CHAPTER V

MONOCYTE ACTIVATION STATUS IN PKDL

Introduction

Monocytes are bone marrow derived circulating leucocytes, responsible for mounting an inflammatory response against microbes by killing pathogens via phagocytosis, production of reactive oxygen species (ROS), nitric oxide (NO) along with inflammatory cytokines. Generally monocytes are fortified with several antimicrobial weapons such as ROS, NO, co-stimulatory molecules (CD40, CD80, and CD86), toll like receptors (TLRs), cytokines etc. They are also capable of polarizing T cell responses, suppress and/or stimulate T cell responses in infectious and autoimmune diseases [Reviewed in Saha and Geissmann, 2011]. The *Leishmania* parasite must ensure its survival within monocytes/macrophages by deviously inhibiting generation of an oxidative burst. Therefore, to achieve parasite elimination, activation of macrophages via preferential production of pro-inflammatory cytokines and production of NO would be beneficial [Reviewed in Van Assche et al. 2011].

Monocytes were initially subdivided based on CD16 expression as either $CD14^{+}16^{-}$ and $CD14^{+}16^{+}$ and recognized as pro-inflammatory and anti-inflammatory respectively [Zeigler-Helbrock H.W, 1996] However, according to the new nomenclature $CD16^{+}$ expressing monocytes can be further subdivided into two populations, $CD16^{dim}$ and $CD16^{bright}$, but their functional differences remains elusive [Reviewed in Woong et al. 2012; Sheel and Engwerda 2012]. Studies revealed that monocytes are probably the first human cells where *Leishmania* parasite reside and multiply in blood [de Almeida MC 2002]. Monocytes infected with *Leishmania* have been studied using *in vitro* and *in vivo* models along with data from human leishmaniasis. However, the results obtained are extremely diverse and may be attributable to the variable origin of the monocytes/macrophages and even the different parasite species used and additionally even the dose of the injected parasite influences the disease profile [de Almeida MC 2002, de Almeida et al. 2003, Seifert et al. 2010]. For example, studies in mice (C57BL/6) having a resistant background against leishmaniasis revealed unaltered expression of co-stimulatory molecules upon infection with *L.donovani*, whereas infection in the susceptible BALB/c mouse model marginally increased CD54 but failed to influence CD80 [Saha et al. 1995, Kaye et al. 1994]. Similarly, infection with *L. major* in human macrophages or in C57BL/6 mice showed no changes in the levels of CD80 or the CD86 [Von Stebut et al. 1998, Brodskyn et al. 2001]. Infection with *L. chagasi* in human macrophages enhances expression of CD86 along with decreased HLA-DR [de Almeida et al. 2003]. Taken together, the take home message is that information from one model or parasite species cannot be extrapolated, emphasizing the need for each parasite and monocyte population to be individually evaluated.

As *in vitro* immunological studies do not necessarily mimic *in vivo* changes, several studies have been conducted to establish the status in humans. In human cutaneous leishmaniasis (CL), an increased expression of CD16 correlated with increased lesion size

[Soares et al. 2006]. In a recent study, Freitas-Teixeira et al. (2012) showed that expression of HLA-DR and CD23 was increased. The immunoregulatory role of monocytes has been investigated in CL wherein circulating monocytes showed a decreased expression of CD80, CD86 co-stimulatory molecules [Vieira et al. 2013]. However, Carrada et al. revealed no changes in either CD40 or CD80 expression in patients with CL and diffuse cutaneous leishmaniasis (DCL) but expression of CD86 was augmented in monocytes in patients with CL and DCL [Carrada et al. 2007]. In contrast to CL, studies regarding monocyte function in VL is notably limited and has so far been limited to measurement of the activities of NADPH oxidase, myeloperoxidase, arginase, lysozyme, nitrite, superoxide and H_2O_2. Importantly all these parameters were found to be significantly lowered in VL patients compared to healthy controls which corroborated with the reported immunosuppression in VL [Kumar et al. 2001, Kumar et al. 2002, Kumar et al. 2012].

Study objectives

As Post kala-azar dermal leishmaniasis (PKDL) is a dermal disease with involvement of systemic immunity [Ganguly et al. 2010b], study of the monocyte functions is warranted. Herein, our aim was to study the monocyte function is terms of generation of NO, ROS, superoxide, expression of co-stimulatory molecules, status of adhesion molecules, expression of HLA-DR, TLR 2 and 4, intramonocyte generation of pro-inflammatory cytokines (IL-6, IL-1β, IL-8, IL-12p40, TNF-α) along with immunoregulatory cytokines (IL-10 and LAP-TGF-β1).

Materials and Methods

Immunophenotyping of peripheral blood leukocytes

Peripheral blood (100 µl) was surface stained with fluorochrome conjugated antibodies to CD14 Fluorescein isothiocyanate or FITC, CD16 Phycoerythrin or PE, CD23 Allophycocyanin or APC, CD40 PE, CD54 APC, CD80 PE, CD86 FITC, and HLA-DR PE along with appropriate isotype controls as described in chapter 2.

Isolation of peripheral blood mononuclear cells (PBMC) and monocytes

Peripheral blood was carefully layered over HiSep 1073 (2:3) for isolation of monocytes and centrifuged (400 g x 30 min). The monocyte rich interface was washed twice in phosphate buffered saline (PBS, 0.01 M, pH 7.2) and resuspended in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% heat inactivated FBS and treated with or without *Leishmania* antigen for flow cytometry and mRNA expression as described in chapter 2.

Determination of Toll like receptor 2/4 (TLR 2/4) expression on monocytes

Monocytes, they ($1x10^6$) were surface stained with anti human CD14 Peridinin chlorophyll protein (PerCP) followed by fixation and permeabilization, after which cells were stained with anti human TLR-2 PE and TLR-4 FITC for 15 minutes. Cells were washed and acquired on a flow cytometer as described in chapter 2.

Determination of intracellular NO in monocytes

Monocytes ($5x10^5$) were centrifuged (400 g × 5 min) stained with 4,5-Diaminofluorescein diacetate [DAF-2DA, 2 μM, Sarkar et al. 2011] for 30 minutes at 37°C. Cells were acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA) as described in chapter 2.

Measurement of the oxidant status of monocytes

Generation of ROS was measured in monocytes (5×10^5) that were centrifuged (400g X 5 minutes), stained with 5-(and-6)-carboxy-2',7'-dichloro dihydrofluorescein diacetate (CMH_2DCFDA, 2.5 μM) for 30 minutes at 37°C; cells were acquired on a flow cytometer.

