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Immunohistochemical Evaluation of TNF-α, IL-1, IL-12, IL-17, IL-23 Expression and Investigation of the Effect of Demodex in Patients with Discoid Lupus Erythematosus

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Abstract

Objective: Discoid lupus erythematosus (DLE) is a chronic inflammatory skin disease that can be triggered by several factors although its etiology is not yet known. Hypotheses have been reported that the demodex mites may be involved in the etiopathogenesis of DLE. In this study, we aimed to investigate the potential relationship between the frequency of immunohistochemical staining of tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-12, IL-17 and IL-23 cytokines obtained from cutaneous biopsy of DLE patients and disease severity.

Materials and Methods: Biopsy tissues of patients who were previously diagnosed with DLE in the dermatology outpatient clinic, which were also supported histopathologically, were re-sectioned and subjected to immunohistochemical examination for TNF-α, IL-1, IL-12, IL-17 and IL-23, and their staining scores were obtained. These immunohistochemical staining scores were compared with disease severity. The presence and density of demodex were evaluated in standard skin surface biopsy taken from the lesions at the time of diagnosis.

Results: In the comparison of immunohistochemical staining scores with DLE-skin score (DLE-SS), a statistically significant positive correlation was found between DLE-SS and TNF-α (p=0.003), DLE-SS and IL-17 (p=0.002). There was no difference between the presence or absence of demodex and DLE-SS (p=0.9). There was no correlation between demodex density and disease severity in demodex-positive cases (p=0.34).

Conclusion: In line with the data obtained from our study, TNF-α and IL-17 seem to be more associated with the disease severity in DLE. The fact that demodex positivity/negativity and demodex density are independent of disease severity supports that demodex mite is an etiopathogenetic factor rather than overlap in DLE cases. Further studies on this subject are needed.

Keywords: Discoid lupus erythematosus, immunohistochemistry, TNF-α, IL-1, IL-12, IL-17, IL-23

Introduction

Discoid lupus erythematosus (DLE) is a permanent inflammatory skin illness that can be provoked by multiple factors (1). Chronic inflammation in DLE poses a hazard for squamous cell cancer, albeit rarely over time (2). For this reason, elucidating the unknown aspects in etiopathogenesis, early diagnosis and treatment improve the prognosis (2,3). Although its etiopathogenesis has not been elucidated, hereditary and environmental factors are considered to have a role. Ultraviolet (UV) has a key role in the pathogenesis of DLE. It triggers keratinocyte apoptosis, ensures the transport of nucleoprotein autobodies to the keratinocyte cell surface, and leads to an increase in inflammatory cytokines [interleukin (IL)-1, IL-6, IL-8, IL-10, IL-17, tumor necrosis factor (TNF)-α, interferons (IFN)] (4).

Increased TNF-α in DLE is responsible for chronic inflammation. However, the reduction of TNF-α with an anti-TNF-α drug can trigger DLE-like lesions (4). TNF-α arranges B-cells, rises the production of inflammatory

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molecules, and hinders the output of IFN-α. The rise in the inflammatory cytokines IL-1, IL-6, IL-10, IL-12, IL-17, and IL-18 augments the inflammation in DLE (5). The relationship of TNF-α and IL-17 with disease activation and clinical course in patients with systemic lupus erythematosus (SLE) has been demonstrated in studies (4-7). There has been no previous study in the literature examining the immunohistochemical expression of TNF-α, IL-1, IL-12, IL-17 and IL-23 cytokines in DLE cases. In our study, we planned to detect TNF-α, IL-1, IL-12, IL-17, IL-23 expression immunohistochemically in DLE cases and to evaluate its relationship with the activation of the disease.

