CHAPTER 5

PERIPHERAL
CELL-MEDIATED IMMUNITY
IN INDIAN PKDL
Introduction

Impaired CMI responses can result from functional defects in any or all constituents of the cellular arm and can either, in ironic fashion, benefit the host by promoting survival of tissue transplants or, following infection by pathogenic microorganisms, render the host more susceptible to rampant pathology (Wing and Remington, 1977). Infections by *Leishmania* parasites are no exception, invariably resulting in either the typically self-resolving, localized dermal lesions of cutaneous leishmaniasis (CL) or the often fatal, systemic infection of visceral leishmaniasis (VL). In both forms, pathogenesis is attributed to the interplay between constituents of the host’s innate and acquired immune responses including neutrophils, macrophages, dendritic cells, lymphocytes and natural killer cells (Scott, 2005). Clinical cure hinges on the activation of parasitized macrophages, which in turn depends on a robust T-helper cell type 1 (Th1) response orchestrated by cross-talk between the antigen-presenting dendritic cells and the responder CD4 and CD8 T lymphocytes culminating in the latter secreting proinflammatory cytokines like IL-12, IFN-γ and TNF-α (Scott, 2005). Counter-regulatory cytokines such as IL-4, IL-10, IL-13 and TGF-β comprise the Th2 response, functioning to deactivate macrophages thereby, limiting production of potentially deleterious levels of proinflammatory cytokines (Scott, 2005). However, by doing so, the Th2 response facilitates intracellular infection.

Cutaneous leishmaniasis

The cell-mediated immune pathology of *Leishmania* infections as gleaned from the most extensively reviewed murine experimental model of infection with *Leishmania major* predicates a down regulation of T helper 1 (Th1)-mediated cellular immunity and up regulation of the T helper 2 (Th2) response, thereby sustaining the infection (Locksley and Scott, 1991). The cell-mediated response rather than the humoral immune response determines cure; with primary activation of T-cell subsets being critical to the development of Th1 and Th2 responses and the course of infection (Sacks and Noben-Trauth, 2002).

In case of infection in humans, even though epidemiological data points to a Th1/Th2 dichotomy as seen in the experimental murine model, it is now generally
appreciated that the human immunological response cannot be defined by distinct Th1/Th2 subsets (Alexander and Bryson, 2005). In this context, lymphocytes from patients with the self-resolving localized cutaneous leishmaniasis (LCL) respond to *Leishmania* antigen by proliferating and producing Th1 cytokines like IFN-γ (Pirmez et al., 1993; Ribeiro-de-Jesus et al., 1998). The scenario is similar in lymphocytes from cured CL patients, upon exposure to either crude *L. major* antigens or the major surface protease gp63 (Kemp et al., 1994a, 1994b). Correspondingly, proliferation and IFN-γ production in response to *Leishmania major* antigen was much higher in patients with clinically mild CL as opposed to severe CL (Gaafar et al., 1995). In contrast, *Leishmania*-stimulated PBMC of patients with chronic lesions produce minimal IFN-γ but high concentrations of IL-4 (Ajdary et al., 2000). Similarly, patients with diffuse cutaneous leishmaniasis (DCL) exhibit a predominantly Th2 peripheral CMI response that is associated with non-responsiveness to *Leishmania* antigen (Bomfim et al., 1996). On the other hand, patients with the non-resolving mucosal leishmaniasis are reported to mount both Th1 and Th2 cytokine responses with raised expression of IL-2, IL-4, IL-5 and TNF-α (Pirmez et al., 1993; Ribeiro-de-Jesus et al., 1998; Amato et al., 2003), which could account for the non-resolving nature of the disease, as Th2 responses dominate over Th1 responses in a state of co-activation (Scott et al., 2005).

**Visceral leishmaniasis**

Since the ability of the mammalian host to resist leishmanial infection is contingent on effector Th1 immune responses and resultant leishmanicidal activity of parasitized macrophages, production of IL-12 by antigen-presenting cells and IFN-γ by T lymphocytes is a requisite for controlling parasite replication as also for conferring acquired resistance in VL (Bacellar et al., 1996; 2000).

