CHAPTER 6

LESIONAL

CELL-MEDIATED IMMUNITY

IN INDIAN PKDL
Chapter 6 – Lesional CMI in Indian PKDL

Introduction

Mammalian hosts typically respond to infection by *Leishmania* parasites by mounting a prominent Th1 response, thus potentiating the leishmanicidal machinery of parasitized macrophages. Much of what is known about the immunological mechanisms underlying *Leishmania* infection is derived from the enormous body of experimental work with *L. major* infection in susceptible BALB/c mice. According to the model that has emerged, a strong IL-4 driven Th2 response favours parasite persistence while conversely, a sustained Th1 response accounts for efficient parasite clearance (Sacks and Noben-Trauth, 2002). However, a dominant Th2 response does not fully account for the non-resolving pathology of cutaneous leishmaniasis in humans, the systemic pathology of human visceral leishmaniasis or the chronic pathology of the reactivated post kala-azar dermal leishmaniasis.

Cutaneous leishmaniasis

Control and resolution of *L. major* infection in mice is attributed to the pro-inflammatory activities of IFN-γ and TNF-α, while parasite persistence and therefore, pathology is accounted for by the anti-inflammatory functions of IL-4, IL-5, IL-10, and TGF-β (Alexander and Bryson, 2005). On the other hand, in humans, the immune milieu of chronic, localized lesions of cutaneous leishmaniasis is characterized by high levels of both pro-inflammatory cytokines and the deactivating cytokine, IL-10 with low or undetectable amounts of Th2-associated cytokines like IL-4. For instance, localized lesions of cutaneous leishmaniasis caused by *L. mexicana* have mixed and not polarized cytokine profiles with both pro-inflammatory (IFN-γ, TNF-α, IL-1 α, IL-6) and anti-inflammatory cytokines (IL-10 and TGF- β) being detected (Melby et al., 1994). In cutaneous lesions due to infection with *L. braziliensis*, gene expression analyses revealed abundance of IFN-γ and IL-2 while in mucosal lesions, IL-4 was predominant followed by IL-5 and IL-10, causative species remaining the same (Pirmez et al., 1993). In localized cutaneous leishmaniasis caused by *L. major*, cytokines with detectable mRNA expression included TNF-α, IL-6, IFN-γ, IL-12, IL-10 and to a lesser extent, IL-4 (Louzir et al., 1998). Taken together, IL-10 and TGF- β, by virtue of their immunosuppressive
effects on macrophage function, are assumed to play important roles in the immunopathogenesis of chronic cutaneous leishmaniasis.

Visceral leishmaniasis

In the visceralising form, concomitantly raised mRNA levels of the antagonistic cytokines, IFN-γ and IL-10, are a feature of the parasitized spleen, liver, bone marrow and even lymph nodes, when involved (Nylén and Sacks, 2007). In a study by Kenney et al. (1998), cytokine gene expression was evaluated in splenic aspirates from patients with visceral leishmaniasis before and after treatment with IFN-γ, sodium antimony gluconate (SAG), or amphotericin B lipid complex (ABLC). At presentation, high levels of both IFN-γ and IL-10 were detected in most patients and IL-4 in a few patients; when treated with IFN-γ, expression of IFN-γ was reduced while there were no differences in IL-10 mRNA levels; however, treatment with SAG or ABLC effected a significant reduction in both IFN-γ and IL-10 mRNA levels (Kenney et al., 1998). Localised cytokine profiles in bone marrow aspirates from patients with VL are similarly characterized by significantly
upregulated expression of both IL-10 and IFN-γ at disease presentation and showed a reduction in IL-10 levels following treatment (Karp et al., 1993). Increased IL-10 mRNA levels are a feature of epitrochlear lymph nodes in patients with visceral leishmaniasis while IFN-γ and IL-2 expression at the same site was observed both at presentation and after treatment, suggesting that IL-10 plays an important role in regulating immune responsiveness during visceral leishmaniasis (Ghalib et al., 1993). Taken together, in VL, distinct from the Th1-Th2 dichotomy observed in CL, polarized Th2 responses are not a feature as a mixed Th1-Th2 response prevails during active disease, which involutes following cure. Further, IL-10, a potent deactivator of macrophages and thus promoter of Leishmania growth within the macrophages, is actively involved in the pathogenesis of visceral leishmaniasis.

