Enhanced Lesional Foxp3 Expression and Peripheral Anergic Lymphocytes Indicate a Role for Regulatory T Cells in Indian Post-Kala-Azar Dermal Leishmaniasis

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Indian post-kala-azar dermal leishmaniasis (PKDL) is a low-frequency (5–10%) dermal sequela of visceral leishmaniasis (VL) caused by *Leishmania donovani*; importantly, affected individuals are speculated to be parasite reservoirs. Insight into its immunopathogenesis could translate into rational immunomodulatory therapeutic approaches against leishmaniases. In patients with PKDL ($n=21$), peripheral lymphocytes were analyzed for surface markers, intracellular cytokines, and lymphoproliferative responses using flow cytometry. In lesional tissue biopsies ($n=12$), expression of counter-regulatory cytokines (IFN-γ and IL-10) and the T-regulatory transcription factor forkhead box protein 3 (Foxp3) was analyzed using reverse transcriptase-PCR, along with immunohistochemical detection ($n=8$) of CD3 and Foxp3 positivity. In patients with PKDL, circulating $CD8^+CD28^-$ and antigen-induced $IL\text{-}10^+CD3^+$ lymphocytes were increased and receded with treatment. $CD8^+$ lymphocytes showed impaired proliferative responses to *L. donovani* antigen (LDA) and phytohemagglutinin, which were reinstated after treatment. At presentation, the upregulated lesional IFN-γ and IL-10 messenger RNA (mRNA), Foxp3 mRNA, and protein were curtailed after treatment. In Indian patients with PKDL, increased frequency of the $CD8^+CD28^-$ phenotype, enhanced antigen-specific IL-10 production, and accompanying anergy of circulating lymphocytes suggest their regulatory nature. Furthermore, the concomitantly elevated lesional expression of Foxp3 suggests their possible recruitment into the lesional site, which would sustain disease pathology.

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INTRODUCTION

Post-kala-azar dermal leishmaniasis (PKDL), first described by Brahmachari (1922), presents as a dermal rash after cure of visceral leishmaniasis (VL) and is caused by the protozoan parasite *Leishmania donovani* (Zijlstra *et al.*, 2000; Ramesh *et al.*, 2007). PKDL is primarily reported from two VL-endemic regions, the Indian subcontinent and Sudan and its adjoining areas. In the Indian variant, only 5–10% of patients with VL acquire PKDL, as opposed to 50–60% in Sudan (Zijlstra *et al.*, 2003; Ramesh *et al.*, 2007); importantly, with zoonotic hosts yet to be defined, patients with PKDL are the proposed parasite reservoirs (Zijlstra *et al.*, 2003).

In the Indian subcontinent, immunological factors predisposing patients with VL to PKDL remain poorly characterized, with the disease pathology often being attributed to parasite-specific cell-mediated immune (CMI) responses (Ramesh *et al.*, 2007). Sudanese patients with PKDL respond to *Leishmania* antigen (Ismail *et al.*, 1999), whereas studies on immune responses in Indian PKDL are not so clearly defined and even contradictory (Haldar *et al.*, 1983; Neogy *et al.*, 1988). Our previous study on intracellular cytokine expression within circulating lymphocytes of patients with PKDL indicated that generalized CMI was restored but antigen-specific CMI was restricted to $CD3^+CD8^+$ lymphocytes producing IL-10 (Ganguly *et al.*, 2008). In this study, we describe downstream CMI responses in patients with PKDL

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Abbreviations: Foxp3, forkhead box protein 3; LDA, Leishmania donovani *antigen; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; PerCP, peridinin chlorophyll protein; PKDL, post-kala-azar dermal leishmaniasis; VL, visceral leishmaniasis*

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vis-à-vis cured VL patients and healthy controls by measuring mitogen-induced and antigen-specific proliferation in circulating $CD4^+$ and $CD8^+$ lymphocytes.

Lesions in PKDL typically present as hypopigmented macules, nodules, and papules. In Sudanese PKDL, the incidence of papular or nodular rash is maximal followed by maculopapular, micropapular, and macular lesions (Zijlstra *et al.*, 2000), whereas Indian patients report erythema and induration on the face or numerous, hypopigmented macules (Ramesh and Mukherjee, 1995; Zijlstra *et al.*, 2003). Regardless of the differences, the nodular, macular, and maculopapular lesions predominate and are construed as hallmarks of PKDL (Zijlstra *et al.*, 2003).

The sustained lesional expression of counter-regulatory cytokines in patients with PKDL (Ansari *et al.*, 2006) suggests a role for regulatory T cells (T_{Regs}) who by maintaining immune homeostasis can regulate immunity to infection (Sakaguchi, 2004). T_{Regs} comprise 5–10% of the circulating $CD4^+$ T-cell population and uniquely express the transcription factor, Forkhead Box P3 (Foxp3), which is indispensable for their development and function (Shevach *et al.*, 2006). In acute infections, T_{Regs} benefit the host by thwarting immune-mediated pathology after pathogen eradication, whereas in chronic infections their activity is detrimental to the host, as they promote parasite survival amidst an active immune response (Belkaid, 2007). Therefore, in PKDL, the chronic nature of the disease warrants an analysis into the possible role of T_{Regs}.

RESULTS

Study population

Patients with PKDL ($n=21$) were classified as polymorphic, macular, or papulonodular. All patients tested positive with the rK39 test and ELISA for anti-leishmanial antibodies; only two patients (one macular and one papulonodular) tested negative for *L. donovani* bodies in skin smears. The majority presented with polymorphic ($n=18$, 85%) followed by papulonodular ($n=2$, 10%) and macular lesions ($n=1$, 5%) (Table 1); five patients (24%) gave no previous history of VL attributable to poor documentation, inability to recall past incidence of VL, or the subclinical nature of VL. However, they were included as they had demonstrable *L. donovani* bodies and tested positive by rK39 and ELISA. In the remaining 16 patients, the time interval between cure of VL and onset of PKDL ranged from 1 to 49 years (Table 1). The time interval often extends to decades (Ramesh and Mukherjee, 1995); only one patient reported an interval of 49 years, four had an interval within 17–19 years, whereas the majority ($n=11$) reported an interval from 1 to 3 years. However, no correlation was evident between time interval and lesional profiles.

