CHAPTER IV

CYTOKINE RESPONSES IN INDIAN PKDL

Introduction

Advances in our knowledge of the role of cytokines in maturation of the immune system and its regulation have helped scientists to determine which cytokines, are synthesised in response to infection can cause disease pathology and/or modulate disease progression. Among the various classes of cytokines, T-helper 1 cells (Th1) along with pro-inflammatory cytokines are considered as immunostimulatory, positive regulators for cell mediated immunity whereas T-helper 2 cells (Th2) and anti-inflammatory cytokines are immunoregulatory/immunosuppressive as well as regulate humoral immunity [Alexander and Brombacher, 2012]. A substantial proportion of knowledge regarding *Leishmania* infections has been through animal models. Cytokines are produced by immune or infected cells and exert their function by activating other cells to release molecules that inhibit or favour the growth of *Leishmania*. In most parasitic and helminthic diseases, a predominantly cellular Th1 or humoral Th2 immune response respectively offers the best control over pathogens.

The outcome of leishmanial infections is generally determined by reciprocal regulation by 2 functionally distinct Th cell populations, namely, a pro-inflammatory Th1 (e.g. interleukin 2 or IL-2, interferon γ or IFN-γ, interleukin 6 or IL-6, and tumor necrosis factor α or TNF-α) and an anti-inflammatory Th2 population (e.g., interleukin 4 or IL-4, interleukin 10 or IL-10, interleukin 13 or IL-13). In murine model of infection with *L. major* (animal model for cutaneous leishmaniasis or CL), a clear dichotomy is observed between cytokine productions either by draining lymph nodes of susceptible vs. resistant mouse strains. Infection with *L. major* in a resistant mouse strain (e.g. C57BL/6) results in the development of a protective Th1 response with high levels of IFN-γ and resistance to reinfection. In contrast, infection of susceptible mouse strains (e.g. BALB/c) leads to the development of a Th2 immune response characterized by the production of IL-4 by draining lymph node cells [Himmelrich et al. 2000]. Targeted disruption of the IFN-γ gene in resistant C57BL/6 mice causes these animals to become highly susceptible to the parasites [Wang et al. 1994] while disruption of IL-4 gene in susceptible BALB/c mice causes these animals to become highly resistant to infection by the parasites [Kopf et al. 1996].In human CL, however the Th1-Th2 dichotomy has not been demonstrated as in localized CL Th1 cells predominates over Th2 whereas in diffuse CL reverse holds true [Reviewed in Sharma and Singh, 2009].

However in visceral leishmaniasis (VL), the disease is associated with marked impairment of macrophage functions [Tripathi et al. 2007] but the Th1/Th2 dichotomy does not hold true in VL, as a mixed Th1/Th2 immune profile has been reported [Reviewed in Goto and Prianti, 2009]. Both in human and experimental VL, the immune response in spleen is characterized by a mixed regulatory and inflammatory response as IL-10 and TNF-α production is elevated in spleen [Reviewed in Kumar and Nylen, 2012]. The association

between cure and relative Th1:Th2 bias has been difficult to detect and it was noticed that in experimental VL that IFN-γ and IL-4 producing cells are found at a ratio of 2-3:1, with minor variations with time and in different organs [Reviewed in Kaye et al. 2004].

Both Th1 and Th2 cytokines play an important role in L-Arginine (L-Arg) metabolism and especially in *Leishmania* infection, L-Arg metabolism is particularly relevant. L-Arg in macrophages can either be catabolized by inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO, final stable product is nitrite) or by arginase for polyamine synthesis, depending on the type of extracellular stimuli. When macrophages are exposed to Th1 cytokines (IFN-γ, IL-6, IL-8, TNF-α), the expression of iNOS enzyme is upregulated resulting in NO production. In contrast Th2 cytokines (IL-4, IL-10, IL-13 and TGF-β) preferentially induce expression and activity of arginase which converts L-Arg to L-ornithine which ultimately facilitate parasite growth and multiplication [Figure 4.1, Bogdan, 2001, Vincendeau et al. 2003, Wanasen and Soong 2008].

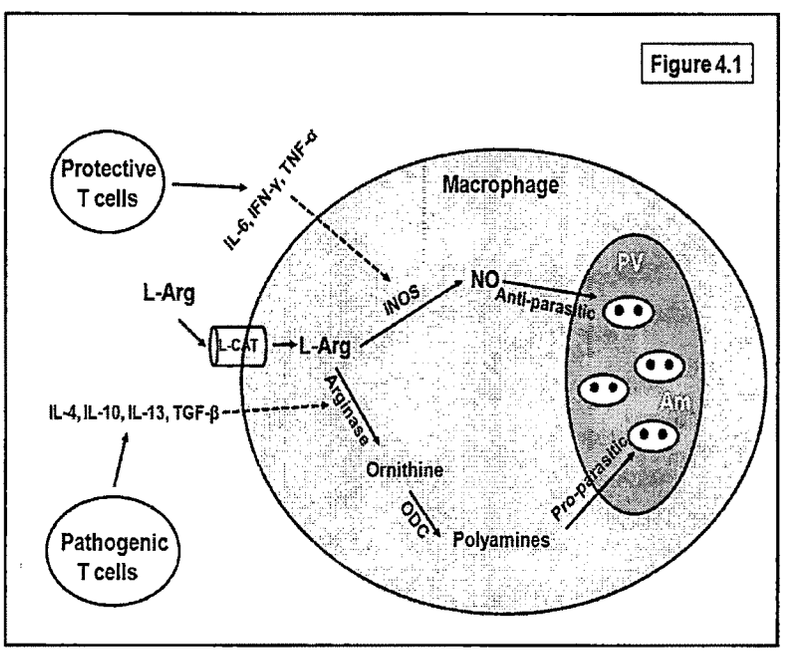

Figure 4.1: Impact of L-Arg metabolic pathways in *Leishmania* infection. Activation of macrophages by Th1 cytokines (IL-6, IFN-γ, TNF-α) from protective T cells results in an enhanced expression of inducible nitric oxide (iNOS), ultimately leading to parasite killing via increased generation of NO. Conversely, Th2 (IL-4, IL-10, IL-13 and TGF-β) mediated activation of macrophages enhances arginase activity, causing parasite growth and multiplication.

