

Proteome Study of Cutaneous Lupus Erythematosus (CLE) and Dermatomyositis Skin Lesions Reveals IL-16 Is Differentially Upregulated in CLE

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Abstract

Background: The objective of the study was to explore disease pathways activated in the inflammatory foci of skin lesions in cutaneous lupus erythematosus (CLE) and dermatomyositis (DM).

Methods: Skin biopsies acquired from active CLE and DM lesions, patient (PC) and also healthy controls (HC) were investigated. Biopsy sections were examined by a pathologist and inflammatory foci were laser micro-dissected, captured and proteins within captured tissue were detected in a hypothesis free manner by mass-spectrometry. Protein pathway analysis was performed by string-db.org platform. Findings of interest were confirmed by immunohistochemistry (IHC).

Results: Proteome investigation identified interleukin (IL)-16 to be the only detectable and abundant cytokine differentially expressed in CLE compared to DM. Caspase-3, enzyme that cleaves IL-16 into its active form, was detected in low levels. Significantly higher proportion of IL-16 and Caspase-3 positive cells were identified in CLE lesions in comparison to DM, PC and HC. Interferon-regulated proteins (IRP) were abundant in both CLE and DM. Proteomic results indicate more abundant complement deposition in CLE skin lesions.

Conclusions: Using hypothesis free mass-spectrometry investigation of CLE inflammatory infiltrates, we identified that IL-16 is the only detectable and highly abundant cytokine, while IRP was a common feature of both CLE and DM. IHC confirmed high expression of IL-16 and caspase-3 in CLE.

Our novel molecular findings indicate that IL-16 detection could be useful in differential diagnostics between the two conditions that can display similar histopathological appearance. Potentially, IL-16 could be of interest as a future therapeutic target for CLE.

Background

Systemic lupus erythematosus (SLE) is an autoimmune disease with a range of clinical manifestations including systemic inflammation, circulating autoantibodies against nuclear antigens and other intracellular molecules, and frequent involvement of the skin, joints, and also renal and hematological systems. If not treated, autoimmune inflammation may lead to organ damage, and in severe cases vital organ involvement can be life-threatening. Cutaneous lupus erythematosus (CLE) is one of the specific manifestations of SLE, but also may develop in patients without systemic disease. Still, some of the CLE patients will later progress to SLE[1, 2]. The autoimmune inflammation observed in SLE and CLE is a result of activated inflammatory pathways within both the innate and adaptive immune systems, and interferons (IFNs) are thought to be the key mediators[3].

Dermatomyositis (DM) is another systemic autoimmune disease, primarily affecting muscles and skin. DM can also affect the lungs and myocardium among other systems, and the vital organ involvement in this disease can also be lethal[4]. In DM, anti-nuclear antibodies are common, as are autoantibodies directed against tRNA synthetases[5].

Genome wide association studies revealed that many genes within IFN pathway could determine susceptibility to either cutaneous or systemic LE, and also dermatomyositis[6–8]. Upregulation of IFN-regulated proteins is observed in both peripheral circulation and at the target organ inflammation in CLE, SLE, and DM[7–9]. The exact reason and molecular mechanism of the activation of IFN system is not known. Disturbances in cell death, clearance of unviable cell debris, as well as activation and deposition of complement components are known to occur in skin lesions in CLE, and muscle fibers in DM. These factors could activate the IFN pathway via pattern recognition receptors and further lead to autoimmune inflammation [9, 10].

The typical histopathologic patterns of CLE and DM skin lesions are surprisingly similar[4]. Specifically, the microscopic patterns include interface dermatitis, apoptosis of keratinocytes, perivascular and perifollicular lymphohistiocytic inflammation, and increased dermal mucin. Despite the similarities on skin biopsy, CLE and DM are very different clinically in respect to distribution of rash and the organ system involvement. Interleukin (IL)-18 has been implicated as a cytokine differentially expressed in DM, but not CLE lesions[11]. It is not clear what other molecular differences underlie the clinical differences and histopathologic similarities between CLE and DM.

In the current study we investigated the total proteome of laser micro-excised inflammatory foci of CLE and DM lesions in a hypothesis free manner in order to identify differential disease specific molecular pathways.

Methods

Patients

Patients with cutaneous lupus erythematosus (CLE) rash (n = 13) were included at disease exacerbation at either the Rheumatology Department at Karolinska University Hospital (KS) or the Dermatology Department at Danderyds Hospital (DS), Stockholm. Patients with dermatomyositis were recruited at diagnosis or disease exacerbation at KS (n = 7). All subjects gave informed written consent at inclusion. Study was approved by the Swedish Ethical Review Agency and was conducted in compliance with the Helsinki Declaration.

