CHAPTER VI

MACROPHAGE POLARIZATION IN PKDL
Introduction

Monocytes and macrophages exhibit great plasticity, which allows them to shift between different modes of activation, and are generally driven by the immediate microenvironment. They perform divergent functions that include patrolling their surroundings and maintaining homeostasis (resident macrophages), combating invading pathogens and tumor cells (classically activated or M1), orchestrating wound healing and immunosuppression (alternatively activated or M2) or as resolution macrophages involved in restoring homeostasis after an inflammatory response [Reviewed in Rahat et al. 2011]. Mirroring the Th1 and Th2 nomenclature in T cells, polarized macrophage subsets were originally referred to as M1 or M2; M2 based on different stimuli were further divided into M2a, M2b, M2c as well as tumor associated macrophages [TAM, Reviewed in Gordon S, 2003 and Martinez et al. 2008]. Later, Mosser and Edwards (2008) reassigned these macrophage subsets to three different classes, namely classically activated or M1 or CAM, alternatively activated or M2a or AAM and regulatory macrophages or M2b/c. These classes are best considered as a continuum of functional states that encompasses a broad range of macrophage phenotypes with interchangeable characteristics.

Differential expression of surface and intracellular markers and microenvironmental stimuli are used to identify monocyte and macrophage subpopulations. The classically activated macrophages are typically induced by the Th1 cytokine IFN-γ, lipopolysaccharide or LPS, granulocyte-macrophage colony stimulating factor or GM-CSF and other pro-inflammatory stimuli (Figure 6.1). The resulting phenotype characteristically displays high IL-12, low IL-10, combined with high reactive oxygen/nitrogen radical generation (ROS and RNS) along with enhanced secretion of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α and IL-8 (Figure 6.1). Therefore, these macrophages efficiently kill bacteria, viruses, parasites and others, along with causing extensive tissue damage. On the contrary, the Th2 immunoregulatory cytokines IL-4, IL-13, IL-10 and TGF-β produced during injury and infection or induction by M-CSF and vitamin D, generate AAM which contrary to CAMs, dampen the inflammatory response, by producing IL-10 and TGF-β. They also promote tissue repair and fibrosis by inducing arginase-I as also express various surface markers including the mannose receptor (MR) or CD206, scavenger receptors concomitant with suppression of production of pro-inflammatory cytokines [Figure 6.1, Reviewed in Hoeksema et al. 2012, Liu and Yang 2013, Sica and Mantovani 2012, Gordon and Martinez 2010, Mantovani et al. 2004].
Monocyte/macrophage polarization in infectious diseases

Recent studies have shown that apart from its role in cancers, cardiovascular diseases, metabolic diseases, diabetes and other autoimmune diseases, macrophage/macrophage polarization plays a pivotal role in infectious diseases [Reviewed in Sica and Mantovani, 2012, Biswas et al. 2012a, Mege et al. 2011, Benoist et al. 2008, Raes et al. 2007, Noel et al. 2004].

M1 polarization and infectious pathogenesis

M1 macrophages/monocytes are generally considered responsible for resistance against intracellular pathogens and certain acute bacterial infections. In patients with acute
typhoid fever, transcriptomic analysis of circulating cells revealed enrichment of M1 polarization related genes. Similarly in H. Pylori infection and L. monocytogenes associated disease, polarization of monocyte/macrophage subsets towards M1 type killed the bacteria thus favouring the host [Reviewed in Mege et al. 2011, Benoit et al. 2008]. On the contrary, uncontrolled M1 polarization can also lead to disease associated with host tissue damage and inflammation as evident in acute infection with E. coli, Streptococcus sp causing gastroenteritis, urinary tract infections and sepsis [Reviewed in Sica and Mantovani 2012, Benoit et al. 2008]. The M1-M2 switch observed during the transition from acute to chronic infection may provide protection against overwhelming uncontrolled inflammation; however, such a phenotype switch can also favour pathogens that have evolved strategies to interfere with M1 associated killing example being Salmonella Dublin protein mediated inhibition of IL-12p70, OMP-25 mediated inhibition of TNF-α by Brucella suis, early secreted antigenic target protein-6 (ESAT-6) mediated inhibition of NF-κB and interferon regulatory factors by Mycobacterium tuberculosis [Reviewed in Benoit et al. 2008].