Materials and Methods

Patient Selection and Study Design

We chose patients who admitted to our outpatient clinic between 2016 and 2021 with the complaint of scaly, itchy red spots or plaques on the face and were diagnosed with new-onset DLE as a result of histopathological evaluation of the tissue obtained from cutaneous biopsy. Disease severity discoid lupus erythematosus skin score (DLE-SS), anti-nuclear antibody (ANA) profiles, presence and number of demodex mites obtained by standardized skin surface biopsy (SSSB) were recorded by scanning the files of the cases. As exclusion criteria, those under 18 years of age or pregnant patients who did not have stable course of these inflammatory cytokines were excluded. To avoid possible histopathological and immunohistochemical changes, cases who had taken hydroxychloroquine, corticosteroid, azathioprine, methotrexate and cyclosporine in the last 3 months were also excluded. Re-sections were made from the blocks of cutaneous biopsy tissue materials at the time of diagnosis, immunohistochemical TNF-α, IL-1, IL-12, IL-17 and IL-23 stains were performed, and staining scores were recorded. The study was approved by the Local Ethics Committee of Necmettin Erbakan University (date: 16.04.2021, no: 2021/3191).

Disease Severity Discoid Lupus Erythematosus Skin Score

DLE-SS is the scoring system evaluating the erythema, induration, squam and atrophy in the lesions of DLE cases and assigning a value between 0 and 4 for each, in which a total of 0-16 results is obtained (6).

Standardized Skin Surface Biopsy

SSSB is a non-invasive quantitative method used to evaluate demodex density in a standard 1 cm^2 field on a microscope slide (1). A bead of cyanoacrylate adhesive was accommodated on a 1 cm^2 area that was drawn by a waterproof pen, on a slide glass and the adhesive face was applied to the skin of the lesion. After allowing the adhesive to dry on the skin (approximately 1 minute), the slide was peeled off gently (8). A drop of immersion oil was dropped on it and the examples slides were examined microscopically at x40 magnification. The presence and density of demodex were evaluated in standard skin surface biopsy taken from the lesions at the time of diagnosis, all evaluations were made by the same dermatologist to decrease errors in technique and microscopic examinations.

Immunohistochemical Staining and Evaluation

Paraffin-embedded tissue blocks of patients with histopathological diagnosis of DLE were obtained from the archives of the pathology laboratory. Sections of 4-micron thickness were taken on positively charged slides using a rotary microtome. Sections were kept in an autoclave at 80°C for an average of 5 minutes to melt the paraffin. Slides were transferred to the Ventana XT instrument for immunohistochemical studies. Antibodies against TNF-α (1:100; St John's Lab's), IL-12A (1:150; St John's Lab's), IL-1R11 (1:150; St John's Lab's), IL-17B (1:50; St John's Lab's), and IL-23 (P19) (1:100; Biolegend) were applied. Stained slides were investigated under an Olympus BX46 light microscope by a single pathologist. The count of positively stained cells [keratinocytes, inflammatory cells (lymphocytes and neutrophils), endothelial cells] and staining consistency were evaluated (Figure 1a-c).

The definitions by Morar et al. (9) were based on the histochemical evaluation. A semi-quantitative assessment was performed for both the count of positively stained cells (keratinocytes, lymphocytes, endothelial cells, and fibroblasts) and the consistency of the immunostaining. The count of positive cells was graded with a score from 0 to 6 (count of stained cells), the immunostaining consistency was graded from 0 to 3. To calculate the total score in each microscopic area analyzed, the score for the count of positively stained cells was multiplied by the corresponding immunostaining intensity score. The immunohistochemical staining score for each patient was calculated as follows: Immunohistochemical staining score = count of positively stained cells score x staining consistency. Stained cell counts (0: No stained cells, 1: 1-5 cells, 2: 6-10 cells, 3: 11-50 cells, 4: 51-100 cells, 5: 101-150 cells, 6: more than 150 cells) and staining intensities (0: No staining, 1: Slight pale staining, 2: Moderate staining, 3: Strong staining) were graded. Immunohistochemical staining score evaluation is shown in Figures 1-3.

