CHAPTER II

MATERIALS AND METHODS

Reagents

Immunological reagents were from BD Biosciences (TLR-2 Fluorescein isothiocyanate or FITC, TLR-4 Phycoerythrin or PE, CD14 Peridinin chlorophyll protein or PerCP, CD80 PE, CD86 FITC, TNF-α PE, IL-10 Allophycocyanin or APC, CD3 PerCP and FACS lysing solution, San Jose, USA) except anti-human CD16 PE and CD14 FITC, CD54 APC, HLA-DR PE, CD71 APC, CD19 APC, ELISA kits for IL-4, IL-6, IL-8, IL-10, IL-13 and TNF-α (Immunotools, Friesoythe, Germany), Quantikine Immunoassay kit for IL-1β and TGF- β (R&D systems, Minneapolis, USA), IL-6 PE and IL-1β PE (eBioscience. Inc., San Diego, CA, USA), anti human Latency associated peptide (LAP)-TGF-β1 APC, IL-12p40 PE, IL-8 APC, CD23 APC, CD 206 Alexa 488 (Biolegend, San Diego, CA, USA), anti human CD206, anti human Arginase-1 (Protein tech, Manchester, UK) anti human CD68 (Abcam, Cambridge, UK), anti mouse Alexa594, anti rabbit Alexa488, Pro-long Gold anti-fade (Invitrogen, Life Technologies Ltd., Paisley, UK). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) except protease inhibitors and 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS, Roche Applied Science, Penzberg, Germany) rK39 immunochromatographic test strips (InBios International, Seattle, USA), N-(1-napthyl) ethylenediamine dihydrochloride (NED, Loba Chemie, Mumbai, India), Trichloro acetic acid (TCA), Sulphanilamide, L-Arginine, Manganese chloride, para nitro phenyl phosphate (PNPP) and Urea (Sisco Research Laboratories, Mumbai, India), HiSep 1073, tetra methyl benzidine (TMB) from Genei, Bangalore, and foetal bovine serum (FBS) from Himedia, Mumbai, India (Himedia, Mumbai, India), polystyrene coated maxisorp strips were obtained from Nunc Immunomodules, Roskilde, Denmark, Target retrieval solution (citrate buffer, pH-6.0, Dako, Cambridge, UK). 4, 5- diaminofluorescein diacetate (DAF-2DA, Cayman Chemicals, Ann Arbor, Michigan, USA), 5-chloromethylfluorescein diacetate (CMFDA), Calcein acetoxymethyl ester (Calcein-AM), 5-(and-6)-carboxy-2',7'-dichloro dihydrofluorescein diacetate, acetyl ester (CMH_2DCFDA, Molecular Probes, Carlsbad, CA, USA), 25 hydroxyvitamin D radio immunoassay kit (DiaSorin, Stillwater, Minnesota, USA), plasma iron was assayed by IRON and Total Iron binding capacity (TIBC) kit (Crest Biosystems, Goa, India), Ferritin using Ferritin ELISA kit (Demeditech Diagnostics, Germany) and Transferrin using Transferrin assay kit (DiaSys Diagnostic System, Germany), RNAqueous-4PCR Kit (Ambion, Life technologies, Carlsbad, CA, USA), One step QiAmp rt PCR kit (Qiagen, Hilden, Germany).

Study population

Recruitment

A total of 57 patients clinically diagnosed with PKDL were recruited from the outpatient unit of Departments of Dermatology, School of Tropical Medicine, Kolkata and

Medical College, Kolkata, West Bengal between 2008-2010. The study was approved by the Institutional Ethical Committee (Annexure I). All patients provided informed written consent (in case of a minor, the parent/guardian provided the same) and the study received approval from the Institutional Ethical Committee of School of Tropical Medicine, Kolkata, India and Institute of Post Graduate Medical Education & Research, Kolkata, India.

Diagnosis

Diagnosis for PKDL was made by the dermatologist(s) in attendance, based on clinical features and a past history of VL, corroborated serologically by rK39 strip test [Sundar et al., 2007] and ELISA [Chatterjee et al., 1998] and histopathological examination for presence of Leishman Donovan (LD) bodies in dermal lesions. Additionally, in cases where the interval between onset of VL and appearance of PKDL was short, we confirmed our diagnosis by doing an ITS1 based PCR from dermal biopsies. In cases where parasite isolates were obtained, they were typed as *L. donovani* [Ghosh et al. 2012]. None of the patients suffered from any other infections or had any pre- existing disease. As controls, age and sex-matched healthy volunteers (n = 15) were recruited from non-endemic areas and were seronegative for anti-leishmanial antibodies.

