Qualitative and quantitative study of immune cells in various types of cutaneous lichen planus

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Received: 24 October 2018
Accepted: 29 January 2019

Background: Lichen planus (LP) is a common inflammatory skin disease. Pathogenesis of LP includes two possible mechanisms; related or unrelated to antigen. Regarding different clinical features of cutaneous and mucosal types of LP, for the first time, we decided to perform a qualitative and quantitative study of immune cells in different types of cutaneous LP and in comparison with normal skin.

Methods: A total of 88 specimens (60 cases of cutaneous LP, 28 cases of normal skin) were selected from 2016 to 2017 in Kerman, Iran. Evaluation of immune cells was carried out based on qualitative and quantitative analysis. These findings were statistically calculated by descriptive statistical tests including frequency and mean ± standard deviation. Quantitative data were analyzed by independent t-test, chi-square, and analysis of variance (ANOVA). Data were analyzed using SPSS16 (SPSS Inc., Chicago, IL, USA). A p value less than 0.05 was considered statistically significant.

Results: Our study demonstrated that the mean number of immune cells was significantly higher in lichen planus group in comparison with the control group. Number and staining intensity of Langerhans cells (LCs) in the LP group were significantly greater in epidermal than dermal region. Mastocytes were located mostly within the deep dermis in the LP group. Hypertrophic and atrophic LP had the highest and the lowest number of immune cells (i.e., mastocytes, LCs, and CD3 positive cells), respectively, with a significant difference.

Conclusion: Our study demonstrated that immune cells were seen in larger numbers in the hypertrophic type of cutaneous LP which is consistent with the chronicity of this disease.

Keywords: Cutaneous lichen planus, immune cells, Immunohistochemistry (IHC)

Iran J Dermatol 2019; 22: 30-39

INTRODUCTION

Lichen planus (LP) is an inflammatory skin disease related to cellular immunity that involves the skin, mucosa, nail, and hair. Prevalence of the disease is variable between 0.22% and 5% according to geographical region. This disease is most commonly seen in the middle-aged population [1-3]. Clinically, classic type of cutaneous LP is characterized by violaceous pruritic papules with delicate white scales on the surface of the lesion (Wickham's striae). Autoimmunity, drugs, sensitization to chemicals, viral infection, and stress have been proposed as the pathogenesis of the disease [1].

Cutaneous type of LP is classified to classic,

hypertrophic, atrophic, bullous, linear, annular, eruptive, pigmentosus, ulcerative, actinic, and lichen planoplaris [4]. Atrophic type is a rare variant of cutaneous LP that is usually developed on the previous ulcerative or annular lesions. Lesions appear as brown or white-bluish colored papules or plaques with the atrophic surface. Lower leg, axillary, glans of penis, and trunk are the most common sites of involvement [1,4].

Hypertrophic type or verrucous LP presents as red-brown thickened papules or plaques. The most common site of involvement is the lower leg while the least common sites are the upper limb and trunk. Lesions have a verrucous or hyperkeratotic surface with more pruritus and longstanding duration. This type can rarely progress to squamous cell carcinoma. It has been proposed that chronic inflammation can participate in the progression of the lesion to malignant transformation [1,4].

Bullous type is a rare variant with the development of blister on violaceous lesions of LP. Lower leg, trunk, and dorsal of hand and foot are the most common sites of involvement. Severe vacuolar degeneration in the basal layer has a role in the development of the lesions [1,4].

Diagnosis of the disease mainly is based on clinical appearance. A skin biopsy can be used in order to confirm the diagnosis in suspicious cases [1]. Pathological features of LP include hyperkeratosis, focal hypergranulosis, acanthosis, liquefactive degeneration of basal layer, colloid body, band-like infiltration of lymphohistiocytic cells in the papillary dermis including dermal-epidermal junction, and melanophages in the upper dermis [5].

Pathogenesis of LP has been described as two possible mechanisms; i.e., related or unrelated to antigen [6]. In the antigen-related mechanism, altered keratinocyte surface antigen due to exogenous factors such as a virus, drugs, vaccines, and contact sensitization in a genetically susceptible person can lead to activation of Langerhans cells (LCs). Pathogenic antigen on LCs or keratinocytes is presented through major histocompatibility (MHC) II to CD4 T helper lymphocytes that can lead to the release of T helper I (THI) cytokines including interlukine2 and interferon. Furthermore, an antigen on keratinocytes is presented to CD8 cytotoxic T cells through MHCI. Finally, CD8 T lymphocytes are activated by MHC I and inflammatory cytokines released from CD4T lymphocytes [3,7].

In nonspecific antigen mechanism, inflammatory mediators such as histamine, TNFα, and chymase release from mastocytes stimulate lymphocytes [8]. Finally, both of these mechanisms result in basal keratinocytes apoptosis by CD8 positive lymphocytes through secretion of perforin, granzyme, TNFα, and binding of CD95 receptor on T lymphocyte to its ligand on the surface of the keratinocyte [9].