Statistical Analysis

The SPSS 25.0 statistical schedule (SPSS Inc., Chicago, IL) was employed to analyze the data. Mean ± standard

deviation and median (min-max) values were employed to summarize numerical data. Number (n) and percent (%) distributions were employed to summarize categorical data. Relationships between numerical data were evaluated with the Student's t-test for independent samples when normality expectations were met and with non-parametric equivalents of the same tests when normality was not met. The Pearson correlation analysis test was applied for the relationship between parameters. For the p-value, <0.05 was stated as statistically significant.

Results

The mean age of the patients included in the study was 42.9 ± 9.4 years. Of the 44 patients included in the study, 22 (50%) were female. ANA results of 27 patients (61.4%) were negative. Demodex mite was not detected in

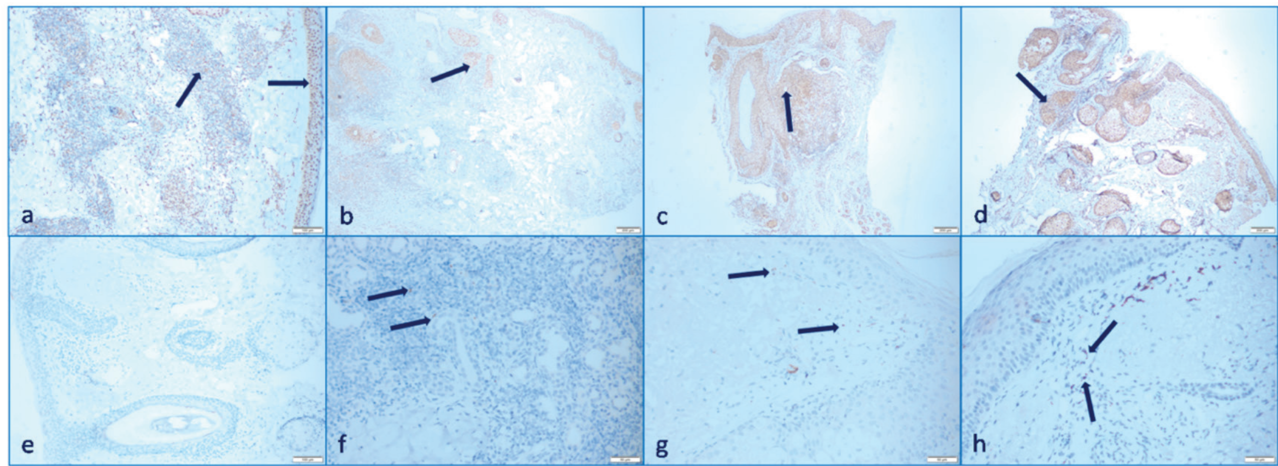

Figure 1. a-d: Immunohistochemical TNF, epidermis cells, endothelial cells and lymphocytes showing positive expression in different density and distribution, a and b: (2+), **c** and d: (3+), **e-h:** Immunohistochemical IL-17. (e: negative expression; f-h: lymphocytes with positive expression in different density and distribution (1, 2 and 3 + expression, respectively).
TNF: Tumor necrosis factor