M2 polarization in chronic infectious diseases

The gradual evolution of chronic infectious diseases is thought to be associated with monocyte/macrophage reprogramming towards a M2 profile as evident in leprosy, Whipple’s disease, Q fever, tuberculosis and in brucellosis. Persistence of the intracellular pathogen in M2 polarized monocytes/macrophages occurs mainly due to suppression of the antimicrobial defense system by immunoregulatory IL-10 and TGF-β; neutralization of both these cytokines can reverse the infection into cure [Reviewed in Mege et al. 2011, Benoit et al. 2008]. Accordingly, M2 polarization is desirable in situations like autoimmunity, diabetes and cardiovascular diseases and sepsis [Biswas et al. 2012a]. Similarly, M2 polarization is also known to play a key role in chronic helminthic infections [Noel et al. 2004].

Effect of M1-M2 switching in parasitic diseases

In experimental and human parasitic infections, macrophages generally undergo a dynamic switch toward M2 polarization. This is because during infections with protozoan parasites such as Leishmania, Trypanosoma, Plasmodium or Toxoplasma, an IFN-γ dependent pro-inflammatory response, triggered development of M1 that would control parasitemia especially in the acute phase of infection. However, depending on parasite virulence, host genotype and stage of infection, the host can also elicit a type 2 or anti-inflammatory response. The major advantage for favouring M2 polarization in chronic parasitic infections is that type 2 cytokines (IL-4 and IL-13) antagonize generation of iNOS mediated nitric oxide (NO) and instead induce arginase-I which helps in polyamine synthesis, necessary for parasite
growth and multiplication [Reviewed in Raes et al. 2007, Das et al. 2010]. The recurrent association of M2 polarization with parasitic infections does not necessarily imply a generalized relevance in pathogenesis. For instance, lineage specific ablation of IL-4Ra caused a dramatic increase in susceptibility to *S. Mansoni*, but delayed disease progression in experimental leishmaniasis with *L. major*, whereas the effect is minimal in case of *Nippostrongylus* infection [Reviewed in Sica and Mantovani 2012, Raes et al. 2007].

This high plasticity of macrophages, complexity of the in vivo environments along with polarized macrophage activation states as defined in vitro, represent extremes of a continuum. However, in vivo, the monocyte/macrophage subsets probably exhibit varying degrees of heterogeneity and represent a mixed activation status. This differential activation of macrophages in vivo remains a largely unresolved issue and therefore evaluating the molecular networks that orchestrate these mixed phenotypes needs to be addressed and carefully dissected on an individual case by case basis in diseased conditions.

**Study objectives**

Considering the complexity and plasticity of monocytes/macrophages and the fact that its relevance in the PKDL immune scenario is not known, this study examined polarization of circulating monocytes and/or dermal macrophages towards alternative activation. We have assessed the expression of arginase-1 and mannose receptor, two most potent markers associated with alternative activation along with peroxisome proliferator activator-γ (PPAR-γ) in monocytes and macrophages. In search of the signaling that triggers this switch, we have measured plasma vitamin D3 and expression of gene (CYP27B1, vitamin D receptor and cathelicidin or LL-37). Finally we have confirmed the regulatory and immunosuppressive nature of these monocytes by measuring intracellular levels of IL-10 and TGF-β.

**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.
Immunofluorescence staining for detection of Arginase-1 and Mannose receptor

Immunofluorescent staining was done on paraffin embedded skin biopsies which were mounted on glass slides followed by deparaffinisation. After antigen retrieval, slides were blocked with blocking buffer followed by overnight staining at 4°C with the primary antibody (mouse anti human CD68, 1: 500 dilution in PBS and/or rabbit anti human arginase-1, 1: 50 in PBS, rabbit anti human CD206, 1:100 in PBS, anti nitric oxide synthase 2 or NOS2 conjugated with Tetramethyl Rhodamine Isothiocyanate or TRITC along with appropriate isotype matched control antibodies. Slides were then washed with wash buffer and binding detected using secondary antibodies (anti mouse Alexa594, anti rabbit Alexa488, 1:200 in PBS for arginase-1 staining and anti mouse Alexa488, anti rabbit Alexa647 for MR staining) for 1 h at room temperature in dark. Following washings, DAPI was added, incubated for 10 minutes and finally, slides mounted with Pro-long Gold anti-fade overnight at 4°C as described in chapter 2. Images were captured in inverted LSM 710 Confocal microscope (Carl Zeiss Microimaging, Cambridge, UK) and analyzed via LSM 7500 software.

