CONCLUSIONS
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Post Kala-azar dermal leishmaniasis (PKDL) is a perplexing disease notably for the shift in parasite tropism after the onset of apparent cure from visceral leishmaniasis (VL) and also for the geographical variation in clinical presentation; furthermore, the lack of an animal model precludes cross-extrapolation of information. As transmission of VL in India is anthroponotic, PKDL patients have been proposed as parasite reservoirs especially during interepidemic periods. Eradication of VL, particularly in India, is therefore contingent on our success in containing PKDL, which to date is limited by our poor understanding of its immunopathogenesis.

Among the patients with PKDL (n = 57), the majority presented with polymorphic lesions (papules or nodules along with macules, n = 41) while the rest were macular (n = 16). The diagnosis of PKDL was primarily clinical and was corroborated with a prior history of VL or residence in an endemic zone for VL; the diagnosis was confirmed by rK39 strip test and/or demonstration of LD bodies by Giemsa staining. Additionally, we confirmed our diagnosis by doing an ITS1 based PCR from dermal biopsies. Patients were randomly allocated to receive either SAG (20 mg/kg body weight/day IM for 4 months) or miltefosine (100 mg/day po for 4 months). Five patients (8.77%) gave no prior history of VL (4 were macular and 1 polymorphic) whereas in the remaining 52, the time interval between cure of VL and onset of PKDL ranged from 3 to 12 yrs, highlighting the logistical hurdles in designing longitudinal studies regarding the transition of PKDL from VL in India.

Mice and humans infected by *Leishmania* exhibit high levels of parasite specific IgG but do not provide protective immunity, because these antibodies are ineffective at killing parasites hiding inside host macrophages. In this study, we found an elevated total antileishmanial antibody response in patients with PKDL in terms of total Ig, IgG and IgE (Chapter 3, Figure 3.1). Furthermore, the response was elevated in polymorphic patients (Chapter 3, Figure 3.2) indicating that in polymorphic PKDL, the humoral response was stronger than in the macular variant, possibly attributable to the relatively longer duration of disease or higher antigen load. Our study also showed raised IgG1 and IgG3 in PKDL patients (Chapter 3, Figures 3.4 and 3.5). As PKDL is a sequel of VL, the problem of inferring from the serological response is limited to some extent as antibodies being stable up to many years may have been generated during their episode of VL. To resolve this problem, we tested the IgG avidity and expression of CD19. In the polymorphic group whose disease profile is more chronic, the IgG avidity was higher than macular PKDL (Chapter 3, Figure 3.7). Interestingly, expression of CD19, the loss of which entails conversion of B cells to antibody secreting plasma cells, was significantly lower in polymorphic PKDL when compared with healthy controls (Chapter 3, Figure 3.8).
As the serological response is tightly regulated by cytokines secreted from either lymphocytes or monocytes, we measured levels of plasma cytokines in patients with PKDL. Co-expression of counter-regulatory cytokines in circulation is a paradoxical feature of leishmaniasis as both IL-10 and IFN-γ are raised. In PKDL, the plasma levels of major pro-inflammatory cytokines (IL-6, TNF-α, IL-1β and IL-8) was evaluated; among them TNF-α and IL-8 were significantly higher (Chapter 4, Figure 4.2), while among the anti-inflammatory cytokines (IL-10, IL-4, IL-13 and TGF-β), levels of IL-4, IL-10 and TGF-β were significantly higher (Chapter 4, Figure 4.2). This led us to conclude that in PKDL, the immunity is a mixed Th1-Th2, but tilting more towards a Th2 type. Importantly, while both SAG and Miltefosine decrease IL-10, treatment with Miltefosine but not SAG significantly induced the pro-inflammatory cytokines (Chapter 4, Figure 4.3). Increased levels of pro-inflammatory TNF-α and IL-8 along with raised levels of IL-4, IL-10 and TGF-β prompted us to measure the plasma nitrite levels and arginase activity, as both these molecules are induced by pro-inflammatory and anti-inflammatory cytokines respectively. Both plasma nitrite and arginase activity are increased at disease presentation (Chapter 4, Figure 4.4) whereas with treatment, there was a curtailment in anti-inflammatory cytokines and increment in pro-inflammatory cytokines; alongside, there was a decrease in plasma arginase activity and rise in nitrite levels (Chapter 4, Figure 4.4).