At inclusion, each patient was examined by a physician. Activity of the skin rash in CLE patients was scored using Cutaneous Lupus Disease Area and Severity Index (CLASI)[12]. Activity of DM rash was assessed using Cutaneous Dermatomyositis Disease Area and Severity Index (CDASI)[13]. SLE activity was assessed using systemic lupus assessment measure (SLAM) and its definitions[14]. All patients had their CLE diagnosis confirmed by a dermatopathologist and majority patients had a diagnosis of SLE at inclusion. The diagnosis of dermatomyositis was based by cutaneous and muscular histopathologic features, as well as serological and blood chemistry tests indicating muscle damage by elevation of muscle enzymes. Biopsies from active skin lesions (CLE or DM) distributed on extremities, trunk or scalp were acquired in local anesthesia using punch technique. Biopsies from uninvolved skin were acquired

from UV-non exposed buttock skin from 5 of the included CLE patients (PC). Five healthy controls (HC) were recruited at the Dermatology Department, DS while undergoing excision of dysplastic naevus (extremities or trunk). Demographic and clinical characteristics of the cohort are presented in Table 1. Information on autoantibodies and medications is presented in Additional Table 1.

Laser capture microdissection and proteomics

Skin biopsies were acquired using 4 mm punch method and fixed in formalin, thereafter embedded in paraffin and sectioned. Skin sections were examined by an experienced dermatopathologist (Dr. J. Lehman) who confirmed histopathological features of CLE and DM respectively. Laser capture microdissection-assisted liquid chromatography-based tandem mass spectrometry was performed as follows. Ten μm thick sections of formalin-fixed, paraffin-embedded skin specimens were mounted onto polyethylene naphthalate membrane slides and stained with Congo Red stain (Sigma-Aldrich, St. Louis, MO) for microanatomic visualization. Laser capture microdissection was used to isolate the inflammatory foci within the dermis. A total of $1,000,000\mu\text{m}^2$ of tissue per case was dissected; tissue elements were collected in digestion buffer in 0.5 ml caps. After processing and digest, each sample was analyzed by nano-LC tandem mass spectrometry at Mayo Clinic Medical Genome Facility - Proteomics Core.

Immunohistochemistry (IHC)

In order to verify proteomics findings, we employed IHC technique. Sequential skin biopsies were processed and stained for IL-16 and caspase-3. First, sections were heated overnight at $60\text{ }^\circ\text{C}$, thereafter deparaffinized in xylene and washed in ethanol. Antigen retrieval was performed by boiling the sections for 30 minutes in 10 mM citrate buffer (pH = 6). Permeabilization was performed by washing in 1M PBS buffer, pH 7.4, containing 0.1% saponin (PBS-S). Non-specific tissue binding was blocked by incubating the sections in 3% hydrogen peroxide (H_2O_2) in PBS-S for 20–30 minutes, which was followed by blocking using an Avidin/Biotin blocking kit (SP-2001, Vector Laboratories) and thereafter by incubating in 2% bovine serum albumin (BSA) and 2% milk in PBS-S. The rabbit polyclonal anti-human IL-16 (HPA018467, Sigma) and anti-caspase-3 antibodies (HPA018467, Sigma) were diluted 1:500 and 1:1250, respectively, in 1% BSA and 1% milk in PBS-S solution, applied to the sections and incubated overnight at room temperature. For controls stains, we either omitted primary antibody or primary antibodies were replaced by rabbit immunoglobulin fraction (X0936, Dako, 1:1000). Next, sections were washed in the buffer 5 min, 3 times and blocked with 2% normal goat serum (NGS) in 20 min. The secondary antibody, a biotinylated polyclonal goat anti-rabbit IgG (B8895, Sigma, 1:500), was applied on the sections in buffer containing 1% NGS and incubated for 60 minutes. Sections were developed using extravidin peroxidase (E2886, Sigma, 1:400), and DAB-HRP substrate kit (SK-4100, Vector Lab). The glasses were counterstained with hematoxylin and mounted with Mountex (Histolab).

Data collection

The sections were visualized using a Leica DMR XA2 light microscope. Semiquantitative assessment of the staining was performed in a blinded manner by two investigators (AH and TSM), and supervised in

case of discrepancy by a third investigator (VO). Images in 20x amplification were scored and obtained of at least two representative infiltrated upper-dermal areas in each LE and DM section. At least one representative stained dermal area in each control skin section was photographed in a similar manner. Standardized visual settings were used throughout the photographing process.

The total number of present/infiltrating cells in each image was counted using a standardized counting method. Each image file was opened in QuPath v0.1.2 digital pathology image analysis software and all stained cells were evaluated individually and marked manually in a systematic manner using the counting tool[15]. The visual settings of the computer screen in use were standardized throughout the process. Positive staining was defined as brown cytoplasmic staining. Infiltrating or tissue cell was defined as a cell located in the extracellular matrix of dermal connective tissue outside of dermal appendages and blood vessels.

Data analysis and statistics

All data were analyzed in following groups: CLE, DM, HC and for IHC uninvolved skin of CLE patients was also included (PC).

For the mass spectroscopy data, total spectral counts were used to calculate fold change ratios between patient groups and controls. We investigated proteins that were differentially expressed (> 5-fold expression; $p < 0.05$; false discovery rate < 1%) between each comparison group. These protein lists were entered into the string-db.org program for pathway analysis and visualization (Fig. 1).