Cutaneous LP is usually a self-limited disease, but the mucosal type has a chronic and progressive course and accompanied with an increased risk of malignant change especially in erosive form [1,3]. So, regarding distinctive clinical features and course of disease in cutaneous LP relative to oral LP, pathogenesis of the disease can be different between them. Although there are many studies about the oral type of LP, only a few of them have evaluated pathogenesis of cutaneous LP while pathogenesis of LP is still not well-documented. Furthermore, there is no study to evaluate the distribution of immune cells in different types of cutaneous LP. So, for the first time, we decided to evaluate the distribution and quantity of immune cells in various types of cutaneous LP and in comparison with normal skin.

MATERIAL AND METHODS

This is a cross-sectional study in Afzalipour Hospital of Kerman University of Medical Sciences in Iran with the approval code of IR.KMU.REC.1394.370 and ethical code of 1655. A total of 88 formalin-fixed paraffin-embedded specimens including 60 cases of cutaneous LP and 28 cases of normal skin were selected from an archive of Pathology Department from 2016 to 2017. The sample size was calculated based on a previous study (α=0.05, power=0.80, P1=0.80, and P2=0.46) [10]. Hematoxylin-eosin- stained slides were reviewed by a pathologist for confirmation of diagnosis and classification of LP. Inclusion criteria for lichen planus were the existence of all of the following features: hypergranulosis, vacuolar degeneration of basal layer, and band-like infiltration of lymphocytes. Control group was including samples with similar histology to normal skin and without any inflammation.

For the immunochemistry study (IHC), 3-µm histologic sections were prepared from paraffin

blocks. Samples were deparaffinized with xylene and then rehydrated with water and alcohols and then IHC study was done with the streptavidin-biotin complex. Monoclonal antibodies of our study were CD1a (Dako M 3571, 1:50, Denmark); CD68 (Dako M 814, 1:100, Denmark); CD3 (Dako A0452, 1:100, Denmark); CD8-α (Dako M- 7103, 1:50, Denmark); CD4 (Biosystem NCL–L–CD 4368, 1:20, Denmark); and CD20 (Dako M 9755, 1:100, Denmark). Antigen retrieval was performed with Tris-EDTA (PH=9) buffer and then sections were microwaved for 10 minutes. Subsequently, they were immersed in peroxidase at room temperature for 30 minutes. Diaminobenzidine chromogen was used in order to characterize antigen-antibody bonds. Slide backgrounds were stained with hematoxylin.

Positive slides in both case and control groups were examined and quantitative and qualitative analyses were done with a light microscope (Olympus, Bx53 series). Digital photomicrographs were taken with high quality and resolution with a microscope camera. Each photograph received a code number and viewed by two dermatopathologists. Counting the immune cells was done by digitally calibrated grid measuring at 250 µm.

Qualitative analysis included staining pattern, intensity, and distribution of immune cells. The pattern of staining was categorized to membranous, cytoplasmic, and mix of membranous and cytoplasmic. The intensity of staining was classified to four groups including severe staining (staining in more than 50% of positive cells), moderate staining (between 10% and 50%), weak staining (less than 10%), and no staining.

Counting of CD1a positive LCs was done in 4 different high power fields (at X 400 magnification) and then their mean number in 4 fields was recorded. The proportion of positive inflammatory cells with IHC staining including histiocytes (CD68), T lymphocyte (i.e., CD3, CD4, and CD8) and B lymphocyte (i.e., CD20) was calculated in 10 different fields (X100 magnifications) and mean percentage of them was recorded. Giemsa staining was utilized for evaluation of mastocytes. Counting of these cells was done in 10 different high power fields (X 400 magnifications) within the two areas including band-like infiltration and deep dermis and their mean in 10 fields was recorded.

Statistical methods

Data were analyzed using SPSS16 (SPSS Inc., Chicago, IL, USA). These findings were statistically calculated by descriptive statistical tests including frequency, relative frequency, and mean ± standard deviation. Independent T-test was used for quantitative analysis. Pearson correlation test was applied in order to evaluate the relationship between different cell types. Analysis of variance (ANOVA) was used for comparison between the mean number and percentage of the cells. Chi-square was utilized for comparison of the qualitative data. A p value less than 0.05 was considered statistically significant.

RESULTS

A total of 88 skin biopsies including 60 LP cases (28 females and 32 males) and 28 control cases (14 females and 14 males) were evaluated (P=0.51). Mean age of patients in the LP group and control group was 41.06 ± 2.14 and 29.40 ± 3.89, respectively (P=0.3). Upper limbs were the most prevalent sites (33.8%) of biopsy (Table 1). The most common type of cutaneous LP was the classic type (63.3%). Other types in descending order were atrophic (15%), bullous (11.7%), and hypertrophic (10%).