Circulating cytokines are known to modulate immune cells, particularly lymphocyte and monocyte subsets. Although in PKDL, studies regarding circulating lymphocyte functions have been documented, studies regarding the functional status of monocytes remain a grey area. Peripheral-blood monocytes are functionally divided as the ‘CD14+CD16’ having an inflammatory property or ‘CD14+16’ as an anti-inflammatory type. At presentation, patients with PKDL showed an increased proportion of CD14+CD16+ i.e. an anti-inflammatory phenotype and decreased proportion of CD14+CD16~ monocytes i.e. a pro-inflammatory phenotype which was completely reversed after treatment (Chapter 5, Figure 5.1, and Table 5.1). Studies have suggested that the leishmanicidal activity of macrophages is dependent on a functional IFN-γ receptor or CD23 ligation with IgE, as both pathways enhance NO production in infected macrophages. Our study showed a significant decrease in the expression of CD23 in patients at presentation as compared to healthy controls which importantly was restored with treatment (Chapter 5, Figure 5.1 and Table 5.1). We have also assessed activation of monocyte subsets in terms of CD54 and HLA-DR expression. CD54 or Intracellular adhesion molecule 1 (ICAM-1) is expressed on monocytes and during antigen presentation is involved in the interaction with LFA-1 (Lymphocyte function associated antigen) while HLA-DR is the representative class II antigen presenting molecule. HLA-DR expression was significantly lowered in patients but not so with CD54 while treatment caused a significant increase in both these molecules (Chapter 5, Figure 5.1 and Table 5.1).
Exploitation of the ‘code of conduct’ of co-stimulation pathways provides an evolutionary incentive to intracellular pathogens like Mtb, *Listeria* sp., *Leishmania* sp. The major co-stimulatory pathways involved in leishmaniasis comprise the CD80/CD86 and CD40 pathway which interacts either with CD28 or CD154 of T cells for their activation. We observed a significant decrease in CD86 not in CD80 (Chapter 5, Figure 5.1 and Table 5.1). Importantly, the expression of CD86 reverted to normal following treatment, thus emphasizing its importance in disease resolution (Chapter 5, Figure 5.1 and Table 5.1). With regard to CD40, we found a significantly lowered expression of CD40 in terms of GMFC (not in terms of percentage of cells, Chapter 5, Figure 5.1), collectively indicating that a severely compromised expression of co-stimulatory molecules on the monocyte surface was concomitant with disease progression.

Signaling through Toll like receptors (TLRs) often leads to the generation of reactive oxygen and nitrogen species (ROS, NO) along with production of cytokines. The expression of both TLR-2 and TLR-4 was down regulated on the monocyte surface indicating that these receptors play a definite role in PKDL (Chapter 5, Figure 5.2). Furthermore, restoration of these receptors after treatment strengthens their role in disease resolution (Chapter 5, Figure 5.2). As we found two major types of receptors (TLR-2/4 and CD23) associated with generation of NO and ROS within monocytes to be downregulated, we felt it pertinent to measure the intracellular generation of these reactive radicals in PKDL. A significant curtailment in generation of NO and ROS was evident (Figures 11 and 12A, B); however, treatment caused a significant increase in generation of NO not in ROS which highlighted the importance of NO over ROS in leishmaniasis (Chapter 5, Figures 5.3 and 5.4A-C). Furthermore, lowering of superoxide generation (Chapter 5, Figure 5.4D) reinforced the notion of a relationship between TLRs and NO/ROS. Often in an *in vivo* condition, modulation of pro-oxidants is also accompanied by changes in the anti-oxidant status. To check whether a redox imbalance is created within monocytes of PKDL patients or not, we measured the levels of non protein thiols (mainly comprise of glutathione) and observed a massive increase in levels of non protein thiols at disease presentation followed by a significant reduction with treatment (Chapter 5, Figures 5.4E-G).