Post kala-azar dermal leishmaniasis (PKDL)

As part of an earlier study on localized CMI in VL, lesions from one Sudanese PKDL patient were analysed for mRNA expression, wherein both IL-10 and IFN-γ were detected (Ghalib et al., 1993). Subsequently, lesional CMI responses have been better profiled in Sudanese PKDL. In lesions from these patients, a dense inflammatory infiltrate consisting of a heterogeneous population of macrophages, lymphocytes, and plasma cells was observed and occasionally, epithelioid granulomas were also seen. A majority of the cells comprising the infiltrate were characterized as CD3+ T lymphocytes while IL-10 was detected prominently in all lesions followed by IFN-γ and IL-4 (Ismail et al., 1999). In another study by Gasim et al. (1998), Sudanese VL patients who later developed PKDL were found to have increased IL-10 expression in keratinocytes and/or sweat glands while in those patients who did not develop PKDL, there was no such expression, leading the authors to propose the utility of IL-10 as a predictor of disease. Studies that followed have looked at immune cell phenotypes and adhesion molecules and concluded that CD3+ T lymphocytes comprised a majority within the cellular infiltrate, with a predominance of CD4+ lymphocytes over CD8+ cells. Further, degenerating basal keratinocytes were shown to be positive for HLA-DR, ICAM-1 and Leishmania antigen while interacting closely with CD4+ T lymphocytes (Ismail et al., 2006a).
Information on the lesional immunology in Indian PKDL is scant, corresponding to the few studies on Indian PKDL, which is further attributable to the low incidence of the disease. The first such study on the lesional immune profile in Indian PKDL reported a transition from sparsely populated CD4+ and CD8+ cells in the early hypopigmented or macular lesions to prolific infiltration of both subsets, particularly the CD8+ cells, in the granulomatous nodular lesions, even though the CD4+ cells outnumbered the CD8+ cells in circulation (1.9:1), similar to healthy individuals (Ghosh et al., 1995b). Lymph nodes from patients presenting with lymphadenopathy in addition to PKDL were characterized by T cell- infiltrated cortical areas with CD8+ cells again predominating over CD4+ cells. Summarising their findings, the authors implicated CD8+ lymphocytes in conjunction with relevant CD4+ subsets in the pathogenesis of PKDL (Ghosh et al., 1995b). In corroboration, Rathi et al. (2005a) reported a predominance of CD8+ lymphocytes (calling them T suppressor cells) over CD4+ T helper cells in both hypopigmented macular and nodular or plaque lesional tissue from Indian patients with PKDL, laying emphasis on the need for more studies on larger groups of patients, focusing on T-cell profiles both locally and in circulation for a better understanding of the pathogenesis of PKDL.

Subsequently, a study by Ansari et al. (2006b) examined the lesional cytokine profile in Indian PKDL and reported increased expression of both pro-inflammatory (IFN-γ, TNF-α, IL-6) and anti-inflammatory (IL-10, TGF-β, IL-4) cytokines with concomitantly reduced IFN-γ R1 receptor expression. In demonstrating elevated mRNA levels of IL-10 and TGF-β in PKDL dermal lesions, the authors proposed a role for these cytokines in disease pathogenesis, mediated by a suppression of Th1 responses. In a recent study, the same group analysed TNF-α receptor expression (TNFR1 and TNFR2) in PKDL lesions, demonstrating a down-regulation of TNFR1 transcripts. Taken together with high TNF-α expression, impairment of Th1 immune responses could thus be explained by the reduced TNFR1 expression (Ansari et al., 2008).

