CLINICAL SCIENCE

IL-2, IL-5, TNF-α and IFN-γ mRNA expression in epidermal keratinocytes of systemic lupus erythematosus skin lesions

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OBJECTIVE: To analyze cytokine gene expression in keratinocytes from patients with systemic lupus erythematosus (SLE).

INTRODUCTION: Keratinocytes represent 95% of epidermal cells and can secrete several cytokines.

METHODS: Keratinocytes were obtained by laser microdissection from 21 patients with SLE (10 discoid and 11 acute lesions) at involved and uninvolved sites. All patients were receiving a low/moderate prednisone dose and 18 were receiving chloroquine diphosphate. IL-2, IL-5, TNF-α and IFN-γ gene expression was evaluated by real-time PCR and expressed as the ratio (R) to a pool of skin samples from 12 healthy volunteers.

RESULTS: Heterogeneity in cytokine gene expression was found among patients with SLE. Eighteen of 38 valid SLE samples (47%) presented overexpression ($R>1$) of at least one cytokine. Lesional skin samples tended to show higher cytokine expression than samples from uninvolved skin ($p=0.06$). IL-5 and IFN-γ were the most commonly overexpressed cytokines. Samples with cytokine overexpression corresponded to more extensive and severe lesions. Prednisone dose did not differ between samples without cytokine overexpression (15.71 ± 3.45 mg/day) and those with overexpressed cytokines (12.68 ± 5.41 mg/day) ($p=0.216$). Samples from all patients not receiving diphosphate chloroquine had at least one overexpressed cytokine.

CONCLUSIONS: The heterogeneous keratinocyte cytokine gene expression reflects the complex immunological and inflammatory background in SLE. Patients with severe/extensive skin lesions showed a higher frequency of cytokine gene overexpression. Increased IFN-γ and IL-5 expression suggests that Th1 and Th2 cells are involved in SLE skin inflammation. The possibility that prednisone and antimalarial drugs may have contributed to low cytokine gene expression in some samples cannot be ruled out.

KEYWORDS: Cytokines; Gene expression; Interleukins; Lupus.

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INTRODUCTION

Skin lesions occur in roughly 80% of patients with systemic lupus erythematosus (SLE), with variable severity and morbidity.[1] The most common forms of skin involvement in SLE are acute exanthema, discoid lupus, photosensitive erythema and subacute cutaneous lupus erythematosus. The clinical and histopathologic features of each of these lesions have been extensively defined.[1,2] Immunologic disorders in several inflammatory diseases have been characterized according to the dominant cytokine pattern of the infiltrating $CD4^+$ T cells. A Th1 pattern is characterized by predominance of interleukin 2 (IL-2) and interferon-γ (IFN-γ), whereas a Th2 pattern is characterized by predominance of IL-4, IL-5, IL-6, IL-10 and IL-13. The type and intensity of the immune disorder may vary from organ to organ in each pathologic condition. Keratinocytes represent 95% of the cells in the epidermis and have been shown to be involved in the production of a variety of cytokines, such as IL-1, tumor necrosis factor-α (TNF-α), IL-6 and granulocyte macrophage-colony-stimulating factor. A Th1 pattern has been well documented in skin lesions from patients with tuberculoid leprosy and psoriasis.[3,4]

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Conversely, a Th2 pattern has been demonstrated in skin lesions of lepromatous leprosy and Sezary syndrome.[3,5]

The cytokine pattern in skin lesions from patients with SLE has already been discussed in previous studies. Nürnberg et al. examined protein and messenger RNA (mRNA) expression for IL-6 in the epidermal layer of affected and non-affected skin in patients with acute, subacute and discoid lupus erythematosus.[6] Increased expression of IL-6 and its mRNA was observed in the basal layer of keratinocytes in most samples of affected skin but not in non-affected samples.[6] Using reverse transcriptase polymerase chain reaction (RT-PCR), Stein et al. studied mRNA production for several cytokines in whole skin samples from healthy volunteers and from acute, subacute cutaneous and discoid lesions of patients with SLE. IFN-γ and IL-5 were detected in the majority of samples from patients with SLE, IL-10 was present in half the samples, IL-2 in a few samples and IL-4 was not detected. Normal samples expressed mRNA only for IFN-γ and IL-10.[7] Using a similar approach, Toro et al. demonstrated elevated levels of mRNA for IFN-γ, IL-2 and TNF-α in whole skin samples from discoid lupus lesions. No transcripts for IL-1 and IL-4 were detected in these lesions.[8]

