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Changes in lesional skin of patients with atopic dermatitis following topical treatment using pimecrolimus 1%: A histopathologic, histometric and immunohistochemical study

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Abstract

Background: Atopic dermatitis (AD) is a chronic, relapsing, pruritic and inflammatory skin disease. Several treatments are available for this condition including pimecrolimus.

Aim of Work: To study the histopathologic, histometric and immunohistochemical (IH) changes in lesional skin of AD patients following topical pimecrolimus 1% cream.

Subjects and Methods: The study included 10 patients with AD, aged between 2.5 to 11 years. Skin biopsies from affected areas were obtained before and after treatment. Biopsy sections were utilized for routine H&E as well as for IH staining for CD markers 3, 4, 8, 20 and 68 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

Results: Post-treatment biopsies showed insignificant ($p=0.06$) histopathologic changes such as mild decrease in the degree of spongiosis, acanthosis, infiltrate and mean epidermal thickness. However, immunohistochemical changes were striking. The number of CD3, CD4, CD8 and CD68 positive cells in the dermal infiltrate showed a significant decline ($p=0.04$) after treatment. In addition, the number of TRAIL positive cells in the dermal infiltrate showed a highly significant decline ($p<0.0001$) after treatment.

Conclusion: Pimecrolimus exerts its therapeutic effects in AD through modulation of the immunological network underling AD lesions, in particular,

through inhibiting lymphocyte function as well as reducing the number of TRAIL-positive inflammatory cells.

Introduction

Atopic dermatitis (AD) is a chronic, relapsing, highly pruritic, and inflammatory skin disease [1]. It is caused by complex interaction of many different genes & multiple environmental factors affecting their expression [2]. The pathogenesis of AD is not completely understood and involves a complex series of interactions between resident and infiltrating cells orchestrated by pro-inflammatory cytokines and chemokines [3]. In the dermis of AD lesions, there is a marked perivascular infiltrate in which both CD4+ and CD8+ T cells are present. The majority of these cells are of the CD45 Ro+ memory /effector phenotype and express the selective skin-homing receptor, cutaneous lymphocyte -associated antigen (CLA) [4,5].

Several treatments are available for this condition including the macrolide immunomodulator pimecrolimus. Pimecrolimus is a safe and effective treatment in reducing the severity of AD symptoms in children and adults. It affects mainly T lymphocytes by interacting with a cyclophilin-like cytoplasmic protein, and this complex in turn inhibits calcineurin, [6] a molecule required for initiation of cytokine gene transcription. This results in inhibition of gene transcription of multiple cytokines with a considerable importance in inflammation pathway (mainly IL-2, IL-4, and IL-5). [7]

The present study aims to study the histopathologic, histometric and immunohistochemical (IH) changes that occur in lesional skin of patients with AD following topical treatment using pimecrolimus 1% cream, for better understanding of the mechanism of action of this medication.

Subjects and Methods

The present study has been conducted on 10 patients with atopic dermatitis, attending the Dermatology outpatient clinic of Al-Minya University Hospital. Eight patients (80%) were males and two (20%) were females, their age ranged from 2.5 to 11 years with a mean and standard deviation (SD) of 8.5 ± 3.2 . All patients satisfied the UK Working Party's refinement of the diagnostic criteria of the Hanifin and Rajka for atopic dermatitis [8]. Patients received no topical or systemic treatment for at least 1 month before being enrolled in the study. Pimecrolimus 1% cream was applied to skin lesions once daily till apparent complete clinical clearance.

Skin Biopsies

Skin biopsies from treated areas were obtained before treatment and after apparent complete clinical clearance, usually after 3 weeks, using sterile 4 mm punches after taking informed consents. Each biopsy was immediately fixed in 10% formalin, embedded in a paraffin block and sectioned into 5 μ m thick sections. These sections were utilized for routine hematoxylin and eosin (H&E) as well as for IH staining.

Histometry

The thickness of the epidermis was studied in both pre- and post-treatment skin biopsies. These histological measurements (histometry) were carried out on standard H&E stained sections. The distances between the outermost surface of the epidermis, excluding the horny layer, and the dermo-epidermal junction were measured at different points throughout the section using an ocular micrometer. The mean of these measurements was taken as representative of the mean epidermal thickness (MET).