**PKDL immunopathology: Can regulatory T cells (Tregs) step in?**

Regulatory T cells (Tregs) representing 5 – 10 % of the CD4+ T cell population and characterized by the expression of the transcription factor Foxp3 (Shevach et al.,
2006) are involved not only in control of immune responses to self-antigens and maintenance of immune homeostasis but also in the regulation of immunity to infection (Sakaguchi, 2004). Tregs mitigate immune-mediated pathology after pathogens have been successfully cleared and are therefore beneficial to the host in acute infections. However, this functionality acts as a double-edged sword in that parasite persistence in the midst of an active immune response is favoured, which is detrimental to the host in chronic infections. A widely expected role for natural Tregs in visceral leishmaniasis was rejected in a study on Indian VL patients (Nylen et al., 2007), which documented IL-10 expression in splenic T cells other than purified CD4+CD25+Foxp3+ Tregs. However, in contrast to VL, PKDL being chronic in nature, Tregs can possibly occupy a functional niche in the former.

Study objectives

A thrust area of this investigation was to examine the role, if any, of regulatory T cells (Tregs) in the localised PKDL immune milieu, considering the complicity of Tregs in states of infection and given the important fact that nothing is known on their functional relevance in the PKDL immune scenario. Since histopathological staining alone gives a picture of the nucleated cells in the inflammatory infiltrate in skin lesions, immunohistochemical staining was also performed to estimate the percentage of CD3 positive T lymphocytes within the lesional site. The gene expression profiles of the pro- and anti-inflammatory cytokines usually active in immunological cross-talk were studied at the lesional site before and after treatment with two different antileishmanial agents, sodium antimony gluconate (SAG) and miltefosine, to check whether differences in immunomodulatory capacity of the drugs could impact on the post-treatment lesional cytokine profile and importantly, to partially validate the therapeutic response of Indian PKDL patients to miltefosine vis-à-vis sodium antimony gluconate (SAG).

Materials and Methods

Collection of lesional tissue

Dermal biopsies were extracted from lesions of patients with PKDL and were either stored in an RNA stabilization reagent for isolation of RNA or kept in buffered formalin to be processed for immunohistochemistry, as previously described.
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**Isolation of RNA and RT-PCR**

RNA was isolated from lesional tissue, subjected to semi-quantitative RT-PCR using gene-specific primers for β-actin, IFN-γ, IL-10 and Foxp3 (Table 1), and gene expression quantified by densitometric analysis, as described in Materials and Methods.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded lesional tissue sections were processed for optimal immunohistochemical staining with antibodies against CD3 and Foxp3, as described in Materials and Methods. The percentage of positive cells per microscopic field was calculated with three representative fields being counted per section, and average values were used to allocate a positivity score on the basis of a scoring index, as detailed in Materials and Methods.

**Results**

**Upregulated lesional IFN-γ and IL-10 regressed with treatment**

To evaluate the effect of treatment on counter-regulatory cytokine profiles in PKDL lesions, we quantitated gene expression of IFN-γ and IL-10 by RT-PCR. In PKDL patients at presentation (n = 10), densitometric analysis after normalization to β-actin expression revealed significantly upregulated IFN-γ and IL-10 mRNA levels as compared to healthy controls (p < 0.05), who showed minimal expression of IFN-γ with undetectable IL-10 (Figure 6.1 A).

Treatment with sodium antimony gluconate (SAG) reduced expression of both IFN-γ and IL-10 (p < 0.01, compared to pre-treatment), the post-treatment IFN-γ expression regressing to control levels (Figure 6.1 A - C). However, IL-10 expression was not affected by treatment, with post-treatment values remaining higher than controls (p < 0.05, Figure 6.1 C).