Treatment

Patients (n = 57, polymorphic, n = 41, Figures 2.1A, B and macular, n = 16, Figures 2.1C and D) were randomly allocated to receive either sodium antimony gluconate (SAG; 20 mg/kg body weight/day intramuscular for 4 months) or miltefosine (100 mg/day per oral for 4 months). Among them, 28 were followed up successfully and received SAG (n = 14) or miltefosine (n = 14) and were either polymorphic, n = 22 or macular, n = 6. Samples were collected at disease presentation and on completion of treatment. Assessment of cure was based on remission of clinical features. Samples were collected at disease presentation and one month after completion of treatment.

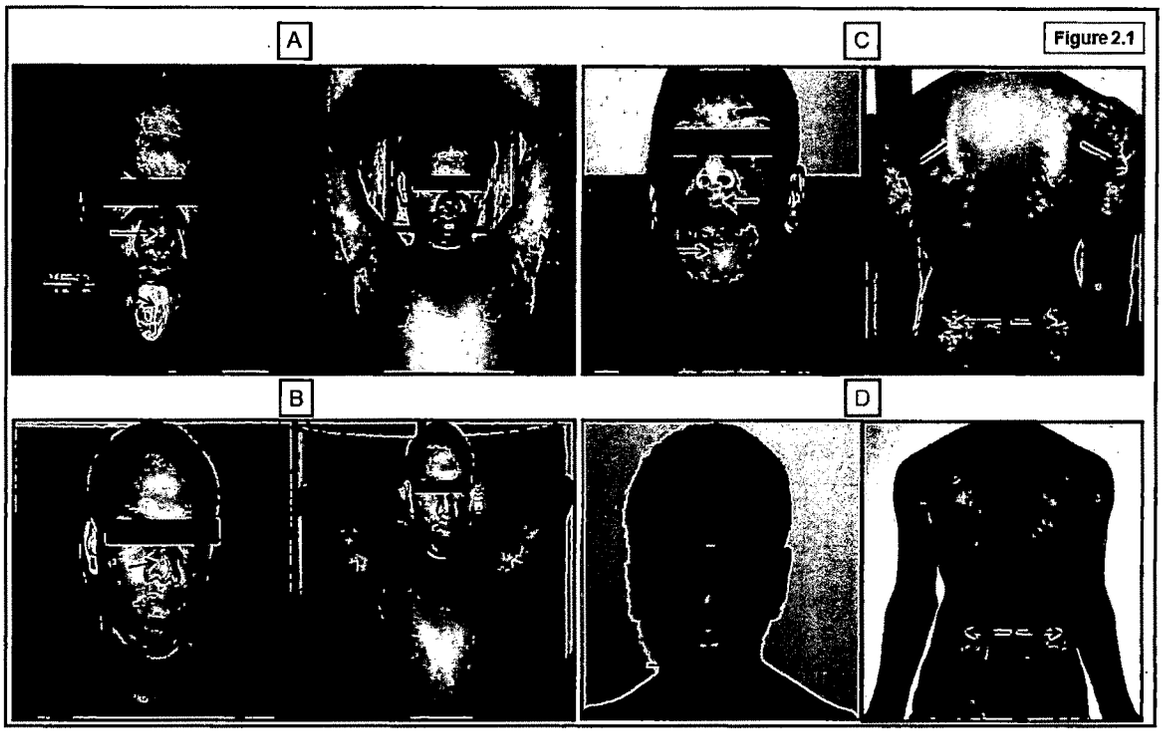

Figure 2.1: Clinical presentation of patients with PKDL

A. Patient with polymorphic PKDL at disease presentation having characteristic papulonodular lesions in tongue, mucosal areas. Appearance of macules in hypopigmentation and erythematous papuloplaque occupying the axillary fold and sparing the vault of axilla.

B. Regression of papules and nodules following treatment (with Miltefosine) in the polymorphic PKDL. However macules in the axillary fold remain unchanged.

C. Patient with macular PKDL at disease presentation with characteristic symmetrically distributed hypopigmented macular lesions in the trunk and in the face covering the mucosal and area and chin.

D. Following treatment (with Miltefosine) curtailment of macules and in hypopigmentation is visible in the face; however, the hypopigmentation in trunks are partially regressed.

