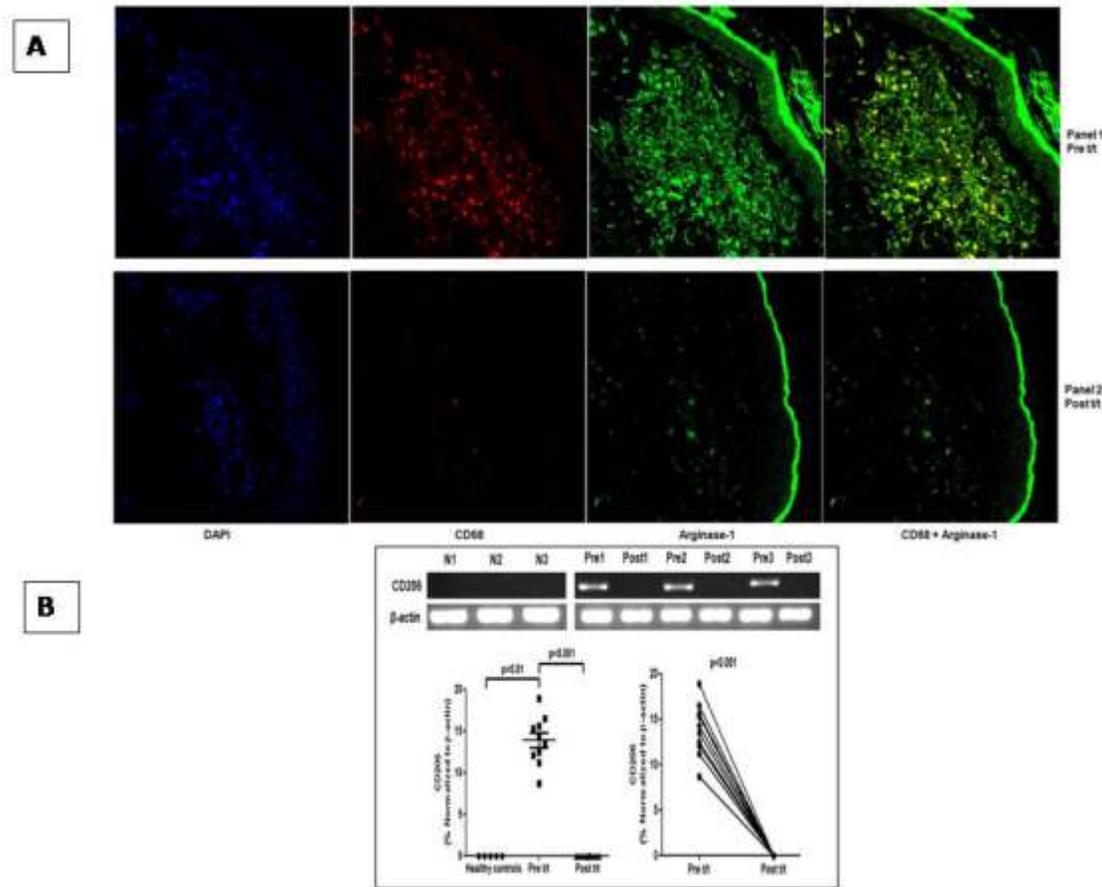


Fig. 4: Lesional macrophages showed a raised expression of PPAR- γ , arginase-1 and mannose receptor

A: Expression of arginase-1 (green, panel 1 and 2 third from left) in CD68⁺ macrophages (red, panel 1 and 2 second from left) at the lesional site of a patient with PKDL pre (Pre t/t) and post treatment (Post t/t). Nuclei are shown in blue (DAPI, panel 1 and 2, left most). Co-localization of macrophage and arginase-1 was shown in right most figure in each panel where co-localization of red and green appears as yellow. Figures were captured in 400X magnification.

B: Representative mRNA expression profile of CD206 in dermal lesions of healthy controls (N 1-3), and patients with PKDL pre (Pre 1-3) and post (Post 1-3) treatment; scatter plots are as in A-B, above.