Scenario of circulating cytokines in CL

CL is a widespread disease and caused by different species of parasites confined to the geographical regions. In India, it is caused by *Leishmania tropica* whereas in Mediterranean countries it is caused by *L. major* whereas in South America, the major CL causing parasite is *L. braziliensis and L. amazonensis* [WHO Technical report 2010]. Importantly, the disease outcome and immunological response is known to vary with the type of parasite [Reviewed in Sharma and Singh, 2009]. In CL, chemotherapy is accompanied by higher circulating levels of IL-6, IL-1β, TNF-α and IL-8 [Kocyigit et al. 2002]. Simultaneous

production of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-10 by antigen stimulated peripheral blood mononuclear cells (PBMCs) from patients with active lesions [Trujillo et al. 2002] as also increased mRNA expression of IL-2, IL-4, IL-5, IL-10 and IFN-γ was demonstrated [Louzir et al. 1998, Pirmez et al. 1993]. Castellano et al. (2009) demonstrated in patients with CL, higher TNF-α, IFN-γ, IL-12, IL-4 and IL-10 production comprising a mixed Th1-Th2 response, while treatment caused clinical cure associated with a sustained Th1 response characterized by elevated levels of IFN-γ levels and down modulation of IL-4, IL-10 production [Castellano et al. 2009]. In India, patients with CL showed increased levels of IL-8 and monocyte chemoattractant protein-1 (MCP-1) at presentation while treatment caused curtailment of IL-8, but not MCP-1, suggesting that IL-8 is an effector immunodeterminants of disease progression [Kumar et al. 2010]. Treatment with antimonials or rifampicin caused a marked modulation in the cytokine status with rifampicin causing more immunomodulation than antimonials [Kumar et al. 2010]. In CL, the plasma nitrite levels are higher and importantly remained higher with treatment which correlated with their antileishmanial activity [Erel et al. 1999, Serarslan et al. 2005, Kumar et al. 2010]. Study regarding the status of arginase activity in CL is limited and Abebe et al. (2012) reported that in periphery arginase activity is unaltered as compared to healthy controls, wherein lesional arginase activity is increased, and proposed to play a role in the immunopathogenesis of the CL.

Circulating cytokine response in VL

The status of circulating cytokines in VL is even more complicated than in CL where the key players are IL-10 for disease progression and IFN-γ, IL-12p40 for disease cure [Nylen and Gautam 2010]. In active VL, although IL-10 and IFN-γ levels are high, the PBMCs showed a decreased ability to produce antigen specific IFN-γ and IL-12p40 production, implying that the source of the raised plasma cytokines could well be the lymphoid organs. Indeed, studies have shown that mRNA expression in bone marrow, spleen and lymph nodes for IFN-γ and IL-10 is higher [Reviewed in Bhattacharya and Ali, 2013, Reviewed in Goto and Prianti, 2009]. Recently, another study confirmed that the high levels of pro-inflammatory cytokines in serum of VL patients could be attributed to increased LPS levels in blood [Santos-Oliveira et al. 2011]. Additionally, damage to the gut as evidenced by the increased plasma intestinal fatty acid binding protein (IFABP) leads to increased translocation of microbes and their microbial end products into the blood stream which may account for the observed cytokine storm in the patients [Santos-Oliveira et al. 2011]. In Indian VL, Ansari et al. (2006b) showed increased levels of IFN-γ, IL-10, IL-6 in circulation. In an another study, Hailu et al. similarly showed that in VL elevated IFN-γ, IFN-γ inducible protein 10 (IP-10),

IL-15, IL-18, IL-12p40 and CXC chemokines levels are present indicating that in VL, production of type 1 cytokines is not depressed; instead unresponsiveness to the type 1 cytokines and/or increased antagonizing type 2 cytokines account for the disease progression [Hailu et al. 2004]. This was proved by, Dasgupta et al. (2003) who have demonstrated a reduced expression of IFN-γ receptor on monocytes in VL patients which gets increased with treatment. Several studies have reported that cultured PBMCs of patients with VL secrete high levels of IL-4, IL-10 along with a reduced capability of secreting IL-2 and granulocyte macrophage colony stimulating factor [GM-CSF, Raziuddin et al. 1994, Mondal et al. 2010]. Importantly, treatment caused reversal of these cytokines [Cillari et al. 1995, Mondal et al. 2010]. The mixed circulating cytokine response in VL consistently accompanied by high levels of plasma nitrite [Ansari et al. 2007, Khambu et al. 2007]. However, study regarding arginase activity in VL is controversial, as Kumar et al. (2012) showed decreased arginase activity whereas Abebe et al. (2013) showed an increased arginase activity in PBMCs of patients, but no alteration in plasma arginase activity.

Study objectives

Studies regarding major circulating pro and anti-inflammatory cytokines along with plasma nitrite and arginase activity in Post Kala-azar Dermal Leishmaniasis (PKDL) are a grey area and accordingly we aimed to study these markers in patients with PKDL at disease presentation and after treatment (sodium antimony gluconate (SAG) or Miltefosine).