Qualitative analysis

Regarding the staining pattern, both patterns of membranous and cytoplasmic were observed

Table 1. Demographic features in the patient and control groups

Variables	Frequency (%)		*P*
	LP group	Control group	
Age			
0-20	7 (11.7)	7 (25)	
21-40	22 (36.7)	12 (42.9)	
41-60	24 (40)	7 (25)	0.304
61-80	6 (10)	1 (3.6)	
Older than 80	1 (1.7)	1 (3.6)	
Sex			
Female	31 (51.7)	16 (59.3)	0.511
Male	29 (48.3)	11 (40.7)	
Site of biopsy			
Head and neck	6 (11.5)	4 (16)	
Upper Limb	19 (36.5)	7 (28)	
Lower Limb	17 (32.7)	8 (32)	0.914
Genitalia	4 (7.7)	3 (12)	
Trunk	6 (11.5)	3 (12)	

in lymphocytes and LCs, but the membranous pattern was seen predominantly. The most frequent pattern of staining for histiocytes was cytoplasmic.

The intensity of staining for lymphocytes and LCs in the LP group was severe and strong. In the control group, these cells were weakly-stained or with no staining at all.

In the LP group, LCs were distributed with more density in the epidermis than dermis. Furthermore, T lymphocyte cells were scattered in the epidermis but they were located in large numbers within the upper dermis. B lymphocytes were seen only occasionally within the dermis. Also, Mastocytes were situated largely in deep dermis under the inflammatory infiltration. Histiocytes were scattered in the upper and lower dermis.

In the control group, LCs and lymphocytes were observed only in limited numbers within the epidermis and upper dermis, respectively. Mastocytes were located with a considerable amount in perivascular and periadnexal areas in the control group. Histiocytes were scattered in the upper and lower dermis.

Quantitative Analysis

Mean number of LCs within the epidermis was greater in the LP group and in the control group (Figure 1). The average number of LCs in dermis was 15.46 ± 1.19 in LP group, while there were no LCs within the dermis in the control group (Table 2). The difference in LCs in both epidermis and dermis was remarkably significant between two groups of LP and control (P<0.001).

The mean number of mastocytes was significantly higher in the LP group than the control group (p<0.001). Mastocytes were observed more abundant within the deep dermis below the

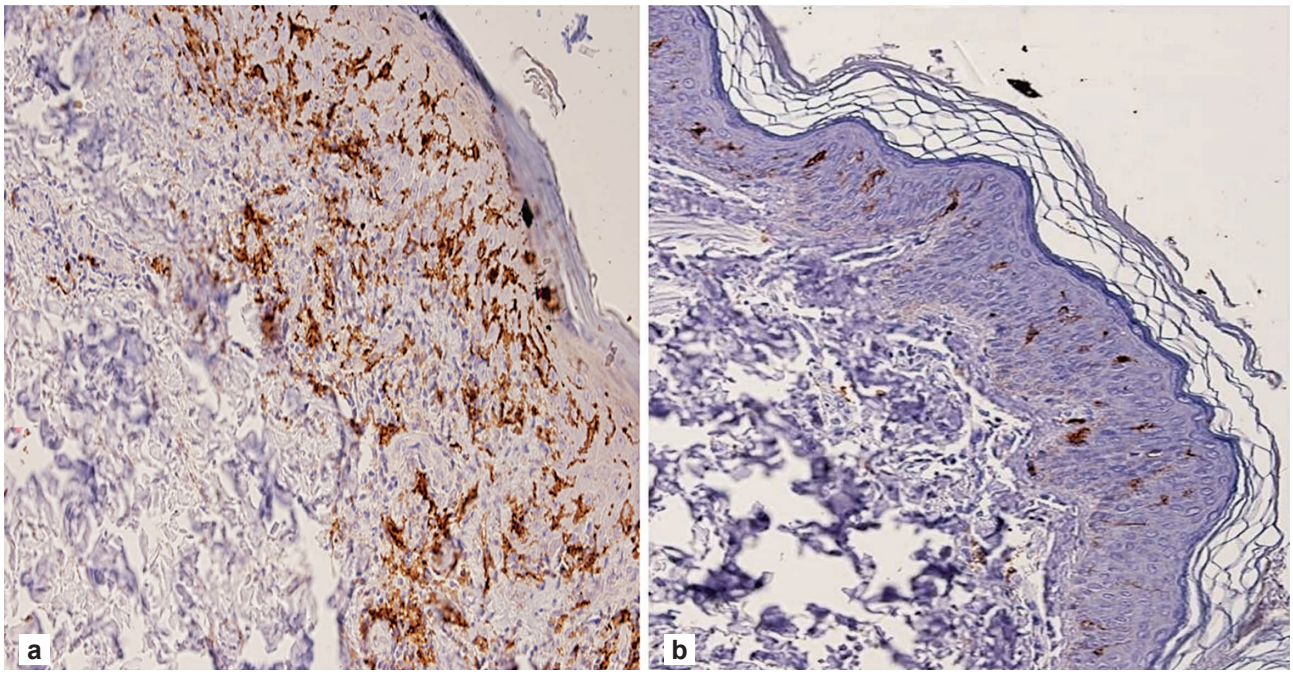

Figure 1. Immunohistochemical staining of CD1a^{+} Langerhans cells in cutaneous LP (a) and control group (b) (×100 magnification) demonstrates a significantly higher number of Langerhans cells in the LP group than the control group.