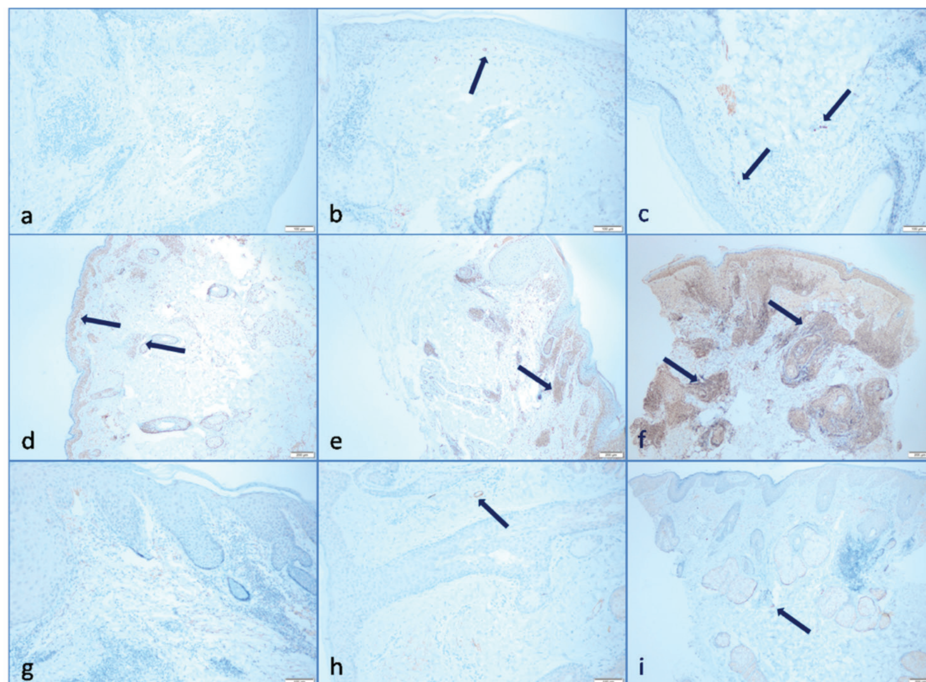

Figure 2. a-c: Immunohistochemical IL-1, [a: Negative expression; b and c: Lymphocytes with positive expression in different density and distribution (2 and 3+, respectively)], **d-f:** Immunohistochemical IL-12, epidermis cells, [endothelial cells and lymphocytes showing positive expression in different densities and distribution (1, 2 and 3+, respectively)], **g-i:** Immunohistochemical IL-23 [g: Negative expression; b: Positive expression in endothelium (2+), positive expression in lymphocytes (1+)].
IL: Interleukin

23 patients in the SSSB taken from the lesions (52.3%). Demodex positive cases were found to have an average of 9.6 ± 4.3 (min: 4, max: 20) demodex mites per cm^2. The mean DLE-SS score of the subjects included in the study, which were evaluated at the time of diagnosis, was 8.5 ± 3.3 (min: 2, max: 16). There was no statistically significant difference between disease activation score and gender (p=0.62), ANA positivity (p=0.84), demodex positivity (p=0.9). There was no correlation between demodex density and illness severity in demodex-positive cases (p=0.34).

When the correlation of immunohistochemical staining scores and DLE-SS was evaluated, a positive correlation was found between TNF-α staining score and disease severity (p=0.003). Similarly, a positive correlation was found between IL-17 staining score and disease severity (p=0.002). Figure 3 shows the correlation graph between TNF-α staining score and DLE-SS, and Figure 4 shows the correlation between IL-17 staining score and DLE-SS. No significant correlation was found between IL-1 staining score (p=0.28), IL-12 staining score (p=0.18), IL-23 staining scores (p=0.22) and disease severity.

No statistically significant correlation was found between demodex density and staining scores in demodex-positive cases. When demodex positive and negative cases were compared, no significant difference was found between the two groups in terms of TNF-α, IL-1, IL-12, IL-17 and IL-23 staining scores.