Materials and Methods

Determination of plasma cytokines

Plasma cytokines (TNF-α, IL-6, IL-1β, IL-8, IL-4, IL-10, IL-13 and TGF-β) were measured using commercially available kits (R&D systems, Minneapolis, USA and Immunotools, Friesoythe, Germany) according to the manufacturer's instructions as described in Materials and Methods (chapter 2).

Isolation of peripheral blood mononuclear cells (PBMC) and production of cytokines

Peripheral blood mononuclear cells were carefully isolated and purified using Histopaque 1077 and seeded (1×10^6 cells/ml) in multiwell tissue culture plates with or without *Leishmania donovani* crude antigen (LDA) at 37°C, 5% CO_2 for 6 days to determine their status of pro and anti inflammatory cytokines; cells were then centrifuged (4000 rpm x 5 min.), supernatants were collected and stored at -20°C.

Determination of serum levels of nitric oxide (NO)

The serum levels of nitrite, a stable representative of NO was determined by the Griess method with slight modifications as described in Materials and Methods (chapter 2).

Measurement of serum Arginase activity

Arginase activity was measured spectrophotometrically by estimating the urea released using isonitrosopropiophenone as described in Materials and Methods (chapter 2).

Results:

Raised levels of TNF-α, IL-8, IL-4, IL-10 and TGF-β in PKDL

Plasma levels of pro-inflammatory cytokines TNF-α, IL-6, IL-1β and IL-8, based on their primary source being monocytes and macrophages were quantified. At presentation, levels of TNF-α were significantly elevated by 11.6 fold as compared to healthy controls ($p<0.001$, Figure 4.2A) which following treatment increased further ($p<0.001$, Figure 4.2A) . With regard to IL-6 and IL-1β, levels at presentation were comparable with controls (Figures 4.2B and C) while treatment caused a significant 4 fold increase in both cytokines ($p<0.05$, $p<0.01$ respectively, Figures 4.2B and C). On the other hand IL-8, was substantially higher (11.3 fold) at disease presentation than healthy controls ($p<0.05$, Figure 4.2D). Treatment caused a significant increase in than presentation ($p<0.05$, Figure 4.2D).

With regard to anti-inflammatory cytokines, IL-4, IL-10, IL-13 and TGF-β were enumerated by sandwich ELISA. IL-10 was increased significantly by 2.7 fold more in patients with PKDL than healthy controls ($p<0.05$, Figure 4.2E) and treatment caused significant curtailment ($p<0.05$, Figure 4.2E). At presentation, IL-4 was 2.2 fold higher in PKDL patients ($p<0.05$, Figure 4.2F) and treatment caused a marginal decrease (Figure 4.2F). TGF-β was also significantly increased at presentation vis á vis healthy controls ($p<0.05$, Figure 4.2H) while treatment caused nominal changes (Figure 4.2H). IL-13 was also found to be substantially higher in patients with PKDL than compared with healthy controls (Figure 4.2G) which again like IL-4 and TGF-β decreased marginally (Figure 4.2G).