NKT cells (CD3/CD56), T regulatory cells (CD4/CD25), B cells (CD19) and monocytes (CD14). In PKDL patients, both before and after treatment, the percentages of T lymphocytes, T helper cells, NK cells, NK T cells, T regulatory cells, B lymphocytes and monocytes were comparable with normal individuals (16). The only change reported from peripheral blood in PKDL patients was a small but significant

increase in the percentage of CD3/CD8 lymphocytes as compared to healthy controls which was retained even after treatment (16). Their responses to phytohemagglutinin was no different from healthy controls as the percentages of IFN- γ , IL-2, IL-4 and IL-10 expressing lymphocytes was comparable to controls, and was in contrast to VL patients who demonstrated reduced proportions of IFN- γ and

IL-2 expressing cells, along with an increase in IL-10 positive cells (16). The important difference in their T cell responses was in their response to *L. donovani* antigen as PKDL patients showed an 8 fold increase in the percentage of IL-10 expressing CD3/CD8 lymphocytes as compared to controls which decreased with treatment and accordingly, it was proposed that IL-10 producing CD3/CD8 lymphocytes are important protagonists in the immunopathogenesis of Indian PKDL (16).

Furthermore, our group reported that in patients with PKDL, there is an increased proportion of circulating CD8⁺CD28⁻ and antigen-induced IL-10⁺CD3⁺ lymphocytes which receded with treatment (17). Importantly, these CD8⁺ lymphocytes demonstrated impaired proliferative responses to *Leishmania donovani* antigen and phytohemagglutinin, and were reinstated following treatment. At presentation, the upregulated lesional IFN- γ and IL-10 mRNA, Foxp3 mRNA and protein was curtailed following treatment. Accordingly, it was proposed that the increased frequency of the CD8⁺CD28⁻ phenotype, enhanced antigen-specific IL-10 production and accompanying anergy of circulating lymphocytes suggested their regulatory nature. This was endorsed by the elevated lesional expression of Foxp3 and their recruitment into the lesional site sustained disease pathology (17).

In peripheral blood, the functional status of CD8⁺ T-cells was examined in terms of activation markers, CD127 and CD69 along with markers of cytotoxicity, perforin and granzyme. During active disease, the frequency of CD127 within CD8⁺ T-cells was significantly decreased as compared to healthy controls and treatment translated into a significant increase (Chatterjee M, personal communication). However, the frequency of CD69, perforin and granzyme of CD8⁺ T-cells remained unchanged, and was comparable with healthy controls suggesting that CD8⁺ T-cells in peripheral blood remain immunocompetent. However, in dermal lesions, there was a conspicuous absence of

CD4⁺ T-cells whereas there was an enhanced infiltration of CD8⁺ T-cells. Importantly, these CD8⁺ T-cells demonstrated an absence of perforin, granzyme and Zap-70. Concomitantly, dermal lesions showed an enhanced expression of PD-1 which suggested exhaustion of CD8⁺ T-cells. In addition, lesions demonstrated an increased proportion of CD8⁺CCR4⁺ T-cells and CCL17/CCL22 and it was proposed that dermal homing of anergic/exhausted CD8⁺ T-cells was a feature of Indian PKDL (Chatterjee M, personal communication).

In the skin, dendritic cells (DCs) are the key immune sentinels, via their ability to respond to microbial signals and by subsequently activating naïve T-cells, play a pivotal role in initiating antimicrobial immunity (18). Accordingly, we delineated the lesional immunopathology in terms of tissue macrophages (M \emptyset), Langerhans cells, granuloma formation along with mRNA expression of IL-12p40 and IL-10. We collected lesional punch biopsies serially at disease presentation and completion of treatment. Immunohistochemical analysis was performed for M \emptyset (CD68), while dermal dendritic cells (dDCs) and Langerhans cells (LCs) were identified using CD1a, CD207 and HLA-DR.