Collection of samples

Phlebotomy by venipuncture of patients before and after treatment as well as healthy controls was followed for collection of 12 ml peripheral blood in heparin (100 units / ml). Plasma was stored in aliquots at -20°C until use. Dermal biopsies were extracted from the lesions by the dermatologist(s) using a biopsy punch under local anaesthesia. The biopsied tissue was split into three parts on a sterile glass slide using a surgical blade. The first part was aseptically introduced into a vial containing 1 ml of Schneider's insect medium containing supplemented with 20% heat-inactivated fetal calf serum (HIFCS). After transport to the lab, this tissue was macerated under a laminar air flow, the original medium with the cellular suspension transferred into a fresh tissue culture flask and stored at 24°C to check for transformation of parasites. The second part was transferred to a vial containing RNA*Later* (Ambion Inc.), an RNA stabilization reagent, and after arrival at the laboratory, stored at -20°C until the day of the RNA isolation procedure. The final part was immersed in a vial of

10 % formalin (v/v in 0.01 M phosphate buffered saline, PBS) and subsequently, stored at 4°C until processing for immunofluorescence study.

The rK39 strip test

Approximately 20 µl of serum was transferred by pipette on the absorbent pad of the strip and allowed to migrate up the strip under the influence of capillary action for 25- 40 seconds. Subsequently, 2 drops of supplied chase buffer were placed on the pad. After 5 - 10 minutes, the strip was checked visually for presence of control and test bands with test-results defined as follows:

1) Positive: Presence of both control and test bands.

2) Negative: Presence of the control band alone.

3) Invalid test: Absence of both control and test bands or presence of test band alone.

Preparation of *Leishmania donovani* antigen (LDA)

Crude *Leishmania* antigen was prepared from a *L. donovani* strain, MHOM/IN/90/GE1F8R [Chatterjee et al., 1998]. Promastigotes were routinely passaged in M-199 medium supplemented with 10% heat inactivated fetal calf serum (HIFCS), 100 IU penicillin G and 100 µg/ml streptomycin and stored at 24°C. Promastigotes were maintained in their log-phase of growth by adding fresh medium every 2 days. When an optimal cell density (>1 × 10^7 cells/ml) was attained, promastigotes were harvested, spun down (700 g x 5 min.) and washed twice with PBS. The pellet was resuspended in 250 µl lysis buffer (20 mM Tris-HCl, 40 mM NaCl, pH 7.4) containing EDTA (5 mM), protease inhibitor cocktail (Roche) and Triton X-100 (0.2% v/v). After vigourous vortexing, the cells were subjected to four freeze-thaw cycles for effective lysis, which was confirmed microscopically. Protein content of the lysate was estimated using Lowry's method [Lowry et al. 1955] and several aliquots of the lysate (to prevent protein degradation due to repeated freeze-thawing) were stored at -20°C until use.

ELISA for total antileishmanial immunoglobulin

LDA was added to polystyrene coated wells, incubated overnight at 4°C and followed by three washings with phosphate buffer saline (PBS) supplemented with 0.05% Tween-20 (PBS-T); the nonspecific binding sites were blocked by PBS supplemented with 2% fetal bovine serum (PBS-FBS) for 2 h (200 µl/well); sera (diluted 1:500 in PBS-FBS; 100 µl/well) was added and incubated overnight at 4°C. The wells were finally incubated with horse radish peroxidase (HRP) conjugated protein-A (diluted 1:5000 in PBS-FBS, 100 µl/well) at 37°C for 30 min. and after five washes with PBS-T, binding was detected using ABTS (100 µl/well),

optical density (OD_{405} nm) being measured on an ELISA reader (Model 680, Bio-Rad, California, USA).

Determination of antileishmanial IgG, IgM and IgE

ELISA for antileishmanial IgG, IgM and IgE was measured using LDA as the coating antigen in the polystyrene coated wells; after an overnight incubation at 4°C followed by three washings with PBS-T, the remaining reactive sites were blocked with PBS-FBS (200 µl/well) for 2 h; sera (diluted 1:500 for IgG/IgM and 1:5 for IgE in PBS-FBS, 100 µl/well) were added and incubated overnight at 4°C. After three washes with PBS-T, the wells were incubated with anti human HRP-IgG or IgM (diluted 1:50,000 and 1:25,000 respectively in PBS-FBS, 100 µl/well) or with alkaline phosphatase conjugated anti human IgE (diluted 1:5000 in PBS-FBS, 100 µl/well) at room temperature (20°-25°C) for 30 min.; following extensive washing with PBS-T, binding was detected using ABTS (100 µl/well) for IgG and IgM and with PNPP (100 µl/well) for IgE; absorbances were measured at 405 nm being measured on an ELISA reader (Model 680, Bio-Rad, California, USA).