Table 2. Mean number of the immune cells in the LP group and control group

Immune cell/Site	Lp Group		Control Group		*P*
	Mean±SD	Min/Max	Mean±SD	Min/Max	
Total number of mastocytes	10.68±0.75	3/34	2.85±0.26	1/6	<0.001
Mastocytes intra- infiltration	3.60±0.57	0/30	0	0:0	<0.001
Mastocytes in the deep dermis	7.53±0.50	2/20	2.85±0.26	1:6	<0.001
Langerhans cells in the epidermis	30.98±1.45	2/51	7±2.44	2/11	<0.001
Langerhans cells in the dermis	15.46±1.19	0/33	0	0	<0.001

band-like infiltration in the LP group rather than intra-inflammatory infiltration (Table 2, Figure 2).

The average percentage of immune cells including histiocytes and B and T lymphocytes were statistically greater in the LP group than the control group (P<0.001) (Table 3, Figure 3). In the LP group, the largest percentage of immune cells belonged to T lymphocytes, while B lymphocyte cells had the lowest quantity. CD4/CD8 ratio was 0.56 ± 0.14 in the epidermis and 1.87 ± 0.11 within the dermis. Therefore, the most prevalent intra-epidermal inflammatory cells were CD8 positive lymphocytes. Besides, CD4 positive cells were the most prevalent intra-dermal inflammatory cells. Among different types of LP, hypertrophic LP had the most frequent inflammatory cells that were statistically significant (P<0.05). Other types of cutaneous LP in a descending order regarding immune cells count were bullous, classic, and atrophic types. Only CD8 T lymphocytes were observed most commonly in bullous type than hypertrophic type, but the difference was not significant (P=0.02) (Tables 4 and 5; Figures 4 and 5).

There was a positive significant relationship between mastocytes with T lymphocytes and LCs. Also, there was a direct and strong connection between T lymphocyte cells and LCs (Table 6).