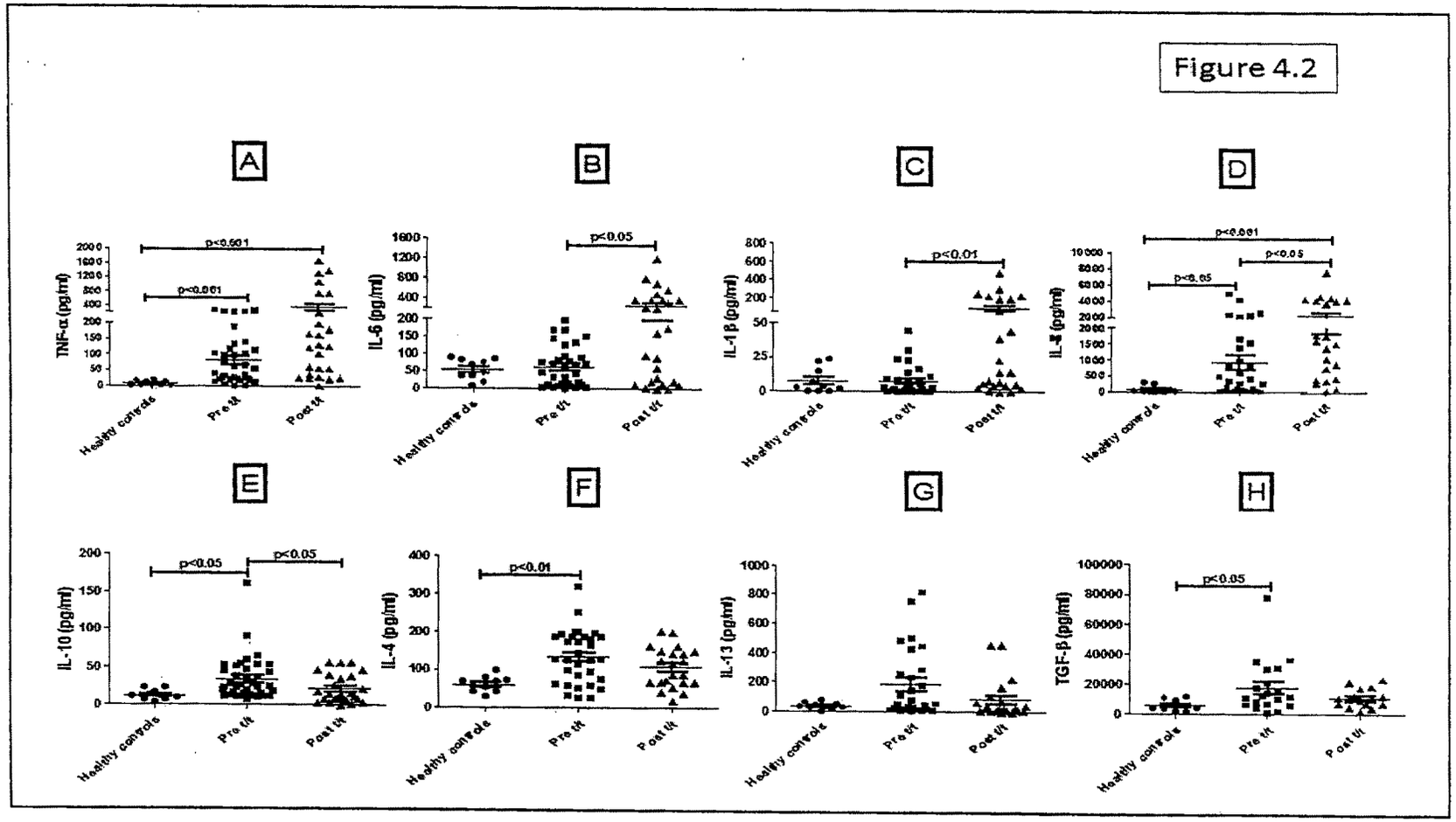

Figure 4.2: Scatter plots showing circulating cytokine levels in patients with PKDL. Levels of TNF-α (**A**), IL-6 (**B**), IL-1β (**C**), IL-8 (**D**), IL-10 (**E**), IL-4 (**F**), IL-13 (**G**), TGF-β (**H**) between healthy controls (n = 15,●), patients with PKDL at presentation (Pre t/t, n = 35, ■) and Post treatment(Post t/t, n = 30, ▲).

Circulating cytokine distribution among polymorphic and macular PKDL

In polymorphic PKDL levels of TNF-α and IL-8 were a significant 12.4 and 7 fold higher than healthy controls (Table 4.1) while IL-6 and IL-1β levels were higher but the differences were not significant (Table 4.1). However, macular PKDL had significant 28.3 fold higher levels of IL-8 than compared to healthy controls (Table 4.1). Importantly, no differences were observed between polymorphic and macular PKDL in terms of pro-inflammatory cytokines.

With regard to anti-inflammatory and immunoregulatory cytokines, polymorphic PKDL had significantly higher levels of IL-10 and TGF-β than healthy controls (Table 4.1); levels of IL-4 and IL-13 were higher but the differences were not significant (Table 4.1). However, macular PKDL had significantly higher levels of IL-4 (Table 4.1) but their levels of IL-10, IL-13 and TGF-β were not significantly altered (Table 4.1).

Table 4.1: Circulatory cytokine distribution among polymorphic and macular PKDL

Cytokines	Healthy controls (n = 10) (pg/ml)	Polymorphic PKDL (n = 30) (pg/ml)	Macular PKDL (n = 10) (pg/ml)
TNF-α	7.00 ± 2.44 4.10 (0.00-13.34)	86.94 ± 16.64* 60.40 (19.34-135.10)	75.55 ± 23.30 53.17 (21.54-117.80)
IL-6	53.94 ± 9.26 57.41 (30.47-81.55)	90.13 ± 23.94 45.91 (6.65-141.40)	127.7 ± 46.72 82.21 (7.09-145.50)
IL-1β	7.20 ± 2.87 2.68 (0.00-16.04)	18.09 ± 8.22 2.44 (0.20-22.52)	29.85 ± 22.79 8.25 (1.75-14.83)
IL-8	82.98 ± 35.34 32.83 (15.77-143.80)	581.40 ± 144.80* 333.30 (64.05-874.70)	2349.00 ± 809.70** 2266.00 (263.90-4402.00)
IL-10	12.68 ± 2.05 11.69 (7.14-18.22)	38.18 ± 5.91** 28.02 (17.31-52.99)	20.76 ± 4.85 13.82 (11.18-28.97)
IL-4	61.35 ± 6.36 62.19 (43.7-74.77)	121.00 ± 14.36 123.00 (57.41-184.60)	149.30 ± 25.87* 13.82 (11.18-28.97)
IL-13	35.13 ± 6.86 33.40 (18.78-52.79)	173.40 ± 50.08 36.73 (12.70-352.50)	221.50 ± 111.20 139.70 (42.93-361.80)
TGF-β	5980.00 ± 1436.00 4540.00 (2369.00-10561.00)	20136.00 ± 4783.00* 14380.00 (6496.00-30846.00)	9224.00 ± 2657.00 10252.00 (3701.00-13719.00)

Serum cytokine levels were measured in healthy controls and in patients with polymorphic or macular PKDL at presentation as described in Materials and methods.

Values are stated as mean ± SEM. Median and IQR values are in parentheses, *$p<0.05$, **$p<0.01$ significantly different than healthy controls.

Miltefosine exerts a greater immunomodulatory action than SAG on circulatory cytokines

The levels of pro and anti-inflammatory cytokines were compared in patients treated with SAG or Miltefosine. Twelve paired samples were taken in each arm for evaluation of cytokine modulation. In terms of pro-inflammatory cytokines Miltefosine exerted a far greater effect as except for TNF-α, it synergistically increased all the pro-inflammatory cytokines (IL-6, IL-1β and IL-8) as compared to active disease ($p<0.01$, $p<0.05$, $p<0.05$ respectively, Figure 4.3) whereas SAG induced pro-inflammatory cytokine levels but the changes were not statistically significant (Figure 4.3).