Superoxide production was measured using cytochrome c reduction assay [Kundu et al. 2011]. Isolated monocyte enriched PBMCs (1×10^6 cells/ml) were incubated with cytochrome c and absorbances of the supernatants were measured at 550 nm. The amount of superoxide generated in 1 ml of the reaction mixture was calculated as follows: O_2^- (nmol) = $47.7 \times A_{(superoxide)}$ (OD at 550 nm).

To measure the status of non protein thiols we used 5-Chloromethylfluorescein Diacetate (CMFDA); monocytes (5×10^5) were stained with CMFDA (50 nM, 15 minutes, 37°C) after ATP depletion and then acquired on a flow cytometer.

Intracellular cytokine staining

Monocytes (1×10^6 cells/well/ml) were incubated for 16 h in the presence or absence of *Leishmania donovani* crude antigen (LDA, 10 μg/ml, prepared as previously described, Ganguly et al. 2008) and further incubated with LDA (10 μg/ml) and Brefeldin A (1 μg/ml) for 4 h for measurement of intracellular cytokines. Cells were harvested, surface stained with CD14 FITC, fixed and permeabilized for staining with IL-6 PE, IL-1β PE, TNF-α PE, IL-8 APC and IL-12p40 PE, along with their appropriate isotype antibodies, therefore acquired on a flow cytometer as described in chapter 2.

Results

Expression of activation and adhesion markers of monocytes in patients with PKDL

To establish whether monocytes in PKDL have a pro-inflammatory nature ($CD14^+CD16^+$) or anti-inflammatory ($CD14^+CD16^-$) immunophenotyping was performed. At disease presentation there was a significant expansion of the anti-inflammatory $CD14^+CD16^-$ monocytes along with decreased levels of pro-inflammatory $CD14^+CD16^+$ monocytes than healthy controls ($p<0.05$, Figure 5.1 and Table 5.1). Treatment caused reversal of the monocyte phenotype as evidenced by reduction of $CD14^+CD16^-$ monocytes, concomitant with expansion of $CD14^+CD16^+$ monocytes ($p<0.05$ and $p<0.01$ respectively, Figures 5.1, Table 5.1).

CD23, a low affinity IgE receptor, is expressed on the monocyte surface following an increase in IL-4, IL-13 and IFN-γ and upon ligation with IgE or CD21 it induces generation of NO within monocytes [Vouldoukis et al. 1995]. The surface expression of CD23 on monocytes was a significant 8.9 fold lower in patients with PKDL when compared to healthy controls ($p<0.01$, Figure 5.1, Table 5.1). Treatment caused a significant 11.3 fold increase in the expression of CD23 ($p<0.01$, Figure 5.1, Table 5.1).

With regard to CD54, its expression at disease presentation was lower than healthy controls (Figure 5.1 and Table 5.1) but was significantly enhanced after treatment ($p<0.01$, Figure 5.1 and Table 5.1). Similarly, the expression of HLA-DR was significantly compromised at disease presentation in comparison with healthy controls ($p<0.05$, Figure 5.1 and Table 5.1) and post treatment, its resulted in a significant expansion ($p<0.001$, Figures 5.1, Table 5.1).

Expression of co-stimulatory molecules (CD80, CD86 and CD40) on monocytes in patients with PKDL

Immunophenotypic analysis of these co-stimulatory molecules revealed that expression of CD86 on $CD14^+$ monocytes was significantly decreased at presentation as compared to healthy controls ($p<0.01$, Figure 5.1, Table 5.1), and reverted following treatment ($p<0.01$, Figure 5.1, Table 5.1); however another co-stimulatory molecule CD80 showed no changes (Figure 5.1, Table 5.1).

With regard to CD40 expression, there was no alteration in the percentage of $CD40^+$ monocytes (data not shown) between patients with PKDL and healthy controls, but when measured in terms of geometric mean fluorescence (GMFC) it was significantly lowered at disease presentation as compared to healthy controls (34.36 ± 5.51 vs. 64.22 ± 7.21, $p<0.05$,

Figure 5.1). Treatment caused an increase in expression of CD40 when compared to naive patients (89.59 ± 13.84, $p<0.01$, Figure 5.1).