Even though there are reports of circulating lymphocytes from children with subclinical VL producing IL-2, IFN-γ and IL-12 in response to *Leishmania* antigen (Carvalho et al., 1992), patients with VL are generally characterized by the anergic response of their circulating lymphocytes (Sacks et al., 1987; Ghalib et al., 1995). However, cured VL patients are not only resistant to reinfection but also display strong antigen-specific responses *in vitro* by producing IFN-γ, negating the possibility of any
inherent defect in antigen-driven Th1 immunity (White et al., 1992; Garg et al., 2005; Nylen and Sacks, 2007). Further, the mitogenic non-responsiveness of lymphocytes from VL patients is generally encountered only in the advanced stages of the disease, which are also associated with a marked drop in T cell proportions within the total circulating lymphocyte population (Cillari et al., 1991).

Usually, a strong immunoregulatory type response prevails during active infection with \textit{L. donovani}. Total impairment of T-cell reactivity to \textit{Leishmania} antigens (Ho et al., 1983; Carvalho et al., 1985), raised levels of IL-4 (Zwingenberger et al., 1990) and pronounced polyclonal hyperglobulinemia due to B-cell activation (Galvao-Castro et al., 1984) are characteristic features of VL. Further, high plasma levels of IL-10 (Cillari et al., 1995) and increased production of IL-10 by peripheral lymphocytes isolated from kala-azar patients [Ghalib et al., 1993; Holaday et al., 1993] have been documented.

In Ethiopian VL, CD3 and CD4 lymphocytopenia together with depleted numbers of memory CD4+ T cells was observed as compared to cured VL patients or subclinical cases. Further, proportions of circulating CD4+ and CD8+ T lymphocytes that produced IFN-\(\gamma\) and IL-4 after stimulation with the protein kinase C analog, Phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore, Ionomycin were minimal in VL patients when compared to subclinical cases or healthy controls (Hailu et al., 2005). In Kenyan VL patients, successful treatment restored the proliferative capacity and IFN-\(\gamma\) production of circulating T cells in response to \textit{L. donovani} antigens (Kurtzhals et al., 1994).

In the Indian context, proportions of IFN-\(\gamma\) producing T helper lymphocytes were greater in VL patients who responded to treatment with SAG than in non-responders while conversely, IL-4 producing cells were greater in non-responders vis-a-vis responders, suggesting a skewing of the cytokine profile in drug-resistant cases of Indian VL towards IL-4 or Th2 driven responses (Thakur et al., 2003).

\textbf{Post kala-azar dermal leishmaniasis (PKDL)}

The immunological features of VL (the “forerunner” with systemic involvement) differ from those of PKDL (the “follower” with only dermal involvement) in several aspects. The immunopathology of VL is characterized by a pronounced suppression of cell-mediated immunity (CMI) with concurrently enhanced humoral immunity...
manifested as hypergammaglobulinemia and formation of immune complexes. Successful treatment restores CMI and confers resistance to re-infection (Reed and Scott, 1993).

PKDL develops in a small percentage of VL patients following apparent cure but the predisposing immunological causes remain open to question (Ramesh et al., 2007). Indian studies have differed on the status of CMI responses in PKDL, with one study indicating a more significant response in newly acquired PKDL as compared to chronic PKDL (Halder et al., 1983) while another study recorded no specific responses in all 10 patients (Neogy et al., 1988). In contrast, Sudanese PKDL patients have reportedly shown uniform proliferative responses to *Leishmania* antigen, producing IFN-γ and in some cases, IL-10, as well (Ismail et al., 1999).

Building on a hypothesis that onset of PKDL in Sudan is determined even before the start of VL treatment and depends upon the interactions between the parasite and the host immune system during the early stage of the infection, a study by Gasim et al. (1998) demonstrated that IL-10 was present in keratinocytes and in high levels in the plasma of VL patients who went on to acquire PKDL, but notably absent in the VL patients who did not acquire PKDL. Thus, important longitudinal studies from Sudan have implicated the involvement of IL-10 in the pathogenesis of PKDL and established that IL-10 levels in the skin and plasma could actually serve as a predictor of PKDL onset in VL patients. Furthermore, these data indicated that PKDL follows as the outcome of a direct immunological onslaught on the parasites and that there is a strong association between heightened T-cell responsiveness to *Leishmania* antigens and onset of PKDL (Kharazmi et al., 1999).