Despite the absence of a mature granuloma, there was heavy infiltration of CD68⁺ M \emptyset *vis-a-vis* healthy controls, which decreased with treatment (Fig. 5A). DCs in the epidermis were significantly decreased in PKDL when compared with healthy donors, and were morphologically altered, as demonstrated by loss of elongated cellular protrusions (Fig. 5B). These LCs did not migrate to the dermis as 6/9 patients showed a near total absence of dDCs (Fig. 5C), which remained unchanged with treatment (Fig. 5C) (19). The lesional mRNA expression of IL-10 during active disease was significantly upregulated as compared to healthy individuals and strengthened our proposition that in PKDL, this decrease in LCs along with increased IL-10 caused immune inactivation that allowed for disease sustenance,

Figure 5 A-C

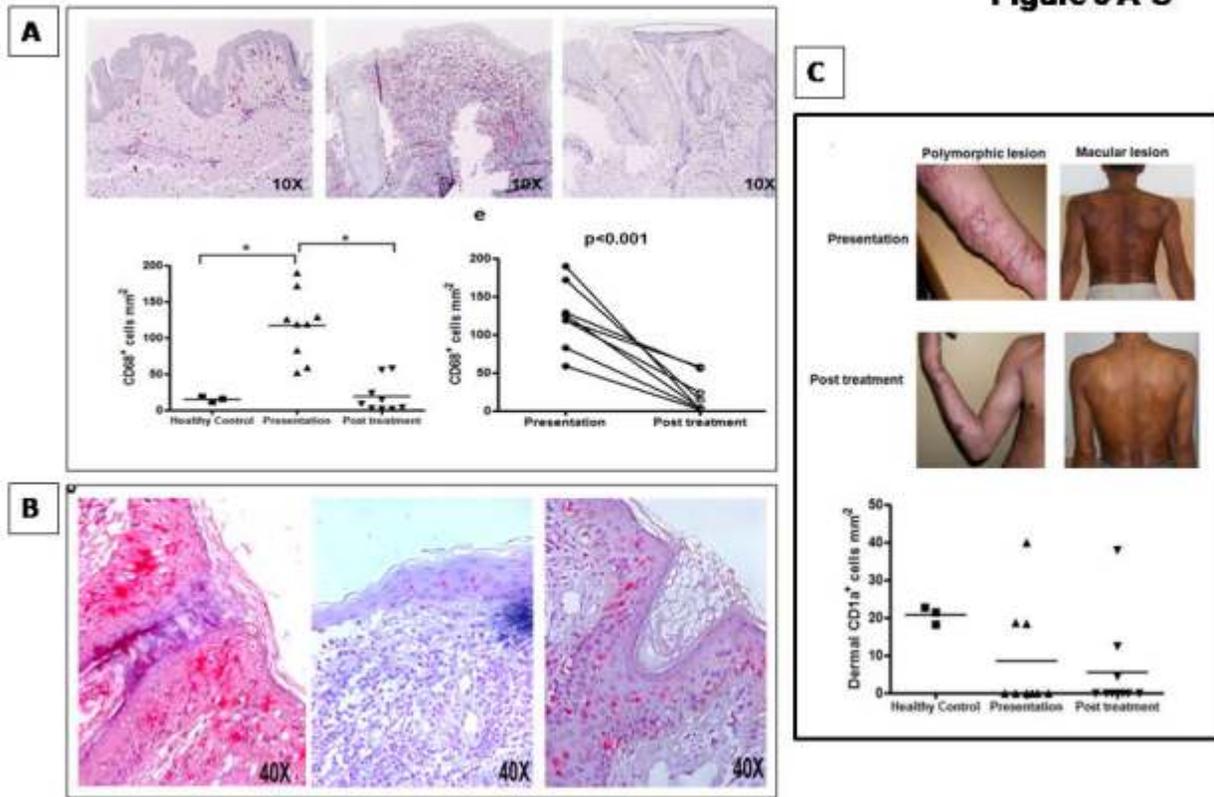


Fig. 5: Dermal infiltration in patients with PKDL

A: Representative IHC profile of CD68⁺ cells in the dermal biopsy of a healthy control, a patient with PKDL at disease presentation and post treatment (magnification, 10X objective).

Comparison of CD68⁺ cells of healthy controls (■), patients with PKDL at disease presentation (▲) and post treatment (▼), *p<0.05. Each horizontal bar represents the median value.

B: Representative IHC profile showing distribution of CD207⁺ cells in the dermal biopsy of a healthy control, patient with PKDL at disease presentation and post treatment under (magnification, 40X objective). Representative IHC profile showing distribution of CD1a⁺ cells in the dermal biopsy of a healthy control, patient with PKDL at disease presentation and post treatment (magnification, 10X and 40X objectives).