Measurement of antileishmanial IgG subclasses

For determination of IgG subclasses, LDA coated polystyrene wells after being blocked with PBS-FBS (200 µl) were incubated overnight with human sera (diluted 1:50 in PBS-FBS) followed by incubation with mouse anti human IgG1 (diluted 1:5000) and biotinylated anti human IgG2, IgG3 and IgG4 (diluted 1:30,000, 1:4000 and 1:15,000 respectively in PBS-FBS). Binding was detected using HRP conjugated anti mouse Ig (diluted 1:10,000 in PBS-FBS) for IgG1 and streptavidin-HRP (1:5000 in PBS-FBS) for IgG2, IgG3 and IgG4 using ABTS (100 µl) as the substrate and OD_{405} nm being measured on an ELISA reader (Model 680, Bio-Rad, California, USA).

Determination of IgG avidity

The avidity of antileishmanial IgG was measured by indirect ELISA using polystyrene coated wells; briefly, LDA (1.0 µg/100 µl in bicarbonate buffer, pH 9.2) served as the coating antigen and after an overnight incubation at 37°C, were washed thrice with PBS-T. The non specific binding sites were blocked with PBS-FBS at room temperature (20-25°C) for 1 h, the plates were again washed thrice with PBS-T and the plates incubated with sera (diluted 1:100 in PBS, 200 µl) in duplicate in 2 sets, Rows A and B, i.e. quadruplets at 37°C for 1 h). Following three washings with PBS-T, 200 µl of dissociation buffer (6 M urea in PBS-T) was added to all wells in Row B, while in Row A, 200 µl of PBS-T alone was added and incubated at 37°C for 10 min. Following three washings with PBS-T, the wells

were incubated with anti-human IgG-HRP (diluted 1:5000, 100 µl, 37°C for 30 min and after extensive washing with PBS-T binding was detected using 100 µl of TMB for 30 min at 20-25°C; the reaction was stopped by addition of 1N H_2SO_4 and absorbances measured at 450 nm on an ELISA reader (Model 680, Bio-Rad, California, USA). The IgG avidity of each sample was calculated as the ratio between its OD_{450} in Row B/O.D_{450} in Row A.

Cytokine ELISA

Circulating and culture supernatant levels of TNF-α, IL-6, IL-8, IL-4, IL-10 and IL-13 were quantified using commercially available kits from Immunotools, Germany, according to the manufacturer's instructions. Briefly, individual cytokine antibodies were coated on ELISA plates; following blocking of nonspecific sites with PBS-2% FBS, particular cytokine standards/ plasma/ culture supernatants were added to designated wells and binding detected by using biotinylated anti cytokine antibodies, followed by addition of peroxidase conjugated streptavidin (1:5000) with ABTS as the substrate, and OD_{405} nm measured in a micro plate reader (BioRad; model 680, California, USA).

The levels of TGF-β and IL-1β were quantified using commercially available ELISA kits from R&D system, according to the manufacturer's instructions. Briefly, to the cytokine antibody-coated plates, cytokine standards (TGF-β and IL-1β) or plasma were added, incubated for 2 h at room temperature and binding detected using appropriate secondary antibodies and substrates and absorbance measured at 450 nm on an ELISA reader (Model 680, Bio-Rad, California, USA).

Determination of plasma levels of nitrite

The plasma levels of nitrite, a stable representative of nitric oxide (NO) were determined by the Griess method with slight modifications [Ghasemi et al. 2007]. Briefly serum was mixed with 5% TCA in the ratio of 1:9 and centrifuged at 8000 rpm for 5 minutes. Supernatants were mixed with equal volume of Griess reagent (mixture of 0.1% NED in water and 1% sulphanilamide in 5% phosphoric acid, 1:1 ratio) and incubated for 30 minutes at 37°C. The absorbance was measured at 546 nm using spectrophotometer. Specific OD_{546} for serum samples were calculated by subtracting the OD_{546} of TCA from the OD_{546} of serum. The concentration of nitrite was determined using a standard curve of sodium nitrite (0-100 µM).