Similarly, with regard to anti-inflammatory cytokines, Miltefosine again proved to be more effective as it decreased IL-10, IL-4 and TGF-β significantly from disease presentation ($p<0.05$, $p<0.01$, $p<0.05$ respectively, Figure 4.3) whereas the effect of SAG were not statistically significant (Figure 4.3), IL-13 remained unaltered by both treatments.

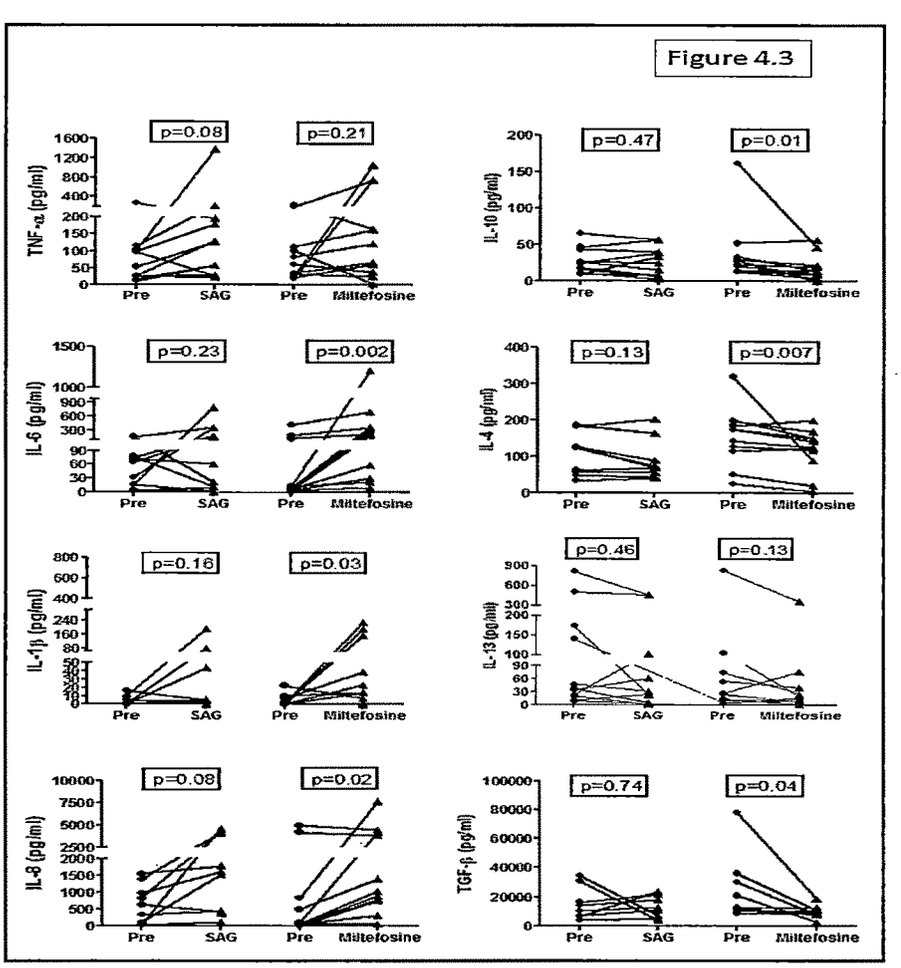

Figure 4.3: Before and after plots of serum levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β and IL-8) in patients with PKDL at presentation (n = 12, •) and after completion of treatment (▲). Before and after plots of serum levels of anti-inflammatory cytokines (IL-10, TGF-β, IL-4 and IL-13) in patients with PKDL at presentation (n = 12, •) and after completion of treatment (▲).

Production of cytokines by cultured PBMCs

The production of cytokines following stimulation with *Leishmania* antigen in PBMCs when evaluated showed that only IL-6 was significantly increased at presentation, both in the stimulated and unstimulated populations (Table 4.2) and treatment caused minimal changes, the levels remaining significantly higher than healthy controls in both SAG and Miltefosine treated groups (Table 4.2). Production of TNF-α was increased at presentation both in unstimulated and stimulated conditions but the differences were not significant, while Miltefosine induced its production significantly via an antigen specific manner (Table 4.2).With reference to IL-8, its production was higher at presentation in both a antigen specific and non specific manner (Table 4.2) but SAG induced its secretion more in the antigen specific population while Miltefosine induced it in the antigen specific and non specific population (Table 4.2).

In the anti-inflammatory cytokine milieu, the significantly raised levels of IL-10 at presentation (Table 4.2) were effectively decreased by Miltefosine irrespective of antigenic stimulation; whereas SAG mediated curtailment was evident only in the unstimulated population. With respect to IL-4 and IL-13, changes were minimal, both at presentation as well as following treatment (Table 4.2).

Table 4.2: Cytokine levels in culture supernatants of patients with PKDL at presentation and after treatment