C: Distribution of CD1a⁺ cells in the epidermal and dermal compartment respectively of healthy controls (■), patients with PKDL at disease presentation (▲) and post treatment (▼), *p<0.05. Each horizontal bar represents the median value.

emphasizing the importance of immunomodulation as a chemotherapeutic strategy against leishmaniasis.

This increasing incidence of unresponsiveness to antimonials (20) led to an invaluable spin off, as it saw the introduction of miltefosine, the first orally effective anti-leishmanial drug (21) along with amphotericin B

(22). Furthermore, in the backdrop of a strong political and administrative commitment the governments of India, Nepal, and Bangladesh, in collaboration with World Health Organization (WHO) and later joined by Bhutan and Thailand, developed a strategic framework to eliminate visceral leishmaniasis as a public health problem (23,24). The programme has three phases: attack (bring down cases to below 1/10,000 by 2017),

consolidation (case levels below 1/10,000 for three years, from 2017–2019), and maintenance (case levels below 1/10,000 beyond 2020) (http://www.who.int/neglected_diseases/London_Declaration_NTDs.pdf). Fortunately, it seems achievable as 2015 was the third year in a row that Nepal has been consistently below that target (25, 26), and in Bangladesh, only a few districts (*upazilas*) remain above the target (26). The Indian burden persists possibly attributable to the higher prevalence of the disease at the beginning.

The focused efforts in implementation of the VL elimination programme translated into a considerable reduction in the incidence by over 75% in the Indian subcontinent. However, in 2015, 90 of the 456 endemic blocks (20%) in India continue to remain endemic for VL and more importantly, new foci have emerged in non-endemic regions (26, 27). Additionally, a neglected component of the elimination programme has been PKDL, which is possibly the most intriguing clinically and scientifically, as it generally develops after apparent successful cure from VL (28, 29). PKDL is confined to South Asia and East Africa, mainly Sudan (1, 30), wherein the Sudanese variant presents with papular or nodular lesions while in South Asia, the polymorphic variant (co-existence of macules/patches along with papulo-nodules) is more prevalent. Although mortality from PKDL is minimal, it is a stigmatizing disease that carries a huge socio-economic burden, further amplified by a reluctance to obtain treatment or due to non compliance. Lesions, especially the papulo-nodules are parasite-rich, fuelling speculation that PKDL plays a pivotal role in the inter-epidemic transmission of VL and in South Asia where VL is anthroponotic, patients with PKDL are the strongest contenders to be the disease reservoir (31). Accordingly, PKDL has been recognised as a major barrier to the current VL elimination efforts and its elimination is now an essential component of the elimination programme currently targeted for 2020 (1).

Two critical factors that contributed

towards the reduction of VL cases were the prompt diagnosis by the rK-39 strip test along with the single dose treatment with liposomal amphotericin B, (LAmB) which revolutionized the chemotherapy of VL. However, the management of PKDL remained neglected by lack of a definitive test, as rK39 positivity can be attributed to a past infection with VL. Ideally, the demonstration of parasites in smears, culture or by PCR is logistically not feasible, but an even bigger hurdle is to motivate patients with PKDL to actively seek treatment, as the disease has practically no mortality and seeking treatment would be purely for cosmetic reasons. This has translated into a conspicuous absence of clinical trials. The treatment for PKDL is prolonged and as there is always a lurking danger of drug resistance, modalities to monitor the parasite load was the challenge we undertook. A treatment regimen for LAmB in PKDL was empirically set at 30 mg/kg b.w. for three weeks, we developed quantifiable approaches for measuring the parasite burden not merely at disease presentation but at the end of the treatment.

Our study population included 184 patients clinically diagnosed with PKDL whom we recruited via two types of surveillance (32) namely (i) *Passive surveillance* where patients presented at the dermatology outpatient departments of School of Tropical Medicine/Calcutta Medical College/Institute of PG Medical Education & Research, Kolkata, West Bengal or (ii) *Active surveillance* via field surveys conducted in endemic districts of West Bengal (Malda, Dakshin Dinajpur, Murshidabad and Birbhum) by a camp approach. This involved a house-to-house survey conducted by Kala azar technical supervisors using standard case definitions and defined risk factors e.g. living in an endemic area and having an epidemiological link (past history of VL). Subsequently, these cases were examined at medical camps wherein cases with hypopigmented macules were considered as macular PKDL, whereas an assortment of papules, nodules, macules, and/or plaques was

termed as polymorphic PKDL (29).