Table 1. Clinical features of study population

	PKDL ($n=21$)
Age (years)	30.43 ± 14.28
(Median; range)	(30; 12–65)
Sex ratio (M:F)	18:3
Lesional type	Polymorphic: 18
	Macular: 1
	Papulonodular: 2
History of VL	76% (16/21)
Interval between cure of VL and onset (year) (median; range)	9.57 ± 13.21 (2; 1–49)

Abbreviations: F, female; M, male; PKDL, post-kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.
Values are mean ± standard deviation. Median and range values are in parentheses.

Circulating $CD8^+CD28^-$ lymphocytes elevated in patients with PKDL

In patients with PKDL, proportions of circulating $CD4^+CD25^+$ lymphocytes, representing natural T regulatory cells, were comparable with healthy controls (Table 2). However, $CD8^+CD28^-$ regulatory lymphocytes were significantly raised in patients with PKDL when compared with healthy controls; treatment effected a significant decrease, values being comparable with healthy controls (Table 2). With regard to the costimulatory molecule CD28, both $CD4^+$ and $CD8^+$ lymphocytes in patients with PKDL showed significant downregulation at presentation when

Table 2. Surface marker and intracellular cytokine profiles of patients with PKDL

Surface marker/ intracellular cytokine	PKDL		Healthy controls
	Pretreatment	Post treatment	
Percentage of gated lymphocytes			
$CD4^+CD25^+$[1]	3.72 ± 2.38	3.41 ± 1.74	2.72 ± 1.40
$CD4^+CD28^+$[1]	22.18 ± 3.86*	33.03 ± 7.18$^{@@}$	30.58 ± 6.91
$CD8^+CD28^+$[1]	11.63 ± 3.64**	16.67 ± 3.70	27.88 ± 3.55
$CD8^+CD28^-$[1]	25.64 ± 4.64***	17.95 ± 8.19$^{@}$	12.05 ± 5.23
$CD3^+$IFN-γ^+ (LAC stimulated)[2]	13.86 ± 2.38	13.11 ± 3.26	ND
$CD3^+$IFN-γ^+ (LDA stimulated)[2]	0.45 ± 0.21	0.14 ± 0.04	ND
$CD3^+$IL-10^+ (LAC stimulated)[2]	15.26 ± 5.21	11.65 ± 3.71	ND
$CD3^+$IL-10^+ (LDA stimulated)[2]	17.63 ± 4.34	6.29 ± 1.49$^{@}$	ND

Abbreviations: LAC, leukocyte activation cocktail; LDA, *Leishmania donovani* antigen; ND, not determined; PBMC, peripheral blood mononuclear cell; PKDL, post-kala-azar dermal leishmaniasis.
[1]Peripheral blood from patients with PKDL (n=21) and healthy controls (n=6) was stained with surface marker antibodies.
[2]PBMCs were fixed, permeabilized, and stained with cytokine antibodies. Cells were acquired using flow cytometry as described in Materials and Methods, with lymphocytes gated by forward and side scatter.
Values are mean ± standard deviation. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ when compared with controls; $^{@}P<0.05$ and $^{@@}P<0.01$ when compared with pretreatment.

compared with healthy controls (Table 2). After treatment, restoration of CD28 expression was restricted to the $CD4^+$ subset (Table 3).

Peripheral lymphocytes from patients with PKDL were unresponsive to *L. donovani* antigen (LDA)

Specific and generalized CMI were analyzed in terms of lymphoproliferative responses to LDA and phytohemagglutinin (PHA), respectively. Proliferating cells showed an expected dilution in carboxyfluorescein diacetate succinimidyl ester fluorescence, whereas unstimulated cells retained maximum fluorescence, indicating absence of proliferation. With regard to LDA-induced proliferation, patients with PKDL showed a poor proliferation index (PI) in both $CD4^+$ (1.30 ± 0.15) and $CD8^+$ lymphocytes (1.18 ± 0.03). After treatment, $CD4^+$ lymphocytes showed significantly greater proliferation than healthy controls (1.95 ± 0.43 vs 1.02 ± 0.01, $P<0.05$), comparable with cured VL patients (2.13 ± 0.64). In the $CD8^+$ subset, the post-treatment PI were more pronounced relative to healthy controls (3.27 ± 1.06 vs 1.01 ± 0.01, $P<0.01$) and cured VL patients (1.47 ± 0.41, Figure 1a and c).

In response to PHA, $CD4^+$ lymphocytes from healthy individuals proliferated strongly, with the PI being 6.70 ± 0.23 (Figure 1b and d). Similarly, patients with PKDL showed comparable PI before (3.60 ± 0.32) and after treatment (4.26 ± 0.94), whereas patients with VL who had been cured showed restoration of their characteristically impaired PI (2.85 ± 0.52). In patients with PKDL, the $CD8^+$ subset showed impaired proliferation when compared with controls (4.35 ± 0.65 vs 9.66 ± 0.63, $P<0.05$), which increased to 7.99 ± 3.17 with treatment, whereas in cured VL patients the PI remained lower than controls (4.48 ± 1.57, $P=0.09$; Figure 1b and d).