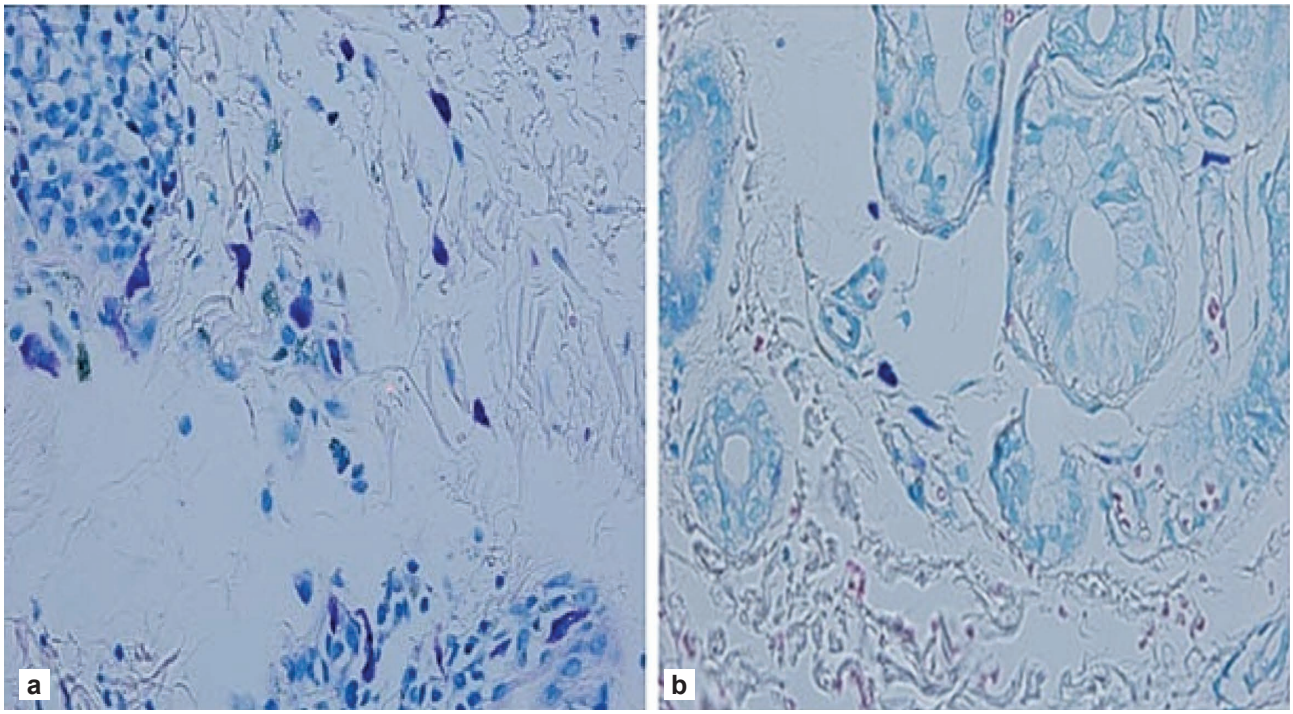

Figure 2. Mastocytes in hypertrophic (a) and atrophic cutaneous LP (b) (×400) magnification, Giemsa stain demonstrates an increased number of mastocytes in hypertrophic than atrophic cutaneous LP group.

Table 3. Mean percentage of the immune cells in the LP and control group

Antibody/Site	LP Group		Control Group		*P*
	Min/Max	Mean±SD	Min/Max	Mean±SD	
CD3 within the epidermis	0/40	10.81±0.83	0/0	0±0	<0.001
CD3 within the dermis	40/90	65.08±1.33	0/15	8.14±0.76	<0.001
CD4 within the epidermis	0/50	4.15±0.94	0/0	0±0	<0.001
CD4 within the dermis	1/70	40.78±1.55	0/8	2.85±0.45	<0.001
CD8 within the epidermis	0/30	7.90±0.60	0/0	0±0	<0.001
CD8 within the dermis	10/35	23.75±0.79	0/12	5.28±0.67	<0.001
CD4/CD8Ratio in the epidermis	7.5/0	0.56±0.14	0/0	0±0	<0.001
CD4/CD8Ratio in the dermis	4.67/0.04	1.87±0.11	0/2	0.44±0.10	<0.001
B Cell	5/15	10.83±0.44	0/5	1.85±0.42	<0.001
Histiocyte	5/50	24.25±1.28	0/15	7.60±0.64	<0.001

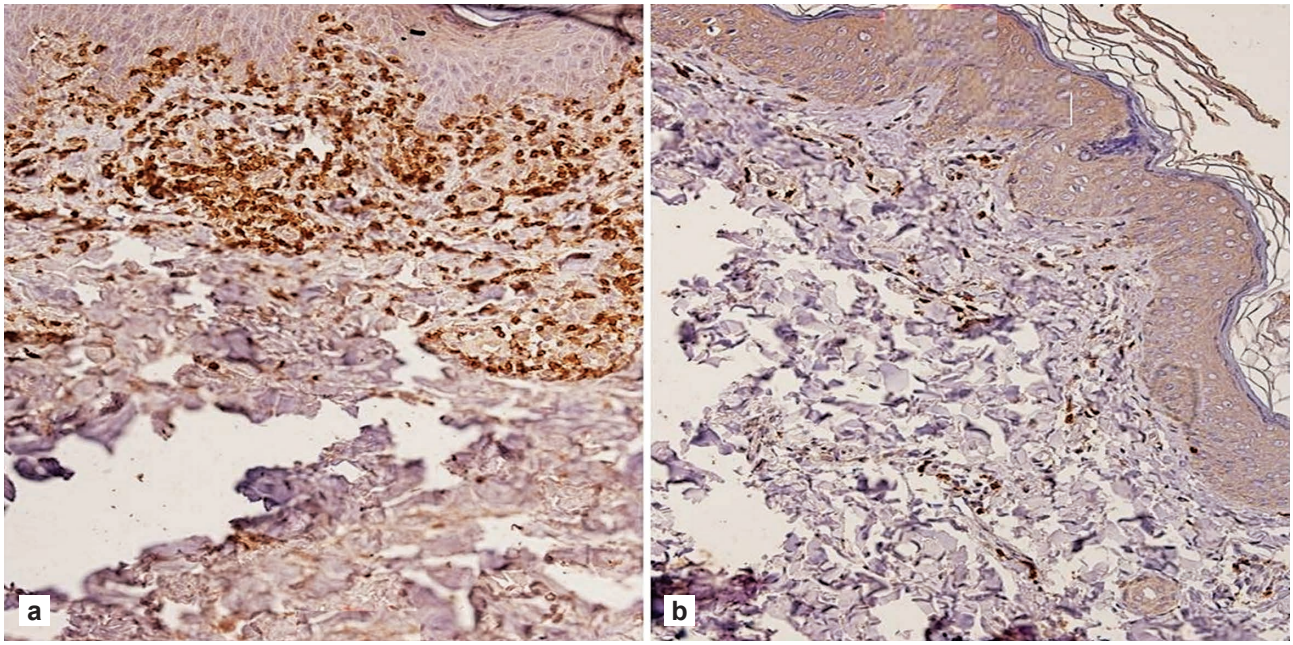

Figure 3. Immunohistochemical staining of $CD3^+$ T lymphocytes in cutaneous LP (a) and control group (b) (×100 magnification) demonstrates a higher number of $CD3^+$ T lymphocytes in LP group than the control group.