Measurement of plasma Arginase activity

Arginase activity was measured as previously described [Del Ara et al. 2002]; briefly, the assay was performed using a reaction mixture containing 0.5 M L-arginine, 1.0 mM

$MnCl_2$ and 100 µl of plasma (diluted 1:1 with PBS). Following 20 min. incubation at 37°C, the reaction was stopped by addition of an acidic solution containing H_2SO_4, H_3PO_4, H_2O (1:3:7 v/v, 800 µl). This was followed by addition of 100 µl 3% isonitrosopropiophenone (dissolved in absolute ethanol), samples were heated at 95°C for 40 min. in the dark and absorbance measured at 540 nm. The calibration curve was prepared with increasing amounts of urea (1-200 µg). The arginase activity was expressed in units per liter wherein one unit (U) of enzymatic activity was defined as the amount of enzyme that catalyzes the formation of 1.0 µmol urea/minute.

Immunophenotyping of peripheral blood leukocytes

Peripheral blood (100 µl) was surface stained with fluorochrome conjugated antibodies to CD3 PerCP, CD14 FITC, CD14 PerCP, CD16 PE, CD19 APC, CD23 APC, CD40 PE, CD54 APC, CD71 APC, CD80 PE, CD86 FITC, CD206 Alexa fluor 488 and HLA-DR PE along with appropriate isotype controls. Following incubation for 15 min. at room temperature, BD FACS lysing solution (2 ml, BD Biosciences, CA, USA) was added and incubated for an additional 10 min. at room temperature. Cells were centrifuged (400 g x 5 min), the resultant pellet was washed with PBS and acquired on a FACS Calibur (BD Biosciences, CA, USA). Cells were gated on the basis of characteristic linear forward and side scatter features of lymphocytes and monocytes with fluorescence measured on a logarithmic scale, using CellQuest Pro software (BD Biosciences, CA, USA).

Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was carefully layered over Ficoll-Hypaque (1:1, Histopaque-1077) for isolation of PBMCs or for isolation of monocytes, 3 ml of blood was diluted 1:1 with PBS (0.01M, pH 7.2) followed by layered over 3 ml. of HiSep 1073 (1:1) and centrifuged (400 g x 30 min) to yield a PBMC rich or monocyte rich interface. This layer was washed twice in phosphate buffered saline (PBS, 0.01 M, pH 7.2) and the cell pellet was resuspended in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% heat inactivated fetal calf serum (HIFCS). Cell viability was confirmed microscopically by trypan blue exclusion with more than 95 % cells negative for trypan blue stain considered as a viable population. These cells were seeded (1×10^6 cells/ml) in multi-well cell-culture plates and incubated in a CO_2 (5%) incubator set at 37°C for different time-periods according to the experimental plan.

Determination of expression of Toll like receptors (TLRs)

After isolation of monocytes, they were surface stained with anti human CD14 PerCP and incubated for 30 minutes at room temperature (RT). The cells were washed twice with

PBS followed by fixation and permeabilization with fix-perm buffer (2% paraformaldehyde + 0.05% saponin + 3% FBS in PBS) for 20 minutes at RT after which cells were stained with anti human TLR-2 PE and TLR-4 FITC for 15 minutes. Cells were finally washed twice and resuspended in PBS-2% FBS and were acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).

Determination of intracellular NO in monocytes

Monocytes ($5x10^5$) were centrifuged (4000 rpm x 5 minutes) and then resuspended in 250 µl of PBS; following staining with DAF-2DA (2 µM, Sarkar et al. 2011) for 30 minutes at 37°C, 100 µl of PBS was further added and cells acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).

Measurement of the oxidant status of monocytes

Generation of ROS was measured in monocytes (5×10^5/ml) that were centrifuged (4000 rpm X 5 minutes), resuspended in PBS (500 µl) and stained with CMH_2DCFDA (2.5 µM dissolved in cell culture tested Di methyl sulfoxide or DMSO) for 30 minutes at 37°C; fluorescence was then acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).

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 (1.0 mg/ml) and incubated for 30 min. at 37°C. The tubes were then placed on ice, centrifugation (400 g × 5 min, 4°C) and absorbances of the supernatants were measured at 550 nm, being representative of the reduced cytochrome c ($A_{superoxide}$). 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 CMFDA; as it is an established substrate for drug efflux pumps in mammalian cells, monocytes (5×10^5) were initially resuspended in ATP depletion buffer (500 µl, Sarkar et al. 2009) and incubated for 30 minutes at 37°C to inactivate the pump activity, if any. Cells were then washed twice with PBS, stained with CMFDA (50 Nm dissolved in DMSO, 15 minutes, 37°C) and then acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).