An equally significant reduction in expression of IFN-γ and IL-10 was seen with miltefosine treatment (p < 0.01, compared to pre-treatment, Figures 6.1 A and B). However, unlike SAG, post-treatment expression of both IFN-γ and IL-10 continued to be higher than controls (p < 0.05, Figures 6.1 A and C).
Figure 6.1. Effect of Miltefosine or sodium antimony gluconate (SAG) treatment on lesional expression of IFN-γ and IL-10 in Indian PKDL.

RNA was isolated from biopsies of dermal lesions of PKDL patients before and after treatment and from healthy skin of controls. RT-PCR products were resolved by agarose gel electrophoresis and bands specific for β-actin, IFN-γ and IL-10 visualized by UV transillumination. Lesional expression of IFN-γ and IL-10 was quantified by densitometric analysis, as described in Materials and Methods.
Scatter plots of expression values for IFN-γ and IL-10 in patients before (▲) and after treatment (△), and from patients who received SAG, before (■) and after treatment (□).

* p < 0.05, significantly different from controls; ** p < 0.01, significantly different from respective pre-treatment group.
Increased lesional Foxp3 expression receded following cure

To check whether upregulated IL-10 expression could be attributed to T regulatory (Treg) cells within the lesional milieu, gene expression of the Treg transcription factor, Foxp3, was quantitated by densitometric analysis. Patients with PKDL had significantly raised Foxp3 transcripts in lesional tissue, as compared to healthy controls (p < 0.05, Figures 6.2 A and C).

Following treatment, 7 patients across the two treatment groups showed a distinct reduction in lesional Foxp3 mRNA whereas 2 showed no changes and one in fact showed increased mRNA expression (Figures 6.2 A and B). On an individual basis, treatment with SAG effectively down regulated Foxp3 expression in 4/5 patients, their post-treatment values being comparable with healthy controls (Figure 6.2 B). After receiving Miltefosine, 3/5 patients showed a reduction in lesional Foxp3 transcripts but their post-treatment Foxp3 mRNA levels were not comparable to control levels (p < 0.05, Figure 6.2 C).

Figure 6.2. Lesional Foxp3 expression in Indian PKDL at presentation and after treatment with Miltefosine or SAG.

Foxp3 expression in lesional mRNA from PKDL patients was quantified by RT-PCR and subsequent densitometric analysis, as described in Materials and Methods.
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Plots depict β-actin normalized expression values for lesional Foxp3 from 5 PKDL patients before (△) and after treatment (△) with Miltefosine vs. 5 patients before (■) and after treatment with SAG (□).

Scatter plot showing lesional Foxp3 expression in patients before (△) and after treatment (△) with Miltefosine, before (■) and after treatment (□) with SAG and in healthy controls (♦). Horizontal lines indicate mean values. * p < 0.05, significantly different from controls.
Accumulation of lesional T regulatory cells regressed with treatment

To ascertain whether the Foxp3 gene expression data from PKDL lesional tissue reflected in the translated protein, immunohistochemistry was performed on formalin-fixed paraffin-embedded sections of lesional tissue from 8 patients (Miltefosine group, n = 4; SAG group, n = 4) and 3 healthy controls using monoclonal antibodies against Foxp3. Additionally, sections were stained for CD3 to verify infiltration of T lymphocytes within dermal lesions, of which T regulatory cells (Foxp3+) constitute a small fraction.

Microscopic analysis indicated an accumulation of Foxp3+ cells in the lymphocytic infiltrate that characterizes PKDL histopathology (Figures 6.3 and 6.4) while healthy control skin had virtually no Foxp3+ cells, scant CD3+ lymphocytes and no marked cellular infiltrate (data not shown).

In the Miltefosine group, at presentation, two patients reported scores of 1 – 5 %, one was within 5 – 10 %, and one showed 10 – 20 % positivity. Following treatment, Foxp3+ cells were reduced in 3 patients, evident in their lower post-treatment scores whereas one continued to have a high score (Table 6.1, Figure 6.3). CD3 positivity at presentation ranged from 20 – 50 % (n = 1) to > 50 % (n = 3), which showed only slight reduction with treatment (Table 6.1, Figure 6.3).