As analysis of plasma cytokines does not pinpoint the source(s), we measured the intracellular status of cytokines in monocytes. Assessment of pro-inflammatory cytokines in monocytes of PKDL revealed that except IL-12 and TNF-α, other proinflammatory cytokines were decreased in patients with PKDL as compared to healthy controls while treatment significantly induced them (Chapter 5, Figure 5.5). Surprisingly, IL-12, although expressed very minimally, was significantly higher in patients with PKDL, both at a protein and mRNA level, as also remained on the higher side even after treatment (Chapter 5, Figures 5.5A and
B). With respect to TNF-α, marginal levels were detected in healthy controls and patients with PKDL, suggesting that an alternative source of TNF-α is present.

The functional plasticity and diversity of macrophages can be attributed to their ability to respond to different micro-environmental signals and two main macrophage subsets are in the mainstream focus, mirroring the Th1/Th2 polarization scheme. These include the classically activated or M1 mimicking the Th1 effector response and the alternatively activated or M2 subtypes (AAMΦs) that mirror the Th2 response. As our data has established that there is an enhanced anti-inflammatory milieu in PKDL, we proceeded to evaluate the status of macrophage polarization by evaluating the expression of Arg-1, PPARγ and mannose receptor (MR) within monocyte enriched PBMCs and at the lesional site of patients with PKDL. The expression of Arg-1 was significantly elevated at both a mRNA and protein level at disease presentation and decreased with treatment (Chapter 6, Figures 6.2); this corroborated with decreased generation of NO within monocytes in patients with PKDL (Chapter 5, Figure 5.2). Global mRNA and protein repertoire analysis revealed that mannose receptor (MR) is the most potent marker for macrophage alternative activation. An enhanced expression of MR was evident in lesional macrophages and monocytes which reverted dramatically to normal levels with treatment (Chapter 6, Figure 6.3). PPARγ is a transcription factor of the nuclear hormone receptor family and is known to cause an upregulation of Th2 responses along with downregulation of Th1 responses. We found a significantly increased expression of PPARγ at disease presentation and again a significant curtailment after treatment, both in circulation and at the disease site (Chapter 6, Figure 6.7) corroborating that in PKDL there is an altered monocyte-macrophage phenotype akin to the M2 phenotype.

As vitamin D3 is also known to induce monocyte polarization towards M2 we investigated the role of vitamin D3 in PKDL, if any. Measurement of plasma levels of 25(OH)D3 were significantly increased in patients with PKDL which decreased with treatment (Chapter 6, Figure 6.4). Thereafter we checked the mRNA expression of CYP27B1, VDR and finally LL-37. Importantly we observed that all these genes are expressed at a significantly high level in patients with PKDL when compared to healthy controls and were restored successfully after treatment in circulation and at the dermal site (Chapter 6, Figures 6.5 and 6). This led us to conclude that increased serum 25(OH)D3 and subsequent activation of downstream signaling could be a molecular switch for monocyte polarization towards M2 type in PKDL.

The role of immunosuppressive IL-10 and TGF-β in leishmaniasis is well explained and it has been found that macrophages could be one of the potent sources for these two cytokines in leishmaniasis. M2 monocytes are known producers of these cytokines owing to their immunoregulatory and immunosuppressive role and these two cytokines are considered as a functional marker of macrophage polarization. Previously in PKDL, CD8+ T cells were
demonstrated to be one of the sources of IL-10, but the role of monocytes had not been studied. In this study, we have established that monocytes are a potent source of IL-10 and TGF-β in patients with PKDL (Chapter 6, Figure 6.8) further boosting our hypothesis that monocytes are being alternatively polarized in PKDL.