Intracellular cytokine staining

Monocytes (1×10^6 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) at 37°C, 5% CO_2; they were further incubated with LDA (10 µg/ml) and Brefeldin A (1 µg/ml) for 4 h at 37°C, 5% CO_2. Cells were harvested; surface

stained with CD14 FITC and was incubated for 30 minutes. The cells were washed twice with PBS+2% FBS followed by fixation and permeabilization with fix-perm buffer (2% paraformaldehyde + 0.05% saponin + 3% FBS in PBS) for 20 minutes at RT. Cells are then washed with perm wash buffer (0.05% saponin + 3% FBS in PBS)and resuspended in perm wash buffer for staining with either IL-6 PE, IL-1β PE, IL-8 APC, IL-12p40 PE, TNF-α PE, LAP-TGF-β1 APC or IL-10 APC along with their appropriate isotype antibodies for 15 minutes at RT in dark. After washing twice with PBS-2% FBS, finally cells were resuspended in PBS-2% FBS, and acquired on a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).

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 in xylene followed by rehydration in graded alcohols. For antigen retrieval, slides were placed in a pre-warmed antigen retrieval solution (S1699 DAKO citrate buffer pH 6.0, diluted 1:10) and incubated in a water bath (30 minutes, 95°C). The slides were then left at room temperature for 20 minutes and after being washed with PBS were blocked with blocking buffer (PBS + 5% goat serum) for 30 minutes to be 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 thrice with wash buffer (PBS + 0.05% BSA) 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 three washings, DAPI (1μg/ml, 200 μl) was added and incubated for 10 minutes and finally, slides were mounted with Pro-long Gold anti-fade overnight at 4°C. The 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 ($1x10^6$ cells) or from punch biopsies taken from the PKDL patients 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 D_3 1-alpha-hydroxylase (CYP27B1), LL-37 (human cathelicidin, a antimicrobial peptide), ferritin, transferrin, transferrin receptor, Haeme oxygenase 1 (HO-1), CD163 (haptoglobin/haemoglobin receptor) and β-actin (Table 2.1). For reverse transcription, samples were subjected to an initial incubation at 50°C for 30 minutes followed by an initial PCR activation (95°C for 15 minutes). The amplification cycle comprised 35 cycles of denaturing (94°C for 30 seconds), annealing for 30 seconds (varying temperature for each primer set; Table 2.1), extension (72°C for 60 seconds), and a final extension at 72°C (10 minutes). 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.

Table 2.1: Primers and amplification conditions for RT-PCR

Name	Sequence (5'⟶3')	Annealing temperature (° C)	Product Size (bp)
IL-12p40 (F)	GCCGTTCACAAGCTCAAGTATG	66	79
IL-12p40 (R)	TCTTGGGTGGGTCAGGTTTG		
Arginasel (F)	ACTGGAGAGCTCAAGTGCAGCAA	61	308
Arginasel (R)	AGCCTTGGCTGAGATCACGAGCC		
Mannose receptor (F)	GGCGGTCGGATGGATGGCTC	62	610
Mannose receptor (R)	TGTGAGGTCACCGCCTTCCT		
PPAR-γ (F)	TCTGGCCCACCAACTTTGGG	59	340
PPAR-γ (R)	CTTCACAAGCATGAACTCCA		
CYP27B1 (F)	GGTCAGGGCCGCCTCACACT	60	396
CYP27B1 (R)	GGCCACAGGTGCCACCCAATC		
Vitamin D3 Receptor (F)	CCAGTTCGTGTGAATGATGG	55	384
Vitamin D3 Receptor (R)	GTCGTCCATGGTGAAGGACT		
LL-37 (F)	GGACCCAGACACGCCAAA	60	51
LL-37 (R)	GCACACTGTCTCCTTCACTGTGA		
Ferritin (F)	AATCCAAGACAGCCACACCTT	55	450
Ferritin (R)	TTGGGAAAGCTGCCCACTAA		
Transferrin (F)	AGGTGATCAGTGGGACGAGT	58	320
Transferrin (R)	GGAAAGGCACCCAGACACC		
Transferrin Receptor (F)	CAGCAGAGACCAGCCCTTAG	55	430
Transferrin Receptor (R)	TGCCTTGTGTGTTGTTTTCGT		
CD163 (F)	ACCTCTTCAACAGACCCCCAGTGAA	58	710
CD163 (R)	GAGGACTGAGAGCTCTTCTGGCATT		
HO-1 (F)	TTCTCTCCCAACCCTGCTTGCGT	60	500
HO-1 (R)	AGGTGGGCAGACCAAGGTTCAA		
β-actin (F)	CCCAAGGCCAACCGCGAGAAGAT	74	224
β-actin (R)	GTCCCGGCCAGCCAGGTCCAG		