In the SAG group, proportions of Foxp3+ cells at presentation varied between 1 - 5 % (n = 2) and 5 - 10 % (n = 2). Following treatment, all four patients reported a decrease in their Foxp3 staining scores (Table 6.1, Figure 6.4). With regard to CD3 positivity, 2/4 patients at presentation had > 50% positivity while two had 20 – 50 % T lymphocytes. Treatment showed no decrease in CD3 scores, although the number of cells comprising the lymphocytic infiltrate decreased following treatment.
Table 6.1. Foxp3 and CD3 positivity scores in PKDL patients before and after treatment with Miltefosine or SAG.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Treatment received</th>
<th>Foxp3 score</th>
<th>CD3 score</th>
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<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>1</td>
<td>Miltefosine</td>
<td>*** ±</td>
<td>++++</td>
</tr>
<tr>
<td>2</td>
<td>Miltefosine</td>
<td>++ +</td>
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<td>3</td>
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<td>4</td>
<td>Miltefosine</td>
<td>+ 0</td>
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<tr>
<td>5</td>
<td>SAG</td>
<td>++ ±</td>
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<tr>
<td>6</td>
<td>SAG</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>SAG</td>
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Paraffin embedded sections of lesional tissue from PKDL patients at presentation and after treatment with Miltefosine or SAG were stained for Foxp3 and CD3 by immunohistochemistry and scored, as described in Materials and Methods.
Figure 6.3. Immunohistochemical staining of lesional sections from PKDL patients before and after treatment with Miltefosine.

Formalin-fixed paraffin embedded sections of dermal lesional biopsies from PKDL patients were stained for Foxp3 and CD3 by immunohistochemistry, as described in Materials and Methods. A representative profile of lesional CD3 and Foxp3 staining in a PKDL patient before and after treatment with Miltefosine. Arrows indicate Foxp3 positive cells within the lymphocytic infiltrate.
Formalin-fixed paraffin embedded sections of dermal lesional biopsies from PKDL patients were stained for Foxp3 and CD3 by immunohistochemistry, as described in Materials and Methods. A representative profile of lesional CD3 and Foxp3 staining in a PKDL patient before and after treatment with SAG. Arrows indicate Foxp3 positive cells within the lymphocytic infiltrate.
Knowledge of the interplay of cytokines within the dermal lesional site is critical to our understanding of the immunopathology of PKDL. In that context, our gene expression studies on lesional tissue from patients with PKDL (before and after treatment with miltefosine or sodium antimony gluconate) vs. dermal tissue from healthy individuals, aimed at elaborating on the roles of the two mutually counteracting cytokines, IFN-γ and IL-10, in PKDL pathogenesis.

In the past, only one group has studied the localized immune profile in Indian PKDL, wherein it was reported that as compared to the VL lesional profile, IFN-γ, TNF-α, IL-6 were highly expressed and could be responsible for pathogenesis (Ansari et al., 2006b). Further, when compared to healthy human skin, in addition to the above three cytokines, the deactivating cytokines, IL-10 and TGF-β, and the Th2 cytokine, IL-4 were also over expressed while IFN-γ receptor 1 (R1) was minimally expressed in PKDL lesions. It was therefore suggested that the over-expressed pro-inflammatory cytokines, IFN-γ and TNF-α, were compensated for by the co-expression of IL-10 and TGF-β along with down regulation of IFN-γ R1 (Ansari et al., 2006b).

Our findings, corroborating the previously reported high intralesional co-expression of IFN-γ and IL-10 in Indian PKDL, further demonstrate that the reduction in cytokine mRNA levels following treatment is independent of the drug administered (Figure 6.1).