Antigen-specific IL-10 production by proliferating $CD3^+$ lymphocytes

Generalized and antigen-specific intracellular cytokines in $CD3^+$ lymphocytes of patients with PKDL were studied. No differences were observed between pre- and post-treatment proportions of IFN-γ and IL-10 expressing $CD3^+$ cells after leukocyte activation cocktail stimulation (Table 2). At presentation, stimulation with LDA in the presence of Brefeldin A caused minimal production of IFN-γ, whereas

Table 3. Immunohistochemistry scores and lesional profiles of patients with PKDL before and after treatment

Patient no.	Clinical features at presentation	Foxp3 score[1]		CD3 score[1]	
		Before	After	Before	After
1	Papulonodular lesions emerged chronologically on the face, trunk, and lower extremities and hypopigmented macules on the trunk and extremities over a period of 7 years.	+++ (11.72 ± 2.21)	± (0.30 ± 0.23)	+++++ (52.58 ± 1.25)	++++ (41.8 ± 1.35)
2	Erythematous papules and nodules on the face for 1 year; papules and macules on extremities for 2–3 months.	++ (5.24 ± 1.99)	+ (1.12 ± 0.64)	+++++ (56.4 ± 0.84)	++++ (46.91 ± 0.26)
3	Papulonodules appeared chronologically on the feet, face, and upper arms and hypopigmented macules on the trunk and back over a 7-year period.	+ (4.19 ± 0.79)	+++ (10.32 ± 2.32)	+++++ (53.09 ± 0.73)	+++++ (52.58 ± 1.09)
4	Nodular lesions with papules and few disseminated macules over face, extremities, and upper back for the past 10 years.	+ (1.86 ± 0.24)	0	++++ (49.72 ± 1.13)	++++ (46.51 ± 1.39)
5	Erythematous plaques and nodules on face, trunk, extremities, and genitalia for the past 2 and a half years; hypopigmented macules on trunk and back without sensory abnormality for the past 2 years.	++ (9.55 ± 1.07)	± (0.42 ± 0.43)	+++++ (51.04 ± 3.47)	+++++ (53.23 ± 1.86)
6	Hypopigmented macules dispersed all over the body, prominently on the face, trunk, and extremities for the past year; micropapules and a few nodules on the facial region.	+ (1.10 ± 0.55)	0	++++ (49.19 ± 1.58)	++++ (45.82 ± 1.71)
7	Nodules and papules emerged on the nose, chin, and pinnae 3 years ago.	+ (2.21 ± 0.65)	0	++++ (41.64 ± 0.89)	++++ (26.88 ± 0.84)
8	Warty nodules and hypopigmented macular lesions on the back of the neck, trunk, and upper extremities for the past 2 years.	++ (6.15 ± 0.83)	0	+++++ (51.05 ± 0.5)	+++++ (50.32 ± 0.69)

Abbreviations: Foxp3, forkhead box protein 3; PKDL, post-kala-azar dermal leishmaniasis.
[1]Paraffin-embedded sections of lesional tissue from patients with PKDL at presentation and after treatment were stained for Foxp3 and CD3 using immunohistochemistry and scored, as described in Materials and Methods. A positivity score for Foxp3/CD3 was allocated on the basis of the following scoring index: 0, negative; ±, <1% positive cells; +, 1–5% positive cells; ++, 5–10% positive cells; +++, 10–20% positive cells; ++++, 20–50% positive cells; and +++++, >50% positive cells.