CMI responses in Indian PKDL: subject of speculation

During visceral leishmaniasis, the immune response to *Leishmania* parasites is either totally impaired or skewed towards a pro-parasitic, anti-inflammatory response (Kemp et al., 1996). With treatment, the immune responses either shift into a pro-inflammatory mode or a mixed response with no clear endpoint (Kemp et al., 1993b, 1996). The immunological factors predisposing the small percentage of cured VL patients to PKDL in India remain to be characterized, although disease pathology is often
attributed to cell-mediated immune (CMI) responses against the parasite (Ramesh et al., 2007).

While uniform lymphoproliferative responses to *Leishmania* antigen have been reported in Sudanese PKDL, 20% of patients producing both IFN-γ and IL-10 and intracellular IFN-γ detected in CD4+ lymphocytes of all patients studied (Ismail et al., 1999), studies on immune responses in Indian PKDL are not only limited, but also contradictory (Haldar et al., 1983; Neogy et al., 1988). Accordingly, detailed analysis of the responses of circulating effector cells may enhance our to-date limited understanding of the immunopathology of Indian PKDL.

**Study objectives**

In light of the limited information on the immunological mechanisms responsible for PKDL in India and the fact that data from the more researched Sudanese form cannot be extrapolated to the distinct Indian variant, a detailed analysis of the functions of circulating effector immune cells from Indian patients with PKDL was attempted. Accordingly, we have analysed cytokine expression at a single cell level in peripheral lymphocytes from patients with PKDL and VL, both at disease presentation and after treatment with two different anti-leishmanial drugs, vis-à-vis healthy controls as a measure of CMI (antigen-specific and generalised). Further, lymphocyte proliferation responses, both antigen-specific and mitogen-induced, were also studied in patients and controls to help draw a more informed picture on the peripheral CMI responses prevalent in Indian PKDL.

**Materials and Methods**

**Isolation of peripheral blood mononuclear cells (PBMC)**

PBMC were isolated by density gradient centrifugation as described in Materials and Methods.

**Intracellular cytokine analysis**

Intracellular cytokines in PBMC from patients with PKDL or VL (before and after treatment) along with healthy controls, stimulated with phytohemagglutinin (PHA) or *Leishmania donovani* antigen (LDA), were detected using flow cytometry after
permeabilization and staining with fluorochrome-conjugated anti-cytokine monoclonal antibodies, as described in Materials and Methods.

**Lymphocyte proliferation assay**

Proliferation of peripheral CD4⁺ or CD8⁺ T lymphocytes in response to 6-day stimulation with a mitogen, PHA or LDA was assayed by dilution of carboxyfluorescein diacetate, succinimidyl ester (CFSE) and measured on a flow cytometer, as detailed in Materials and Methods. Proliferation indices were computed by curve-fitting analysis on the ModFit LT (Verity Software House, USA) software platform (Representative analyses shown in Figure 5.7).
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Results

Generalized cell mediated immune (CMI) responses of PKDL and VL patients

PBMC from patients with PKDL or VL (pre- and post-treatment) as also healthy individuals were assayed for their CMI response to PHA. In PBMC from PKDL patients at presentation, and following cure, the CMI response as judged from the % of lymphocytes expressing Th1 cytokines, IFN-γ and IL-2 expression was comparable to healthy controls (Table 5.1, Figure 5.1). Contrastingly, in VL patients, a pronounced impairment of CMI was recorded with significantly lowered IFN-γ (2.51 ± 0.73 %, p = 0.009) and IL-2 expression (7.94 ± 3.39 %, p = 0.074); normal levels were restored after cure (Table 5.1, Figure 5.1).