The contribution of epidermal keratinocytes to the cytokine milieu in the various SLE skin lesions has not been fully elucidated since most studies used whole skin as source of mRNA. It is well known that epidermal keratinocytes are considerably affected by ultraviolet radiation and that this environmental factor is associated with triggering or exacerbation of SLE. This study was designed to analyze the cytokine expression pattern in isolated epidermal keratinocytes in patients with acute skin lesions and discoid SLE. Based on previous literature findings, we selected for study Th2 cytokine IL-5 and Th1 cytokines IL-2 and IFN-γ. Owing to the remarkable response of SLE skin lesions to drugs that interfere with TNF-α (e.g., thalidomide), we analyzed the expression of this cytokine also.

MATERIALS AND METHODS

Two skin 4 mm punch biopsy samples (one from affected and one from non-affected skin) were obtained from each of 21 sequentially selected adults with active SLE (11 with acute lesions and 10 with discoid lesions)—that is, 22 samples from patients with acute lesions and 20 from patients with discoid lesion. Skin 4 mm punch biopsy samples were also obtained from 12 healthy volunteers. Patients with SLE were classified according to the criteria established by the American College of Rheumatology.[9] Patients using immunosuppressive drugs other than glucocorticosteroids were excluded. All subjects signed an informed consent form previously approved by the institutional ethics committee. Samples were immediately frozen in liquid nitrogen and stored at −80℃ until use. Frozen samples were subjected to microdissection under optical microscopy[10] and 5–10 µg of the epidermal layer was minced and used for total RNA extraction with the Trizol® reagent (Invitrogen, Carlsbad, CA, USA). The RNA pellet was solubilized in 10 µL DEPC-treated distilled H_2O. Reverse transcription was performed with RNA samples shown by UV spectrophotometry to be free of protein and phenol. Briefly, 0.5 µg total RNA and 2 µL oligo dT 0.5 µg/mL in DEPC-treated H_2O qsp 14 µL were incubated at 65℃ for 5 minutes and immediately quenched on ice. After adding 4 µL dithiothreitol 10 mM, 2 µL dNTP 10 mM and 8 µL First Strand buffer (Pharmacia, Uppsala, Sweden), the solution was brought to 55℃ and completed with 200 U Superscript II® (Pharmacia). The solution was then incubated at 55℃ for 60 minutes and at 85℃ for 15 minutes. The cDNA obtained was stored at -80℃ until processing for PCR.

Primers were designed with the aid of the software PrimerExpress (Applied Biosystems Inc, Foster City, CA, USA) for TNF-α (sense, CCG AGG CAG TCA GAT CAT CTT; anti-sense, AGC TGC CCC TCA GCT TGA), IL-2 (sense, AAG AAT CCC AAA CTA ACC AGG AT; anti-sense, TCT AGA CAT GAA GAT GTT TCA GTT CTC), IL-5 (sense, AAG AGA CCT TGG CAC TGC TTT C; anti-sense, GGA ACA GGA ATC CTC AGA GTC TCA) and IFN-γ (sense, TGT AGC GGA TAA TGG AAC TCT TTT; anti-sense, AAT TTG GCT CTG CAT TAT T). The predicted sizes of the amplicons were 85 bp, 86 bp, 73 bp and 85 bp, respectively. Real-time PCR was carried out with 15 ng cDNA and 12.5 µL Sybr Green PCR Master Mix (Applied Biosystems Inc) in an ABI 5700 Sequence Detection System (Applied Biosystems Inc) with the following protocol: 2 minutes at 50℃, 10 minutes at 95℃ and 45 cycles of 15 seconds at 95℃ and 1 minute at 60℃. The primer concentration was optimized as 200 nM for TNF-α and IFN-γ, 600 nM for IL-2 and 900 nM for IL-5. Amplification of the reference gene β-actin (sense TAA TGT CAC GCA CGA TTT CCC and anti-sense TCA CCG AGC GCG GCT at 300 nM) was performed for each sample. All PCRs were carried out in duplicate for each sample. When differing results were obtained the duplicate run was repeated. In addition, each run contained duplicate wells with no cDNA (non-template control) and wells with a pool of cDNA from the 12 normal controls. The specificity of each primer was verified both by assessing the band size on agarose gels and by measuring the melting profile of the amplicon. All reactions were subjected to dissociation curve analysis to confirm that results were specific to one single amplified product. The melting temperature for each of the amplicons was 77℃ for IL-2, 79℃ for IL-5, 83℃ for TNF-α, 78℃ for IFN-γ and 84℃ for β-actin. The relative quantification of cytokine transcripts was calculated according to Pfaffl's mathematical model:[11]