Immunohistochemistry (IH)

IH staining techniques were used to demonstrate some CD markers for T and B-lymphocytes and histiocytes as well as to demonstrate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression in skin samples before and after treatment. A ready-to-use detection system (lab EnVision + System, Peroxidase (DAB), lab vision®, Cat# TP-015-HD) was used. Primary antibodies used to demonstrate the different CD markers and their dilutions are shown in **table 1**. The primary antibody for TRAIL was the monoclonal mouse anti-human TRAIL/TNFSF10 antibody (R&D system Corporation®, Cat# 375). It was used in a dilution of 25µg/ml.

CD marker	Antibody	Dilution	Company & Cat# number
CD3 (Pan T lymphocyte marker)	Mouse monoclonal anti-human CD3	01:50	Bio Care Medical®, Cat# CM110C
CD4 (T-helper lymphocyte marker)	Mouse monoclonal anti-human CD4 antibody	ready-to-use	Lab vision Corporation®, Cat# MS-1528
CD8 (T-suppressor/cytotoxic lymphocyte marker)	Mouse monoclonal anti-human CD8 antibody	ready-to-use	Lab vision Corporation®, Cat# RM-9116
CD20 (B lymphocyte marker)	Mouse monoclonal clone L26 anti-human CD20 antibody	0.1805556	Daco Cytomation®, Cat# M 0755
CD68 (Histiocyte marker)	Mouse monoclonal anti-human CD68 antibody Ab-3 (KP1)	01:30	NeoMarkers®, Cat# MS-397-P

Table 1: List of the 1ry antibodies, dilutions and commercial sources for CD markers used in the study.

The expression of CD markers in the dermal infiltrate was evaluated according to the system previously described [9] as shown in **table 2**. The expression of TRAIL in the cells of the dermal infiltrate was evaluated according to the number of positively stained cells/H.P.F. as shown in **table 3**.

<i>Number of positive cells/H.P.F.</i>	<i>Score</i>
No staining	0 (-)
Staining < 25%	1 (+)
26 – 50 %	2 (++)
51 – 75 %	3 (+++)
> 75 %	4 (++++)

Table 2: Scoring of CD markers expression in dermal cellular infiltrate.

<i>Number of positive cells/ H.P.F.</i>	<i>Score</i>
No positively stained cells	0
1- 25 cells	1
26 – 50 cells	2
51 – 75 cells	3
75-100 cells	4
> 100 cells	5

Table 3: Scoring system for quantifying TRAIL expression in the cells of the dermal infiltrate.