Irrespective of the type of surveillance, diagnosis of PKDL was confirmed by the rK39 strip test from blood along with an ITS-1 PCR (33). After confirmation by ITS-1 PCR, cases were randomly allocated to receive miltefosine (50 mg p.o. twice daily for 12 weeks) or LAmB (5 mg/kg body IV, twice weekly, for 3 weeks) as per recommended guidelines. Peripheral blood and a 4 mm skin biopsy were collected at three time points, namely disease presentation, on completion of treatment and six months later.

The clinical outcome was assessed by a dermatologist and they were considered cured based on total regression of papules/nodules, no new lesion(s) and considerable regression of macular lesions.

For measurement of parasite load, a standard curve was generated by adding a defined number of *Leishmania* parasites sourced from a *L. donovani* strain (ranging from 10 to 1×10^5) to blood (180 μ l) from a healthy control (34) and DNA was eluted in 50 μ L of DNA elution buffer. Real-time PCR was performed using

Figure 6 A-C

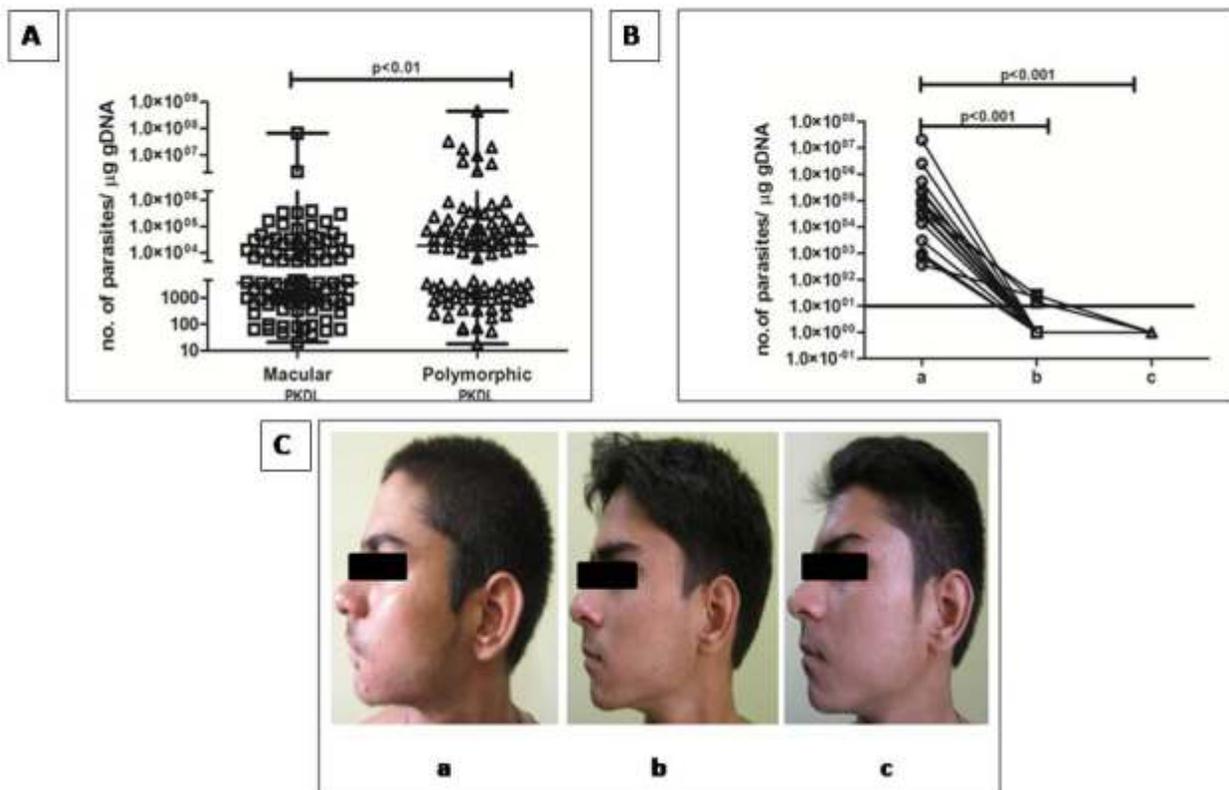


Fig. 6: Status of parasite load in patients with PKDL

A: Scatter plot showing the parasite load as median (range) in patients with PKDL on a lesional basis as macular (■, n=91) or polymorphic (▲, n=93).