Isolation of RNA and Reverse transcriptase-PCR from peripheral blood mononuclear cells

Total RNA was isolated from monocyte enriched PBMCs (1x10⁶ cells) according to the manufacturer’s instructions Reverse transcriptase-PCR was performed on isolated RNA (50 ng) with a one-step reverse transcriptase-PCR kit using gene-specific primers for IL-12p40, Arginase-1 (Arg-1), Mannose receptor (CD206), Peroxisome proliferator-Activated Receptor-gamma (PPARγ), Vitamin D₃ Receptor (VDR), 25-Hydroxyvitamin D₃ 1-alpha-hydroxylase (CYP27B1), LL-37 (human cathelicidin, a antimicrobial peptide), ferritin, transferrin, transferrin receptor, HO-1, CD163 and β-actin as described in chapter 2. Products were resolved on agarose gels (2%) containing ethidium bromide (0.5 mg/ml), observed and analyzed in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools (Version 4.01.04) software, values being normalized to β-actin.

Immunophenotyping of peripheral blood leukocytes

Peripheral blood (100 μl) was surface stained with fluorochrome conjugated antibodies to CD14 Peridinin Chlorophyll protein or PerCP and CD206 (mannose receptor) Alexa 488 along with appropriate isotype controls as described in chapter 2.

Measurement of plasma 25(OH) Vitamin D₃

Plasma concentrations of 25(OH) vitamin D₃ were assayed with a radioimmunoassay kit, according to the manufacturer’s instructions.
Intracellular cytokine staining

Monocytes (1 x 10^6 cells/well/ml) after being 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 was further incubated with LDA (10 µg/ml) and Brefeldin A (1 µg/ml) for 4 h for intracellular cytokine measurement. Cells were harvested; surface stained with CD14 FITC, fixed and permeabilized for staining with IL-10 APC, TGF-β APC along with their appropriate isotype antibodies, therefore acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).
Chapter VI: Macrophage polarization in PKDL

Results

Enhanced expression of arginase 1 in patients with PKDL

A raised level of arginase is a hallmark of M2 monocytes/macrophages [Martinez et al. 2008] and in patients with PKDL increased levels of plasma arginase has been observed [chapter 4, Figure 4.4], but its source needs to be pinpointed. It is present in two isoforms and is released from neutrophils, monocytes or hepatocytes [Munder 2009]. Lesions of patients with PKDL showed a significantly increased mRNA expression of Arg-1 (34.35 ± 3.74 vs. 4.56 ± 0.70, p<0.001, Figure 6.2A) which decreased with treatment (19.64 ± 2.83, Figure 6.2A). Analysis on an individual basis showed that treatment caused a significant curtailment in Arg-1 expression (p<0.05, Figure 6.2A). This increase in Arg-1 expression at the lesional site was confirmed by multicolour fluorescence on formalin-fixed paraffin-embedded dermal tissue sections from patients with PKDL. Confocal microscopic analysis revealed increased proportions of Arg-1 expressing CD68+ macrophages in the inflammatory infiltrate of patients with PKDL at disease presentation which decreased substantially with treatment (Figure 6.2B).

Alongside, we have checked the mRNA expression of Arg-1 in monocyte enriched PBMCs of patients with PKDL and observed a similar increase, being 5.29 fold higher at disease presentation than healthy controls (42.71 ± 4.46 vs. 8.08 ± 2.51, p<0.01, Figure 6.2C); similarly treatment caused a significant 2.63 fold reduction in the expression of Arg-1 (16.22 ± 2.05, p<0.05, Figure 6.2C).
Chapter VI: Macrophage polarization in PKbL

Figure 6.2
A. Representative profile of mRNA expression of arginase-1 and P-actin in lesions isolated from patients with PKDL before (Prel-Pre3) and after treatment (Postl-Post3) and from healthy controls (N1-N3). Scatter diagram for expression of arginase-1, after normalization to P-actin levels in healthy controls (n=5), patients with PKDL at presentation (Pre t/t, n=10) and after treatment (Post t/t, n=10) quantified by densitometric analysis of RT-PCR products. Before and after plots (n=10).

B. Expression of arginase-1 (green) in CD68+ macrophages (white) at the lesional site of patients with PKDL before and after treatment. Figures were taken using 40X magnification.