Measurement of plasma 25(OH) Vitamin D_3

Plasma concentrations of 25(OH) vitamin D_3 were assayed with a radioimmunoassay kit (DiaSorin, Stillwater, Minnesota, USA), according to the manufacturer's instructions. Briefly, following extraction of 25(OH) vitamin D_3 using donkey anti-goat precipitating complex, the samples were assayed according to the equilibrium radioimmunoassay procedure. The reference range for the assay was 9.0–37.6 ng/ml. and its sensitivity was 1.5 ng/ml.

Determination of intracellular iron in monocytes

Monocytes ($5x10^5$/ml) were centrifuged (4000 rpm X 5 minutes) and cells resuspended in 500 µl PBS, stained with Calcein-AM (1.25 nM, DMSO) and incubated for 30 minutes at 37°C. Fluorescence was acquired a flow cytometer (BD FACS Calibur, BD Biosciences, San Jose, CA, USA).

Measurement of plasma Iron

Plasma level of iron was measured according to manufacturer's instructions. Briefly, plasma (100 µl) was treated with an acidic buffer to release the iron bound to transferrin which was then reduced to the ferrous form. Then, ferrozine was added to the sample to form a violet coloured complex which is directly proportional to the total iron content of the sample and absorbances measured at 570 nm using Spectramax M2e (Molecular devices, LLC, Orleans Drive Sunnyvale, CA, USA). The concentration of iron in plasma was calculated against a supplied standard (100 µg/dl).

Measurement of plasma ferritin

Plasma ferritin level was measured by an enzyme linked immunosorbent assay (ELISA) based approach according to the manufacturer's instructions. Briefly, to the anti human ferritin pre-coated wells; samples (25 µl) were added and incubated for 30 minutes followed by three washings. Binding was measured after adding the enzyme conjugate for 15 minutes and detected using Tetra methyl benzidine as the substrate for 15 minutes. Following the addition of stop solution (2N HCl), absorbances was measured at 450 nm using a microplate reader (BioRad Model 680, Hercules, CA, USA).

Determination of plasma Transferrin

Plasma Transferrin was measured by an immunoturbidometric method according to manufacturer's instructions. Briefly, 2 µl of plasma was mixed with 250 µl of Reagent 1 (100 mM Tris, 180 mM NaCl, Polyethylene glycol), incubated for 5 minutes; Reagent 2 (100 mM

Tris, 300 mM NaCl, goat anti human Trf antibody) was then added and incubated for an additional 5 minutes. Absorbances were taken at 570 nm using Spectramax M2e (Molecular devices, LLC, Orleans Drive Sunnyvale, CA, USA) and the concentration of transferrin was calculated against a supplied standard of 180 mg/dl.

Flow cytometry

Monocytes ($5x10^5$ or $1x10^6$) were gated on their forward vs. side scatter characteristics followed by fluorescence; 5000 monocytes were acquired and analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA). Fluorescence was evaluated and quantified through FL-1 channel (equipped with a 530/30 nm band pass filter) for FITC, Alexa 488, triazolic product of DAF (DAF-2T), CM-DCF, CMF and Calcein, FL-2 channel (having a 585/42 nm band pass filter) for PE, FL-3 channel (having 670 nm long pass filter) for PerCP and FL-4 channel (equipped with a 661/16 nm band pass filter) for APC. Analysis was done for fluorescence also by Cell-Quest pro software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data was analyzed between 3 groups by Kruskal-Wallis test followed by Dunn's multiple comparison tests for non-parametric data for analysis of variance or by Mann Whitney t test where 2 groups were compared. Correlation was calculated using Spearman's Rank correlation and paired data were analyzed by either Wilcoxon signed rank test for non parametric data or paired t test for parametric data using GraphPad Prism software (version 5.0) was used for analysis, $p< 0.05$ being considered as significant.