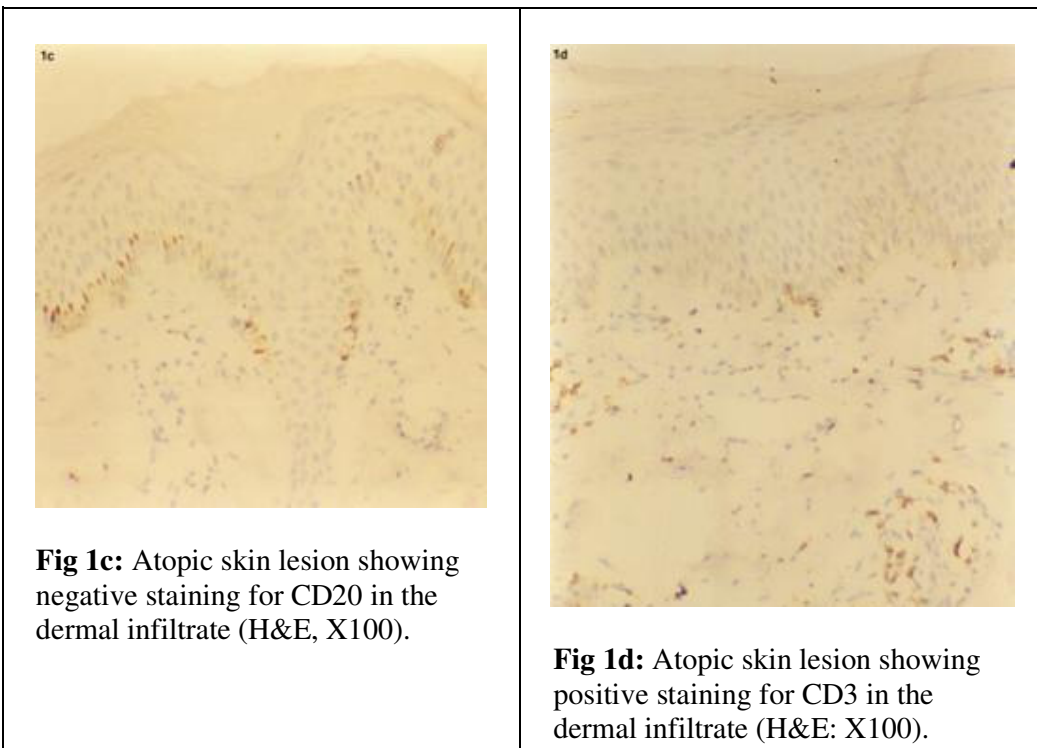
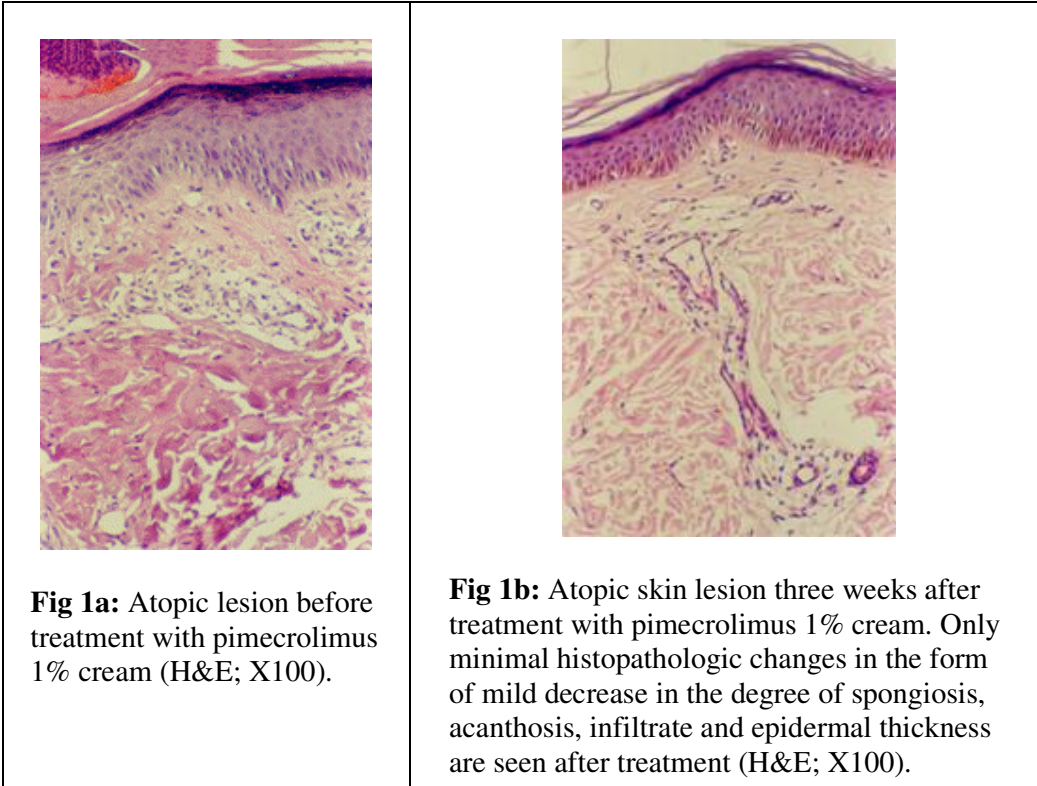
Statistical analysis

Data were collected and tabulated using Excel Software. Statistical analysis was done using SPSS (version 11.0). The numerical data was expressed as mean \pm SD. Student-t test was used to compare numerical values. The (t-test) values were expressed in terms of p-value. P value was considered significant when it is < 0.05 and highly significant when it is < 0.001.

Results

Histopathologic changes

Biopsies taken 3 weeks after treatment with pimecrolimus cream 1% showed only minimal histopathologic changes in the form of mild decrease in the degree of spongiosis, acanthosis, infiltrate and mean epidermal thickness. The latter reduced from a mean of $145\mu\text{m}\pm 30$ before treatment to mean of $115\mu\text{m}\pm 5$, however, this difference is statistically not significant ($p=0.06$) (**Fig.1a, b**).