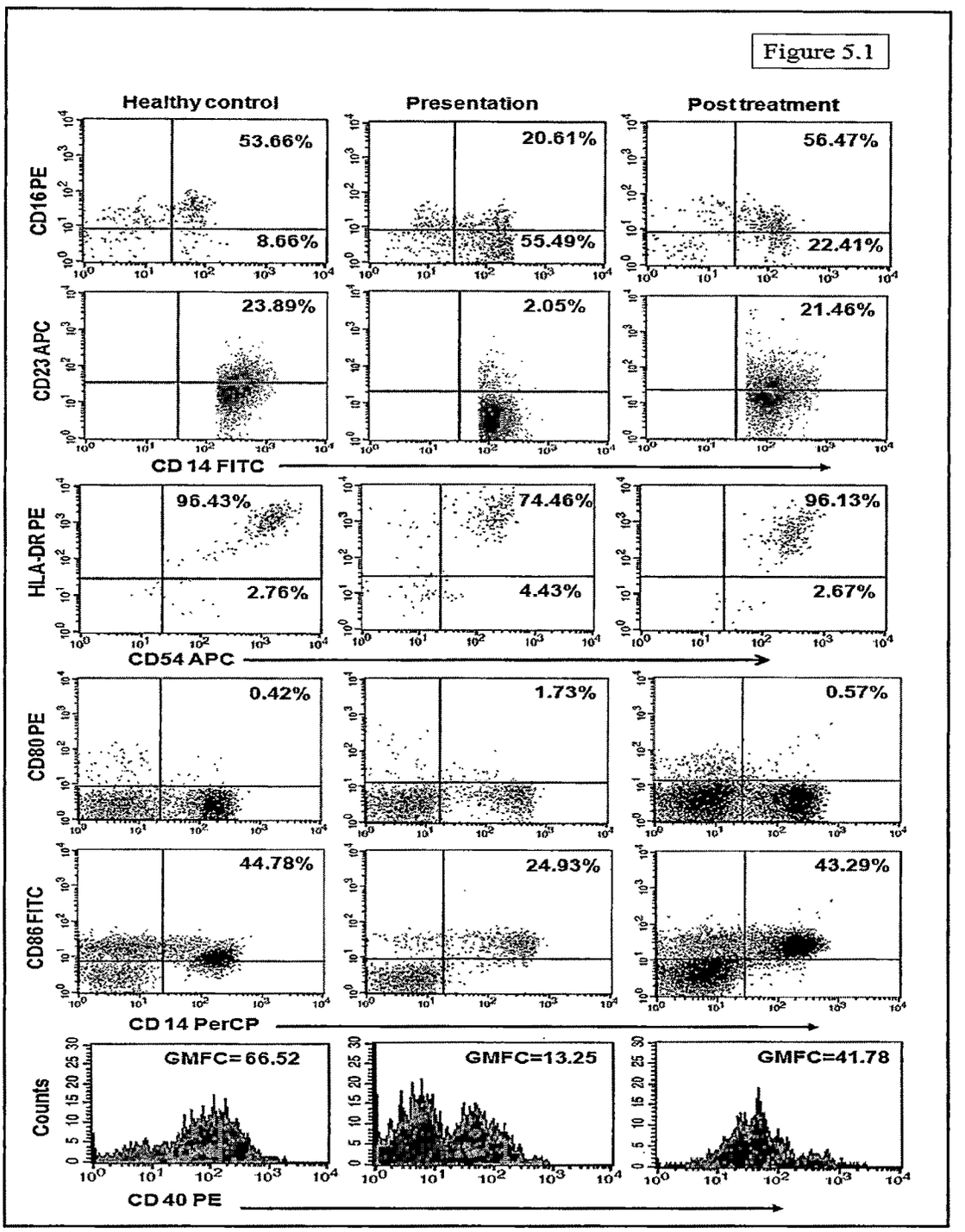

Figure 5.1: Representative profiles of the % of $CD14^+CD16^-$, $CD14^+CD16^+$, $CD14^+CD23^+$, $CD54^+$, $CD54^+HLA\text{-}DR^+$, $CD14^+CD80^+$and $CD14^+CD86^+$ monocytes in a healthy control and patient with PKDL (at presentation and end of treatment). A similar representative profile for GMFC of CD40 has been showed in a healthy control and patient with PKDL (at presentation and end of treatment).
Individual gates were set by using monocyte forward and side scatter characteristics and then with fluorochrome conjugated anti human CD14 FITC/PerCP.

Table 5.1: Surface marker analysis on monocytes

*Phenotype	Healthy controls	PKDL (Presentation)	PKDL (Post treatment)
%$CD14^+CD16^-$	14.23±2.63	41.05±4.34$^{@}$	26.62±5.32$^{\#}$
%$CD14^+CD16^+$	47.89±8.04	24.33±4.26	46.97±6.32$^{\#\#}$
%$CD14^+23^+$	21.19±5.68	2.38±0.71$^{@}$	26.90±6.28$^{\#\#}$
% $CD54^+$	89.45±3.34	81.89±4.25	94.72±1.06$^{\#\#}$
% $CD54^+HLA\text{-}DR^+$	88.83±3.92	74.82±4.71$^{@}$	92.01±1.13$^{\#\#\#}$
%$CD14^+80^+$	1.41±0.88	1.49±0.47	4.16±1,30
%$CD14^+86^+$	52.11±6.58	28.11±3.57$^{@@}$	50.97±3.99$^{\#\#}$

*Data are Mean ± SEM of monocytes stained with surface marker antibodies, and acquired on a flow cytometer as described in Materials and methods.

$^{@}p<0.05$ significantly different from healthy controls; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ significantly different from presentation.

Decreased expression of TLR-2 and TLR-4 on monocytes in Indian PKDL

TLRs are important membrane receptors wherein appropriate ligand binding signals the cells to become activated, a step mandatory to eliminate the pathogen. In patients with PKDL, expression of both TLR-2 and TLR-4 on $CD14^+$ monocytes was significantly lower than healthy controls, mean ± SEM being 54.70 ± 7.52% vs. 86.32 ± 2.78% ($p<0.01$, Figure 5.2) and 41.26 ± 8.90% vs. 75.95 ± 3.56% ($p<0.05$, Figure 5.2) respectively. With treatment, the levels of TLR-2 significantly increased and reverted to control values (66.07 ± 3.21, Figure 5.2) whereas TLR-4 showed statistically insignificant increase (79.67 ± 3.23, Figure 5.2), suggesting that in PKDL, TLR-2 is the more important determinant.