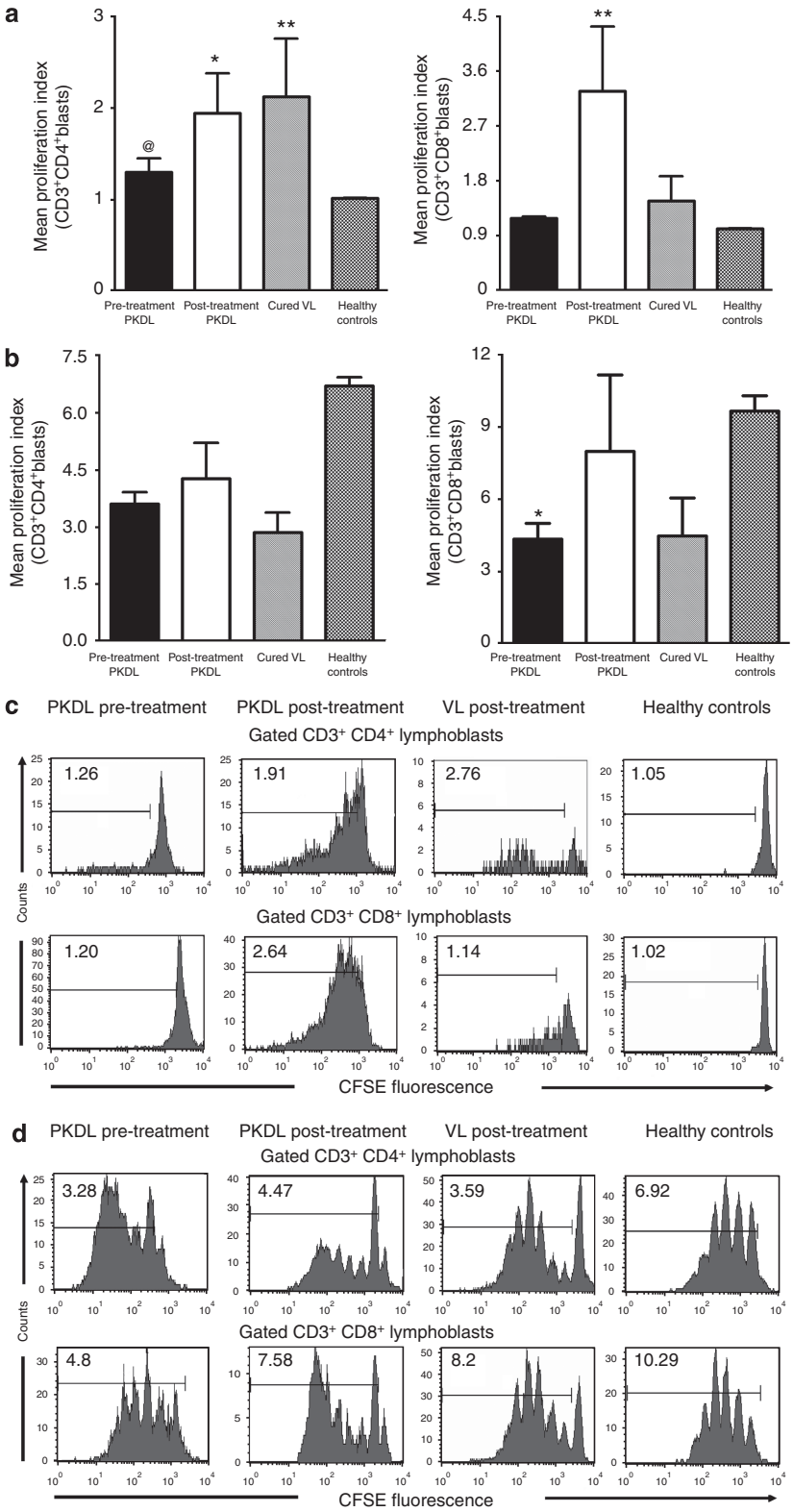

Figure 1. Specific and generalized proliferative responses of lymphocytes. Lymphocytes from patients with post-kala-azar dermal leishmaniasis (PKDL) before (■, $n=21$) and after (□, $n=12$) treatment, from patients with visceral leishmaniasis (VL) after cure (▨, $n=5$), and from healthy controls (▩, $n=6$) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE), incubated with/without *Leishmania donovani* antigen (LDA; 5 μg ml^{-1}, **a**) or phytohemagglutinin (PHA; 10 μg ml^{-1}, **b**), and CFSE fluorescence of gated CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ lymphoblasts was determined using flow cytometry. Columns represent mean proliferation indices of at least five individuals per group, with error bars denoting SEM. *$P<0.05$, **$P<0.01$, significantly different from controls; $^@P<0.05$, significantly different from VL after treatment. (**c**) Representative profile of proliferative responses of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ lymphocytes to LDA in patients with PKDL before and after treatment, cured VL patients, and healthy individuals. (**d**) Representative profile of proliferative responses of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ lymphocytes to PHA in patients with PKDL before and after treatment, cured VL patients, and healthy individuals.

IL-10-producing $CD3^+$ cells were elevated, which were significantly reduced after treatment (Table 2).

Upregulated lesional expression of IFN-γ and IL-10 decreased with treatment

To elucidate counter-regulatory cytokine profiles in lesional tissue from patients with PKDL, messenger (mRNA) expression of IFN-γ and IL-10 was quantitated in paired samples. At presentation ($n=12$), a significant upregulation in IFN-γ expression was observed when compared with healthy controls (1.21 ± 0.08 vs 0.21 ± 0.02, $P<0.001$); similarly, IL-10 transcripts were significantly increased (0.44 ± 0.07 vs 0.005 ± 0.002, $P<0.001$, Figure 2a and c). After treatment, a significant reduction in both IFN-γ (0.50 ± 0.11, $P<0.001$) and IL-10 (0.04 ± 0.01, $P<0.001$) was evident (Figure 2a and b).

Elevated lesional Foxp3 mRNA expression in patients with PKDL

To determine whether increased expression of IL-10 in lesional tissue was associated with an enhanced presence of T_{Reg} cells, lesional mRNA expression of the T_{Reg} transcription factor, Foxp3, was quantitated. Patients with PKDL, when compared with healthy controls, had significantly upregulated Foxp3 transcripts (0.19 ± 0.05 vs 0.02 ± 0.01, $P<0.01$; Figure 3a and c); treatment effected a modest reduction in Foxp3 mRNA levels (0.14 ± 0.05) (Figure 3a–c). On an individual basis, decreased expression was evident in 9 out of 12 patients, the percentage reduction ranging from 6.13 to 86.49%; one patient retained the same level of expression, whereas two showed higher mRNA levels (Figure 3b).

Increased lesional accumulation of T regulatory cells in patients with PKDL regressed with treatment

To confirm whether upregulated expression of Foxp3 translated into increased protein, immunohistochemistry was performed on lesional tissue from patients with PKDL ($n=8$), healthy controls ($n=3$), and one patient with erythema nodosum leprosum; in addition, sections were stained for CD3. In PKDL lesions, microscopic analysis showed an accumulation of $Foxp3^+$ cells within a dense and predominantly $CD3^+$ lymphocytic infiltrate (Figure 4a and b), whereas healthy skin showed virtually no $Foxp3^+$