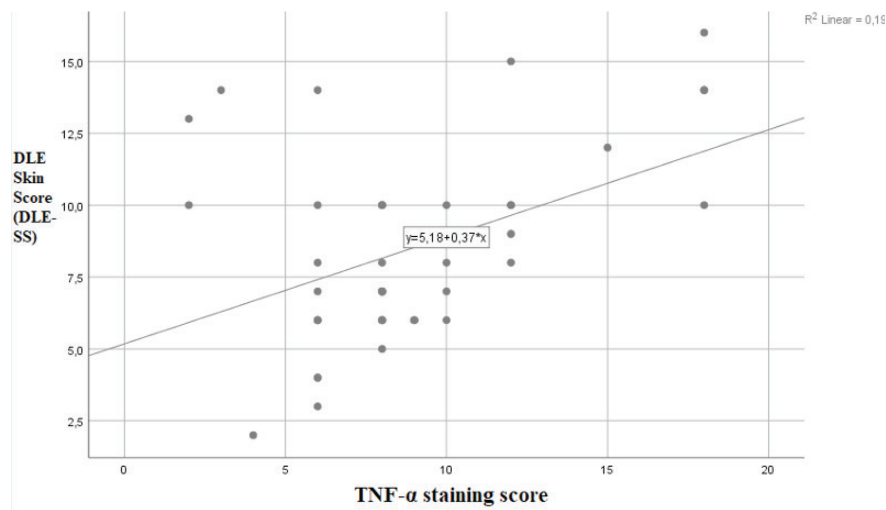

Figure 3. The correlation graph between TNF-α staining score and DLE-SS.
TNF: Tumor necrosis factor, DLE-SS: Discoid lupus erythematosus skin score

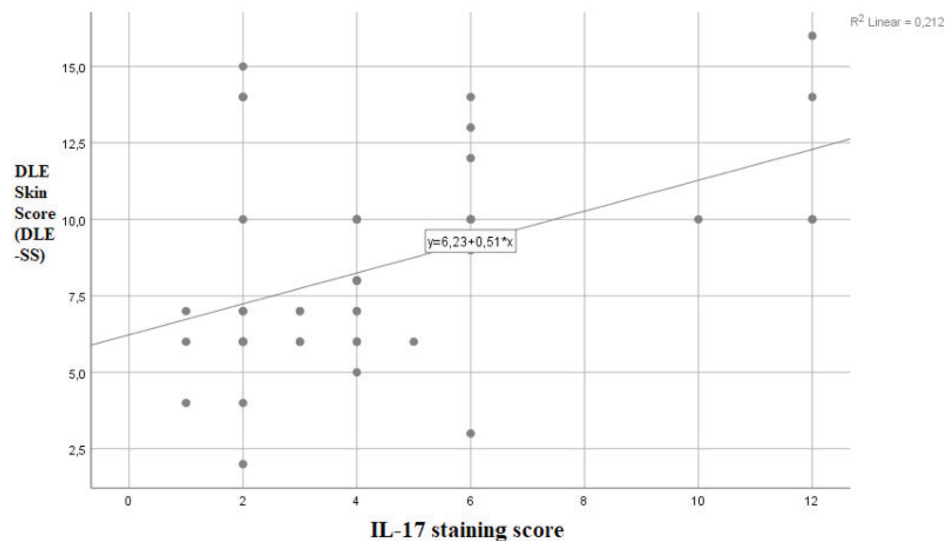

Figure 4. The correlation graph between IL-17 staining score and DLE-SS.
DLE-SS: Discoid lupus erythematosus skin score, IL: Interleukin

Discussion

DLE is the most widespread variant of cutaneous lupus erythematosus (CLE) and presents with lesions on the face, lips and sun-exposed area on the skin with hyperkeratotic, erosive and sometimes follicular prominence and atrophic scars. Genetic and environmental triggering (such as immune dysregulation, inflammation, UV) factors of DLE have been identified (10). The role of the immune system and the inflammatory mechanism in etiopathogenesis has been supported by various studies (11). In a study from the Netherlands in 1991, frozen sections of biopsy samples from 20 DLE patients were examined immunohistochemically (11). It was noted that the inflammatory infiltrates consisted mainly of T lymphocytes and monocytes/macrophages, with only a few Langerhans cells (11). It has also been reported that plasmacytoid dendritic cells (pDCs), CD4 T lymphocytes (Th1 is more dominant than Th2), CD8 T lymphocytes and B lymphocytes are involved in the etiopathogenesis, and in fact, DLE is highly associated with the immune system, beyond being an autoimmune disease (10,12). Type I IFNs (IFN-α and IFN-β) are involved in the pathophysiology of DLE through their connections to pDCs and apoptotic pathways (13). JC O'Brien et al. (14) retrospectively examined skin biopsies of 30 DLE patients in a study they conducted and evaluated inflammatory cells. They observed that $CD3^+$ and $CD4^+$ T-cells were the most predominant inflammatory cells, regardless of the stage of the disease. In the same publication, it was stated that CD8 T lymphocytes were more dominant in the initial phase, leading to keratinocyte apoptosis and caspase activation (14). The predominance of CD4 Th1 cells in DLE enables related pro-inflammatory cytokines to play a role in the etiopathogenesis of DLE. The increased secretion in the inflammatory cytokines IL-1, IL-6, IL-10, IL-12, IL-17, and IL-18 augments the inflammation in DLE (5,15). In a study conducted on 8 patients with a clinical and histopathological diagnosis of DLE, type 1 cytokine levels were investigated (16). TNF-α level was found to be significantly higher in 8 biopsy specimens. This has been attributed to TNF-α being a potent inducer of local inflammation. IL-1β messenger RNA was not found in all 8 specimens (16). In our study, a significant correlation was found between DLE disease severity and TNF-α staining score.