In PKDL patients at presentation, expression of the primary anti-inflammatory cytokine IL-10 was higher compared to healthy controls but was not statistically significant; this trend was retained following cure. VL patients demonstrated a more substantial increase in the % of IL-10 expressing lymphocytes which also persisted following cure (Table 5.1, Figure 5.2). IL-4 was barely detectable in all groups, the highest (1.04 ± 0.77) being in VL patients (Table 5.1, Figure 5.2).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>VL Pre-treatment</th>
<th>VL Post-treatment</th>
<th>PKDL Pre-treatment</th>
<th>PKDL Post-treatment</th>
<th>Healthy control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>2.51 ± 0.73</td>
<td>10.06 ± 2.99</td>
<td>7.99 ± 4.55</td>
<td>12.48 ± 6.3</td>
<td>6.61 ± 1.19</td>
</tr>
<tr>
<td>IL-2</td>
<td>7.94 ± 3.39</td>
<td>17.81 ± 3.68</td>
<td>22.07 ± 6.45</td>
<td>22.75 ± 4.52</td>
<td>24.16 ± 8.52</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.04 ± 0.77</td>
<td>0.24 ± 0.1</td>
<td>0.17 ± 0.12</td>
<td>0.2 ± 0.1</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>30.34 ± 10.79</td>
<td>21.49 ± 7.89</td>
<td>11.27 ± 8.51</td>
<td>12.84 ± 4.54</td>
<td>4.06 ± 1.58</td>
</tr>
</tbody>
</table>

PBMC were stimulated with PHA, restimulated with leukocyte activation cocktail, stained for cytokines, and acquired on a flow cytometer as described in Materials and Methods. Each value is the mean ± SEM of at least 6 individuals in each group. * p < 0.01, significantly different from controls; @ p < 0.05, significantly different from VL, pre-treatment.
Figure 5.1. A representative profile of IFN-γ and IL-2 expression in patients with PKDL, VL (pre- and post-treatment) and healthy controls.

PBMC were stimulated with PHA (10 µg/ml) and intracellular staining for cytokines performed as described in Materials and Methods; cells were analyzed by flow cytometry. Lymphocytes were gated on the basis of their light scatter properties and percentages of cytokine-positive cells obtained from the gated populations.
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Figure 5.2. A representative profile of IL-4 and IL-10 expression in patients with PKDL, VL (pre- and post-treatment) and healthy controls.
Antigen-specific CMI responses of PKDL and VL patients: CD3⁺CD8⁺ lymphocytes are the cellular source of antigen-induced IL-10 in PKDL patients

Since cytokine expression in response to antigenic stimulation is an indicator of antigen-specific CMI, we analysed proportions of cytokine-producing cells, upon exposure to LDA, by flow cytometry. It was consistently observed that T helper cells (CD3⁺CD4⁺) from PKDL patients (before and after treatment) and healthy individuals donors failed to synthesize antigen-driven Th1 cytokines, IFN-γ and IL-2 (Table 5.2); a similar trend was obtained for IL-4 expression. IL-10 expression, though detectable, showed no significant variation amongst the 5 groups (Table 5.2, Figure 5.3). As CD3⁺CD4⁻CD8⁻ lymphocytes were negligible and our prior optimization had correlated cytokine positivity between CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes in a few samples, we considered the CD3⁺CD4⁺ population to primarily represent CD3⁺CD8⁺ lymphocytes. In this subset, expression of IFN-γ, IL-2 and IL-4 was negligible (Table 5.3); however, a key finding that emerged from these assays was the 9.6 fold greater IL-10 positivity exclusively in PKDL patients (p<0.05) as compared to controls, which decreased slightly following treatment (Table 5.3, Figure 5.3). On the other hand, VL patients, both before and after treatment, had comparable IL-10 expression which did not vary significantly from controls.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LDA (5 µg/ml)</th>
<th>(%) cytokine positive CD3⁺CD4⁺ lymphocytes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PKDL Pre treatment</td>
<td>PKDL Post treatment</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.03 ± 0.007</td>
<td>0.02 ± 0.006</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.13 ± 0.07</td>
<td>0.08 ± 0.08</td>
</tr>
<tr>
<td>IL-10</td>
<td>5.21 ± 1.08</td>
<td>5.89 ± 1.54</td>
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</table>

PBMC were stimulated overnight with LDA, treated with Brefeldin A for 4 hours, stained for cytokines and acquired on a flow cytometer as described in Materials and Methods. Each value is the mean ± SEM of at least 6 individuals in each group. ND = Not detected.
### Table 5.3: Specific CMI responses in CD8+ T lymphocytes.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LDA (5 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% cytokine positive CD3+CD8+ lymphocytes)</td>
</tr>
<tr>
<td></td>
<td>PKDL Pre treatment</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.009 ± 0.005</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.006 ± 0.002</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>IL-10</td>
<td>*4.71 ± 1.82</td>
</tr>
</tbody>
</table>