$$R = \frac{(\text{E cytokine})^{\Delta\text{CT cytokine gene (control pool} - \text{sample)}}}{(\text{E } \beta - \text{actin})^{\Delta\text{CT } \beta - \text{actin gene (control pool} - \text{sample)}}}$$

where E is the efficiency of the amplification and CT is the threshold cycle. The normal pool was arbitrarily ascribed an R of 1. Samples with $R>1$ were considered overexpressed and samples with $R<1$ were considered underexpressed for the respective cytokine gene.

Association between qualitative parameters was analyzed by χ^2 test and Fisher's exact test, when appropriate. Comparison of quantitative parameters between two groups was performed by Student's t test. Variance between two groups was assessed by the Levene's test. All analyses were performed using the computer-based statistical software package SPSS, version 13. An inference level of 0.05 was established for all analysis.

RESULTS

In general, demographic, clinical and laboratory features of the studied subjects were equivalent among the groups; however, patients with discoid lesion were slightly older than subjects in the two other groups (Table 1). In addition, patients with acute skin lesion had a higher Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and higher daily prednisone dose than those with discoid lesion. Four of the 42 SLE skin biopsy samples were excluded because there was no amplification signal for any of the tested transcripts and thus these 4 samples were considered as inappropriate RNA isolates (all were from non-affected skin: 2 from patients with discoid lesion and 2 from patients with acute lesion).

There was a wide variation in the expression level of the four cytokines in the 38 valid samples (Figure 1). Among the 80 amplification reactions for the 4 cytokines in the 20 samples from patients with acute lesion (11 from affected skin and 9 from non-affected skin), 12 (15%) showed overexpression and 68 (85%) showed underexpression of the respective cytokine. Among the 72 amplification reactions in the 18 samples from patients with discoid lesion (10 from affected skin and 8 from non-affected skin), 9 (12.5%) showed overexpression and 63 (87.5%) showed underexpression of the respective cytokine. Despite the fact that most of the 152 amplification reactions showed underexpression, 18 (47%) of the 38 valid samples showed overexpression of at least one cytokine and 3 (8%) showed combined overexpression of two cytokines. A comparison of the cytokine expression in affected (acute and discoid lesion) and non-affected skin samples showed a trend towards higher cytokine overexpression in affected skin samples. Among the 84 amplification reactions in affected skin samples (acute and discoid lesion), 16 (19%) showed overexpression of the respective cytokine and among 68 amplification reactions in non-affected skin samples, only 5 (7%) showed overexpression of the respective cytokine ($p = 0.06$). Overexpression of one and two cytokines was observed in 10 (48%) and 3 (14%) of the 21 samples from affected skin, respectively. In comparison, only 5 (29%) samples from the 17 non-affected skin samples showed overexpression of one cytokine and none had overexpression of two cytokines ($p = 0.078$).

The same trend was observed when analyzing patients with discoid and acute lesions separately, although the sample size was too small for statistical analysis. Among the 11 valid samples from affected skin with acute lesion, 2 (18.2%) had two cytokines overexpressed, 5 (45.5%) had one cytokine overexpressed and 4 (36.4%) had all cytokines underexpressed or undetectable. In contrast, among the 9 valid samples from unaffected skin of patients with acute lesion, 3 (33%) had one cytokine overexpressed and 6 (67%) had all cytokines underexpressed or undetectable. For discoid lesions, among the 10 valid samples from affected skin, 1 (10%) had two cytokines overexpressed, 5 (50%) had one cytokine overexpressed and 4 (40%) had all cytokines underexpressed or undetectable. In contrast, among 10 samples from unaffected skin of patients with discoid lesion, 2 (20%) showed one cytokine overexpressed and 8 (80%) showed all cytokines underexpressed or undetectable.

Overexpression was observed for IL-5 in 8 samples (5 affected and 3 non-affected skin), for IFN-γ in 8 samples (7 affected and 1 non-affected skin), for TNF-α in 3 samples (all affected skin) and for IL-2 in 2 samples (1 affected and 1 non-affected skin). Among the 21 samples from affected skin (acute and discoid lesion), two presented overexpression for IL-2, four for IL-5, three for TNF-α and seven for INF- γ. In contrast, among the 17 samples from non-affected skin (from patients with acute and discoid lesion) one showed overexpression for IL-2, three for IL-5, one for INF-γ and none for TNF-α.