C. Representative profiles of mRNA expression of arginase-1 and P-actin in monocyte enriched PBMCs isolated from patients with PKDL before (Prel-Pre3) and after treatment (Postl-Post3) and from healthy controls (N1-N3). Scatter diagram for expression of arginase-1, after normalization to P-actin levels in healthy controls (n=5), patients with PKDL at presentation (Pre t/t, n=5) and after treatment (Post t/t, n=5) quantified by densitometric analysis of RT-PCR products. Before and after plots for the same (n=5).
Increased expression of mannose receptor confirms the alternative activation of monocytes/macrophages in PKDL

The mannose receptor (CD206/MR), considered as the most potent marker of macrophage alternative activation [Sica and Mantovani 2012, Biswas et al. 2012b] is a member of the C-type lectin receptor family, expressed primarily by macrophages and dendritic cells. It is considered as a non canonical pattern recognition receptor (PRR) able to bind pathogen derived molecules to mediate physiological clearance and acts as a bridge between immunity and homeostasis [Gazi et al. 2009]. Accordingly, we measured the expression of MR which was showed a dramatic increase as evidenced by rt PCR and immunofluorescence. The lesional expression of MR showed 13.9 fold increase at presentation as compared to healthy controls whose expression of MR was undetectable (13.86 ± 0.92, p<0.01, Figure 6.3A); with treatment, the expression of MR became non detectable (Figure 6.3A). Immunofluorescence staining in formalin fixed skin biopsies from patients with PKDL validated this finding before and after treatment. Importantly, we have demonstrated that macrophages in the dermal infiltrate are strongly positive for MR staining and numbers were significantly decreased after treatment (p<0.05, Figure 6.3B). Interestingly some non macrophage cells also stained positive for MR and decreased after treatment, suggesting that these cells could well be dendritic cells, capable of expressing MR [Wollenberg et al. 2002].

In monocyte enriched PBMCs, we got a significant 14 fold increase in mRNA expression of MR vs. healthy controls whose expression was undetectable (13.94 ± 1.01, p<0.01, Figure 6.3C). Importantly following treatment, the expression of MR returned to undetectable levels (Figure 6.3C). Analysis on an individual basis showed the same trend (p<0.001, Figure 6.3C). Furthermore, we assessed the surface expression of MR (CD206) on monocytes and observed that in patients with PKDL, the expression of MR was significantly higher than healthy controls (p<0.01, Figure 6.3D), thus corroborating with its raised mRNA expression in monocyte enriched PBMC fractions and dermal macrophages.
Chapter VI: Macrophage polarization in PKbL

Figure 6.3
A. Representative profile of mRNA expression of mannose receptor and β-actin in lesions isolated from patients with PKDL before (Pre1-Pre3) and after treatment (Post1-Post3) and from healthy controls (N1-N3). Scatter diagram for expression of mannose receptor, after normalization with β-actin levels in healthy controls (n = 5), patients with PKDL at presentation (Pre t/t, n = 10) and after treatment (Post t/t, n = 10) quantified by densitometric analysis of RT-PCR products, before and after plots for the same (n = 10).

B. Expression of mannose receptor (white) in CD68+ macrophages (green) at the lesional site of patients with PKDL before and after treatment. Figures were taken in 40X magnification.

C. Representative profile of mRNA expression of mannose receptor and β-actin in monocyte enriched PBMCs isolated from patients with PKDL before and after treatment (Pre1-Pre3 and Post1-Post3) and from healthy controls (N1-N3). Scatter diagram for expression of mannose receptor, after normalization to β-actin levels in healthy controls (n = 5), patients with PKDL at presentation (Pre treatment, n = 5). Patients with PKDL at presentation (Post t/t, n = 5) quantified by densitometric analysis of RT-PCR products, before and after plots for the same (n = 5).

D. A representative profile of the % of CD206+ monocytes in a healthy control and a patient with PKDL at presentation. Scatter diagram for expression of mannose receptor, after normalization to β-actin levels in healthy controls (n = 6), patients with PKDL at presentation (Pre treatment, n = 5).
Raised plasma 1\alpha,25-dihydroxyvitamin D3 levels in patients with PKDL

1\alpha,25-dihydroxyvitamin D3(1\alpha,25(OH)2D3) an active metabolite of vitamin D3 plays an important role in calcium and bone metabolism; additionally it is also known to act as an important regulator of the immune response. 1\alpha,25(OH)2D3 is known to suppress monocyte activation after IFN-\gamma stimulation and is responsible for down regulation of monocyte costimulatory molecules including CD40 and CD86 concomitant with down regulation of TLR-2 and TLR-4 [Griffin et al. 2003, Hart et al. 2011]. Moreover, it has been established that in M2 macrophages, Vitamin D3 along with VDR are up regulated and they together up regulate synthesis of the antimicrobial peptide (Cathelicidin or LL-37). Additionally Vitamin D3 has the ability to up regulate anti-inflammatory genes and down regulate pro-inflammatory genes including TLRs, NOS 2 thus providing for an immunosuppressive milieu [Hart et al. 2011].

