Detection of IL-13, IL-10, and IL-6 in the Leprosy Skin Lesions of Patients during Prednisolone Treatment for Type 1 (T1R) Reactions[1]

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ABSTRACT

This study demonstrates the presence of IL-10 and IL-6, by immunohistochemistry, in the skin lesions of patients with Type 1 reactions. Fifteen patients with Type 1 reaction from Hyderabad, India were included in this study. They were all receiving standardized treatment for Type 1 reactions: a reducing course of daily oral prednisolone for 6 months. Biopsies were taken before treatment and during treatment at weeks 1, 4, and 6 months. IL-13 was observed in the lesions of most patients. By week 4 of treatment, the presence of IL-13, IL-10, and IL-6 in the lesions had decreased significantly.

Although some patients showed significant clinical skin sign improvement within one week of therapy, no concomitant decrease or increase in any of the cytokines was observed at this time point. Interestingly, some cytokine activity within the lesions was observed after 6 months of treatment.

RÉSUMÉ

Cette étude met en évidence par immuno-histochimie la présence d'IL-10 et d'IL-6 au sein de lésions de patients souffrant de réaction de type 1. Quinze patients présentant des réactions de type 1, provenant de Hyderabad aux Indes, ont été recrutés pour cette étude. Ils étaient tous en train de recevoir un traitement standard de la réaction de type 1 : une dose décroissante de prednisolone par voie orale pendant 6 mois. Les biopsies ont été effectuées avant le traitement et aux semaines 1, 4 et 6 mois de traitement. L'IL-13 fut observée dans les lésions de la plupart des patients. Vers 4 semaines de traitement, la présence de l'IL-13, IL-10 et l'IL-6 avaient diminué de façon significative.

Bien que certains patients aient montré une amélioration significative des signes cliniques dans la semaine qui a suivi le début du traitement, il n'y avait pas à ce temps de prélèvement d'évidence suggérant une augmentation ou une diminution d'une de ces cytokines. De façon surprenante, certaines activités de cytokines furent observées après 6 mois de traitement au sein des lésions.

RESUMEN

Se hizo un estudio inmunohistoquímico en las lesiones de la piel de pacientes con reacción leprosa Tipo 1 para buscar la presencia intralesional de IL-13, IL-10 e IL-6. Se incluyeron 15 pacientes de Hyderabad, India, con lepra y reacción leprosa Tipo 1. Todos los pacientes estaban recibiendo el tratamiento estándar para este tipo de reacción: dosis diarias decrecientes de prednisolona durante 6 meses. Se tomaron biopsias antes y durante el tratamiento a las semanas 1, 4 y 24 (6 meses). Al inicio del tratamiento, las lesiones de la mayoría de los pacientes mostraron la presencia de IL-13, sin embargo, cuatro semanas después, la expresión de IL-13, IL-10 e IL-6 en las lesiones había disminuido significativamente.

Aunque algunos pacientes mostraron una notoria mejoría clínica en sus lesiones dérmicas en la primera semana de tratamiento, no se observó ningún cambio concomitante en ninguna de las citocinas. Por otro lado, todavía se observó algo de actividad de las citocinas en las lesiones después de seis meses de tratamiento.

Type 1 reactions (T1R) occur in about 30% of patients with the immunologically unstable borderline forms of leprosy. These reactions are phases of acute inflammation that often lead to nerve damage and are associated with high levels of pro-inflammatory cytokines in the skin and nerve [6]. Six months of treatment using the corticosteroid, prednisolone, reduces skin inflammation and improves nerve function in about 60% of patients [9]. Although T1R episodes are described as phases of delayed-type hypersensitivity (DTH) associated with the clearance of mycobacteria [7], the immunological factors underlying these reactions and specifically nerve damage are not well understood. Research in this field is required to understand the complex immunology underlying T1R so that treatment for these patients may be improved.

Serum levels of both pro- and anti-inflammatory cytokines decrease during multidrug therapy (MDT) but this is reversed during the onset of a T1R [13]. In the skin lesions of these patients, T1R episodes are associated with the expression of many cytokines including many inflammatory: interleukin-12 (IL-12), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and anti-inflammatory cytokines: interleukin-10 (IL-10), T-cell growth factor-β (TGF-β) [6, 11, 12]. The "upgrading" of the protective cell mediated immunity in T1R is paradoxically associated with the neuropathy observed in these patients. In particular, the expression of TNF-α as part of the antimicrobial response is considered to be a key mediator associated with the systemic inflammatory symptoms observed in T1R [6].