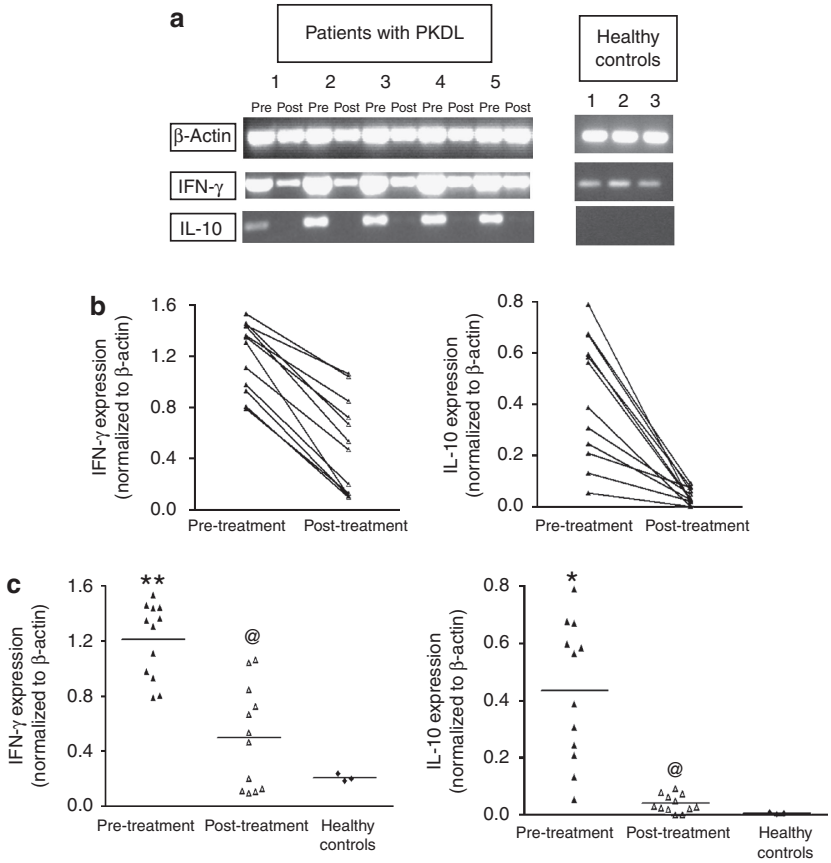

Figure 2. Longitudinal monitoring of lesional expression of IFN-γ and IL-10 in patients with post-kala-azar dermal leishmaniasis (PKDL) before and after treatment. (**a**) Representative profile of mRNA expression of IFN-γ and IL-10 in lesional tissue from patients with PKDL ($n=5$) before and after treatment and normal skin tissue from healthy individuals ($n=3$). Isolated RNA was subjected to reverse transcriptase-PCR (RT-PCR); products were resolved using agarose gel electrophoresis with bands specific for β-actin, IFN-γ, and IL-10, visualized using UV transillumination, and quantified using densitometric analysis, as described in Materials and Methods. (**b**) Before–after plots of IFN- γ and IL-10 expression in patients with PKDL ($n=12$) before (▲) and after completion of treatment (△). (**c**) Scatter plots of expression values for IFN-γ and IL-10 in patients with PKDL ($n=12$) before (▲) and after treatment (△) as well as in healthy controls (◆, $n=3$); horizontal lines indicate mean values. *$P<0.01$, **$P<0.001$, significantly different from controls; @$P<0.001$, significantly different from pre-treatment.

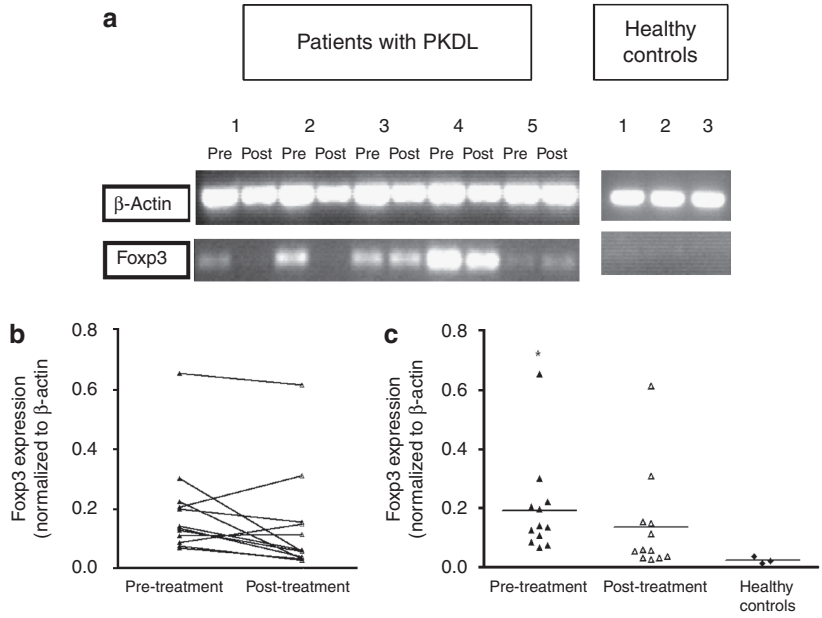

Figure 3. Lesional forkhead box protein 3 (Foxp3) expression in patients with post-kala-azar dermal leishmaniasis (PKDL) at presentation and after treatment. (**a**) A representative profile of Foxp3 mRNA expression in lesional tissue from patients with PKDL ($n=5$) before and after treatment and normal skin tissue from three healthy individuals. Expression was quantified using reverse transcriptase-PCR (RT-PCR) and subsequent densitometric analysis, as described in Materials and Methods. (**b**) Before–after plot of Foxp3 expression in patients with PKDL ($n=12$) before (▲) and after completion of treatment (△). (**c**) Scatter plot showing Foxp3 expression values in patients with PKDL ($n=12$) before (▲) and after treatment (△) and in healthy controls (◆, $n=3$). Horizontal lines indicate mean values. *$P<0.01$, significantly different from controls.

cells, scant $CD3^+$ lymphocytes, and minimal cellular infiltrate (Supplementary Figure S1 online). In the patient with erythema nodosum leprosum, $Foxp3^+$ cells were barely detectable amidst a $CD3^+$ lymphocyte-rich infiltrate (Supplementary Figure S1 online).

At presentation, one patient had a positivity of 10–20% (Figure 4a), three had a score of 5–10% and four out of eight patients had $Foxp3^+$ scores of 1–5% (Figure 4b). After treatment, all patients except one showed a reduction in $Foxp3^+$ positivity to either <1% or undetectable levels (Figure 4a and b and Table 3). At presentation, CD3 positivity ranged from 20 to 50% ($n=3$) and to >50% ($n=5$, Figure 4a and b). After treatment, all except two patients showed no differences in their proportions of $CD3^+$ lymphocytes (Figure 4a and b and Table 3).