In this study, we have measured hydroxylated vitamin D3 in patients with PKDL (a) plasma levels of in monocyte enriched PBMCs and (b) skin biopsies from patients with PKDL the plasma levels of 25 hydroxylated VitaminD3 and mRNA expression of 25-Hydroxyvitamin D3 1-alpha-hydroxylase or CYP27B1, VDR and their downstream LL-37. In patients with PKDL levels of plasma 25 hydroxylated VitaminD3 was significantly higher than healthy controls (16.31 ± 3.79 vs. 3.57 ± 3.45 ng/ml, p<0.05, Figure 6.4A) and after treatment, the levels decreased but not in a statistically significant manner (11.18 ± 3.76 ng/ml, Figure 6.4A). However when analyzed on an individual basis, the decrease was statistically significant (p<0.05, Figure 6.4B).

**Figure 6.4:**

A. Plasma 25(OH)D3 level in from healthy controls, patients with PKDL at presentation (Pre t/t) and after treatment (Post t/t).
B. Before and after plots for the same (n = 6).

**Activation of the vitamin-D signalling pathway accounts for lesional macrophage polarization**

At the lesional site, a 13 fold enhanced mRNA expression of CYP27B1 was evident in patients with PKDL than healthy controls (39.48 ± 7.95 vs. 3.10 ± 0.59, p<0.01, Figures 6.5A and B) which following treatment was decreased 2 fold (19.18 ± 3.32, Figures 6.5A and
B). On an individual basis the difference was also significant as 7 out of 10 patients had a substantial decrease in the expression (p<0.05, Figure 6.5B)

With regard to VDR a significant 11 fold increase at presentation was observed (44.80 ± 5.56, vs. 4.49 ± 0.82, p<0.001, Figures 6.5A and C) which again decreased significantly following treatment (18.34 ± 3.89, p<0.05, Figures 6.5A and C). Similarly, on an individual basis a sharp decrease in the VDR expression was evident with treatment (p<0.01, Figure 6.5C).

In terms of LL-37, the increase was even more dramatic being 45 fold (45.15 ± 3.93, p<0.001, Figures 6.5A and D) as in healthy control it was non detectable. Following treatment significant curtailment in LL-37 expression was evident (6.85 ± 3.54, p<0.01, Figures 6.5A and D). On a paired basis all 10 patients showed a marked reduction in LL-37 expression (p<0.0001, Figure 6.5D).

Figure 6.5

A. Representative profiles of mRNA expression of CYP27B1, VDR, LL-37 and β-actin in lesions isolated from patients with PKDL before (Pre1-Pre3) and after treatment (Post1-Post3) and from healthy individuals (N1-N3, n = 3).

B. Scatter diagram for expression of CYP27B1 after normalization to β-actin levels in healthy controls (n =5), patients with PKDL at presentation (Pre t/t, n =10) and after treatment (Post t/t, n =10). Before and after plots for the same (n = 10).

C. Scatter diagram for expression of VDR after normalization to β-actin levels in healthy controls (n =5), patients with PKDL at presentation (Pre t/t, n =10) and after treatment (Post t/t, n =10). Before and after plots for the same (n = 10).

D. Scatter diagram for expression of LL-37 after normalization to β-actin levels in healthy controls (n =5), patients with PKDL at presentation (Pre t/t, n =10) and after treatment (Post t/t, n =10). Before and after plots for the same (n = 10).
Systemic activation of Vitamin D signaling in PKDL possibly favours polarization of circulating monocyte

In PBMCs, the mRNA expression of CYP27B1, VDR and LL-37 in patients with PKDL was similarly enhanced as compared to healthy controls where the expression are undetectable (20.93 ± 3.59, p<0.01 and 47.07 ± 4.15, p<0.001 and 43.60 ± 0.00, p<0.05 respectively, Figures 6.6A, B and C respectively); with treatment 1.86 fold curtailment of CYP27B1 expression was evident (11.23 ± 2.22, Figures 6.6A and B), a significant 2.62 fold decrease in case of VDR along with a significant 18.17 fold for LL-37 (17.99 ± 4.82, p<0.05, 2.40 ± 2.40, p<0.05, Figure 6.6C and D respectively).