Table 4. Mean number of immune cells in various types of LP

Immune cell/Site	Atrophic	Classic	Hypertrophic	Bullous	*P*
Total number of mastocytes	6.33±0.78	10.23±0.94	17.16±2.72	13.14±0.93	0.002
Mastocytes intra- infiltration	4.33±3.22	2.92±0.45	5.33±1.28	4.85±0.63	0.460
Mastocytes in the deep dermis	5±0.81	7.31±0.61	11.83±2.15	8.28±0.60	0.007
Langerhans cells in the epidermis	17.55±2.62	31.02±1.65	40.16±3.27	40.14±1.83	0.001
Langerhans cells in the dermis	6±1.46	16.31±1.44	21.16±4.32	18.14±2.89	0.004

Table 5. Mean percentage of immune cells in various types of LP

Immune cell marker	Atrophic	Classic	Hypertrophic	Bullous	*P*
CD3	56.66±3.11	65.92±1.69	71.66±2.78	65.71±2.97	0.029
CD4	32.33±4.77	41.4±1.85	51.66±4.59	38.57±1.42	0.018
CD8	21.11±1.38	24.07±1.04	21.66±2.47	27.14±2.14	0.206
CD68	33.11±3.13	23.02±1.58	19.16±2.71	23.85±3.21	0.021

DISCUSSION

Our study indicated that the mean number of immune cells including mastocytes, histiocytes, LCs, and T and B lymphocytes in the LP group were statistically significant in comparison with the control group that was similar to previous studies [11,12]. Also, in our study, most of the LP patients were female with a female to male ratio of 1.06 to 1. In addition, the majority of the patients were between 40 and 60 and the most frequent site of involvement was an upper limb; this result is in line with the findings of other studies [1-4].

Hussein and collogues in Egypt evaluated immune cells on 19 cases of cutaneous LP. In this study, the percentage of CD3 was 58.70% in LP group and 3% in the control group that was almost in line with our results (68.08% in the LP group and 8.18% in the control group). Moreover, in Egypt study, the percentage of histiocytes and B lymphocytes in the LP group was 21.30% and 9%, respectively, which was statistically higher than the control group (4%, 0). In our research, histiocytes and B lymphocytes were 24.25% and 10.83% in the LP group. (7.60% and 1.85% in the control group, respectively) [12].

In the current study, the majority of lymphocytes were T lymphocytes (65.08%). Previous studies

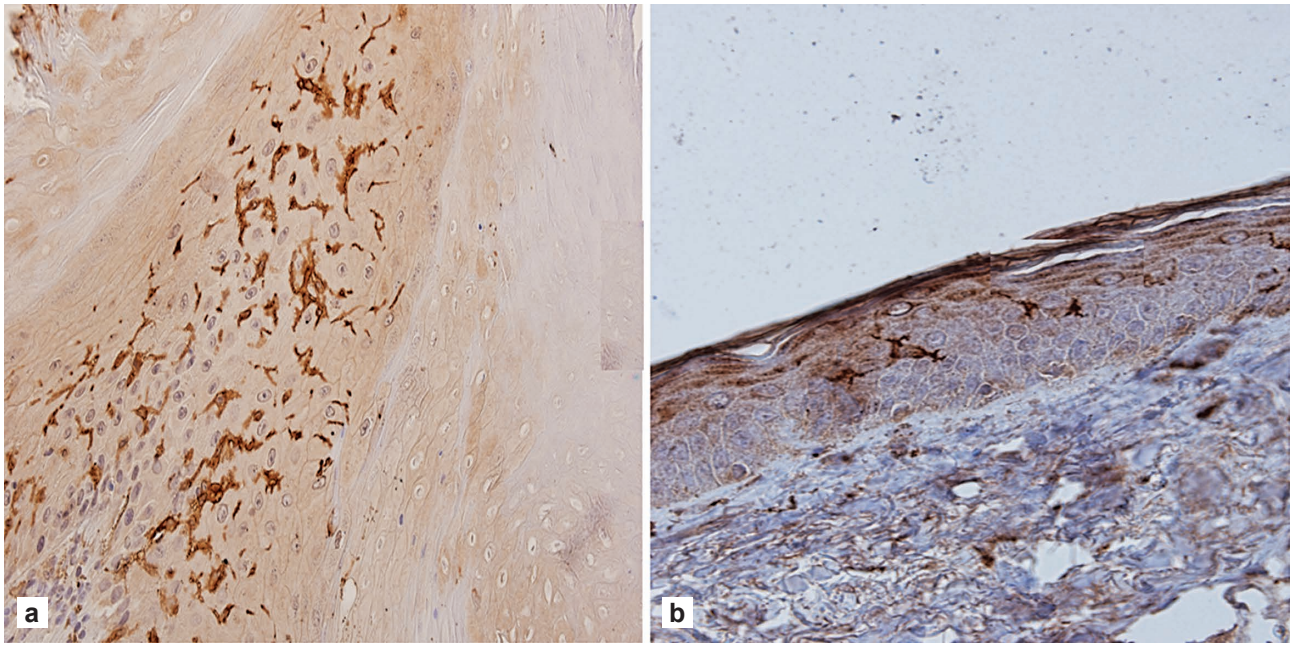

Figure 4. Immunohistochemical staining of $CD1a^+$ Langerhans cells in hypertrophic (a) and atrophic cutaneous LP (b) (× 400 magnification) demonstrates a higher number of $CD1a^+$ Langerhans in hypertrophic than atrophic cutaneous LP group.