IH staining

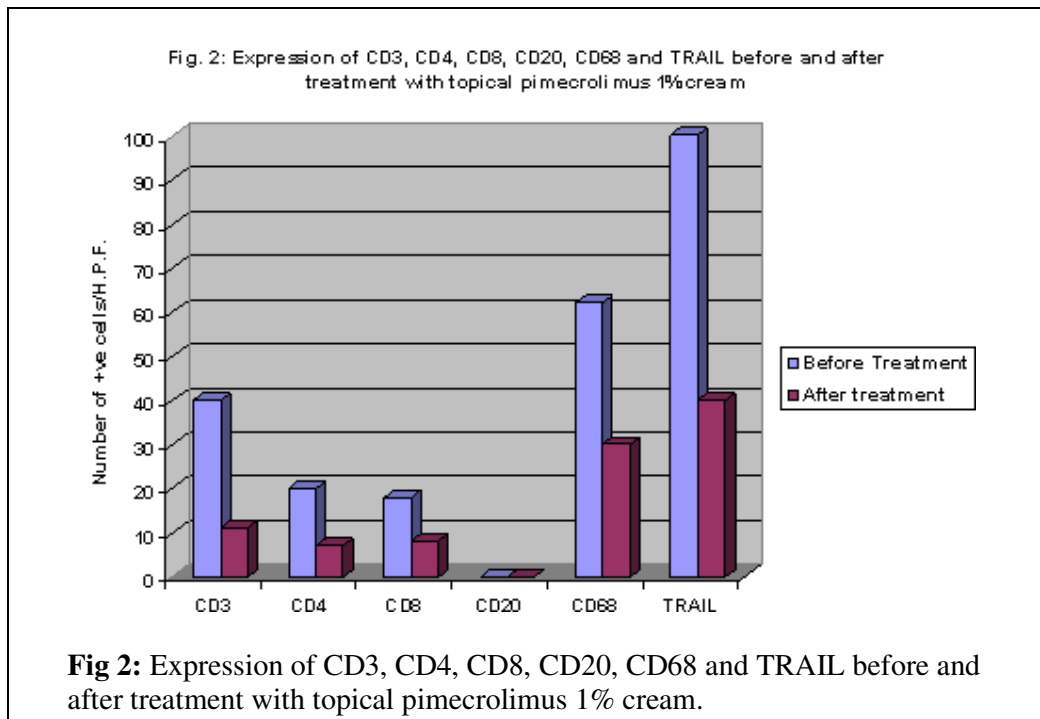
IH staining techniques were performed to study the expression of some CD markers as well as to evaluate TRAIL expression before and after treatment.

CD markers: The markers CD3, CD4, CD8 and CD68 showed positive staining in all pre-treatment biopsies. In post-treatment biopsies the expression of all of these CD markers had declined. This difference is statistically significant ($p=0.04$). The marker CD20 was negative in lesional skin and remained as such even after three weeks of topical pimecrolimus treatment (**Table 4; Fig.1-5**).

CD marker	Before treatment		After treatment	
	Number of positive cells/H.P.F.	Score	Number of positive cells/H.P.F.	Score
CD3	40±2	++	11±1	+
CD4	20±3	+	7±2	+
CD8	18±3	+	8±2	+
CD20	-	-	-	-
CD68	60±2	+++	30±3	++

$p=0.04$

Table 4: Score of CD markers expression before and after treatment with pimecrolimus 1% cream.



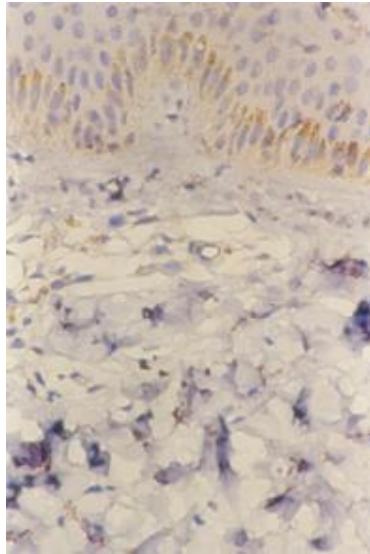


Fig 3a: CD 4 staining in atopic skin lesion before treatment (Immunoperoxidase technique; X200).