Figure 6.6

A. Representative profiles of mRNA expression of CYP27B1, VDR, LL-37 and β-actin in PBMCs isolated from patients with PKDL before (Pre1-Pre3) and after treatment (Post1-Post3) and from healthy individuals (N1-N3, n = 3).

B. Scatter diagram for expression of CYP27B1 after normalization to β-actin levels in healthy controls (n =5), patients with PKDL at presentation (Pre t/t, n =5) and after treatment (Post t/t, n =5). Before and after plots for the same (n = 5).

C. Scatter diagram for expression of VDR after normalization to β-actin levels in healthy controls (n =5), patients with PKDL at presentation (Pre t/t, n =5) and after treatment (Post t/t, n =5). Before and after plots for the same (n = 5).

D. Scatter diagram for expression of LL-37 after normalization to β-actin levels in healthy controls (n =5), patients with PKDL at presentation (Pre t/t, n =5) and after treatment (Post t/t, n =5). Before and after plots for the same (n = 5).
Enhanced mRNA expression of PPARγ in PKDL

A distinct set of signaling molecules and transcription factors are involved in macrophage polarization and includes nuclear receptor PPARγ, an important component which regulates the oxidative metabolism of macrophages and is a well documented transcription factor of M2 subsets [Sica and Mantovani 2012, Chawla 2010]. At the lesional level, a significant 16 fold increase was evident as compared to healthy controls where akin to vitamin D signaling genes, no expression was evident (16.66 ± 1.44, p<0.01, Figure 6.7A) and reverted completely with treatment (Figure 6.7A). In monocyte enriched PBMCs, at disease presentation there was a significant 47.37 fold increase in expression of PPARγ as compared to healthy controls (9.00 ± 2.65 vs. 0.19 ± 0.19, p<0.05, Figure 6.7B). This increased expression of PPARγ dramatically decreased after treatment (p<0.01, Figure 6.7B); thus strengthening our hypothesis of PKDL being associated with alternatively activated macrophages.
Modulation of cytokine expression by the M2 monocytes in patients with PKDL

Among the immunoregulatory cytokines, the most important one which plays a regulatory role in PKDL is IL-10 secreted from CD8+ T cells [Ganguly et al. 2008]. But recently it has also been observed that M2 macrophages were also able to produce IL-10 to suppress immunity [Martinez et al. 2009]. So, to check whether IL-10 was also been produced by the monocytes we have performed intracellular cytokine staining in monocytes and observed that in patients with PKDL these M2 monocytes were capable of producing IL-10 in a significantly higher level than form healthy controls (69.56 ± 9.28% vs. 11.48 ± 6.39%, p<0.01, Figure 6.8A). Treatment effected in a modest reduction of IL-10 level (42.61 ± 12.10% respectively, Figure 6.8A). With regard to another regulatory, anti-inflammatory cytokine TGF-β, we have measured its functional status. TGF-β, after synthesis forms a homodimer and remained bound with LAP stays within the cell or is secreted from the cell as an inactive form. During activation, Matrix metalloproteinases 9 and 2 (MMPs 9 and 2) or other stimulus cleaves the LAP part and the functional TGF-β then binds with its receptor and causes its effect within the cell. So, measurement of LAP-TGF-β complex can gave us the information about the amount of inactive TGF-β within the cell [Yoshinaga et al. 2008]. In patients with PKDL we got significantly lowered expression of LAP-TGF-β1 than compared to healthy control, mean ± SEM being 2.57 ± 1.19% vs. 34.79 ± 8.23%, p<0.05 (Figure 6.8A), which suggested that in patients with PKDL, the functional TGF-β1 level was higher. Akin to IL-10, treatment causes mild effect in increasing the levels of LAP-TGF-β1 i.e. decreasing the functional TGF-β1 level (22.73 ± 6.95%, Figure 6.8A). As increase in LAP-TGFβ1 indicated an indirect measurement of TGF-β expression so we have measured the mRNA expression of TGF-β1 in circulating monocytes by rtPCR. Herein also we have observed a significant 10 fold expression of TGF-β1 in patients with PKDL than from healthy controls (31.05 ± 5.74 vs. 3.00 ± 0.44, p<0.05, Figure 6.8B) and following treatment a mild reduction was observed (16.36 ± 4.84, Figure 6.8B).
Discussion

The polarization of monocyte occurs in leishmaniasis as increased levels of IL-4 and Arg-1 have been reported in the dominant stage of the disease [Osorio et al. 2008]. However in this study, we have provided direct evidence of monocyte alternate activation in PKDL.