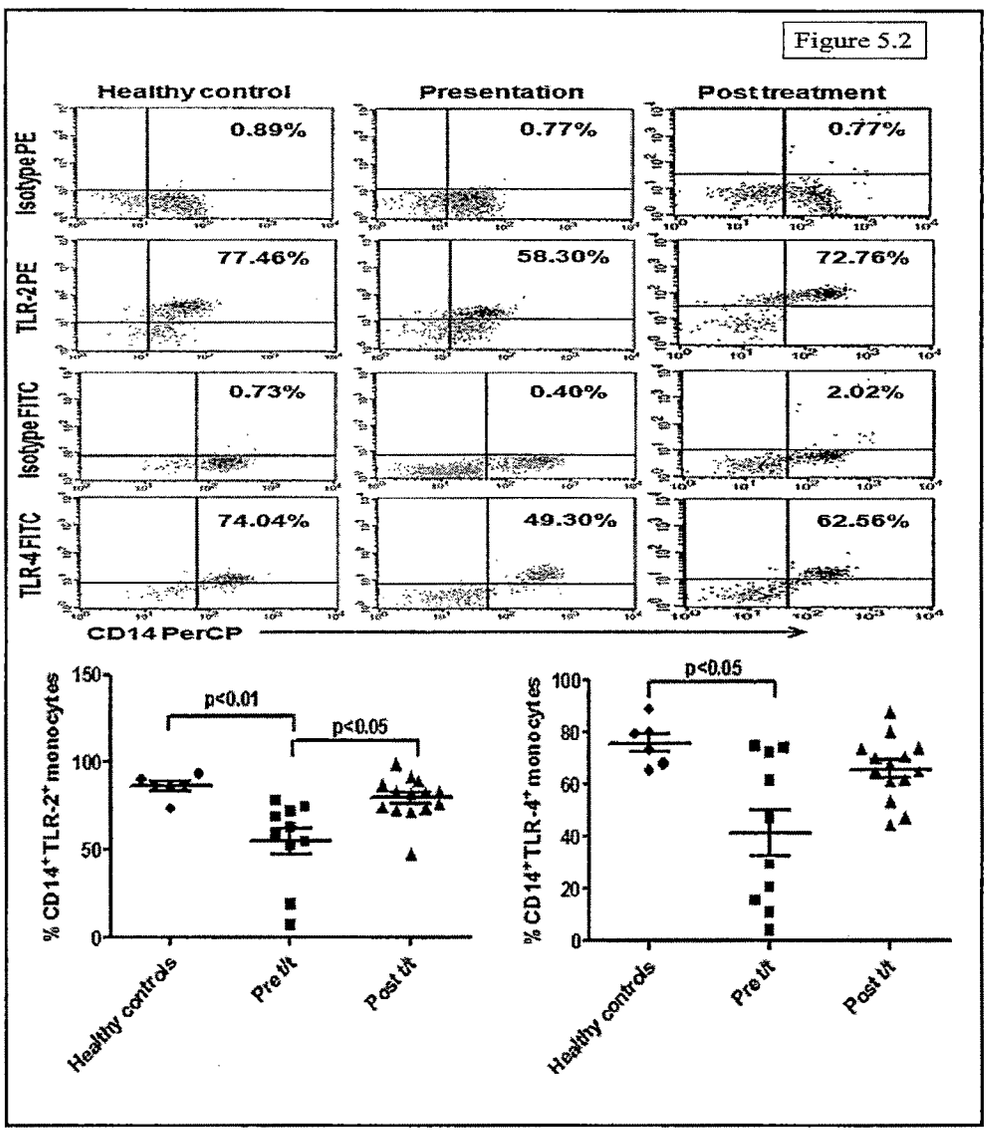

Figure 5.2: Representative profiles of the % of $CD14^+TLR\text{-}2^+$, $CD14^+TLR\text{-}4^+$ monocytes in a healthy control, patients with PKDL (at presentation and end of treatment). Scatter diagram for % of $TLR\text{-}2^+$ and $TLR\text{-}4^+$ in healthy controls (n = 7, •), patients with PKDL at presentation (pre t/t, n = 10, ■) and post treatment (post t/t, n = 14, ▲).
Individual gates were set by using monocyte forward and side scatter characteristics and then with fluorochrome conjugated anti human CD14 antibodies.

Generation of nitric oxides in monocytes

Nitric oxide (NO), is an important second messenger within cells especially within macrophages, where its potent antimicrobicidal property comes into play after phagocytosis of the invading pathogen [Bogdan 2001]. At presentation, levels of NO were significantly attenuated as compared to healthy controls, evident by the decreased GMFC mean ± SEM being 125.40 ± 32.74 vs. 306.30 ± 20.78 ($p<0.01$, Figures 5.3A and B). Treatment translated into patients regaining their ability to generate NO as reflected in the significant increase in their DAF-2T fluorescence (371.50 ± 76.51, $p<0.05$, Figures 5.3A and B). Analysis on an

individual basis corroborated the same, as a significant decrease in generation of NO at presentation increased upon completion of treatment ($p<0.01$, Figure 5.3C).

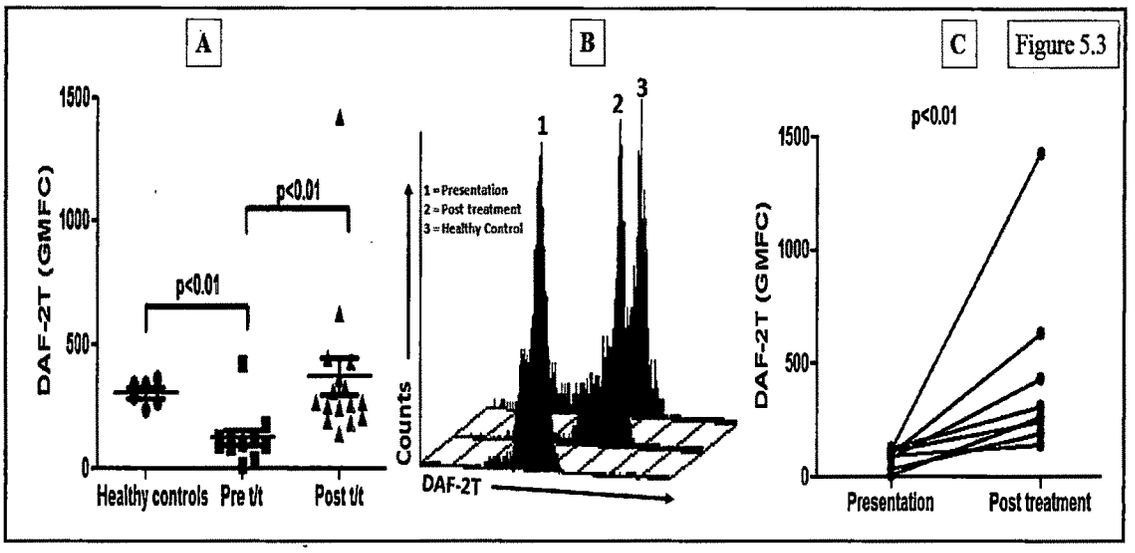

Figure 5.3: Generation of NO in monocytes
A. Scatter plot showing GMFC of DAF-2T in healthy controls (n = 7, •), patients with PKDL at presentation (Pre t/t, n = 10, ■) and post treatment (Post t/t, n = 14, ▲).
B. A representative 3D histogram profile of the DAF-2T fluorescence in monocytes from a healthy control (2), patient with PKDL at presentation (1) and end of treatment (3).
C. Analysis of intramonocyte generation of NO on an individual basis.

A decreased oxidative burst indicate monocyte inactivation

Monocytes upon activation generate an oxidative burst which facilitates elimination of the *Leishmania* parasite, owing to their inherently weakened anti-oxidant system. So, it would logically follow that for the parasite to persist, it would decrease the oxidative burst, making it a safe haven for its survival [Saha et al. 2011]. Accordingly, the oxidative status of monocytes was measured using a non-fluorescent dye CMH_2DCFDA, A decreased level of ROS was generated in monocytes from patients with PKDL as compared to healthy controls (858.70 ± 171.70 vs. 2132.00 ± 259.90, $p<0.01$, Figures 5.4A and B). However with treatment, an enhanced but non significant generation of ROS ensued as compared to disease presentation (1396.00 ± 158.20, Figures 5.4A and B) which remained unchanged when analyzed individually (Figure 5.4C).