*Cytokine	Healthy individuals (Mean ± SEM, pg/ml)		Presentation (Mean ± SEM, pg/ml)		SAG (Mean ± SEM, pg/ml)		Miltefosine (Mean ± SEM, pg/ml)	
	Ag Specific	Ag Nonspecific	Ag Specific	Ag Nonspecific	Ag Specific	Ag Nonspecific	Ag Specific	Ag Nonspecific
TNF-α	337.0 ± 121.06	783.0 ± 201.4	597.6 ± 224.5	1003.1 ± 269.6	580.9 ± 202.7	259.9 ± 54.92	2040.9 ± 715.7$^{@@,\#\#}$	1127.2 ± 355
IL-6	428.0 ± 127.6	165.3 ± 82.5	1054.4 ± 159.7$^{@}$	950.4 ± 144.0$^{@@}$	895.0 ± 372.7	1024.5 ± 350.3$^{@}$	859.3 ± 161.3	806.3 ±165.6$^{@}$
IL-8	4019.7 ± 1063.8	4659.4 ± 2006.2	36669.1 ± 755.2	23997.7 ± 6044.8	96920.9 ± 24348.2$^{@}$	88737.8 ± 27585.5	104599.5 ± 23168.6$^{@@,\#}$	97460.3 ± 21467.8$^{@,\#}$
IL-10	38.0 ± 5.6	35.5 ± 6.7	75.1 ± 6.6$^{@}$	77.1 ± 7.6$^{@}$	57.4 ± 12.3	47.3 ± 7.7$^{\#}$	36.2 ± 11.1$^{\#}$	22.6 ± 9.0$^{\#\#}$
IL-4	10.8 ± 1.7	11.9 ± 1.7	10.1 ± 3.7	16.5 ± 3.2	29.6 ± 6.4	27.4 ± 5.2	11.7 ± 4.0	11.6 ± 2.4
IL-13	4.9 ± 4.9	7.8 ± 4.0	3.6 ± 1.9	5.6 ± 1.5	5.6 ± 3.3	5.7 ± 2.0	6.1 ± 3.2	6.5 ± 1.8

*PBMCs from healthy individuals and patients with PKDL at presentation and after treatment with SAG or Miltefosine were cultured for 6 days, after which culture supernatants were collected and secreted cytokines measured by sandwich ELISA as described in materials and methods.

$^{@}p<0.05$, $^{@@}p<0.01$, as compared with healthy controls; $^{\#}p<0.05$, $^{\#\#}p<0.01$, as compared with presentation.

Elevated levels of plasma nitrite and arginase activity in PKDL was reverted by Miltefosine

In view of the importance of reactive nitrogen intermediates in promoting the macrophage mediated leishmanicidal activity, we evaluated serum nitrite levels in our study population. As shown in figure 4.4A, serum nitrite levels were significantly higher in patients with PKDL than healthy controls (8.82 ± 0.62 μM vs. 6.36 ± 0.19 μM) and remained higher with treatment (9.59 ± 0.80 μM, Figure 4.4A). When analyzed within polymorphic and macular variants we found that only polymorphic PKDL had significantly higher levels of plasma nitrite (8.97 ± 0.80 μM vs. 6.36 ± 0.19 μM, Figure 4.4A) but not macular PKDL (8.42 ± 0.82 μM) possibly owing to their localized inflammation. On an individual basis, treatment with Miltefosine significantly increased serum nitrite levels, whereas SAG tended to decrease nitrite levels (Figure 4.4A)

Arginase is an important immunomodulatory enzyme that modulates macrophage function [Das et al. 2010] and as *Leishmania* resides within macrophages, we felt it pertinent to measure arginase activity. In patients with PKDL at presentation, plasma arginase activity was significantly higher than healthy controls (529.80 ± 41.53 vs. 365.80 ± 28.10 U/L, $p<0.05$, Figure 4.4B) and here also the arginase activity was found to be greater in polymorphic PKDL than healthy controls (542.00 ± 50.02 vs. 365.80 ± 28.10 U/L, Figure 4.4B), as also higher than in macular PKDL (479.60 ± 57.92 U/L, Figure 4.4B). When analyzed on an individual basis, Miltefosine decreased arginase activity significantly while SAG showed no decrease (Figure 4.4B).