All patients in this study were using prednisone in doses varying from 5 to 20 mg/day, 18 (86%) of them were using chloroquine diphosphate and none was receiving immunosuppressant therapy. The seven patients with no overexpressed cytokine were using a prednisone dose of 15.71 ± 3.45 mg/day while the 14 patients with at least one overexpressed cytokine had a mean prednisone dose of 12.68 ± 5.41 mg/day ($p = 0.216$). All three patients not receiving chloroquine diphosphate had at least one overexpressed

Table 1 - Demographic, clinical and laboratory characterization of the studied subjects.

Demographic, clinical and laboratory features	SLE with acute lesions (n = 11)	SLE with discoid lesions (n = 10)	Normal controls (n = 12)	p Value
Age (years), mean ± SD	32.2 ± 9.2	44.0 ± 4.2	36.8 ± 7.97	0.003
Gender (female/male), n	9/1	9/1	11/1	0.628
Ethnic group (white/black), n	6/5	4/6	5/7	0.757
Disease duration (years), mean ± SD	10.7 ± 5.8	15.2 ± 7.8	–	0.181
Articular involvement	10 (91)	9 (90)	–	NS
Alopecia	5 (45)	6 (60)	–	NS
Mucosa ulceration	2 (18)	1 (10)	–	NS
Photosensitivity	11 (100)	10 (100)	–	NS
Cutaneous vasculitis	2 (18)	1 (10)	–	NS
Kidney involvement	6 (55)	4 (40)	–	NS
Hematologic involvement	3 (27)	2 (20)	–	NS
Serositis	1 (9)	1 (10)	–	NS
Neuropsychiatric involvement	2 (18)	0 (0)	–	NS
Antinuclear antibodies	10 (91)	9 (90)	–	NS
Hypocomplementemia	5 (45)	3 (30)	–	NS
SLEDAI, mean ± SD	16.8 ± 4.26	10.0 ± 3.71	–	<0.001
Prednisone dose (mg/day), mean ± SD	15.9 ± 4.36	11.25 ± 4.60	–	0.028
Antimalarial drugs	11 (100)	7 (70)	–	NS

Results are shown as number (%) unless stated otherwise.
SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