Further, we have assessed the production of superoxide by incubating monocytes using the cytochrome c reduction assay, in which cytochrome c reduced by superoxide produces an intense red colour, quantified by its absorbance at 550 nm. In patients with PKDL, the generation of superoxide was significantly lowered than healthy controls, being 2.15 ± 0.41 nM vs. 4.40 ± 0.12 nM ($p<0.05$, Figure 5.4D) which changed minimally following treatment (3.49 ± 0.79 nM, Figure 5.4D).

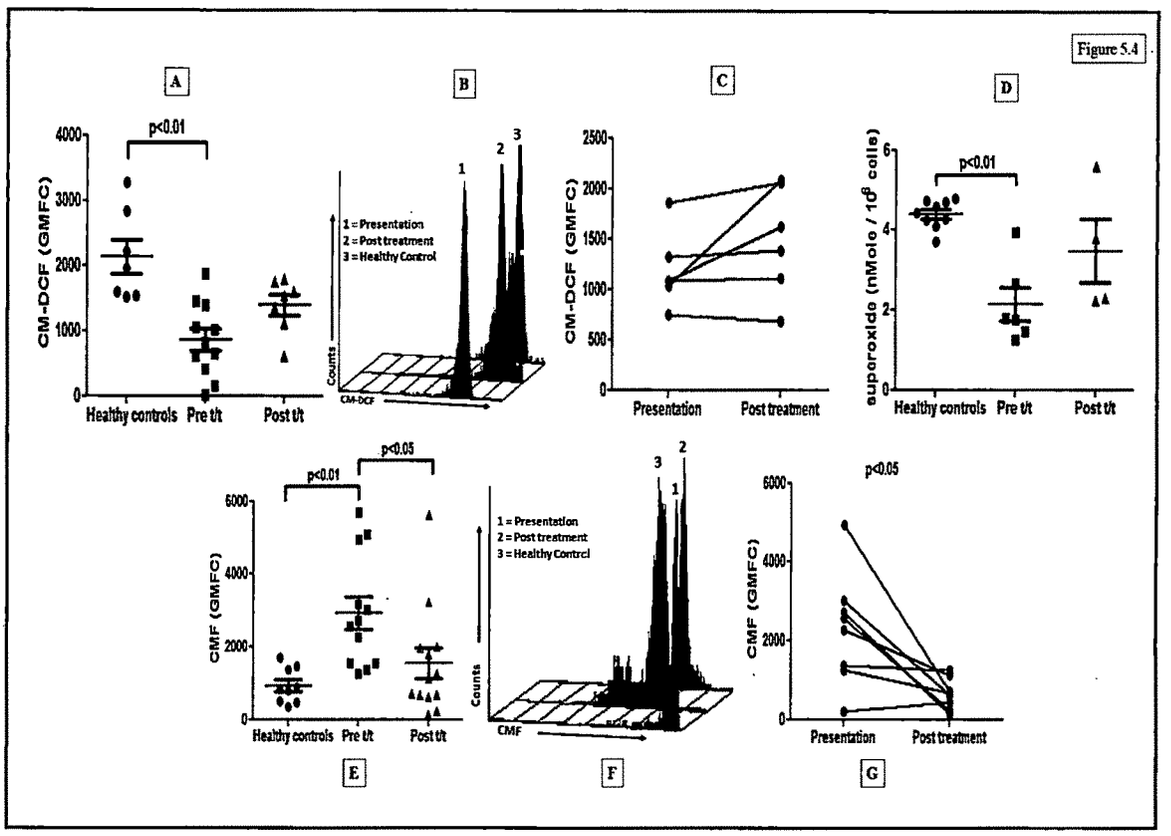

Figure 5.4: Generation of oxidative status in monocytes from patients with PKDL
A. Scatter diagram for GMFC of CM-DCF in healthy controls (n = 7, •), patients with PKDL at presentation (Pre t/t, n = 10, ■) and post treatment (Post t/t, n = 14, ▲).
B. A representative 3D histogram profile of the CM-DCF fluorescence (representative of ROS) in monocytes from a healthy control (3), patient with PKDL at presentation (1) and end of treatment (2).
C. Analysis of intramonocyte ROS generation on an individual basis.
D. Levels of superoxide produced by monocytes in healthy controls (n = 9, •), patients with PKDL [at presentation (n = 8, ■) and post treatment (n = 4, ▲)].
E. Scatter diagram for GMFC of CMF (representative of thiols) in healthy controls (n = 10, •), patients with PKDL at presentation (Pre t/t, n = 10, ■) and post treatment (Post t/t, n = 14, ▲).
F. A representative 3D histogram profile of the CMF fluorescence in monocytes from a healthy control (3) and patients with PKDL at presentation (1) and end of treatment (2).
G. Before and after plots of intramonocyte levels of non protein thiol.

As generation of ROS in monocytes was decreased at presentation, we measured levels of intracellular non protein thiols to ascertain its contribution towards generation of redox imbalance, which too would enable parasite survival within monocytes [Van Assche et al. 2011]. Levels of thiols were significantly increased in patients with PKDL at presentation when compared with healthy controls (2931.00 ± 445.20 vs. 926.00 ± 160.60, $p<0.01$, Figures 5.4E and F), which correlated ($r = -0.57$) with decreased levels of ROS in patients with PKDL. Similarly, after treatment, levels of non protein thiols decreased significantly when compared with patients at disease presentation (1541.00 ± 415.90, $p<0.01$, Figures 5.4E and F), and was supported by data when individually analysed. Here also on an individual basis, a statistically significant decrease in levels of non protein thiols was evident post t/t ($p<0.05$, Figure 5.4G).

Modulation of intracellular cytokine expression in monocytes from patients with PKDL

In this study, expression of both pro and anti-inflammatory cytokines in patients with PKDL in the presence or absence of LDA was assessed. A consistent observation irrespective of the cytokine was that no differences were observed between LDA stimulated and the unstimulated population (data not shown). With regard to IL-6, its expression was decreased at presentation as compared to healthy controls (21.66 ± 6.17% vs. 47.23 ± 11.26%, Figure 5.5A), but increased significantly after treatment (54.15 ± 7.16%, $p<0.01$, Figure 5.5A).