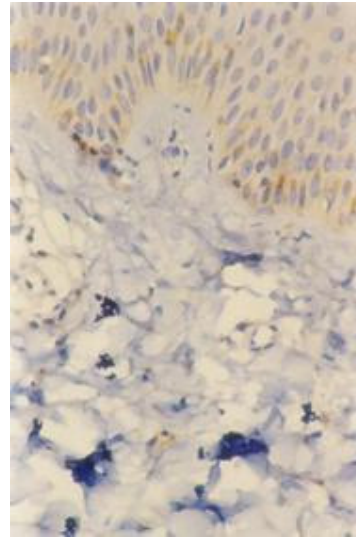


Fig 3b: Atopic skin lesion three weeks after treatment with pimecrolimus 1% cream, showing decrease in the number of CD4+ve cells in the dermal infiltrate after treatment (Immunoperoxidase technique; X400).

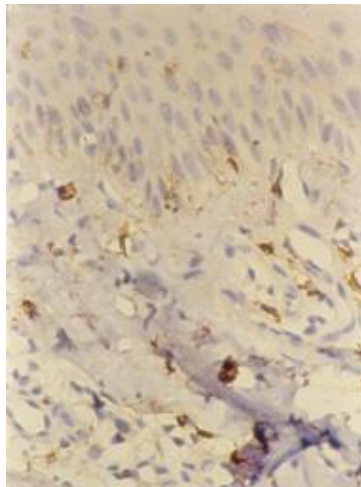


Fig 4a: CD8 staining in atopic skin lesion before treatment (Immunoperoxidase technique; X200).

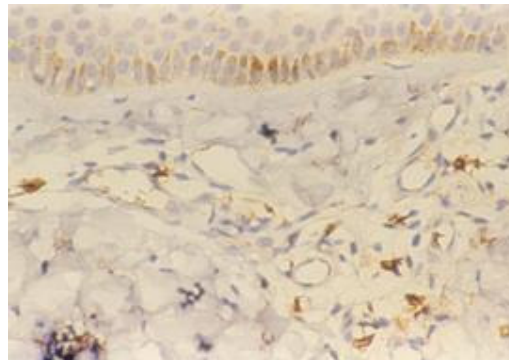


Fig 4b: Atopic skin lesion three weeks after treatment with pimecrolimus 1% cream, showing decrease in the number of CD8+ve cells in the dermal infiltrate after treatment (Immunoperoxidase technique; X200).

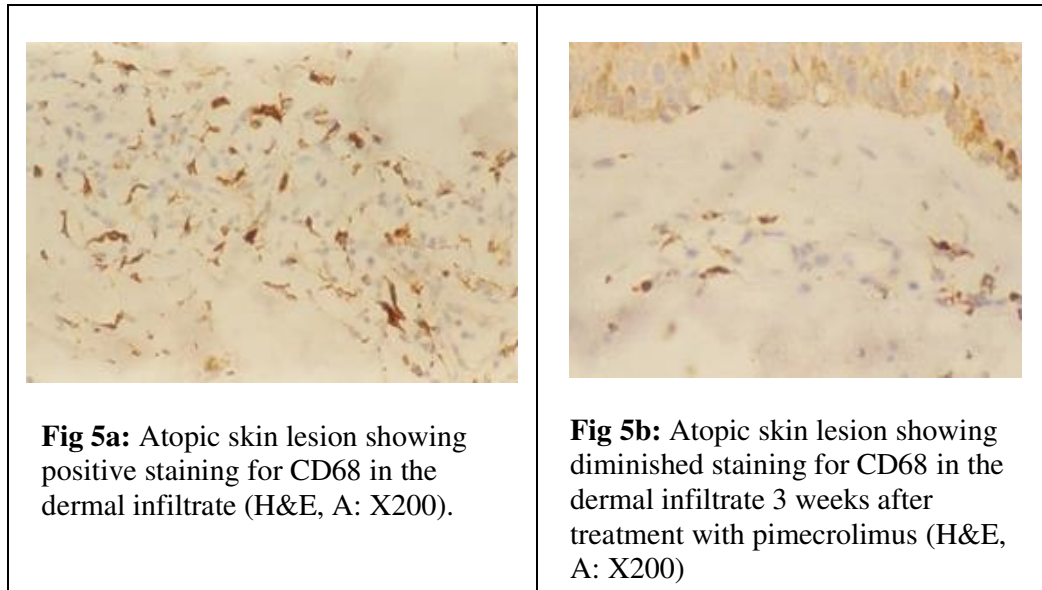


Fig 5a: Atopic skin lesion showing positive staining for CD68 in the dermal infiltrate (H&E, A: X200).