Our final objective was to evaluate the role of iron and expression of genes involved in its uptake and storage based on the evidence that iron is an important molecule required by monocytes/macrophages for production of toxic radicals; alongside, pathogens too need it for their survival. M2 monocytes are known to have a higher labile iron pool (LIP) than M1 monocytes and this was evident in PKDL wherein an increased LIP was evident at presentation which changed marginally with treatment (Chapter 7, Figure 7.2A-B). The plasma iron level was significantly reduced in patients with PKDL which changed marginally with treatment (Chapter 7, Figure 7.2C). As this decreased iron level may occur if iron is complexed with ferritin, we checked the ferritin level in plasma and found that ferritin level was decreased (Chapter 7, Figure 7.3C). To establish whether iron is being transported to monocytes by the transferrin-transferrin receptor pathway, a common iron transport mechanism, we measured the plasma transferrin and transferrin receptor expression on monocytes in patients with PKDL. The elevated transferrin level at presentation decreased after treatment (Chapter 7, Figure 7.3C) but expression of CD71 (transferrin receptor, TfR) was not modulated as for ferritin and transferrin (Chapter 7, Figure 7.3D). At the lesional site, modulation of ferritin, transferrin and TfR expression was evident as both transferrin and TfR were upregulated at presentation possibly to take up iron and decreased following treatment; ferritin expression was triggered after treatment (Chapter 7, Figure 7.3A). To confirm the role of iron in monocyte/macrophage polarization in PKDL, we evaluated the expression of CD163 and HO-1, as these two markers reflect iron-mediated macrophage polarization. As both these markers were raised in PKDL patients at the lesional site and circulation (Chapter 7, Figure 7.4), it strengthened our hypothesis.

Collectively, the key findings in this study (Figure 1) are-

- Patients with PKDL are characterized with high levels of antileishmanial Ig, IgG (mainly IgG1 and IgG3) and IgE which occurs following differentiation of CD19+ B cells to plasma cells (CD19+).
- Miltefosine exerted a greater effect in curtailment of antileishmanial antibody levels as compared to SAG.
- At disease presentation, patients with PKDL have raised circulating levels of TNF-α and IL-8 along with raised levels of IL-4, IL-10 and TGF-β concomitant with increased levels of nitrite and arginase activity.
> In PKDL, chemotherapy with Miltefosine effectively tilted the immune response towards a pro-inflammatory type by enhancing pro-inflammatory cytokines and down-regulating anti-inflammatory cytokines.

> Circulating monocytes were functionally impaired as evident by decreased surface expression of TLR-2/4, CD23, CD40, CD 86 and HLA-DR, as also decreased ability to generate free radicals. Additionally generations of pro-inflammatory cytokines (IL-6, IL-1β and IL-8) within monocytes was lowered.

> In PKDL, circulating monocytes and dermal macrophages are polarized towards an alternatively activated or M2 phenotype as characterized by increased expression of arginase-1, mannose receptor, PPAR-γ which is possibly induced by active vitamin D3 signaling. Additionally, these M2 cells have an increased ability to produce immunosuppressive IL-10 and TGF-β.

> Circulating monocytes in PKDL have an increased labile iron pool which is accompanied with decreased plasma iron. Iron is transported through the transferrin-transferrin receptor and CD163 mediated pathways which were increased in patients.

**Figure 1:** Model for immunosuppression in PKDL

During cure from VL, the T helper cells (Th) generally show an effective memory response and activate memory T-cell mediated classical macrophage activation (CAMφ OR M1); however, some patients show an aberrant immune response due to increased presence of anergic regulatory T cells, increased Vitamin D3 and parasite persistence. This led to an enhanced Th2 response which subsequently polarized the monocyte-macrophage in to an alternatively activated type (AAMφ or M2), which have decreased anti-leishmanial activity and allowed for development of PKDL. Chemotherapy with antileishmanials caused repolarization of AAMφ to CAMφ and resulted in disease elimination.