Similarly with IL-1β, there was a significant 2.57 fold decrease in patients with PKDL in comparison with healthy controls (26.34 ± 7.61% vs. 67.68 ± 8.10%, $p<0.01$, Figure 5.5A). After treatment, IL-1β levels reverted back to control values (50.97 ± 3.82%, $p<0.05$, Figure 5.5A). Akin to IL-6 and IL-1β, the expression of IL-8 was also significantly reduced in patients with PKDL than healthy controls (37.05 ± 6.31% vs. 63.46 ± 6.54% respectively, $p<0.05$, Figure 5.5A) and increased after treatment (58.13 ± 3.40% respectively, $p<0.05$, Figure 5.5A).

In contrast to IL-6, IL-1β and IL-8 another very important pro-inflammatory cytokine IL-12p40 was not decreased in patients with PKDL; rather, there was a significant increase in its expression *vis a vis* healthy controls (2.42 ± 0.52% vs. 0.64 ± 0.18%, $p<0.05$, Figure 5.5A); treatment caused minimal changes, and levels remained higher than in healthy controls (1.70 ± 0.59%, Figure 5.5A). As the levels of IL-12p40 was lower than other pro-inflammatory cytokines, we confirmed whether this data by assessing the mRNA expression in monocytes from patients with PKDL. Akin to the flow cytometric data a weak expression of IL-12p40 was evident in patients with PKDL but absent in healthy controls (6.38 ± 0.75, $p<0.05$, Figure 5.5B) and remains higher after treatment (5.68 ± 0.72, Figure 5.5B).

Similar to IL-12p40, the expression of TNF-α was also very low within the monocytes but importantly in contrast to IL-12p40 no changes was observed between healthy controls and in patients with PKDL (2.49 ± 0.59% vs. 1.13 ± 0.36%, Figure 5.5A). Treatment caused no further change in the expression of TNF-α (1.56 ± 0.40, Figure 5.5A).

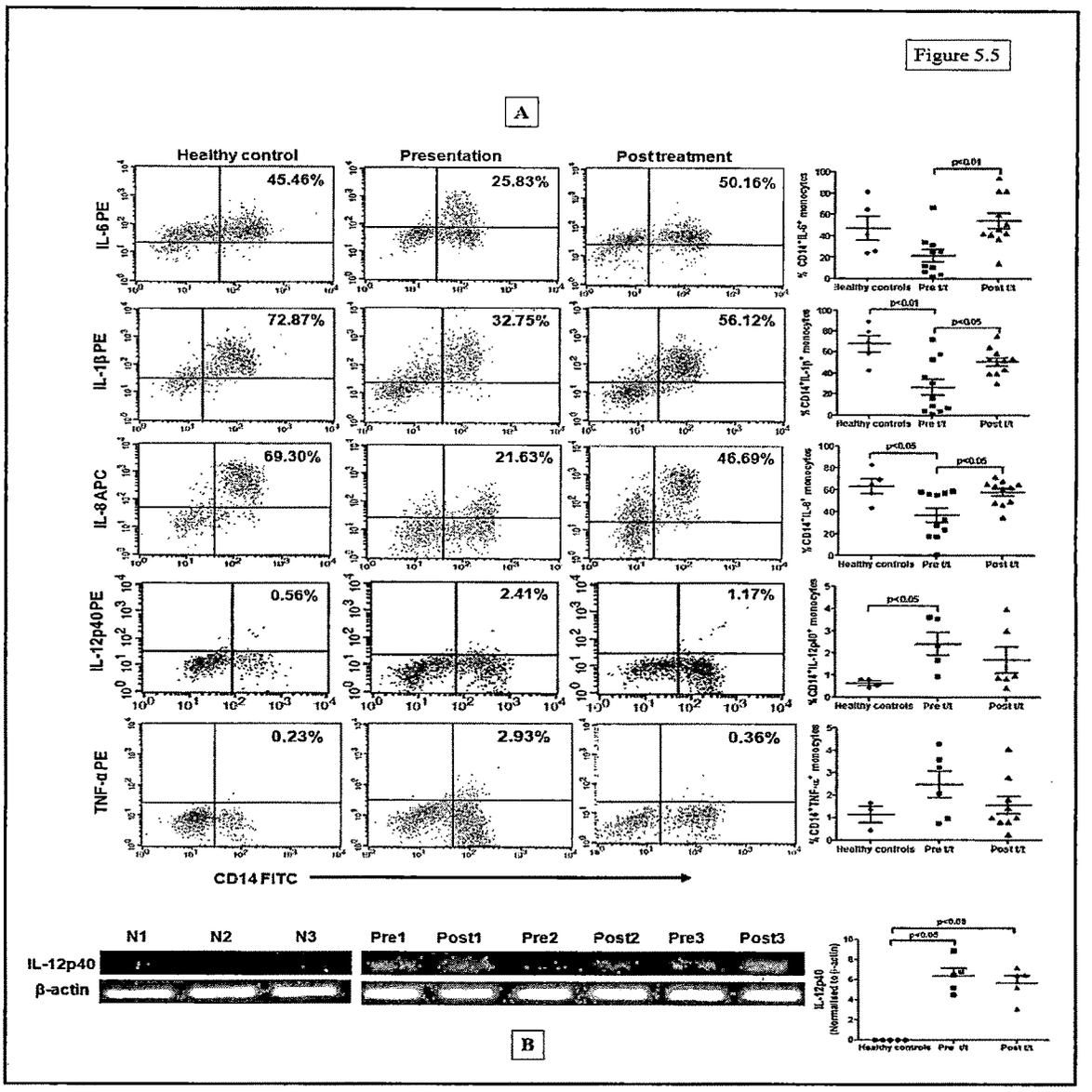

Figure 5.5

A. Representative flow cytometric profiles of the intracellular cytokine analysis of IL-6, IL-1β, IL-8, IL-12p40, TNF-α in monocytes from a healthy control, patient with PKDL at presentation and end of treatment. Scatter plot for intracellular cytokine analysis in monocytes from healthy controls (n = 5, •), patients with PKDL at presentation (n = 10, ■) and post treatment (n = 5, ▲).

B. Representative profile of mRNA expression of IL-12p40 and β-actin in PBMCs of patients with PKDL before and after treatment (Pre1-Pre3 and Post1-Post3, n = 3) and from healthy controls (N1-N3, n = 3). Scatter plot for mRNA expression of IL-12p40 in healthy controls (n = 7, •), patients with PKDL at presentation (n = 5, ■) and post treatment (n = 5, ▲).