B: Before after plots indicating the parasite load at disease presentation (●, n = 19, a), end of treatment with Miltefosine (■, n = 19, b) and six months later (▲, n = 6, c). The minimum detectable parasite number is indicated by a horizontal line.

C: Clinical features of a polymorphic PKDL case at disease presentation (a), following completion of treatment with Miltefosine (b), and six months later (c).

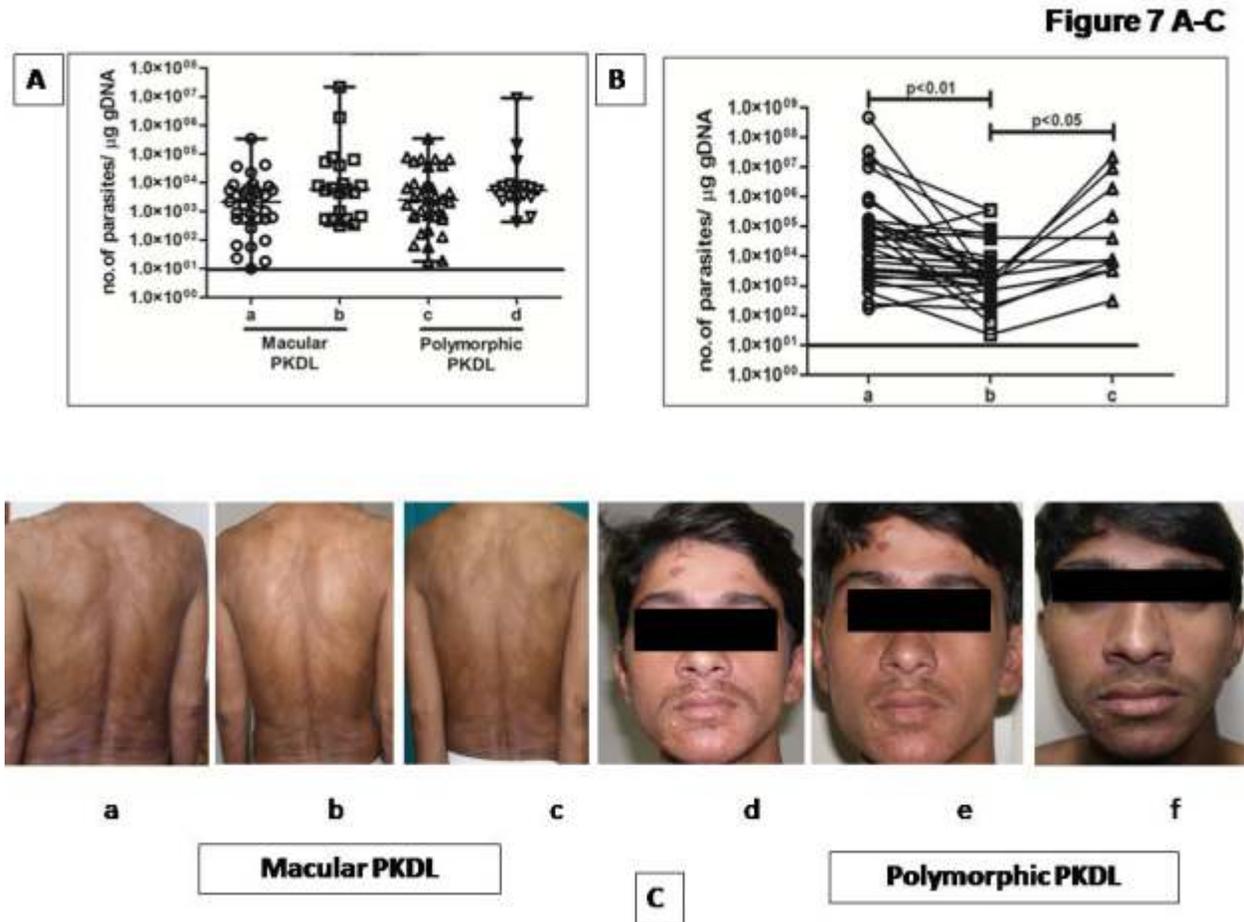


Fig. 7: Effect of three-week treatment with LAmB on median parasite load, macular and polymorphic lesions profile

A: Scatter plot indicating the median parasite load upon completion of three weeks treatment with LAmB presenting with macular lesions (●, n = 34, a) or polymorphic (▲, n = 36, c). They were again evaluated six months later and grouped based on their lesional profile, being macular (■, n= 21, b) or polymorphic (▼, n = 17, d). The minimum detectable parasite number is indicated by a horizontal line.

B: Before after plots indicating the parasite load at disease presentation (●, n = 31, a), end of treatment with LAmB (■, n = 31, b) and six months later (▲, n = 11, c). The minimum detectable parasite number is indicated by a horizontal line.

C: Representative clinical features of two PKDL patients, of macular and polymorphic variant at disease presentation (a, d), following completion of treatment with LAmB (b, e), and their evaluation six months later (c, f).

specific primers for minicircle kDNA; the number of parasites was extrapolated from the standard curve and final parasite load stated as the number/µg genomic DNA. The parasite number when <10 reported a C_t value almost equivalent to NTC, and was accorded an arbitrary value of 1. Upon initial rK39 positivity, the ITS-1 PCR was performed and when positive

(n=184), the parasite burden was quantified by qPCR in macular (n=91) and polymorphic (n=93) PKDL. Their median (IQR) at disease presentation was 5229(896-50898). On examination on a lesional basis, the macular variant had a 5.1 fold lower parasite load as compared to the polymorphic cases being 3665 (615-21528) vs. 18620(1266-93934), p<0.01

(Fig. 6A).