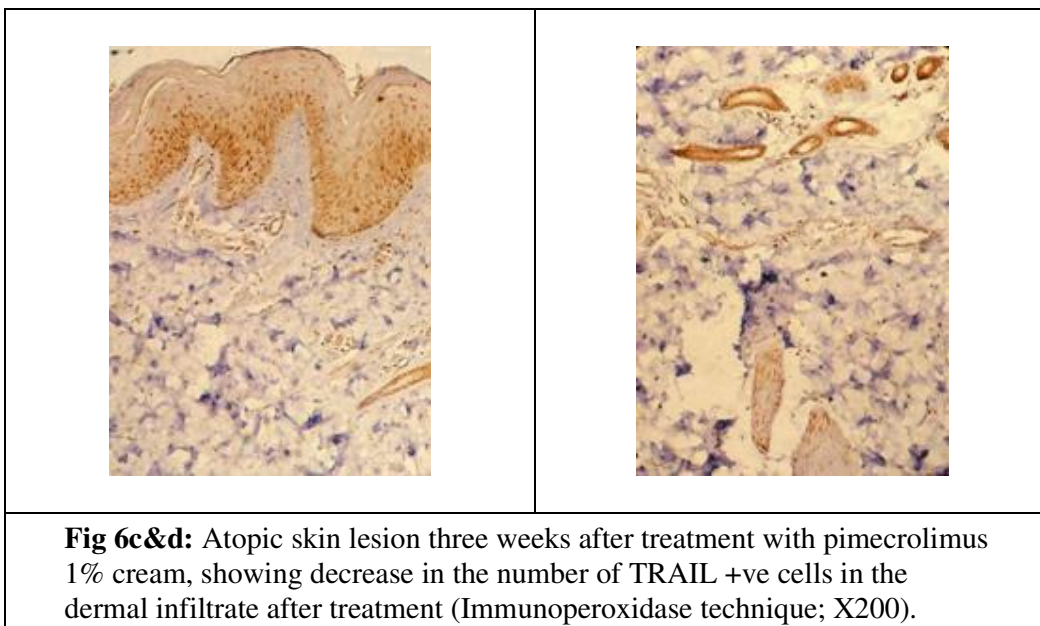
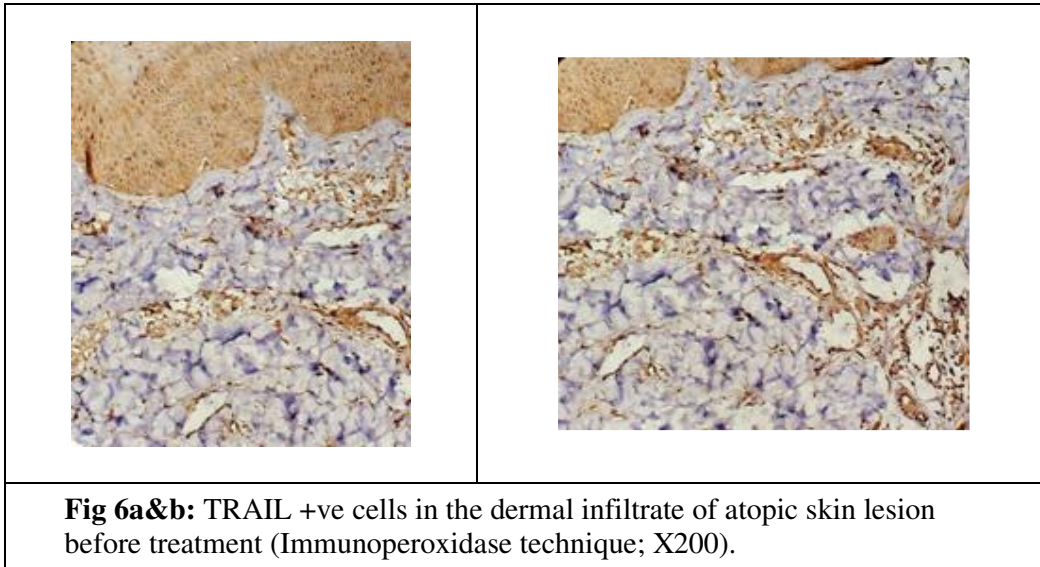
Fig 5b: Atopic skin lesion showing diminished staining for CD68 in the dermal infiltrate 3 weeks after treatment with pimecrolimus (H&E, A: X200)

TRAIL expression: TRAIL expression was found to show marked decrease in biopsies obtained 3 weeks after treatment as compared to pre-treatment biopsies. The expression decreased from a mean of 100±10 cells/H.P.F before treatment to a mean of 40±4 cells/H.P.F after treatment. This difference is statistically highly significant ($p < 0.0001$) (Table 5; Fig.2 and Fig.6a-d).