The role that prednisolone plays in controlling nerve damage is partially due to its capacity to down-regulate the pro-inflammatory cytokines. The effect of prednisolone on pro-inflammatory cytokines has been observed in many severe inflammatory diseases [3, 5]. The down-regulatory effect of prednisolone on inducible nitric oxide synthase (iNOS), IFN-γ, IL-12, TNF-α, and IL-6 in the lesions as been demonstrated [8, 12]. However, the effect of prednisolone on other regulatory cytokines is not clear, although there is some *in vitro* evidence that high doses of methylprednisolone increase LPS-induced IL-10 levels [10]. In leprosy reactions, no reduction in the expression of IL-10 has been observed during treatment [12]. With the effects of prednisolone on inflammatory cytokines well documented, this study aims to determine whether prednisolone effects the expression of the regulatory cytokines IL-10, IL-13, and IL-6 in the skin, and determines whether there is a direct relationship between the expression of these and the clinical skin sign improvement in patients with T1R.

MATERIALS AND METHODS

Patients. Fifteen leprosy patients with T1R (borderline tuberculoid: n = 6, borderline lepromatous: n = 9) from Blue Peter Research Center (BPRC), Hyderabad, India were recruited for this study. Patient status according to Ridley-Jopling [15] scale and MDT treatment is given in The Table and in a previous publication [8]. T1R was defined as the appearance of erythema and oedema in either existing or new leprosy skin lesions within the previous 2 weeks. Histological examination of biopsies was undertaken for all patients. Patient consent was obtained before collection of biopsies. Skin biopsies (6 mm) were taken from the reactive skin lesions at time points during treatment, and snap frozen and stored in liquid nitrogen. The initial biopsy was taken at the time of presentation before prednisolone treatment had commenced (day 0). Subsequent biopsies were taken from the lesion (close to the previous biopsy site) after weeks 1, 4, and 26. Treatment consisted of a standard reducing course of steroids, initially of 30 mg oral prednisolone daily, which was reduced by 5 mg each month for 6 months. Clinical improvement in this study relates to improvement seen in the skin signs of the patients. Skin signs were measured by applying a numerical severity scale that assessed the degree of inflamma-

THE TABLE   *BL-RR, borderline lepromatous leprosy in reversal reaction; BT-RR, borderline tuberculoid leprosy reversal reaction.*[a]

Patient	Class	MDT	Age	Sex	BI	Skin signs				Cell infiltrate				IL-10				IL-13				IL-6			
						0[b]	1[b]	4[b]	26[b]	0	1	4	26	0	1	4	26	0	1	4	26	0	1	4	26
143	BT-RR	PB(3)	23	M	0	1	0	0		2	1	1		3	1	0		0	0	0		3	1	1	
162	BT-RR	PB(3)	40	F	0	5	0	0		3	2	2		3	4	1		2	2	0		3	2	1	
179	BT-RR	PB(2)	21	M	0	5	4	0	0	3	3	3	2	2	4	2	3	1	3	2	2	3	4	3	3
303	BT-RR	MB(3)	—	M	0	2	2	2	0	3	3	1		3	3	2	1	2	1	0	0	4	3	3	1
342	BT-RR	PB(3)	—	F	0	3	4	0	0	3	3	1	0	3	3	3	1	0	0	0	0	3	3	3	1
380	BT-RR	PB(3)	—	F	0	3	0	0		3	3	1		3	3	1		3	1	0		3	3	2	
186	BL-RR	PB(3)	40	F	1.2	4	4	0		2	1	1		3	3	1		0	0	0		3	2	0	
198	BL-RR	MB(2)	36	F	3	2	5	0	0	3	3	2	0	3	4	3	1	3	0	3	1	1	4	2	1
141	BL-RR	MB(2)	44	F	2.4	5	0	0	2	3	2	1	2	3	3	2	3	2	0	0	0	4	3	3	4
260	BL-RR	MB(1)	—	M	3.3	5	2	1	1	3	3	1	1	4	4	2	1	0	2	0	0	4	4	3	2
207	BL-RR	PB(1)	50	M	0	4	1	0	1	3	3	0	0	4	4	1	1	4	4	0	0	3	3	0	2
304	BL-RR	MB(3)	—	F	1.2	5	4	0		2	2	0		2	4	1		3	3	1		4	5	1	
343	BL-RR	MB(3)	—	M	1	3	4	0	0	1	2	0	0	3	3	0	0	2	3	1	0	4	4	1	1
131	BL-RR	MB(1)	25	M	3.3	4	3	0	0	2	3	3	1	2	3	3	1	4	3	3	2	3	4	4	1
146	BL-RR	MB(3)	35	F	—	5	0	4		3	2	2		4	4	3		3	3	2		5	5	3	