Following ITS-1 PCR positivity, patients were randomly allocated to receive miltefosine or LAmB. Irrespective of the lesional variant, the repeat ITS-1 PCR at the end of treatment with miltefosine showed no product whereas following treatment with LAmB, a product was consistently obtained. Cases who returned at any time point six months later were examined, wherein patients who received miltefosine demonstrated no ITS-1 PCR product, while with LAmB, a product was always obtained.

In 19 cases that received miltefosine, the parasite load was serially monitored on completion of treatment and six months later. At presentation, their parasite load of 36500(963-197362) decreased significantly with treatment to 1(1-1), $p < 0.001$ (Fig. 6B). Importantly, this decrease was sustained for at least six months as the parasite load remained negligible being 1(1-1), $p < 0.001$ (Fig. 6B) and correlated with clinical features as total disappearance of dermal lesions was evident (Fig. 6C).

The story was less promising with LAmB, as there was a dramatic reduction in polymorphic cases, and less prominent in the macular variant (Fig. 7A). More importantly, in patients who reported six months later ($n=38$), there was an alarming increase in the parasite load, both in macular and polymorphic cases (Fig. 7A). Additionally, the parasite burden was serially monitored in 31 patients who received LAmB. During active disease, the parasite load of 33257(3138-160727) decreased to 2128(731-9172), $p < 0.01$ by 15.6 fold at the end of treatment (Fig. 7B). Eleven patients could be monitored six months later and all consistently showed a dramatic increase in their parasite load (Fig. 7B) and was reflected in persistence of their dermal lesions (Fig. 7C, a-f).

With considerable gains in our understanding of the nature and prevalence of NTDs, including leishmaniasis, especially with successes in improvement of chemotherapy

strategies and other health interventions (1), the NTDs continue to rank high among the world's greatest global health problems. Possibly a multipronged approach is necessary that should include improved drugs, sensitive diagnostic and monitoring tools along with vector control agents. This review has summarized some of the key challenges in translational science emphasizing on approaches to ensure success in our global efforts to eliminate leishmaniasis.

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