Elevated serum IL-17 levels have been found in DLE and SLE patients (17). In another study, serum IL-17A, IL-17F levels and the number of Th17 cells were significantly higher in SLE and DLE patients compared to healthy controls (18). In our study, we found a significant correlation between DLE disease activity and IL-17 staining score. IL-17 stimulates T-cells and increases the production of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-17, IL-22, etc.). In the literature, 9 CLE and 1 DLE patients, for whom ustekinumab, a drug that inhibits the differentiation of T-cells into Th17 through inhibition of IL-12/23, was successfully used in DLE treatment, have been reported (19).

Although demodex infection is usually asymptomatic, demodex mites are thought to play a role in the pathogenesis of some dermatoses such as rosacea and pityriasis folliculorum by causing the formation of inflammatory lesions (1). Increased mite density can cause suppurative and granulomatous inflammation (1,20). In addition, Akilov and Mumcuoglu (21) have hypothesized that NK2 cells are responsible for the destruction of Demodex mites and that the fragmented parts of the mites reason the activation of this lymphocyte subpopulation. In the existence of Cw2 or Cw4 phenotypes, the killing activity may direct the body's own T lymphocytes, which can be thought to cause autoinflammation (21). It is conceivable that the demodex-positive DLE variant may be associated with such a local trigger (1,21). In our study, no significant relationship was found between the presence and density of demodex and the severity of the disease. Our findings suggested that demodex mite was one of the etiopathogenetic factors of DLE, rather than being an overlap condition accompanying severe DLE cases.

DLE is a long-lasting, scarring autoimmune disease, and early effective treatments (such as topical corticosteroids, intralesional steroids, topical tacrolimus, antimalarials, methotrexate, azathioprine, and also anti-TNF, anti-IL inhibitors) improve its clinical course (22). Our study showed that more attention should be paid to TNF-α and IL-17 cytokines in the etiopathogenesis of DLE and severe disease. We think that these cytokines may be target cytokines for treatment in the future.

Conclusion

The fact that the presence of demodex and the density of demodex are independent of disease severity supports that demodex parasite is an etiopathogenetic factor rather than being overlapping one in DLE cases. Our results will shed more light on the treatment with these cytokines in the future by evaluating the relationship between DLE pathogenesis and disease severity of these cytokines, which are important in many autoimmune and inflammatory diseases. This is the first study to examine the immunohistochemical expression of TNF-α, IL-1, IL-12, IL-17 and IL-23 cytokines in DLE cases.

Ethics

Ethics Committee Approval: The study was approved by the Local Ethics Committee of Necmettin Erbakan University (date: 16.04.2021, no: 2021/3191).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: R.D., S.A.T., Concept: R.D., S.A.T., Design: R.D., S.A.T., Data Collection or Processing: R.D., S.A.T., S.F., K.D., P.O., Analysis or Interpretation: R.D., S.A.T., S.F., K.D., P.O., Literature Search: R.D., S.A.T., Writing: R.D., S.A.T.

Conflict of Interest: No conflict of interest was declared by the authors.

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