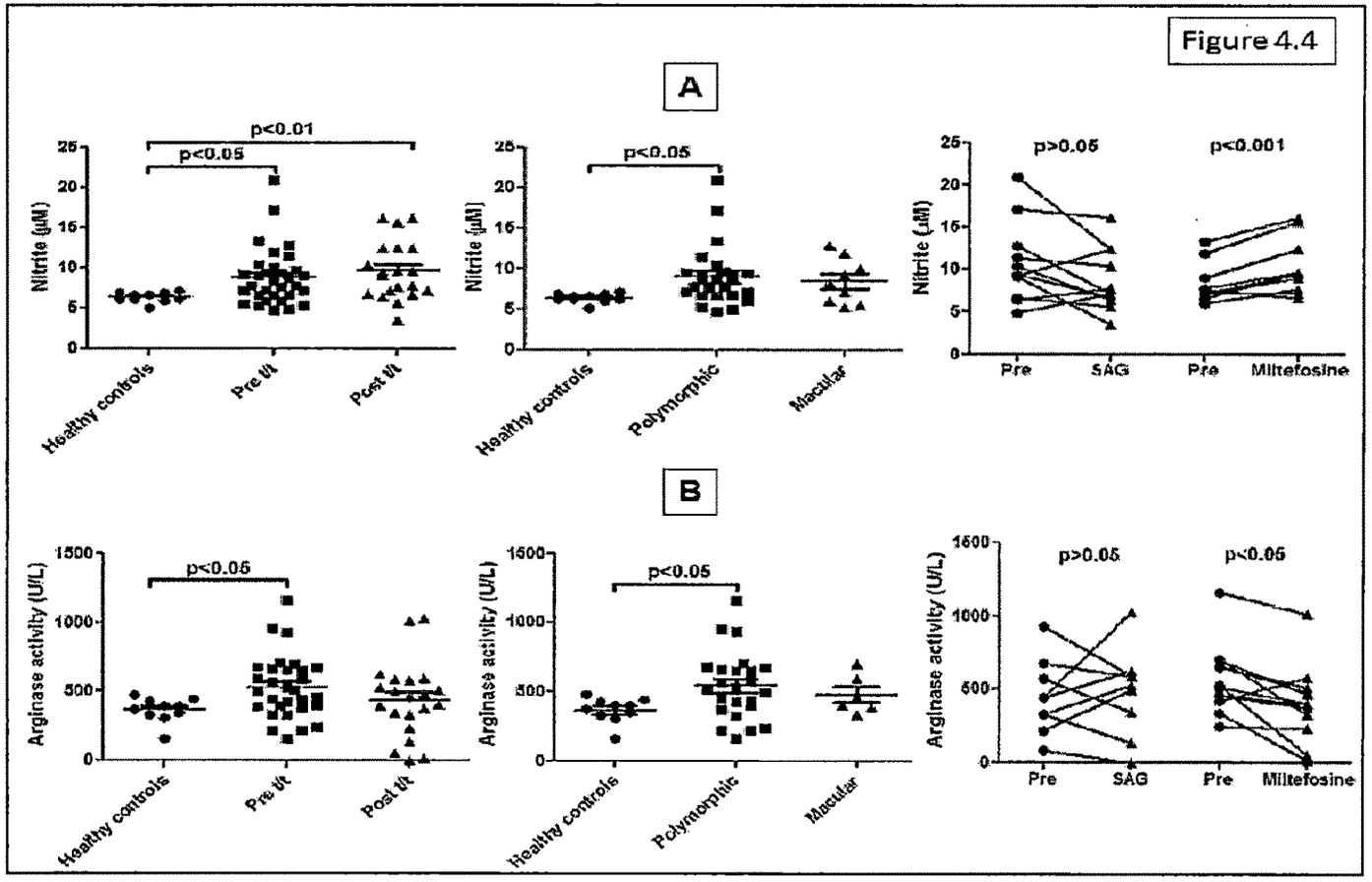

Figure 4.4:
A. Scatter plots showing plasma nitrite levels in healthy controls (n = 10, ●), patients with PKDL at presentation (Pre t/t, n = 35, ■) and Post treatment (Post t/t, n = 25, ▲). Between healthy controls (n = 10, ●), polymorphic PKDL (n = 25, ■) and macular PKDL (n = 10, ▲). Before and after plots of nitrite levels in patients with PKDL at presentation (n = 12, ●) and after completion of treatment with SAG or Miltefosine (n = 12, ▲).
B. Scatter plots showing plasma arginase activity in healthy controls (n = 10, ●), patients with PKDL at presentation (Pre t/t, n = 30, ■) and Post treatment (Post t/t, n = 25, ▲). Between healthy controls (n = 10, ●), polymorphic PKDL (n = 24, ■) and macular PKDL (n = 6, ▲). Before and after plots of arginase activity in patients with PKDL at presentation (n = 12, ●) and after completion of treatment with SAG or Miltefosine (n = 12, ▲).

Discussion

Irrespective of the clinical variant of leishmaniasis, development of a successful parasitic relationship with the host is mandatory for disease pathogenesis. The *Leishmania* parasite ensures its survival within macrophages by deviously inhibiting generation of oxidative burst [Van Assche et al. 2011]. Therefore, it is logical to extrapolate that for parasite elimination, activation of macrophages via preferential production of pro-inflammatory cytokines and production of NO would be beneficial. Indeed pentavalent antimonials, the drug of choice in all forms of leishmaniasis have been reported to induce a pro-inflammatory response in patients with CL [Kocyigit et al. 2002].

In patients with CL, VL and MCL, levels of circulating pro-inflammatory cytokines have been reported to be higher at presentation [Kocyigit et al. 2002, Kumar et al. 2009, Cenini et al. 1993, Ansari et al. 2006b, Da-Cruz et al. 1996, Castes et al. 1993]. With regard to TNF-α, PKDL patients showed a significant increase at presentation (Figure 4.2) akin to previous studies [Ansari et al. 2006b], possibly indicative of the hosts attempt to eliminate the parasite. IL-6 is another pro-inflammatory marker primarily secreted by macrophages [Kocyigit et al. 2002, Nylen and Gautam 2010], whose synthesis can be induced by IL-1β [Kocyigit et al. 2002]. In PKDL, levels at presentation were comparable with healthy controls [Ansari et al. 2006b, Ansari et al. 2007]; post chemotherapy, a 2 fold increase was demonstrated (Figure 4.2). The scenario with IL-1β too was similar, albeit a 3 fold increase in patients with PKDL at presentation (Figure 4.2). IL-8, a potent chemotactic cytokine in polymorphonuclear neutrophils, stimulates chemotaxis and generation of reactive oxygen metabolites, and is additionally synthesized by monocytes/macrophages, chondrocytes, and fibroblasts [Kocyigit et al. 2002]. TNF-α stimulates release of IL-8, which in turn enhances release of pro-inflammatory cytokines IL-1ß, IL-6, and TNF-α, thereby reinforcing the inflammatory cascade [Hirota et al. 1992]. Taken together, the increase in pro-inflammatory cytokines in leishmaniasis suggests that infection *per se* does promote an inflammatory response, but not enough to eliminate the parasites. It may be envisaged that chemotherapy would be effective by exacerbating this enhanced inflammatory response, already triggered by infection. Indeed, Miltefosine provided the necessary boost to the inflammatory response (Figure 4.3). In leishmaniasis, the status of IL-8 appears controversial, as it was initially proposed that IL-8 is beneficial for the host [Teixeira et al. 2006] but subsequently, it was demonstrated that *Leishmania* infected human PMNs secrete more IL-8 which by increasing recruitment of PMNs, eventually aids disease progression [Laufs et al. 2002]. In PKDL, the status of IL-8 was unknown and this study demonstrated a marked increase in IL-8 (Figure 4.2) thus providing corroborative evidence that a host driven pro-inflammatory burst helps in parasite elimination. Animal and human studies have established that antileishmanial

compounds increase pro-inflammatory cytokines [Kocyigit et al. 2002, Sodhi et al. 1990, Kumar et al. 2010] and thus has been endorsed in our study, as all the pro-inflammatory cytokines studied (TNF-α, IL-6, IL-1β and IL-8) increased after treatment, be it SAG or Miltefosine (Figure 4.2). However, when analyzed on an individual basis, Miltefosine appeared to exert a more potent immunostimulatory effect than SAG (Figure 4.3).