To confirm macrophage polarization, the expression of Arg-1, PPARγ and MR was established within monocyte enriched PBMCs and at the lesional site of patients with PKDL. The inclusion of Arg-1 in this panel is controversial as many researchers believe that its
expression is strictly restricted to mouse M2 cells, notably not in humans [Raes et al. 2005, Scotton et al. 2005, Martinez et al. 2006]. A point of note is that all these studies are based on in vitro IL-4 or IL-13 stimulated human monocyte derived macrophages from healthy volunteers. So, this could well be a cytokine induced artefact whereas human monocytes in vitro probably require multiple signals to reveal the full spectrum of alternative activation [Scott 2009]. Moreover, Babu et al. (2009) showed increased Arg-1 expression in M2 monocytes of patients with filariasis. Taken together, as the in vivo condition differs from in vitro polarization, we felt it pertinent to include Arg-1 in our panel of alternative activation markers and found a significantly elevated mRNA expression at disease presentation that decreased with treatment (Figure 6.2). The increased expression of Arg-1 corroborated with decreased generation of NO within monocytes in patients with PKDL (Chapter 5, Figure 5.3). As induction of Arg-1 is known to cause T cell mediated immunosuppression [Stempin et al. 2010], it may be proposed that this increase in Arg-1 in monocytes accounted for the impaired CD8+ T cell activity reported in PKDL [Ganguly et al. 2010a].

Global mRNA and protein repertoire analysis revealed that MR is the most potent marker for macrophage alternative polarization in humans and mice [Gordon and Martinez 2010]. This enhanced expression of MR is implicated in uptake of pathogen via carbohydrate recognition receptors and thereby contributes to infection. Recently, the role of MR has been linked to induce the Th17 pathway in Candida infection [Van de Veerdonk et al. 2009] and in PKDL, an increased Th17 subset of cells and related cytokines has been reported [Katara et al. 2012]. Although the mannose receptor has not been found to play a role in experimental leishmaniasis [Akilov et al. 2007], studies regarding its status in human leishmaniasis are notably absent. Expression of MR is also known be induced by activation of PPARγ [Shalhoub et al. 2011] and is considered as an important transcription factor for polarization of macrophages. We thought that as MR has been associated with macrophage polarization, it is pertinent to study the expression of MR in PKDL where we demonstrated an enhanced expression of MR in lesional macrophages and in monocytes, which dramatically revert to normal levels with treatment [Figure 6.3].

Vitamin D and its active metabolite 1α,25(OH)2D3 plays an important role in immune regulation as evident by expression of VDR in monocytes/macrophages, dendritic cells and activated lymphocytes. Expression of CYP27B1, the 25 hydroxylated vitaminD3 hydroxylating enzyme in monocytes/macrophages entails the special role of Vitamin D3 in these cells [Bikie 2009]. 25(OH)D3 in circulation binds to vitamin D binding protein (VBP) and therefore enter immune cells such as monocytes where the 25(OH)D3 is hydroxylated further by CYP27B1 to be converted into the active 1α,25(OH)2D3. It then forms a complex with VDR and upon entering into the nucleus, the complex binds to the Vitamin D response elements (VDRE) to induce antibacterial peptides like LL-37 and other factors including
TGF-β, Arg-1 etc [Hart et al. 2011]. Its action is further extended as it also down regulates TLR-2, TLR-4 and monocyte co-stimulatory molecules like CD40, CD86 etc [Sadeghi et al. 2006, Hart et al. 2011]. Recently, Zhang et al. (2012) it has also been reported that 1α,25(OH)₂D₃ inhibits production of pro-inflammatory cytokines from monocytes by modulating the MAPK Phosphatase-1; vitamin D₃ is also known to induce monocyte polarization towards M2 type [Mantovani et al. 2005]. Collectively this information encouraged us to investigate the role of vitamin D₃ in PKDL if any.