[a] MDT therapy, multi-drug therapy; PB, paucibacillary bacillary regimen; MB, multibacillary regimen; (1) completed MDT before reaction; (2) on MDT at time of reaction; (3) no previous history MDT and given MDT during study. —, not known. Scoring for skin signs described in text. Scoring for cytokine staining was as follows: 0, negative; 1, <10% of cells staining positive; 2, 10 to 30% of cells staining positive; 3, 50 to 80% of cells staining positive; and 4, 80 to 100% of cells staining positive. Scoring for cellular infiltrate was as follows: 0, no cellular infiltrate; 1, small granulomas; 2, medium-sized granulomas; and 3, large granulomas.

[b] Samples collected on day 0; and after 1 week, 4 weeks, and 26 weeks of treatment. The 26 week time point varied between 25 and 27 weeks and some patients were unavailable for sampling.

tion and percentage of raised and erythematous skin lesions throughout the body. Scoring of the degree of inflamed skin signs (score A) was from 0 to 3, with 0 being no inflamed lesions, 1 erythematous lesions, 2 raised and erythematous lesions and 3 ulcerated lesions. The percentage of all lesions that are raised and inflamed (score B) was scored as 0 being <25%, 1 as 25 to 50%, 2 as 50% to 75% and 3 as >75%. The overall skin sign score was the sum of the scores A and B. Ethical approval was obtained for this study from the BPRC (Blue Peter Research Center, Hyderabad) and London School of Hygiene and Tropical Medicine, London. Patient data is presented in The Table.

Immunostaining. Cryosections (thickness 5 to 6 μm) were adhered to silane (Sigma, Poole, U.K.)-coated slides and acetone or paraformaldehyde (4%, Sigma) fixed. Non-specific protein binding was prevented by incubation in serum-free blocking medium (Dako, Glostrup, Denmark). Staining was refined using the peroxidase-antiperoxidase (PAP) and avidin-biotin complex (ABC) methods (Fig. 1). Sections were incubated with the following antibodies: monoclonal anti-IL-13 (Peprotech, St. James Square, London, U.K.); monoclonal anti-IL-10 (Santa Cruz Biotechnology Ltd., California, U.S.A.); monoclonal anti-IL-6 (Santa Cruz Biotechnology Ltd., California, U.S.A.). An irrelevant murine antibody of the same isotype was included as a negative control. Positive staining was developed using 3, 3′ diaminobenzidine (DAB) chromogen (Sigma) and counterstained in haematoxylin (Sigma). Cellular infiltration and cytokine staining were measured on scales described in The Table legend and previously ([8]). All cytokine staining was evaluated independently by two observers. Differences between all four groups were determined statistically using the Student's *t* test.

RESULTS

Cellular infiltration. Cellular infiltrates were assessed at all time points in all patients (The Table). All patients had well defined granulomas in the initial biopsy. The
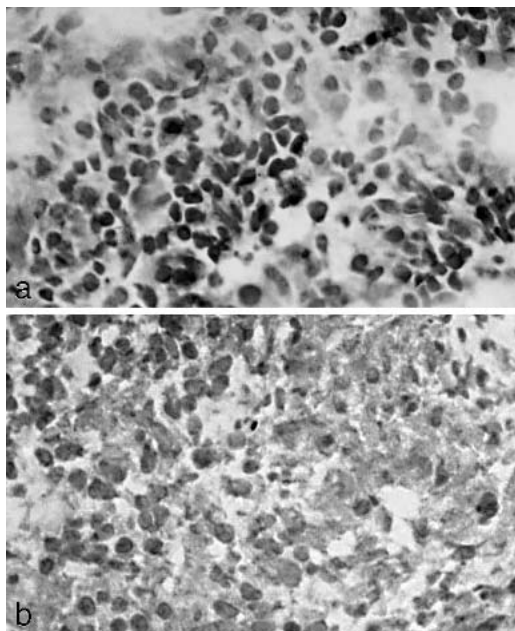
Fig. 1.   Representative photomicrographs of tissue sections taken from lesions of a T1R patients at time point week 0. Sections were stained with immunoperoxidase for IL-13 (top) and IL-10 (bottom). Strong positive staining was seen in both patients (nos. 207 and 260 respectively). 40× objective.

cellularity and size of these granulomas decreased with treatment (Fig. 2). This decrease was significant between day 0 and week 4 (p <0.001) and between week 1 and week 4 (p <0.005) of treatment. No significant difference in cellularity was found within the first week of treatment. No significant difference was found when comparing the borderline lepromatous (BL) and borderline tuberculoid (BT) groups (results not shown).