PBMC were stimulated overnight with LDA, treated with Brefeldin A for 4 hours, stained for cytokines and acquired on a flow cytometer as described in Materials and Methods. Each value is the mean ± SEM of at least 6 individuals in each group. * p < 0.05, significantly different from controls. ND = Not detected.
A representative profile of IL-10 present in LDA stimulated CD4+ T lymphocytes from healthy controls, patients with PKDL (pre- and post-treatment) or VL (pre- and post-treatment). PBMC were stimulated with LDA (5 µg/ml) and intracellular staining for IL-10 performed as described in Materials and Methods; cells were analysed by flow cytometry. T lymphocytes were gated on the basis of their combined characteristic light scatter and FL3 fluorescence using anti-CD3 PerCP and percentages of cytokine positive CD4+ or CD8+ T cells were quantified on the gated populations after setting quadrant markers based on isotype fluorescence.
PBMC from PKDL patients proliferated in response to PHA but not to *L. donovani* antigen

Generalized CMI responses were assessed by studying peripheral lymphocyte proliferation in response to PHA and specific response using LDA. After incubation for 6 days, proliferating cells showed an expected dilution in CFSE fluorescence (successive daughter populations represented by reduced fluorescence peaks) while unstimulated cells retained maximum CFSE fluorescence, indicating absence of any cell proliferation. Lymphocytes from healthy individuals showed robust responses to PHA, with their CD3⁺CD4⁺ blasts recording an average proliferation index of 6.70 ± 0.23 (Figures 5.4 and 5.5). PKDL patients showed proliferative responses to PHA before (3.15 ± 0.29) and after treatment (2.78 ± 0.30) but their respective indices were significantly lower, compared to controls (p ≤ 0.0005), which was also a feature of cured VL patients (2.85 ± 0.52, p < 0.01), who, expectedly, showed no response at disease presentation (data not shown).

With regard to CD3⁺CD8⁺ blasts, PKDL patients again demonstrated lower proliferation as compared to controls (2.52 ± 0.95 vs. 9.66 ± 0.63, p<0.05). However, with treatment, the proliferative index in PKDL patients increased to 6.82 ± 0.77 while in cured VL patients (4.48 ± 1.57), the index remained lower than controls (Figures 5.4 and 5.5).

In response to LDA, proliferation was notably absent in PKDL patients at presentation and after cure, the proliferative indices in CD3⁺CD4⁺ cells being 1.20 ± 0.06 and 1.26 ± 0.06, respectively (Figures 5.4 and 5.6). However, the same T-helper lymphocytes from successfully treated VL patients showed the strongest antigen-specific proliferation, significantly greater than both controls (2.13 ± 0.64 vs. 1.02 ± 0.01, p < 0.01) and PKDL patients (p<0.01), irrespective of their treatment status. The CD3⁺CD8⁺ blasts displayed negligible proliferative responses across all groups, except for cured VL patients (Figures 5.4 and 5.6).
Figure 5.4. Generalised and antigen-specific proliferative responses of circulating lymphocytes.

Lymphocytes from patients with PKDL (n=10) before ( ) and after ( ) treatment, patients with VL after cure (n=5, ) and healthy controls (n=5, , , ) were stained with CFSE, incubated with/without PHA (10 μg/ml, A) or LDA (5 μg/ml, B) and CFSE fluorescence of gated CD3⁺CD4⁺ and CD3⁺CD8⁺ blasts was acquired on a flow cytometer. Columns represent mean proliferation indices of at least 5 individuals per group, error bars denoting SEM. *p<0.05, **p<0.01, ***p<0.0005, significantly different from controls; ® p<0.05, significantly different from VL post-treatment.
Figure 5.5. Representative profile of lymphoproliferative responses to PHA.

Representative profile of proliferative responses of CD3+CD4+ and CD3+CD8+ lymphocytes to phytohemagglutinin (PHA) in patients with PKDL before and after treatment, cured VL patients and healthy individuals. Numerical values denote proliferation indices.
Representative profile of proliferative responses of CD3+CD4+ and CD3+CD8+ lymphocytes to *Leishmania donovani* antigen (LDA) in patients with PKDL before and after treatment, cured VL patients and healthy individuals. Numerical values denote proliferation indices.
Figure 5.7. Calculation of proliferation indices by ModFit peak-plotting software.
Effect of drugs on the cell-mediated immune (CMI) response in PKDL: Miltefosine and sodium antimony gluconate (SAG) are equally effective in reducing proportions of antigen-specific IL-10 expressing T lymphocytes.