Preparation of buffers and solutions

Unless mentioned, reagents were prepared in deionised water or tissue culture grade DMSO.

Phosphate buffered saline (PBS, 0.2 M or 10X)

Ingredients	Weigh/addition
Na_2HPO_4 (MW= 178)	35.6 g
NaH_2PO_4 (MW= 156, 0.2 M)	31.2 g in 1L
NaCl (0.85 %)	85.0 g

Preparation:

Na_2HPO_4 was dissolved in approx. 500 ml of water, NaCl was added to it and the pH was adjusted with NaH_2PO_4 (0.2 M) to pH 6.8; volume was then made up to 1L (it was

observed that addition of NaCl changes the pH slightly, so it was added before); the solution was diluted to 1X, checked for pH (7.2- 7.4) and stored at 4°C.

Preparation of lysis buffer (1 ml)

Ingredients	Addition
Tris-HCl (pH. 7.4, 0.2 M)	100 µl
NaCl (0.4 M)	100 µl
EDTA (0.5 M, pH 8.0)	10 µl
Protease Inhibitor Cocktail (20X)	20 µl
Triton X 100 (0.25%)	2.5 µl
ddH_2O	767.5 µl

Phosphate buffer (PB, 0.02 M, 200 ml)

Ingredients	Addition
Na_2HPO_4 (MW = 178)	0.712 g
NaH_2PO_4 (MW = 156, 0.1M)	0.624 g in 200 ml

Preparation:

0.712 gm of Na_2HPO_4 was dissolved in about 50ml of double distilled water and the pH was adjusted with 0.624 gm of NaH_2PO_4 to 7.8 and the volume was made up to 200ml with double distilled water.

Carbonate buffer (PB, 0.1 M, 100 ml)

Ingredients	Addition
Na_2CO_3 (MW = 106)	0.32 g
$NaHCO_3$ (MW = 156, 0.1M)	0.624 g in 200 ml

Preparation:

0.32 gm of sodium carbonate was dissolved in 50 ml of double distilled water and the pH was made to 9.6 with the help of 0.58 gm of sodium bicarbonate and the final volume was made up to 100 ml with double distilled water.

CMH_2DCFDA: A stock solution of DAF-2DA (1 mM) was prepared in dimethylsulfoxide (DMSO), stored at -20°C, and diluted immediately before use.

DAF-2DA: A stock solution of DAF-2DA (1 mM) was prepared in dimethylsulfoxide (DMSO), stored at -20°C, and diluted immediately before use.

CMFDA: A stock solution of CMFDA (0.5 mM in DMSO) was prepared and stored at -20°C in small aliquots; freeze thawing was avoided.

Griess reagent preparation

- **NED (0.1%):** 0.01 g NED was dissolved in 10 ml water.
- **H_3PO_4 (5%):** 588 µl H_3PO_4 (88%) was added to 9.412 ml water.
- **Sulphanilamide (1%):** 0.1 g sulphanilamide was dissolved in 5 ml 5% H_3PO_4 solution, vortexed vigorously and volume made up to 10 ml. To dissolve bigger particles, the solution was kept at 37° C for a short period (kept on checking).
  Griess reagent was always freshly prepared, NED (0.1%) and Sulphanilamide (1%) in a 1:1 ratio.
- **$NaNO_2$ (MW = 69, 10 mM):** 0.69 mg $NaNO_2$ was dissolved in 1 ml de-ionised water. A working stock of 100 µM was freshly prepared.

Tris-HCl (Tris MW= 121.14, pH. 7.4, 200 mM): 2.42 mg Tris was dissolved in 80 ml of water, thereafter pH was adjusted with HCl (MW = 36.5, 1N) to 7.4, volume made up to 100 ml.

ABTS preparation

Reagents required

- ABTS tablets (stored at 4°C in vacuum desiccators)
- ABTS buffer (10X concentrate containing H_2O_2, stored at -20 °C)
  Single ABTS tablet was dissolved in 45 ml of mili Q water and 5 ml of ABTS buffer to make 50ml of ABTS solution.

PNPP preparation

Reagents required

- PNPP (3mM)
- Sodium carbonate (0.05M)
- Magnesium chloride (0.5M)
- Deionised water

530 mg of sodium carbonate in 100 ml of deionised water and 10 mg of magnesium chloride were mixed in a beaker and to 25 ml of that solution 28 mg of PNPP was added.