DISCUSSION

In PKDL, enhanced secretion of IL-10 and transforming growth factor-β by antigen-stimulated peripheral blood mononuclear cells (PBMCs) has been associated with disease severity (Saha *et al.*, 2007). Subsequently, we proposed IL-10-producing antigen-specific circulating $CD8^+$ lymphocytes together with increased serum IL-10, IgG1, and IgG3 as markers of Indian PKDL (Ganguly *et al.*, 2008). After antigen exposure, IL-10 not only influences induction of peripheral type 1 T_{Reg} cells (Groux *et al.*, 1997; Vieira *et al.*, 2004), but it also serves as the flag bearer of their suppressive activity (Workman *et al.*, 2009). As antigen-induced T_{Regs} exclusively produce IL-10 (O'Garra and Vieira, 2004), the involvement of T_{Regs} in PKDL has previously been proposed (Ganguly *et al.*, 2008). This study was undertaken to show the presence of both circulatory and lesional T_{Regs} in a substantial number of patients with PKDL (Table 1).

In Sudanese PKDL, antigen-specific CMI responses have been documented (Ismail *et al.*, 1999; Gasim *et al.*, 2000), whereas in India, reports are varied. Haldar *et al.* (1983) reported specific CMI in five out of six patients with newly acquired PKDL *vis-à-vis* three out of six patients with chronic PKDL, whereas Neogy *et al.* (1988) recorded an absence of *Leishmania*-specific CMI. In this study, patients with PKDL showed no antigen-induced proliferation of $CD4^+$ lymphocytes that was marginally restored after treatment (Figure 1a and c). However, in cured VL patients, a higher degree of proliferation was observed, attributable to greater proportions of antigen-specific memory and residual effector lymphocytes (Figure 1a and c). With respect to antigen-primed $CD8^+$ lymphocytes, patients with PKDL again showed an impairment that was also restored after treatment, attributable to the elevated proportion of $CD8^+CD28^-$ regulatory lymphocytes (Table 2).

However, no impairment of generalized CMI has been reported in Indian PKDL (Haldar *et al.*, 1983; Neogy *et al.*, 1988), which is corroborated by robust intracellular IFN-γ and IL-2 production (Ganguly *et al.*, 2008). In previous studies, proliferative responses were examined in bulk lymphocyte populations (Haldar *et al.*, 1983; Neogy *et al.*, 1988), thereby overlooking differences in effector lymphocyte subsets. Indeed, whereas Neogy *et al.* (1988) showed an overall PHA-induced proliferation in Indian patients with PKDL, this study, using carboxyfluorescein diacetate

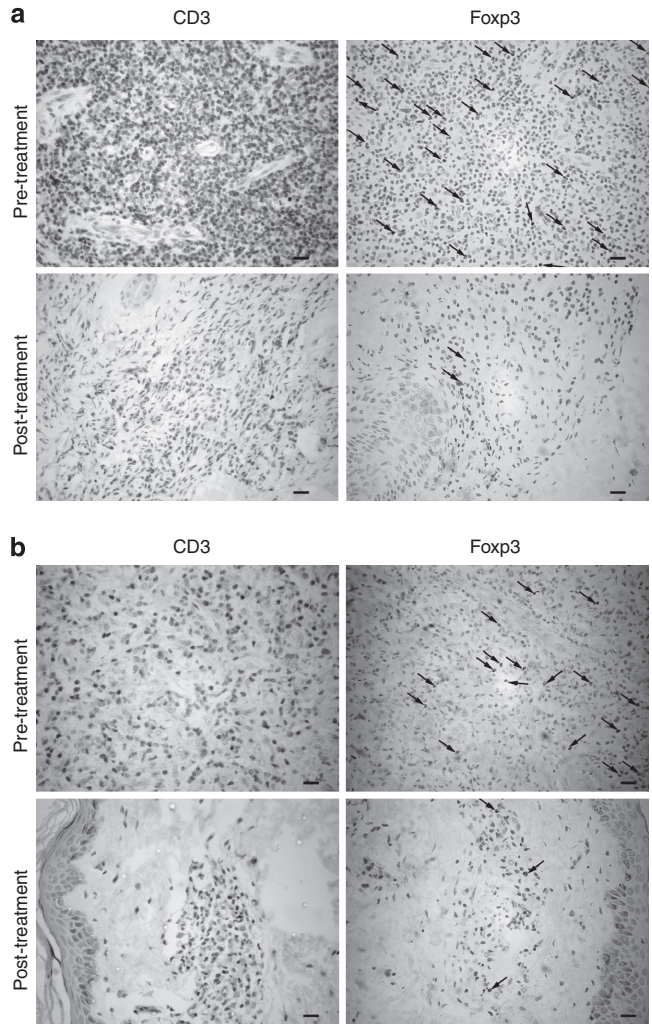

Figure 4. Immunohistochemistry of lesional sections from patients with post-kala-azar dermal leishmaniasis (PKDL). (**a**) A representative profile of CD3 and forkhead box protein 3 (Foxp3; 10–20% positivity) staining in lesional tissue sections from a patient with PKDL before and after treatment. (**b**) A representative profile of CD3 and Foxp3 (1–5% positivity) staining in lesional tissue sections from a patient with PKDL before and after treatment. Arrows indicate Foxp3-positive cells within the lymphocytic infiltrate. Bars = 10 μM.

succinimidyl ester dilution, established differences between the PHA-specific proliferative capacity of $CD4^+$ and $CD8^+$ lymphocytes. Pre- and post-treatment $CD4^+$ responses were comparable with healthy controls and cured VL patients (Figure 1b and d); however, $CD8^+$ lymphocytes proliferated weakly *vis-à-vis* healthy controls, with the impairment being corrected after treatment (Figure 1b and d). As CD28 expression and signaling is instrumental in the CD28/B7 costimulatory pathway preventing induction of clonal anergy (Becker *et al.*, 1995), the elevated $CD8^+CD28^-$ subset at presentation in patients with PKDL with the attendant CD28 downregulation on $CD8^+$ lymphocytes, possibly accounts for the diminished responsiveness of the $CD8^+$ subset to PHA (Table 2).