Cytokine expression. At the first time point all patient biopsies had strong positive staining for IL-6 and IL-10. IL-13 expression was observed in 11 out of 15 patients at the first time point (The Table). Analysis of all the biopsies revealed that the mean expression of IL-6, IL-10, and IL-13 decreased, but only significantly after 4 weeks of treatment (Fig. 3). The mean level of expression of IL-6 decreased significantly between day 0 and weeks 4 (p <0.005) and also between week 1 and week 4 (p <0.002) (Fig. 3). Similarly, the level of expression of IL-10 decreased between day 0 and week 4 (p <0.001), and between week 1 and 4 (p

<0.0001) but no alteration in IL-10 levels was observed after one week of treatment. The percentage of cells that stained positive for IL-13 was lower than that of IL-6 or IL-10 (Fig. 3). In accord with the IL-10 and IL-6 results, the only significant drop in IL-13 cytokine expression was observed between week 1 and week 4 (p <0.05) and between day 0 and week 4 (p <0.005) but not in the first week of treatment or between week 4 and week 26. Two patients (179 and 141) had a high level of expression of IL-10 and IL-6 over the full 6 months of treatment. Taking each time point there was no significant difference between the BT and BL patients for any of the cytokines (results not shown).

Clinical improvement. The clinical improvement of the patients was evaluated by the skin sign grading (The Table). When analyzing all patients, significant skin sign improvement was observed after 4 weeks of treatment and between week 1 week and week 4 (p <0.002). Clinical improvement after 4 weeks of prednisolone treatment corresponds to decreased cellularity and the reduced expression of IL-6, IL-13, and IL-10 observed in the skin biopsies. Nine out of the 15 patients showed significant clinical improvement within the first week of treatment. In the lesions of these patients, no associated difference was observed in the *in vivo* expression of any of the IL-6, IL-10, or IL-13 (Fig. 4a). Six of the 15 patients showed no skin sign improvement in the first week of treatment. These patients showed no associated differential in the observed expression of IL-6, IL-10, or IL-13 (Fig. 4b).

DISCUSSION

Type 1 reactions (T1R) are acute inflammatory events usually occurring in borderline leprosy patients with the immunologically unstable form of leprosy. The expression of inflammatory cytokines, in particular TNF-α, is thought to be responsible for the pathological nerve damage associated with T1R ([6, 8, 17]). To study the role of other cytokines within the skin lesion granulomas of T1R patients, levels of IL-10, IL-13, and IL-6 have been evaluated by immunohistochemistry.