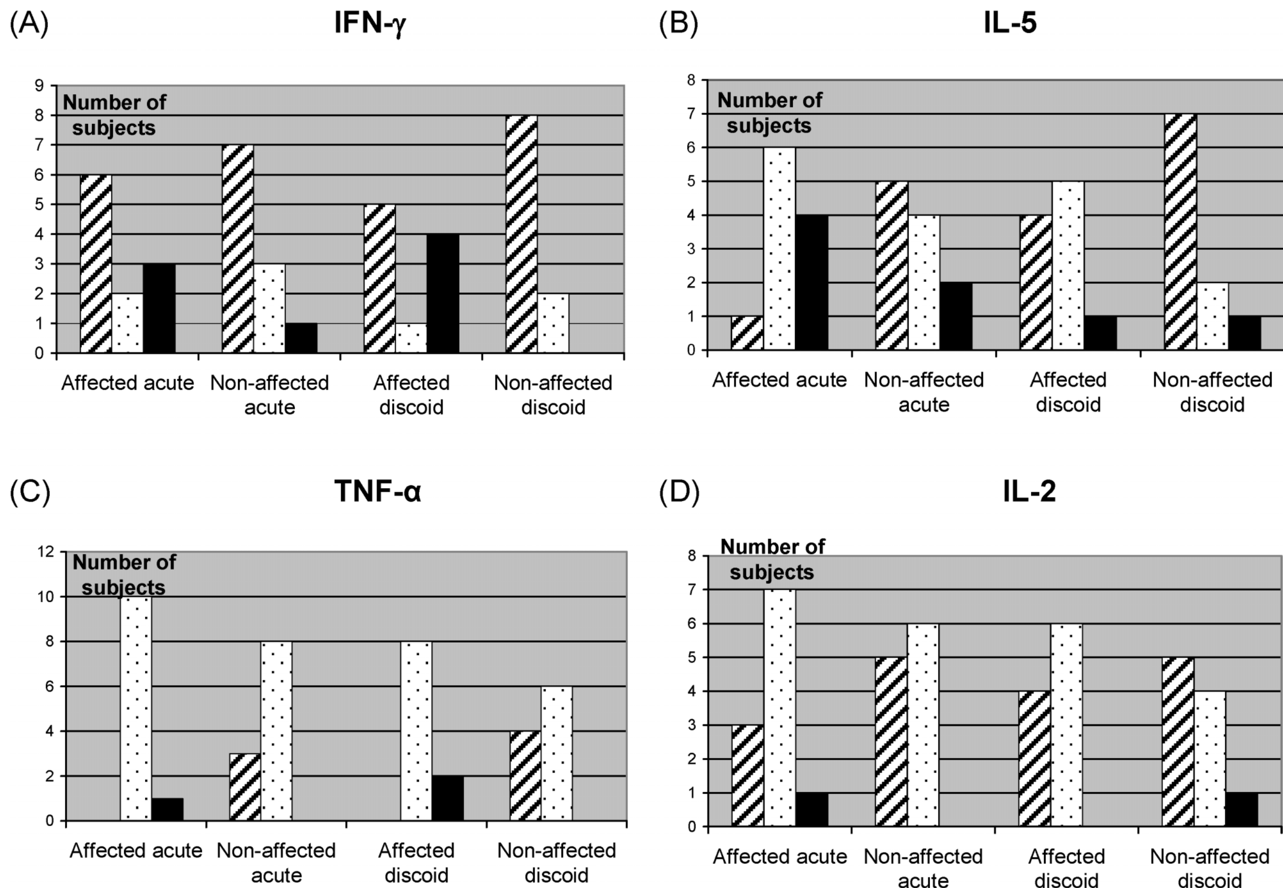

Figure 1 - Distribution of the skin samples from patients with systemic lupus erythematosus (SLE) according to the expression of mRNA for (A) interferon-γ (IFN-γ), (B) interleukin-5 (IL-5), (C) tumor necrosis factor-α (TNF-α) and (D) interleukin-2 (IL-2). Total RNA from the keratinocyte layer of epidermis from acute lesions, discoid lesions and non-affected skin from patients with SLE was subjected to reverse transcription and real-time PCR for the reference gene β-actin and the relevant cytokine genes. Cytokine transcript was quantified relative to gene expression in polled samples from normal keratinocytes (R) and was classified into three groups: ▨ not detected; □ underexpressed: R<1; ■ overexpressed: R>1.

cytokine and one of them had two overexpressed cytokines. Among valid samples from patients receiving chloroquine diphosphate, 15 (40%) had at least one overexpressed cytokine and only 2 (5%) had two overexpressed cytokines. No association was observed between SLEDAI and cytokine expression (data not shown). However, the 3 samples presenting overexpression of more than one cytokine were derived from patients with intense and diffuse skin involvement.

DISCUSSION

This study has shown the considerable heterogeneity in gene expression for IL-2, IFN-γ, IL-5 and TNF-α in keratinocytes from acute and discoid lesions from patients with SLE. Although the majority of the amplification reactions for mRNA extracted from patients with SLE showed underexpression of the respective cytokine in relation to the normal control pool, nearly half the SLE specimens showed overexpression of at least one cytokine. IFN-γ and IL-5 were the most commonly overexpressed cytokines. All patients receiving no drugs showed hyperexpression of at least one cytokine but as their numbers were few, the influence of medication on cytokine expression could not be clearly demonstrated. There was no correlation between SLEDAI and cytokine expression but there was a clear trend for cytokine overexpression in samples from patients with extensive skin involvement.

Keratinocytes represent 95% of cells in the epidermis and have been shown to be involved in the production of a variety of cytokines, such as IL-1, TNF-α, IL-6 and granulocyte macrophage-colony-stimulating factor.[12] Ultraviolet radiation is thought to stimulate cytokine expression by keratinocytes and this is probably relevant to the pathophysiology of local and systemic inflammation in SLE.[13-15] The observed low cytokine expression in epidermal keratinocytes from patients with SLE as compared with the normal keratinocyte pool in this study might be due to the effect of medication and the small number of cells from which mRNA was amplified. Indeed, the high Ct values observed indicate the small amount of transcripts in the original samples. However, the small amount of biological material did not appear to be a limiting factor since the reference gene β-actin mRNA was successfully amplified in the 38 valid samples. In addition, all these samples were normalized for 0.5 μg RNA to prepare the cDNA and there was amplification of at least one cytokine mRNA in each sample. In most of the samples in which cytokine overexpression was present, only one of the four cytokine genes was overexpressed and the others were either underexpressed or not detected at all. Altogether these

observations support the possibility that the low cytokine expression profile observed was due to drug therapy and not to the restricted amount of biological material from the samples.