In comparison with their $CD4^+CD25^+$ major histocompatibility complex class II-restricted counterparts, the class I-restricted $CD8^+$ T_{Regs} remain poorly characterized (Siegmund *et al.*, 2009). Induced or naturally occurring $CD8^+$ T_{Regs} include the $CD8^+CD28^-$ population that suppress T-cell activation, not by cell-cell contact, but by interfering with antigen-presenting cell functions (Liu *et al.*, 1998). Interestingly, the T_{Reg} marker, Foxp3, although not detectable in peripheral $CD8^+$ cells, can be induced by *in vitro* stimulation with anti-CD3 and anti-CD28 or transforming growth factor-β (Siegmund *et al.*, 2009). In mice infected with *L. major*, studies on DNA immunization have proposed an immunoregulatory role for $CD8^+$ lymphocytes (Gurunathan *et al.*, 2000); however, information on clinical leishmaniases remains limited.

At the intracellular level, carboxyfluorescein diacetate succinimidyl ester-stained $CD3^+$ lymphocytes were examined and not $CD8^+$ lymphocytes, owing to a technical limitation in a four-color assay. In patients with PKDL, after 6 days of antigenic stimulation, increased production of the potent regulatory cytokine, IL-10, was observed (Table 2). This finding, in conjunction with our previously reported increase in intracellular IL-10 within the $CD8^+$ subset from patients with PKDL (Ganguly *et al.*, 2008), and impaired $CD8^+$ proliferative responses (Figure 1a and c), supports a regulatory role for peripheral $CD8^+$ lymphocytes in Indian PKDL.

As knowledge of the interplay of cytokines within the lesional site is critical to understanding the immunopathology of PKDL, gene expression studies were undertaken on paired lesional biopsies from patients with PKDL. Our study corroborated reports of high lesional expression of both IFN-γ and IL-10 in Indian PKDL (Ansari *et al.*, 2006); on an individual basis, all 12 patients showed a significant reduction in mRNA levels of both cytokines after treatment (Figure 2), pointing to their involvement in disease pathogenesis, with IFN-γ possibly being directed against skin-resident parasites, whereas IL-10 curtailed excessive IFN-γ-driven immune responses. Gene expression studies cannot establish the cellular source(s) of altered cytokines, more so for a pleiotropic cytokine such as IL-10, which is secreted by a plethora of immune 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 in which its often-dense inflammatory cell infiltrate can theoretically host several immune cells. The question of which cellular subset accounts for the increased lesional IL-10 expression in PKDL has remained unanswered, thus stoking our interest in inducible T_{Regs}.

In chronic infections, T_{Regs} are primary sources of IL-10 (Belkaid, 2007). As no information exists on T_{Reg} involvement in PKDL, we examined the role (if any) of T_{Regs} in the localized immune milieu of PKDL. Expression of the T_{Reg}-exclusive transcription factor, Foxp3, studied both at the mRNA (Figure 3) and protein levels (Figure 4), yielded a finding that T_{Regs} feature prominently in the inflammatory infiltrate (Figures 3 and 4a and b and Table 3); to our knowledge, this finding has not been reported in PKDL.

This accumulation of Foxp3$^+$ T_{Regs} was not simply an accompaniment of infiltrating CD3$^+$ lymphocytes, as lesions of a patient with erythema nodosum leprosum, a granulomatous reversal reaction of leprosy, showed barely detectable Foxp3$^+$ cells in a CD3$^+$ lymphocyte-rich lesional milieu (Supplementary Figure S1 online). Treatment curtailed accumulation of T_{Regs} as evident by the reduced expression of Foxp3 (Figure 4a and b and Table 3) affirming their contribution to lesional pathology, which however was not evident in the marginal decrease in mean lesional Foxp3 mRNA levels, suggesting a deficiency in translational mechanisms. Although natural T_{Regs} have a negligible role in VL with IL-10 expression limited to splenic T cells other than CD4$^+$CD25$^+$Foxp3$^+$ T_{Regs} (Nylen *et al.*, 2007), lesional T_{Regs} downregulate effector T-cell responses in cutaneous leishmaniasis caused by *Leishmania braziliensis*, (Campanelli *et al.*, 2006). In addition, in localized cutaneous leishmaniasis caused by *L. guyanensis*, treatment refractoriness was associated with high lesional Foxp3 expression (Bourreau *et al.*, 2009a) and lesional T_{Regs} were shown to suppress IFN-γ production by antigen-stimulated peripheral CD4$^+$CD25$^-$ T lymphocytes (Bourreau *et al.*, 2009b), suggesting that accumulating T_{Regs} by dictating impairment of local immunity contribute to disease persistence in leishmaniases.

Collectively, we propose that in patients with PKDL, the circulating antigen-specific IL-10-producing CD8$^+$ lymphocytes (Ganguly *et al.*, 2008) are anergic and endowed with regulatory ability. These cells upon infiltration into the lesional site potentially sustain disease progression by producing IL-10 to counter IFN-γ-mediated responses against parasites. However, specialized immunohistology is necessary to pinpoint whether T_{Regs} are the cellular sources of IL-10 in PKDL, considering that Foxp3 is nuclear in location whereas IL-10 is cytoplasmic. Future investigations dealing with their functional profiles, specifically their interaction with target immune cells within the localized PKDL milieu, are necessary for the development of immunomodulatory therapies against PKDL and by extension, the leishmaniases.

MATERIALS AND METHODS

Study subjects

Patients diagnosed with PKDL ($n=21$) or treated for VL ($n=5$) were recruited from the School of Tropical Medicine, Kolkata and Medical College, Kolkata from 2006 to 2009. Diagnosis was based on clinical features, a past history of VL, rK39 positivity (Sundar *et al.*, 2006), ELISA using crude LDA (Chatterjee *et al.*, 1998), and presence of *L. donovani* bodies in skin smears. Patients with PKDL received either sodium stibogluconate (20 mg kg^{-1} day^{-1} intramuscular for 3 months) or miltefosine (100 mg day^{-1} p.o. for 2 months), whereas patients with VL had received amphotericin B (1 mg kg^{-1} day^{-1} intramuscular for 1 month). Remission of clinical features was a primary criterion of cure; samples were collected at presentation and 1 month after completion of treatment after obtaining informed consent from the patient or parent/guardian in case of minors. Non-endemic controls ($n=6$) were included. The study received previous approval from the institutional human ethical committee and was conducted according to the Declaration of Helsinki principles.