Cytokines are strong candidates for surrogate markers of immunity, especially helpful in interpreting the Th1/Th2 paradigm present in the mouse model of leishmaniasis. Macrophages infected with *Leishmania* amastigotes have down regulated expression of Th1 cytokines, along with an increased expression of anti-inflammatory cytokines. Therefore, a consistent feature of all variants of leishmaniasis is an increase in anti-inflammatory cytokines that was also evident in PKDL, as serum levels of IL-10 were higher than healthy controls [Reviewed in Nylen and Akuffo, 2009, Ganguly et al. 2008]. Similarly, raised levels of IL-13 and TGF-β have also been reported in human VL [Reviewed in Nylen and Gautam 2010]. However the status of IL-4 in VL is contradictory as both increased levels [Raziuddin et al. 1994] have been reported, while Ansari et al. reported minimal changes [Ansari et al. 2006b]. Our data indicated that in patients with PKDL at presentation, serum IL-10, TGF-β, and IL-4 were all significantly higher than healthy controls (Figure 4.2), suggesting a Th1/Th2 imbalance. Although antileishmanial compounds have been reported to consistently cause curtailment of anti-inflammatory cytokines, our study showed that Miltefosine effectively decreased all the anti-inflammatory cytokines, once again reinforcing the more potent immunomodulatory action of Miltefosine (Figure 4.3) while SAG effectively decreased only IL-4.

Our understanding of alterations in the immune system during leishmaniasis is better explained by the cytokine production of *in vitro* cultured PBMCs wherein production of pro and anti-inflammatory cytokines was studied. Among the pro-inflammatory cytokines, IL-8 and TNF-α were significantly increased with Miltefosine, independent of antigen stimulation (Table 4.2); strikingly, IL-6 too was significantly increased at presentation and increased further after treatment (Table 4.2), confirming activation of the host immune response. Among the anti-inflammatory cytokines, the effect on IL-10 appeared more prominent than IL-4 and IL-13 which after treatment with SAG or Miltefosine decreased significantly, Miltefosine again being more effective (Table 4.2).

Nitric oxide (NO) is an effector molecule necessary for elimination of intracellular *Leishmania* parasites; understandably, *Leishmania* infection prevents activation of macrophages resulting in decreased production of NO [Bogdan C, 2001, Cunnigham AC, 2002]. Conversely, cure necessitates increased production of NO, which is generally mediated by upregulation of inducible NO synthase (iNOS or NOS2) [Brunet LR, 2001]. As

Miltefosine induced secretion of all pro-inflammatory cytokines in patients with PKDL, we checked the status of plasma nitrite, a stabilized form of NO. The nitrite levels were increased both at presentation and after treatment (Figure 4.4A), the source(s) being not only monocytes/macrophages but also endothelial cells and red blood cells, collectively reflective of the hosts attempt to eliminate the parasite.

Arginase contributes towards parasite persistence, as it not only reduces levels of NO but also helps in polyamine synthesis, necessary for parasite growth [Das et al. 2010]. Furthermore, arginase activity is induced by anti-inflammatory cytokines IL-4, IL-13, IL-10 and TGF-β [Bogdan C, 2001] and increased expression of arginase has been reported in experimental VL [Osorio et al. 2012]. Importantly arginase was also known to suppress T cell proliferation and functionality [Stempin et al. 2010] and previously in PKDL our group had reported that circulating $CD8^+$ T cells were one of the major sources of IL-10 in PKDL [Ganguly et al. 2008] along with decreased antigen specific proliferation of T cells with loss of surface expression of CD28, which made them anergic [Ganguly et al. 2010]. So, this raises the functional impact of raised the arginase activity in patients with PKDL. Indeed, the plasma arginase activity in our study population was 1.5 fold higher than healthy controls and was effectively decreased by Miltefosine (Figure 4.4B), reinforcing our hypothesis of arginase mediated immunosuppression in PKDL and also the greater immunomodulatory potency of Miltefosine. A major source of IL-8 and arginase is neutrophils, but one cannot exclude another important source namely the monocyte-macrophages. As the levels of IL-8 and arginase were quantified in plasma, we cannot pinpoint their cellular source, but it is expected that as macrophage functions are primarily altered in leishmaniasis, the observed changes are reflective of macrophage function, where importantly, a differential secretion of NO and arginase is expected [Bogdan C, 2001].

Studies evaluating the effect of drug treatment on PKDL are limited to three studies wherein emphasis was placed on the lesional pathology, and the status of pro and anti-inflammatory cytokines was measured. These studies consistently indicated an increase in pro-inflammatory cytokines at presentation which decreased after treatment. However, this study has focused on the systemic effects of SAG and Miltefosine upon Th1/Th2 modulation, and established the immunological superiority of Miltefosine over SAG. Future investigations should be directed towards studying the impact of antileishmanial drugs on signaling mechanisms involving host-pathogen interactions; such studies are underway.