Discussion

The host-parasite relationship is a complex phenomenon wherein the parasite manipulates the host for its survival. In terms of leishmaniasis, this relationship assumes even greater complexity as the parasite resides within macrophages, the key sentinels of our immune system. Macrophages can produce anti parasitic reactive oxygen species (ROS) and NO upon encountering the parasite to get rid of it and therefore to reside within the macrophages, the parasite should execute not only immune evasion but also immune

deviation such that it there immunosuppression [Bhardwaj et al. 2010]. Upon infection, inability of the macrophages to kill the parasites following activation of adaptive immune responses occur possibly due to the parasites direct ability to alter essential host immune signalling pathways or by parasite derived product(s) that can successfully alter the host immune system [Shio et al. 2012]. Importantly, although all macrophages are not infected with the parasite, all became functionally impaired as they cannot trigger activation of the immune system to mount a strong antileishmanial response. This bystander inactivation is not very well understood in context of leishmaniasis [Kaye and Scott 2011]. This immune anergy could possibly be caused by parasite secreted products or by small molecules (metabolites or cytokines) from infected cells. The critical role of cytokines needs to be addressed as depending on the cytokine driven micro-environment, macrophages got differentiated into distinct subsets to eliminate parasite survival.

Activated monocytes and macrophages responsible for intracellular killing of parasites are associated with Th1 cells producing IFN-γ and TNF-α [Carrada et al. 2007, Gaze et al. 2006, de Saldanha et al. 2012, Sheel and Engwerda 2012]. The aim of this study was to characterize the activation status of circulating monocytes by studying the frequency of co-stimulatory molecules, generation of reactive radicals, and expression of TLR-2 and TLR-4 along with intracellular cytokine formation among patients with PKDL.

Peripheral-blood monocytes show tremendous heterogeneity and plasticity in terms of surface expression markers. They are mainly typed as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) but due to the limitation to separate the intermediate from non-classical monocytes, they are commonly divided into $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ types [Ziegler-Heitbrock et al. 2010] Functionally the $CD14^{+}CD16^{+}$ are considered as the inflammatory monocyte phenotype along with an increased expression of pro-inflammatory cytokines, enhanced potency in terms of antigen presentation, increased T cell interaction, generation of reactive radicals and are present in a high frequency of inflammatory diseases including rheumatoid arthritis, diabetes, atherosclerosis and kawasaki disease etc [Reviewed in Ziegler-Heitbrock L 2007, Reviewed in Woong et al. 2012]. Additionally, M1 cells are reported to have high levels of CD16 and low levels of CD14 and the reverse holds true for M2 cells [Mantovani et al. 2004]. But in a recent review by Rahat et al. (2011) the consideration of M1 and M2 monocytes based on CD14 and CD16 expression was revisited and they concluded that based on only these two surface markers, it is virtually impossible to divide the monocyte population into M1 or M2 type. In leishmaniasis, information regarding the status of the $CD16^{+}$ monocytes appears contradictory, as patients with CL showed an increased expression of $CD16^{+}$ monocytes at presentation [Soares et al. 2006], which is possibly reflective of the self-limiting nature of CL. In contrast in the nonhealing DCL variant, $CD16^{+}$ monocyte levels were low at presentation [Pereira et al.

2009]. Similarly in PKDL, levels of $CD16^+$ monocytes at presentation were significantly decreased than from healthy controls (Figure 5.1; Table 5.1). The healing lesions of DCL showed an 8-fold increase in circulating $CD16^+$ and $CD14^+$ monocytes, and our results are in agreement because effective chemotherapy caused a 1.9 fold expansion of the $CD16^+$ population (Figure 5.1; Table 5.1).

CD23 and IgE are altered during parasitic infections as studies have suggested that the leishmanicidal activity of macrophages is dependent on either IFN-γ receptor or CD23 ligation as both pathways can enhance production of NO in infected macrophages [Vouldoukis et al. 1995]. Indeed Mossalayi et al. (1999) reviewed the potential role of CD23 signaling in the elimination of *Leishmania* parasites from macrophages by inducing NO generation and it was validated in several human leishmaniasis studies. [Reviewed in Mossalayi et al. 1999]. Patients with PKDL had an increased IgE (Chapter 3) and therefore we felt it pertinent to investigate the expression of CD23. We observed a significant decrease in the expression of CD23 in patients at presentation than healthy controls which importantly was restored with treatment (Figure 5.1, Table 5.1).

We have also assessed activation of the monocyte subsets in terms of CD54 and HLA-DR expression. CD54 or Intracellular adhesion molecule 1 (ICAM-1) is expressed on monocytes and involved in the interaction with LFA-1 (Lymphocyte function associated antigen) during antigen presentation, whereas HLA-DR is the representative class II antigen presenting molecule. Previously, it has been reported that both CD54 and HLA-DR were lowered during *Leishmania* infection in monocytes and restored upon treatment with IFN-γ [Reviewed in de Almeida et al. 2003, Engelhorn et al. 1990]. We too observed that HLA-DR expression was significantly lowered in patients whereas the decrease in CD54 was not significant; however treatment caused a significant increase in both these molecules (Figure 5.1, Table 5.1) thus strengthening the hypothesis that in PKDL, monocytes contributes towards the observed immune anergy [Ganguly et al. 2010b]

Co-stimulatory molecules expressed on antigen presenting cells (APCs) as well as on lymphocytes play a decisive role in the initiation as well as sustenance of immunity. Exploitation of the 'code of conduct' of co-stimulation pathways provides an evolutionary incentive to intracellular pathogens like *Mtb, Listeria sp., Leishmania sp.*, and others [Khan et al. 2012]. These co-stimulators on APCs not only provide unilateral 'help to T cell' signalling, rather the signal is bilateral and effective co-stimulation influences the activity of both T cells and APCs during their interaction [Gowthman et al. 2010, Khan et al. 2012]. The major co-stimulatory pathways that are involved in leishmaniasis comprise the CD80/CD86 and CD40 pathway [Tuladhar et al. 2011]. Apart from a MHC-TCR interaction, the interaction of CD80 and CD86 co-stimulatory molecules with CD28 is necessary for T cell activation [Rudd et al. 2009]. In PKDL, lack of CD28 on circulating $CD8^+$ T cells had been

reported previously by our group [Ganguly et al. 2010b]. Herein, our aim was to investigate the expression of interacting partners of CD28 on circulating monocytes and notably we have observed a significant decrease in CD86 but not in CD80 (Figure 5.1, Table 5.1). Importantly, the expression reverted to normal following treatment, thus emphasising its importance in disease resolution (Figure 5.1, Table 5.1).