The effect (if any) of drug treatment upon antigen-specific and general CMI responses was measured using flow cytometry by studying the status of intracellular cytokines in patients before and after they received treatment with Miltefosine or SAG.

Analysis of cytokine-producing cells after non-specific stimulation with leukocyte activation cocktail (LAC, containing PMA, Ionomycin and Brefeldin A) revealed no major differences in proportions of both IFN-γ and IL-10 positive cells before and after treatment with either drug (Figures 5.8 and 5.9). However, IL-10 expression was inordinately low in the SAG pre-treatment group (1.05 ± 0.04) which increased with treatment (10.01 ± 3.54) but was not statistically significant.

After stimulation with LDA alone, both groups of patients showed minimal expression of IFN-γ, more so in the SAG group, and remained so post-treatment. With respect to antigen-induced IL-10, proportions of CD3+ cells expressing the cytokine were elevated in both groups of patients at presentation (Miltefosine: 19.60 ± 9.81, SAG: 15.54 ± 4.97). Upon treatment, patients receiving Miltefosine showed a 2.7 fold reduction in the percentage of IL-10 producing cells (7.21 ± 3.26), more or less similar to the 1.85 fold decrease in SAG treated patients (8.38 ± 2.18), indicating comparable effectiveness of Miltefosine in abrogation of antigen-specific IL-10 synthesis (Figure 5.8).
Figure 5.8. Generalised and specific CMI responses in peripheral T lymphocytes from patients with PKDL before and after treatment with miltefosine or sodium antimony gluconate (SAG).

PBMC from patients with PKDL before (■) and after (□) treatment with SAG (n=5) or before (●) and after (▲) treatment with miltefosine (n=5) were stimulated with PMA (10 ng/ml) + Ionomycin (1 μM) + Brefeldin A (1 μg/ml) for 4h or incubated overnight with LDA (5 μg/ml) + Brefeldin A (1 μg/ml) for the last 4h and intracellular staining for IFN-γ or IL-10 performed as described in Materials and Methods; percentages of cytokine-positive CD3+ cells were detected by flow cytometry. Columns represent mean percentages of all 5 individuals per group, error bars denoting SEM.
PBMC from patients with PKDL before and after treatment with miltefosine or sodium antimony gluconate (SAG).

Figure 5.9. A representative profile of non-specific IFN-γ and IL-10 expression in PBMC from PKDL patients before and after treatment with Miltefosine or sodium antimony gluconate (SAG).
Discussion

As impaired cell-mediated immunity (CMI) is a consistent feature of VL, we were interested in studying the CMI in Indian PKDL patients, especially since existing knowledge is limited to two small studies that reported the presence of non specific CMI with loss of parasite specific CMI (Haldar et al., 1983; Neogy et al., 1988). Our findings confirmed that an intact CMI response to PHA in PKDL patients was observed and furthermore, we have provided evidence at a single cell level, cytokines serving as beacons of the effector response (Table 5.1, Figures 5.1 and 5.2). The percentages of IFN-γ and IL-2 producing lymphocytes were comparable to those of healthy individuals establishing restoration of CMI in PKDL (Table 5.1, Figure 5.1). In Ethiopian VL, impairment of CMI was reflected in the inability of CD4+ and CD8+ subsets to synthesize both Th1 (IFN-γ) and Th2 (IL-4) cytokines; responsiveness was regained following effective treatment (Hailu et al., 2005). In Indian VL, we report a similar impairment in non specific CMI, evident in the reduced expression of the Th1 cytokines, IFN-γ and IL-2, which was restored following cure (Table 5.1, Figure 5.1). Changes in IL-4 were minimal; an increased % of IL-10 positivity was evident which was retained following cure (Table 5.1, Figure 5.2).