We first measured the serum 25(OH)D₃ level which was significantly raised in patients with PKDL and decreased with treatment (Figure 6.4). Thereafter we have checked the mRNA expression of CYP27B1, VDR and finally LL-37. Importantly we observed that expression of all these genes are significantly raised in patients with PKDL than compared to healthy controls and restored successfully after treatment in circulation and in dermal sites (Figures 6.5 and 6.6). This led us to conclude that increased serum 25(OH)D₃ and subsequent activation of downstream signalling could be a molecular switch for monocyte polarization towards the M2 type in PKDL. Our data corroborated with other studies as in leishmaniasis it has been observed that VDR knockout mice were more resistant to L. major infection than their wild-type counterpart; furthermore treatment of L. major infected macrophages with 1α,25(OH)₂D₃ demonstrated a VDR dependent inhibition of macrophage killing activity, induction of Arg-1 and down regulation of iNOS [Ehrchen et al. 2007].

PPARγ is a transcription factor of the nuclear hormone receptor family involved in lipid metabolism and inflammation. PPARγ expression is both directly and indirectly up regulated by IL-4 [Chawla 2010]. Notably, PPARγ has been demonstrated to cause up regulation of Th2 responses and down regulation of Th1 responses, possibly by interfering with nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and signal transducers and activators of transcription (STAT) at a transcriptional level [Anthony et al. 2012]. On the other hand by its transcriptional activity, it induces the expression of Arg-1 and via its transrepressive action, PPARγ blocks the expression of iNOS and NF-κB mediated transcription of pro-inflammatory mediators [Chan et al. 2012]. In our study, we have observed an increased expression of Arg-1 and decreased generation of NO (Figure 6.2A and Chapter 5). Additionally, PPARγ in the cytosol is found to be associated with protein kinase C-α (PKC-α) and interfere with PKC-α translocation to the cell membrane i.e. its activation. This subsequently causes suppression of NADPH oxidase mediated superoxide generation within macrophages [Brüne et al. 2013]. Corroborative to this phenomenon, our study too showed that in PKDL, generation of superoxide is impaired (Chapter 5). All these findings further necessitated assessing the expression of PPARγ in our study population. The information related to expression of PPARγ in leishmaniasis is very limited and indeed the first report is published by Chan et al. (2012) in an experimental model of VL where they
have found following infection an increased expression of PPARγ in spleen, liver as also in peritoneal cells, not only that they have found that inhibition of PPARγ expression lowered the parasite burden [Chan et al. 2012]. We found a significantly increased expression of PPARγ at disease presentation and again a significant curtailment after treatment both in circulation and the disease site (Figures 6.7A and B) highlighting the altered monocyte phenotype in patients with PKDL.

The role of immunosuppressive IL-10 and TGF-β in Leishmaniasis is well explained and it has also been found that macrophages could be one of the potent sources for these two cytokines in leishmaniasis [Nylen and Gautam 2010]. M2 monocytes are also known to be a producer of these cytokines owing to their immunoregulatory and immunosuppressive role [Martinez et al. 2009] and these two cytokines are considered as a functional marker of macrophage polarization. Previously in PKDL, CD8+ T cells were known to be the source of IL-10 but the roles of monocytes had not been studied [Ganguly et al. 2008]. In this study, we have established that monocytes are also a potent source of IL-10 and TGF-β in patients with PKDL (Figure 6.8) which boosts the fact that monocytes are being alternatively polarized.

Taken together, our study showed evidence for the first time of alternative polarization of monocytes in PKDL, indeed in any form of leishmaniasis. As till date all the studies regarding immunology of human leishmaniasis dealt mainly the function of T cells particularly the role of Tregs if any in the disease pathogenesis [Mougneau et al. 2011], our study is unique in that it showed the importance of monocytes in the disease pathogenesis of PKDL. Our understanding of the immunology of PKDL revealed that circulating monocytes in patients although not infected with parasites are still functionally impaired and mediates immunosuppression in PKDL. This could be driven by parasite antigens that migrate from the dermal site or by Vitamin D3. We also found that after treatment, monocytes are being repolarised to the classically activated type which are possibly responsible for disease elimination. Taken together, it could be concluded that in a chronic infection, it is not always the Tregs that play a major role in immunity, but equally important are alternatively polarized monocytes. Most of our current knowledge of monocyte/macrophage polarization has originated from cytokine induced transcriptome analysis in either mice or from in vitro models [Martinez et al. 2009] but human studies being notably limited.