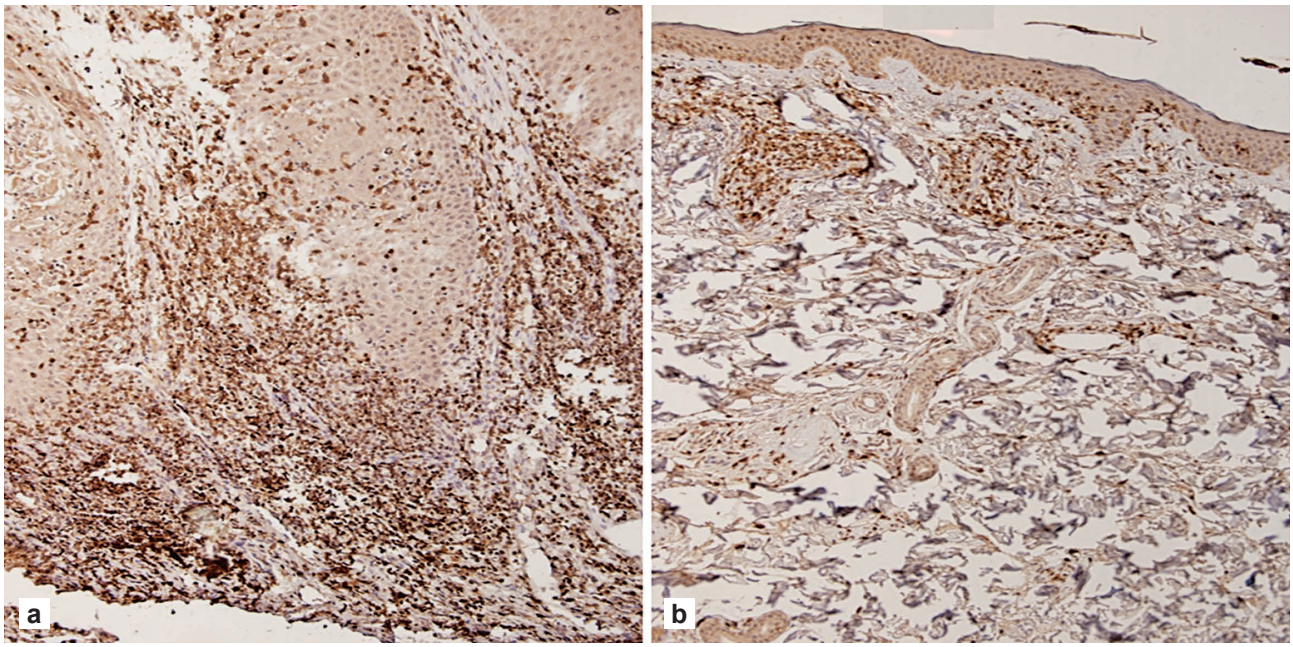

Figure 5. Immunohistochemical staining of $CD3^+$ T lymphocytes in hypertrophic (a) and atrophic cutaneous LP (b) (×100 magnification) demonstrates an increased number of CD3 in hypertrophic than atrophic cutaneous LP group.

Table 6. Correlation between immune cells in the LP group

Immune cell marker	Mast cell	CD3	CD4	CD1a
Mast cell	1	0.341*	0.263*	0.679*
CD3	0.341*	1	0.697*	0.351*
CD4	0.263*	0.697*	1	0.335*
CD1a	0.679*	0.351*	0.335*	1

*P<0.05; Pearson correlation analysis

also revealed that B lymphocytes and plasma cells were reduced in number in LP lesions. So, there is no evidence of humoral immunity in the pathogenesis of LP [3].

This study revealed that intra-epidermal cells mainly consisted of CD8 positive lymphocytes. CD4/CD8 ratio was 0.58 ± 0.14 in the epidermis that was compatible with Popvska's study on mucosal LP lesions (0.51 ± 0.12) [12]. This issue confirmed the role of CD8 cells in keratinocyte apoptosis through programmed death receptors (i.e., FAS and FASL) and release of TNFα, perforin, and granzyme [13].