Absence of antigen-specific CMI is well-documented in VL patients who, in contrast to endemic controls, fail to produce IFN-γ (Sacks et al., 1987; Nylen et al., 2007), as was observed in our study (Table 5.2). Furthermore, IL-10 was not detected in culture supernatants of Leishmania antigen stimulated PBMCs from Indian VL patients (Sacks et al., 1987). Our studies with PBMCs from Indian VL patients partly corroborated these findings as percentages of IL-10 expressing CD3+CD4+ lymphocytes were comparable to healthy controls (Table 5.2, Figure 5.3). Interestingly, in our PKDL patients, increased IL-10 expression at a single cell level was restricted to the CD3+CD8+ lymphocyte subpopulation which regressed following cure, as opposed to VL patients who had moderately raised IL-10 expression at disease presentation and after treatment (Table 5.3, Figure 5.3). Neogy et al. (1988) have previously reported suppression of antigen-specific CMI in Indian PKDL. In contrast, 20% of Sudanese PKDL patients responded to L. donovani antigen by secreting IFN-γ and concomitantly producing IL-10.
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(Ismail et al., 1999) while in a longitudinal study on Sudanese VL patients, onset of PKDL occurred within 6 months and was concurrent with acquisition of Leishmania-specific lymphocyte reactivity (Gasim et al., 2000a).

Our studies on lymphocyte proliferation in response to the mitogen, phytohemagglutinin (PHA) or to Leishmania donovani antigen (LDA) employed the novel CFSE dilution assay, a technique that could quantitate the proliferation indices of CD4+ and CD8+ sub-populations within the peripheral lymphocyte pool. This was a big experimental advance compared to the non-discriminatory tritiated thymidine incorporation assay used in previous studies (Haldar et al., 1983; Neogy et al., 1988). In the study by Neogy et al. (1988), PBMC from patients proliferated only in response to PHA and not to Leishmania antigen, findings that were corroborated by our CFSE dilution data. Importantly, we have been able to show differences in the proliferative capacity of CD4+ and CD8+ lymphocytes, with the latter always enjoying a head start over the former (Figure 5.4). Both lymphocyte subsets from PKDL patients before and after treatment responded adequately to PHA stimulation, compared to healthy controls, also a feature of cured VL patients (Figures 5.4 and 5.5). Antigen-specific proliferation was notably absent in PKDL patients, regardless of treatment, and observed only in the cured VL group, as was expected with recent infection having left behind antigen-specific memory and effector lymphocytes in the circulation (Figures 5.4 and 5.6). In this context, it is tempting to speculate that the IL-10 producing CD8+ T lymphocytes implicated in PKDL have lost their effector function and are functioning as T regulatory cells (Tregs), in conformity with published reports on antigen-driven IL-10 producing Tregs that do not produce any other effector cytokines (O’Garra and Vieira, 2004).

In comparing the effects of two drugs used against leishmaniasis, the conventional sodium antimony gluconate (SAG) and the newer Miltefosine, on the resolution of PKDL, performance was assessed on the basis of improvement in several immunological parameters, one of which was the antigen-specific and general CMI response of peripheral lymphocytes. At the intracellular level, CFSE-stained CD3+ lymphocytes were examined and not CD8+ lymphocytes, owing to a technical limitation in a four-color assay. As was observed earlier in our study (Table 5.1, Figure 5.1), non-specific CMI responses were not impaired in PKDL patients and continued to be so after treatment,
irrespective of the drug used (Figures 5.8 and 5.9). The markedly reduced IL-10 expression in the SAG pre-treatment group could be attributed to the lower expression \textit{per se} in patients of that group as we have earlier shown sufficiently raised IL-10 in our patients at presentation (Table 5.1, Figure 5.2). When responses to \textit{Leishmania donovani} antigen (LDA) were considered, both drugs were seen to be equally effective in reducing proportions of IL-10 producing cells (Figures 5.8 and 5.9). Our observations were in agreement with previous findings on the specific immunopotentiating ability of SAG via effects on cell signaling, cytokines and on immune complex induced granulocyte macrophage colony stimulating factor (GM-CSF) levels (Pathak and Yi, 2001; Elshafie et al., 2007). Taken together, our study answered the need for larger and elaborate studies stressed by earlier case reports (Sundar et al., 2006a; Ramesh et al., 2008) with a view to establish Miltefosine as a frontline drug against PKDL. Our study demonstrates the immunomodulatory capacity of miltefosine against PKDL, comparable to that of SAG. Considering the small number of patients who were administered either drug, larger clinical trials with emphasis on immunological treatment markers would give us a better perspective on the suitability and effectiveness of miltefosine in management of PKDL.