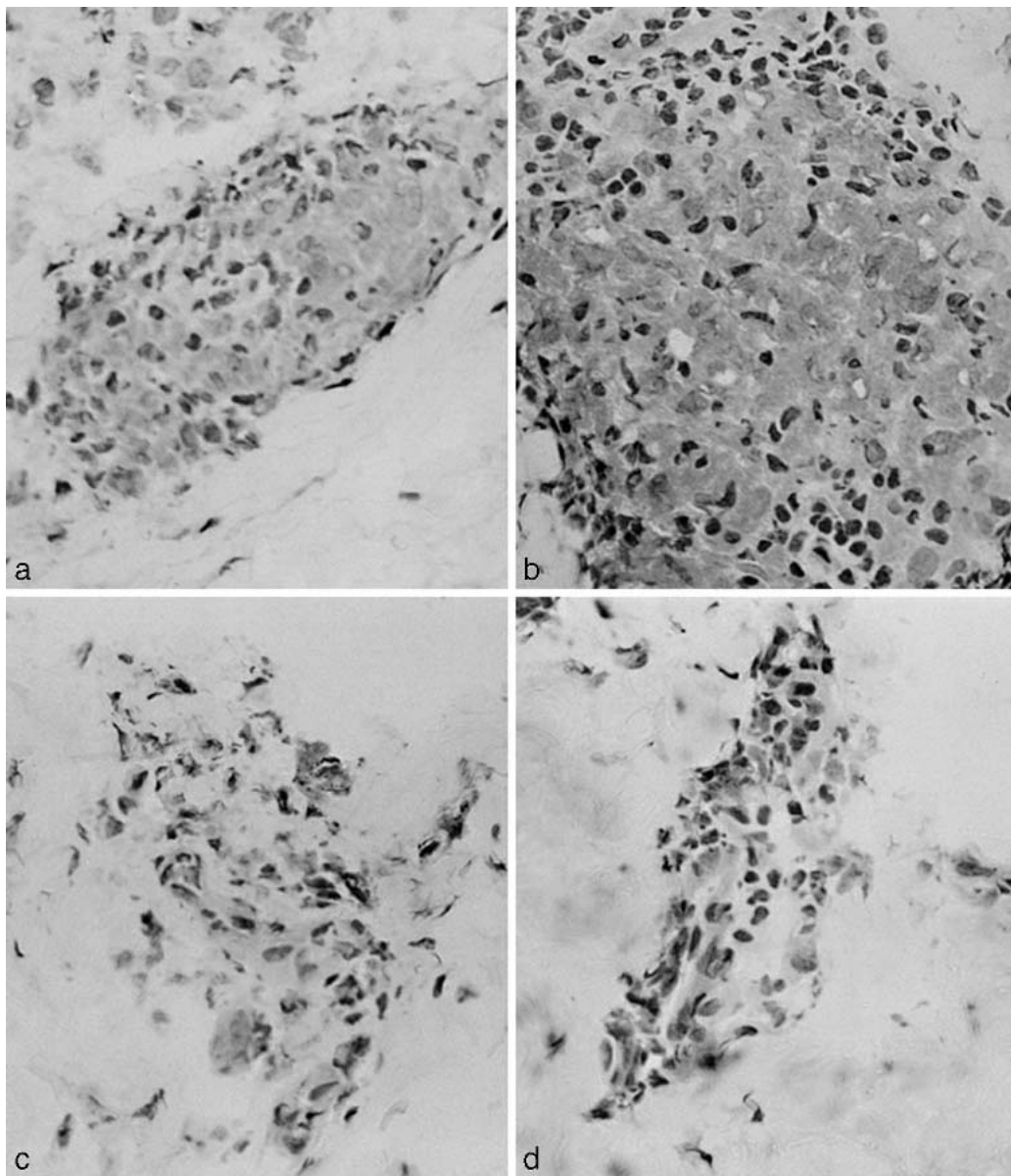Although previous studies have provided evidence of anti-inflammatory cytokines at

FIG. 2.   Representative photomicrographs of tissue sections taken from lesions of a T1R patient (no. 260). Sections were stained with immunoperoxidase for IL-6. Strong positive staining was seen at week 0 Fig. 2a (top-left) and week 1 Fig. 2b (top-right) with less positive staining in weeks 4 Fig. 2c (bottom-left) and 26 Fig. 2d (bottom-right). 40× objective.

the transcriptional level ([12]), this study confirms the presence of IL-10 and IL-13 protein in the granulomas of the skin lesions of patients with T1R. The presence of IL-6 in these lesions has also been confirmed. The presence of both anti- and pro-inflammatory cytokines in these lesions highlights the multiplicity of cytokine expression within the granulomas of these patients and suggests that a simple model of Th1 activation is insufficient to explain reactional pathology. These results also highlight the true complexity of regulatory pathways within the granuloma. Other inflammatory diseases are also characterised by the expression of both pro- and anti-inflammatory cytokines ([16]).