Our investigation demonstrated that CD4 positive lymphocytes were the dominant intra-dermal inflammatory cells (40.78%), which is in line with Sinon's study [10] on mucosal lesions, but is in contrast with Popvska's results [13]. This difference might be explained by performing a skin biopsy in various stages of the disease. Although CD4 lymphocytes are seen in the initial stage of the disease, CD8 positive lymphocytes are the predominant intra-dermal cells [14].

LC is a subtype of dendritic cells that have been recognized as antigen presenting cells in the skin and mucos [15]. In the present study, the number and staining intensity of LCs in the LP group was significantly greater than the control group; consistent with the results of Deguchi on cutaneous LP [10]. Epidermal LCs represented interconnecting networking that can be characterized by stronger antigen presenting activity compared to a control group, with no evidence of this change.

Furthermore, the highest density of LCs was observed within the epidermis; which is contrary to the results of Devi [16]. This difference is due to skin biopsy in different stages of the disease. In normal skin and early stage of the inflammatory disease, LCs are seen in large quantities within the epidermis. Then, LCs following recognition of foreign Ag migrate toward the dermis and then through the lymphatic network to the regional lymph node. So, with the progression of the disease, LCs are seen more commonly within the dermis than theepidermis [16].

Our study indicated an intimate correlation between LCs and T lymphocytes. Thus, it can be evidenced that LCs have a role in lymphocyte augmentation through secretion of IL 12 and binding of CD40 receptor of LCs to its ligand on T lymphocyte cell. Conversely, T lymphocytes influenced the maturation of LCs by the release of TNF and IL_1 [17].

Mastocytes are immune cells containing secretory granules that predominantly are located in perivascular and periadnexal areas. Chronicity of the disease and enhancement of inflammatory reactions have been attributed to these cells [18]. In the present study, mastocytes were observed with a larger number in the LP group than the control group ($P<0.05$). In addition, mastocytes were situated mostly outside the inflammatory infiltration within the deep dermis in LP group; which is concordant with other studies [19,20]. Sharma et al. evaluated mastocytes in oral LP [19]. Mean difference of mastocytes between LP and control group was 6.74 within the band-like infiltration and 5.78 in the deep dermis, which was almost similar to our results (4.68 in the band-like infiltration and 3.60 in the deep dermis).

Our research suggests that there is an obvious connection between mastocytes and T lymphocytes. This result supports that mastocytes encourage chemotaxis of lymphocytes through the release of inflammatory mediators such as histamine, TNF α, and chymase [20].

Initially, histamine secretion led to vasodilatation and increased the permeability of vessels. Then, TNFα will increase adhesion molecules on the endothelial cell surface that can stimulate lymphocytes extravasation. Finally, chymase will activate of metalloproteinase, leading to the migration of T lymphocytes toward upper dermis [21]. Inversely, RANTES secretion from T lymphocytes attracts more mastocytes toward the inflammatory area and triggers degranulation of mast cells [22].

In the current study, LP cases based on clinical and pathologic features are categorized into four groups including classic, atrophic, bullous, and hypertrophic. This paper presents the first study on the distribution of immune cells in various types of cutaneous LP. Our results demonstrated that hypertrophic type had the most prevalent immune cells count and atrophic type had the least number of immune cells. Also, the bullous type was the second type regarding the number of immune cells. To date, no studied has assessed immune cells profile in various types of cutaneous LP, but there are a few studies on mucosal LP with confounding results [23,24].

Sinon et al. (2013) found no significant difference

between hypertrophic and atrophic type in the distribution and density of immune cells [13]. In contrast, Juneja represented the greater number of lymphocytes in the erosive (atrophic) type of mucosal LP. This study also proposed that chronicity and increased malignant change in the atrophic type of mucosal LP can be relevant to the more number of inflammatory infiltrations in these lesions [25]. Consequently, in our study, the increased number of inflammatory cells - especially mastocytes and LCs in hypertrophic cutaneous LP-represents chronicity, refractory course, and increased risk of malignant transformation similar to atrophic mucosal LP. A higher number of mastocytes in the hypertrophic type of cutaneous LP can be explained by more itching sensation in this type. Furthermore, in the present study, the bullous type had the most common type of CD8 positive T lymphocytes, which can be explained by the higher damage of basal layer and separation of epidermis from dermis in this type secondary to the cytotoxic effect of CD8 T lymphocytes. Atrophic type of cutaneous LP can be seen the later stage of other types of cutaneous LP. Therefore, it may explain the lower number of immune cells in this type.

Our study revealed that cutaneous LP significantly had a larger number of immune cells than the control group. Moreover, B lymphocytes were observed in a lower number than T lymphocytes. Thus, the immune mechanism particularly cell-mediated immunity is involved in the pathogenesis of the disease. A larger number of immune cells profile was seen in the hypertrophic type of cutaneous LP; which is consistent with the chronic course of this disease. The least number of immune cells was seen in atrophic types.

Acknowledgment

We wish to thank the Afzalipour Pathology Department for cooperating with the authors of this study.

Conflict of Interest: None declared.

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