The skin layer used for mRNA extraction was obtained by micro-dissection and was thus largely enriched for keratinocytes. However, it is possible that minor amounts of Langerhans cells and T lymphocytes were present in various proportions in the samples. Since these cells are known to secrete a rich panel of cytokines, this prospect should be entertained as an additional possible explanation for the variability in the cytokine profile among the SLE samples.

Glucocorticosteroids can affect the expression of several cytokines, including IL-2, IL-5, TNF-α and IFN-γ.[16-20] This information is important for an appreciation of our results, since all patients were receiving low to moderate doses of prednisone. Brink et al. reported that even low doses of steroids can inhibit cytokine synthesis in patients with SLE.[21] In another study, Swaak et al. observed that patients with SLE taking prednisone at a dose of <15 mg/day showed a reduction in ''*ex-vivo*'' production of IL-6 and TNF-α.[22] Nürnberg et al. have demonstrated that IL-6 protein expression in the epidermal basal layer is increased in patients with SLE but this was not observed in those patients using corticosteroids and antimalarial drugs.[6] In this study patients were continuously receiving prednisone at a mean dose of 11.25 mg/day for those with discoid lesion and 15.9 mg/day for those with acute lesions. Furthermore, 18 patients were receiving chloroquine diphosphate at a daily dose of 250 mg and 14 had used thalidomide 200 mg/day on average 5 months before entering the study.

The literature provides scarce information on the influence of treatment on cytokine mRNA expression in skin lesions of patients with SLE. Increased expression of protein and mRNA for IL-2, IFN-γ and TNF-α, but not IL-1β and IL-4, was observed in 8 skin samples from discoid lupus patients by immunohistochemistry and semiquantitative conventional PCR.[8] Unfortunately, there is no information on the use of medication in that study. Stein et al. studied the presence of IL-2 and IL-5 mRNA by conventional RT-PCR using whole skin from patients with different forms of lupus skin lesions. IL-2 mRNA was not detected in 16 out of the 19 skin samples while IL-5 was detected in all samples from patients with SLE. Again no data are available about treatment in those patients.[7]

In this study the IFN-γ gene was overexpressed in 8 (19%) of the samples, equally distributed between discoid and acute lesions. Most of the samples with IFN-γ overexpression were from involved skin (7 samples) against only one from uninvolved skin. This observation suggests that keratinocyte-derived IFN-γ may play an active role in the pathologic process of discoid and acute SLE skin lesions. Although analyzing mRNA extracted from whole skin, the findings of Stein et al[7] are similar to ours in that IFN-γ was detected by conventional RT-PCR in 84% (16/19) of miscellaneous lupus skin lesions. Similar results were obtained by Toro et al.,[8] who demonstrated increased amounts of mRNA and protein for IL-2 and IFN-γ in whole skin from 8 patients with discoid lupus.

An interesting finding in this study was that the cytokine expression profile was similar in discoid and acute lesions, which suggests that keratinocyte-derived cytokines (IL-2, IL-5, TNF-α and IFN-γ) are important in the pathophysiology of the disease but are not key factors in the differentiation of the type of skin lesion in SLE. Although the cytokine expression did not correlate with SLEDAI, it should be pointed out that the three samples overexpressing more than one cytokine were derived from patients with intense and diffuse skin involvement. These three patients were receiving treatment and it is possible that the therapeutic regimen was insufficient to control the pathologic process, allowing a full-blown expression of proinflammatory cytokines and exuberant inflammation of the skin.

In conclusion, this study showed a heterogeneous cytokine gene expression pattern in keratinocytes derived from patients with SLE with discoid and acute skin lesions receiving treatment with chloroquine diphosphate and low dose prednisone. IFN-γ and IL-5 were the most commonly overexpressed cytokines, which suggests that both Th1 and Th2 $CD4^+$ cells may be involved in the pathophysiology of SLE skin inflammation. IFN-γ was the cytokine whose gene expression was most closely associated with the presence of clinical skin inflammation. Samples from patients with more severe and extensive skin lesions presented higher frequency of cytokine gene overexpression. The use of prednisone and antimalarial drugs by most of the patients may have contributed to the relatively low expression of cytokines by epidermal keratinocytes in some of the samples studied and this subject will be formally investigated in appropriately designed trials.

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Conflict of Interest: None.

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