Gene-expression studies offer limited information in the sense that there are no pointers to the cellular source(s) of the upregulated cytokines. This limitation is felt more in case of a pleiotropic cytokine like IL-10 that is secreted by a plethora of both innate and adaptive immune cells including T lymphocytes (Th1, Th2, Th17 and CD8*), regulatory T cells (Tregs), monocytes, macrophages, dendritic cells and B cells (O’Garra and Vieira, 2007). The magnitude of the problem increases manifold if one considers the cellular profile of the lesional site in PKDL-dermal tissue with its often dense inflammatory cell infiltrate, which can theoretically host each and every one of the above cells and more. Moreover, since IL-10 primarily curbs immune responses of T cells directed against pathogens, it can be speculated that it has a similar role in containing
immunopathology within the PKDL lesion, evident in the elevated IFN-γ expression. However, the question remains as to which immune cell subset is responsible for the increased lesional IL-10 expression in PKDL.

Against this backdrop, a potential source of IL-10 particularly in scenarios of infection could be the T regulatory cells (Tregs), important constituents of the immunoregulatory network that emerge as a response to the infectious process (Belkaid, 2007). Importantly, no information exists on Treg involvement in either variant of PKDL—Indian or Sudanese. Accordingly, we sought to examine the role (if any) of Tregs in the localized immune milieu of PKDL. Analysis of the expression of the Treg-exclusive transcription factor, Forkhead box P3 (Foxp3), studied both at the level of mRNA by RT-PCR (Figure 6.2) and protein by immunohistochemistry (Figures 6.3 and 6.4), yielded a novel finding that Tregs feature prominently in the inflammatory infiltrate within PKDL dermal lesions (Figures 6.3 and 6.4, Table 6.1), concomitant with increased gene expression of Foxp3. In most but not all patients, treatment with miltefosine or SAG curtailed accumulation of Tregs (Figures 6.3 and 6.4, Table 6.1), affirming their contribution to lesional pathology. Due to the substantial heterogeneity in lesional Foxp3 expression at presentation, there was only a marginal decrease in mean Foxp3 mRNA levels, which in conjunction with treatment-induced abrogation of lesional Tregs suggested a deficiency in translational mechanisms (Figure 6.2). Additionally, Foxp3 expression did not correlate with the type or severity of lesions, indicating that infiltrating Tregs are fundamentally involved in immunoregulation of pro-inflammatory cytokines such as IFN-γ, directed against the skin-resident parasites, and are not unique to lesions with a higher degree of inflammation, e.g. nodules.

In the previous chapter on peripheral CMI responses in Indian PKDL, the increased proportions of circulating antigen-specific IL-10+ CD8+ T lymphocytes (Figure 5.3, Table 5.3) detected in PKDL patients were proposed to be regulatory in function that could promote parasite persistence and thus, potentially act as important immunodeterminants in the pathogenesis of PKDL. In cutaneous leishmaniasis caused by *Leishmania braziliensis*, accumulating Tregs in the lesions have been shown to downregulate effector T cell responses (Campanelli et al., 2006). A recent study on localized cutaneous leishmaniasis caused by *L. guyanensis* associated treatment
refractoriness with high lesional Foxp3 expression, further suggesting an impairment of local immune responses by infiltrating T regulatory cells (Bourreau et al., 2009).

Our findings have substantially added to the existing body of knowledge by demonstrating for the first time, accumulation of T regulatory cells at the lesional site in PKDL. It can be proposed that the antigen-driven IL-10 producing CD8+ lymphocytes documented in Indian PKDL patients are anergic, have a regulatory capacity and upon local recruitment, sustain disease pathology by impeding antigen-specific effector cell activity. Future studies focusing on their functional aspects, specifically their interaction with target immune cells within the localized PKDL milieu, could help us formulate newer and effective immunomodulatory strategies against PKDL with a broader view of tackling the spread of visceral leishmaniasis.