The role of CD40 in leishmaniasis has been extensively demonstrated in a mouse model of CL [Reviewed in Tuladhar et al. 2011]. CD40 is associated with cholesterol containing lipid raft and TNF-receptor associated factor (TRAF) molecules to form a signalosome. Depending on the strength of association CD40 reciprocally controls the outcome of the *Leishmania* infection [Mathur et al. 2004]. With high cholesterol, CD40 binds to TRAF 2,3,5, signals through p-38MAPK, induces IL-12p40 to abrogate infection while on the contrary, when it binds to TRAF 6, in a low membrane cholesterol environment, it induces ERK-1/2 mediated IL-10 production, which is beneficial for the parasite [Bhardwaj et al. 2010]. In our study, we found a significantly lowered expression of CD40 in terms of GMFC (but not in terms of percentage of cells expressing it, Figure 5.1), collectively indicating that a severely compromised expression of co-stimulatory molecules on the monocyte surface facilitates disease progression. However, in the context of immunology of PKDL, modulation of these surface markers on circulating monocytes is surprising as in PKDL, parasites are not found in the blood yet, the monocytes are functionally impaired.

TLRs are hallmarks of cellular receptors that recognize pathogen associated molecules and participate in innate responses to infections. Signaling through TLRs influence the generation of ROS, NO and production of cytokines [Faria et al. 2012]. Since TLR recognition is often associated with the production of pro-inflammatory cytokines and generation of NO and ROS, the role of TLRs in immunity against *Leishmania* is unquestionable and indeed *Leishmania* derived molecules have been reported to activate TLRs, particularly TLR-2, TLR-4 and TLR-3 [Tuon et al. 2008, Faria et al. 2012]. Role of TLR-3 mediated signaling play a decisive role in the development of muco-cutaneous leishmaniasis (MCL) because the parasites responsible for MCL, *L. braziliensis* and *L. guyanensis* are known to harbor a RNA virus, which in turn activates a detrimental Th1 mediated inflammation through TLR-3 [Olivier M, 2011]. But the virus has not been reported in the old world strains, particularly in *L. donovani* and so to us, it was more pertinent to study the TLR-2 and TLR-4 in PKDL [Hartley et al. 2012]. The role of TLR-2 and 4 in leishmaniasis is not yet clearly stated and data from experimental studies varies [Reviewed in Tuon et al. 2008, Cezário et al. 2011, Mukherjee et al. 2012, Faria et al. 2012]. However, TLRs are considered as potential vaccine candidate and drug targets [Singh et al. 2012]. 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 (Figure 5.2). Furthermore, restoration of these receptors after treatment strengthens their role in disease resolution (Figure 5.2).

As we found changes in two major receptors (TLR-2/4 and CD23) associated with generation of NO and ROS within monocytes, we felt it pertinent to measure the intracellular generation of these reactive radicals in PKDL. Previously we have reported decreased generation of NO in patients with VL which is restored following treatment [Sarkar et al. 2011]. Importance of these reactive leishmanicidal molecules is very well known [Van Assche et al. 2011] but their relevance in leishmaniasis and more specifically in patient monocytes is limited [Sarkar et al. 2011]. In PKDL, we observed a significant curtailment in generation of both NO and ROS (Figures 5.3 and 5.4) but treatment caused a significant increase in generation of NO but not in ROS which highlighted the greater importance of NO over ROS in leishmaniasis (Figures 5.3 and 5.4). Furthermore, lowering of superoxide generation (Figure 5.4D) reinforced the notion of a relationship between TLRs and NO/ ROS. Often in *in vivo* conditions modulation of pro-oxidants is accompanied by changes in the anti-oxidant status. To check whether a redox imbalance is created within the monocytes from PKDL patients or not, we measured the levels of non protein thiols (mainly glutathione) and observed a massive increase in the non protein thiols at disease presentation followed by a significant reduction with treatment (Figures 5.4 E, F and G). Collectively, the redox imbalance in monocytes helps keep them inactivated and thus contributes to disease progression.

It was well documented that signaling through TLR-2 and TLR-4 produced pro-inflammatory cytokines including IL-6, IL-8, IL-1β, TNF-α and IL-12 [Faria et al. 2012]. Similarly, in leishmaniasis, IL-6, IL-8, IL-1β, TNF-α, IL-12 helps in parasite clearance and disease resolution [Nylen and Gautam 2010]. In patients with PKDL the serum profile of pro and anti-inflammatory cytokines is a mixed one but chemotherapy is effective by exacerbating the inflammatory response [chapter 4]. Analysis of intracellular cytokine expression for pro-inflammatory cytokines in monocytes of PKDL revealed that except IL-12 and TNF-α, rest of the proinflammatory cytokines are decreased in patients with PKDL than healthy controls and significantly induced with treatment (Figure 5.5). Surprisingly we found that IL-12, although expressed very minimally was significantly higher in patients with PKDL both at a protein and mRNA level and remained on the higher side even after treatment (Figures 5.5B and C). The absence in controls may be attributable to the fact that IL-12 is an inducible cytokine and so would be undetected in healthy individuals who are uninfected. In a recent study on experimental VL, Gupta et al. showed that Miltefosine in an infected hamster model increased pro-inflammatory cytokines but they also found that with infection IL-12 is induced [Gupta et al. 2012] which proves that infection *per se* promotes an inflammatory response, but possibly not sufficient to eliminate the parasites. With respect to TNF-α we

found an insignificant level in healthy controls as well as patients with PKDL and suggest that the raised plasma TNF-α sourced from other cells.

Diseases caused by protozoan parasites are endemic in regions of poor socioeconomic status and often complicated due to co-infection with HIV and tuberculosis. This factor, along with limited access to clinical material limits our knowledge on the role of monocytes, neutrophils and dendritic cells. In PKDL our study showed for the first time that monocytes are altered in terms of their phenotype and function. It is yet not known whether these changes associated with monocytes are the cause of the disease or a consequence of the disease. Our finding of decreased $CD14^{+}CD16^{+}$ monocytes at disease presentation also needs to be validated that whether these changes are due to altered surface expression of CD14 and CD16 or the result of differentiation of one monocyte subset to another. The key research area in future will be the identification of the factors responsible for the differentiation of monocyte subsets. Given the increasing importance of monocytes in diseases caused by protozoan parasites, modulation of these factors holds great therapeutic potential.