	Before treatment	After treatment
Number of positively-stained cells/H.P.F.	100±10 cells/H.P.F.	40±4 cells/H.P.F.
Score of positively-stained cells	4	2
Intensity of staining	3	2
Mean score	3.5	2

$p < 0.0001$

Table 5: Score of TRAIL expression before and after treatment with pimecrolimus 1% cream.



Discussion

Atopic dermatitis is one of the most common inflammatory skin disorders [10]. Two main concepts evolved to explain its pathogenesis: excessive T-cell activation in response to an antigen and hyperstimulation of T cells by atopic Langerhans cells [11,12,13]. Topical corticosteroids treatment is a mainstay in treatment of AD; although long-term use is limited by its side effects [14]. However, the evolving understanding of the pathogenesis of AD has allowed researchers to target specific steps in the inflammatory cascade and this has driven the development of more effective, steroid-free therapies to treat AD [13], such as the calcineurin inhibitor pimecrolimus, that has proven to be a novel option for AD treatment [15,16]. This

work aims to study the histopathologic, histometric and IH changes that occur in AD skin lesions following topical pimecrolimus 1% cream.

In the present study post-treatment biopsies has shown only minimal histopathologic changes in the form of mild decrease in the degree of spongiosis, acanthosis and inflammatory infiltrate. In addition, post-treatment biopsies has shown minimal, but insignificant, decrease in the mean epidermal thickness ($p=0.06$). Our observation suggests that topical pimecrolimus 1% cream does not cause epidermal atrophy, a common problem with long-term topical corticosteroid treatment [14]. However, since post-treatment biopsies in our study were obtained after a relatively short treatment period (usually 3 weeks), therefore post-treatment biopsies after longer treatment periods will be required to confirm our observation.

Although histopathologic changes were minimal, IH changes were striking. The number of CD3, CD4, CD8 and CD68 positive cells among cells of the dermal infiltrate showed a significant decline ($p=0.04$) after 3 weeks of topical pimecrolimus treatment (**Table 4**). This suggests that this therapy results in a significant reduction in the number of T-helper cells, suppressor/cytotoxic T cells and histiocytes in treated skin of patients with AD. We suggest that this decline in the number of these inflammatory cells may be an indicator of subsiding of the acute inflammatory phase of AD under the effect of this treatment. Pimecrolimus, being targeting T cells and mast cells, inhibits the production and release of cytokines and other inflammatory mediators as well as the expression of signals essential for the activation of inflammatory T lymphocytes [17] and thus exerts a beneficial effect in AD.

Furthermore, the number of TRAIL positive cells in the dermal infiltrate has shown a highly significant decline ($p<0.0001$) from a mean of 100 ± 10 cells/H.P.F. to a mean of 40 ± 4 cells/H.P.F. after 3 weeks of topical pimecrolimus treatment (**Table 5**). TRAIL is a member of the TNF superfamily, and has been implicated in the regulation of various physiological and pathological immune responses [18]. This might be because of its wide expression among cells of the immune system, including activated T cells [19], B cells [20,21], monocytes [22], dendritic cells [23], natural killer cells [19], and neutrophils [22]. Since TRAIL-positive mononuclear cells are present in greater numbers in lesional skin of patients with AD compared with non-lesional skin and normal controls [24], a decline in the number of TRAIL positive inflammatory cells following topical pimecrolimus treatment again may be an indicator of subsiding of the acute inflammatory phase of the disease under the effect of this treatment. To the best of our knowledge TRAIL expression was not previously evaluated in patients with AD following treatment with pimecrolimus.

In conclusion, the results of the present study suggests that pimecrolimus exerts its therapeutic effects in AD through modulation of the immunological network that underlies AD lesions, in particular, through inhibiting lymphocyte function as well as through reducing the number of TRAIL-expressing inflammatory cells. Further studies on a larger number of patients are recommended to confirm this latter observation.

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