Collection of samples

Heparinized blood (12 ml) was collected and skin biopsies (4 mm) were extracted from lesional site(s) for (a) immunohistochemistry, (b) isolation of RNA stored in RNA*Later* (Ambion, Austin, TX) at −20 °C, and (c) parasite transformation (placed in Schneider's insect medium (Sigma-Aldrich, St Louis, MO) supplemented with 20% heat-inactivated fetal calf serum).

Surface marker analysis

Whole blood (100 μl) was surface stained with fluorochrome-conjugated antibodies (CD4 and CD25, CD8 and CD28); lymphocytes were gated on a linear forward versus side scatter dot plot and fluorescence was measured (Ganguly *et al.*, 2008) using CellQuest Pro software (BD Biosciences, San Jose, CA).

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated as previously described (Ganguly *et al.*, 2008) and resuspended in RPMI-1640 medium supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and 10% heat-inactivated fetal calf serum. Cell viability (>95%) was confirmed using Trypan blue exclusion.

Preparation of *L. donovani* antigen (LDA)

Antigen was prepared as previously described (Ganguly *et al.*, 2008) and stored at −20 °C until use.

Lymphoproliferation assay

Peripheral blood mononuclear cells were incubated with carboxyfluorescein diacetate succinimidyl ester (10 μM in serum-free medium) at 37 °C in 5% carbon dioxide for 10 minutes; the reaction was terminated on ice for 5 minutes, and the cells were washed, resuspended in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (1×10^6 ml well^{-1}), and incubated with PHA (10 μg ml^{-1}) or LDA (5 μg/ml^{-1}) for 6 days at 37 °C in 5% carbon dioxide. They were then harvested, stained with fluorochrome-conjugated antibodies to CD3 and CD4 for 15 minutes, and acquired on a BD FACSCalibur flow cytometer using BD CellQuest Pro software (BD Biosciences). Lymphoblasts were specifically gated on the blast-characteristic population on a forward versus side scatter dot plot and further gated on an FL3 (CD3 fluorescence) versus FL2 (CD4 fluorescence) dot plot and a minimum of 10,000 CD3$^+$ blasts were acquired. Proliferation indices were determined by curve-fitting analysis on a proliferation model of ModFit LT software (Verity Software House, Topsham, ME), in which the marker was set on the parent population in unstimulated cells.

Intracellular cytokine staining

Peripheral blood mononuclear cells, which were proliferating in response to PHA (10 μg ml^{-1}) or LDA (5 μg ml^{-1}), were either restimulated for the last 4 hours of treatment with leukocyte activation cocktail containing phorbol myristate acetate (10 ng ml^{-1}), ionomycin (1 μM), brefeldin A (10 μg ml^{-1}), or exposed to brefeldin A alone. After harvesting, PBMCs were surface-stained with anti-human CD3-PerCP, fixed and permeabilized, stained with fluorochrome-conjugated antibodies against IFN-γ and IL-10, acquired on a flow cytometer, and analyzed as previously described (Ganguly *et al.*, 2008).

Reverse transcriptase-PCR analysis

Total RNA was isolated from skin lesions according to the manufacturer's instructions (RNAqueous-4PCR Kit, Ambion). Reverse transcriptase-PCR was performed on isolated RNA (100 ng) with the one-step reverse transcriptase-PCR kit (Qiagen, Hilden, Germany) using gene-specific primers for β-actin, IFN-γ, IL-10, and Foxp3 (Supplementary Table S1 online). For reverse transcription, samples were subjected to an initial incubation at 50 °C for 30 minutes followed by an initial PCR activation (95 °C for 15 minutes). The amplification cycle comprised 35 cycles of denaturing (94 °C for 30 seconds), annealing for 30 seconds (varying temperatures for each primer set; Supplementary Table S1 online), extension (72 °C for 60 seconds), and a final extension at 72 °C (10 minutes). Products were resolved on agarose gels (2%) containing ethidium bromide (0.5 μg ml^{-1}) and quantified using Total lab Nonlinear Dynamic Image analysis software (Newcastle, UK), with the values being normalized to β-actin.

Immunohistochemistry

Immunohistochemical staining was performed as previously described (Little *et al.*, 2001). In brief, slide-mounted sections were boiled in citrate buffer (pH 6.0; for CD3 staining) or Tris-EDTA buffer (pH 9.0; for Foxp3 staining) for antigen retrieval. Sections were incubated overnight at 4 °C with a 1:75 dilution of anti-human Foxp3 (eBioscience, San Diego, CA) or for 1 hour at room temperature with a 1:100 dilution of anti-human CD3 (Dako, Glostrup, Denmark) and binding was detected by Super Sensitive Polymer-horseradish peroxidase Detection system (BioGenex, San Ramon, CA), following the manufacturer's instructions. After addition of the substrate 3-diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin, the cells that were positive for DAB or hematoxylin were counted, with three representative fields per section. A positivity score for Foxp3/CD3 was allocated on the basis of a scoring index as follows: 0, negative; ±, <1% positive cells; +, 1–5% positive cells; ++, 5–10% positive cells; +++, 10–20% positive cells; ++++, 20–50% positive cells; and +++++, >50% positive cells. Sections were evaluated independently by two observers and all samples were coded to minimize bias.

Statistical analysis

For parametric data (determined with the Shapiro–Wilk normality test), one-way analysis of variance was performed and the differences between groups were analyzed using Bonferroni's multiple comparison test. For non-parametric data, Kruskal–Wallis analysis of variance followed by Dunn's multiple comparison test was used to analyze data for individual groups. All analyses were performed using GraphPad Prism software (GraphPad Software Inc, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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