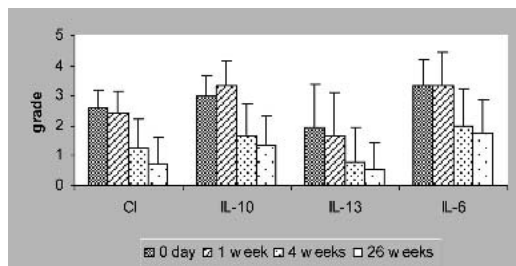Pro-inflammatory cytokines have a num-

FIG. 3.   Graph to show the mean levels of cellular infiltration (CI), IL-10, IL-13, and IL-6 during the course of prednisolone treatment in all patients recruited into the study (N = 15).
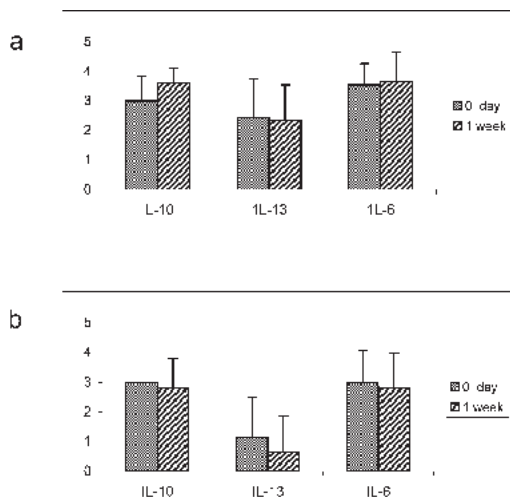

FIG. 4.   Graphs to represent the mean score levels of IL-10, IL-13, and IL-6 expression in the lesions during the first week of treatment in patients with (a) skin sign improvement (N = 9) and (b) no skin sign improvement (N = 6).

ber of effects within the granuloma (i) promoting cellular protective responses, (ii) maintaining granuloma formation ([2]), and (iii) initiating nerve damage ([6]). In contrast, the role of anti-inflammatory cytokines within the granuloma is not well understood. It can be postulated that the anti-inflammatory cytokines play a crucial role in controlling the critical balance between the protective and tissue damaging effects of the pro-inflammatory cytokines. Within the granuloma the control of the network of cytokines appears to be self-regulating. The expression of these anti-inflammatory cytokines within the lesions of patients with T1R highlights the high degree of cellular activity and cytokine expression occurring within the granuloma.

Current treatment for T1R is designed to inhibit the production of pro-inflammatory mediators. Prednisolone is an effective inhibitor of pro-inflammatory cytokines in leprosy reactions and other inflammatory diseases. The effect of prednisolone on anti-inflammatory mediators is not so well understood although it has been demonstrated that prednisolone promotes anti-inflammatory cytokines in asthma patients ([4]). This study provides evidence that prednisolone down regulates the expression of IL-10, IL-6, and IL-13 within the lesions of patients with T1R. This down regulation could be could be due to the decrease in cellular activity during pro-inflammatory down regulation within the granuloma, or via the direct effect of prednisolone on transcription factors such as NF-κB ([1]). The development of an effective treatment should

focus on directing the control and balance of these cytokines.

To detect a correlation between clinical presentation and cytokine expression, the skin sign improvement scale was analyzed against the level of the cytokine expression. Within the first week of treatment, improvement in the clinical skin sign was observed in nine of the fifteen patients. Surprisingly, an associated change in the production of any of the cytokines within the skin lesion granulomas of these patients was not observed until week 4 of treatment. Similarly, deterioration in the clinical skin signs within the first week is not associated with any of the cytokines measured. This suggests that skin sign clinical improvement is not directly mediated by the expression of these cytokines within the granulomas of the skin lesions but that skin sign clinical improvement is mediated by events other than those related to cytokine expression. The skin sign improvement may be associated with other known effects of prednisolone, such as the effect on prostaglandin levels ([4]). Previous work ([8]) has shown that IL-12, IFN-γ, and iNOS protein within the lesions of the same patients reduced in expression in a similar manner as

the cytokines measured in this study. The fact that the expression of all of these cytokines within the granuloma is not seen until 4 weeks after the start of treatment may be due to both the complexity of the cellular organisation and the cytokine pathways network within the granuloma.

Although the level of cytokine activity within the granulomas declines within 4 weeks of therapy, this activity is not completely abrogated by 6 months. IL-12, IFN-γ, and iNOS were also observed in the lesions of some these patients after 6 months of treatment ([8]). This suggests that cytokine activity within the granuloma may be difficult to switch off and/or the granuloma may be semi-pervious to prednisolone or that the circulating levels of prednisolone are not high enough to affect granuloma activity.

These findings have implications for the treatment of reactional patients. If the pro-inflammatory cytokines are involved in the peripheral neuropathy, the need for a fast acting anti-inflammatory treatment is required. Although the up-regulation of pro-inflammatory cytokines is directly involved in reactional lesions, this study provides evidence that the anti-inflammatory cytokines may also play a role in the immunopathology of T1R. In particular, further studies should focus on the use of inhibitors controlling the overall balance of pro- and anti-inflammatory cytokines rather than on specific cytokine inhibitors.

It should be noted that twelve of the patients were receiving MDT which includes the mild anti-inflammatory drug clofazamine. The effect of this drug can not be determined within this sample size.

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