For the immunohistochemistry data, the total number of present and positively stained cells was calculated for each section and protein. The proportion of stained cells out of the total dermal cell number was calculated. The percentages of the stained cells were compared among the groups using Wilcoxon non-parametric or Mann-Whitney tests as appropriate, p values < 0.05 were considered significant. Microsoft Excel 365 and JMP13 software (SAS Institute, Carey, NC, USA) was used for all statistical testing.

Results

Total protein analysis of inflammatory foci in CLE and DM

We analyzed total proteome of excised inflammatory foci of CLE and DM lesions in order to identify the upregulated proteins of major importance. The hypothesis free total proteome analysis identified over 2000 proteins that were detected at a level of at least 5 copies per sample. Proteins in lower abundance were considered to have higher variability and be hits of secondary interest and are not discussed further (Additional Table 2). Ratios of total spectral counts for these 2000 proteins were compared among three groups CLE and DM patients, and HC.

Proteins upregulated 5 fold or more in comparison to HC were regarded as interesting hits and included in further analyses (Fig. 1 and Additional Table 2).

Interleukin-16 (IL-16) was identified as a unique highly abundant cytokine in the CLE lesions

The proteomic analysis identified IL-16 as a single top-upregulated cytokine in the inflammatory foci of CLE. Only limited amount of copies of IL-16 were detected in DM and in HC (Table 2). IL-16 is an intracellular protein. In order for it to become functional, upon T-cell stimulation, it must be cleaved by caspase-3. Caspase-3 was detected in CLE lesions by proteomic analysis, but at low levels (fewer than 5 peptides).

In CLE lesions, high expression of IL-16 coincides with presence of caspase-3

In order to verify our observation, we performed IHC staining for IL-16 on CLE (n = 13) and DM (n = 7) skin lesions, unaffected skin of CLE patients (n = 5) and HC skin (n = 5). We confirmed that a high proportion of infiltrating cells in CLE was positive for IL-16 (Fig. 2).

Semiquantitative analysis of IHC staining revealed that approximately 66% (interquartile ratio (IQR) 46–81) of the cells comprising the inflammatory foci in CLE expressed IL-16 (Figs. 2 and 3). While IL-16 was detected in both DM, PC and HC, but the percentage of cells expressing IL-16 was significantly lower (35% (IQR 25–63), 34% (IQR 16–38) and 34% (29–48), respectively (Fig. 2, 3, Additional Fig. 1), $p = 0.01$. The cells within the same infiltrates were observed to express caspase-3, the enzyme that cleaves IL-16. Up to 27% (IQR 12.5–35.5) of the cells within CLE infiltrates were positive for caspase-3, while in DM it was only 7% (IQR 3–20), and even fewer in PC and HC, respectively 3% (IQR 1.5-6) and 5% (IQR 2-22.5) ($p < 0.001$)(Fig. 2, 3, Additional Fig. 1).

There were no significant correlations between percent of IL-16 or Caspase-3 positive cells and CLASI, CDASI activity or damage indices (data not shown).

High expression of IFN-regulated proteins in the inflammatory foci of CLE and DM lesions

Interferon (IFN) regulated proteins (IRP) were the most abundant molecules within inflammatory foci of both CLE and DM, and in both conditions were upregulated 20 fold or more. In CLE, IFN protein signature was stronger and included even more molecular hits than in DM (Table 2 and Fig. 1).

Other molecular pathways activated in both CLE and DM were antigen peptide transporter (TAP) protein network as well as several tRNA synthases and other common enzymes, including aspartate aminotransferase (GOT1) (Table 2).

Intralesional presence of classical complement activation pathway components is a feature of CLE

In CLE, proteomic analysis identified high expression of almost all components of the classical complement activation pathway including early and terminal activation proteins, as well as complement regulators and receptors. Only C2 was not detected at all, while C4A and C4B as well as C9 were detected, but upregulated only approximately 1.5 fold, in comparison to HC. These molecules were not detected or detected at a low level in DM or control skin samples (Fig. 1 and Table 3).

Discussion

In this study we aimed to explore what proteins are expressed in the inflammatory foci of CLE and DM lesions. Our major finding from tandem mass spectrometry investigation is that IL-16 is the only detectable and highly abundant cytokine in CLE, but not DM lesions.

The study is unique in several aspects. We utilized a novel method – laser capture microdissection which allowed excision of the cells comprising inflammatory foci. The collected tissue was analyzed in a hypothesis-free manner using mass-spectrometry technique. The detected proteins were compared among the groups CLE, DM and HC. Data analysis using string-db.org database revealed the involved protein networks, similarities, and also some major differences between the conditions.

Increased levels of circulating IL-16 in SLE patients has been described before, by us and others[16, 17]. Also, upregulation of IL-16 has been observed in psoriasis, systemic sclerosis, inflammatory bowel disease and several malignancies[17–19]. Our group has previously reported observation that SLE patients with active nephritis or arthritis, had high levels of circulating IL-16, while patients with CLE had lower[17]. The presented data indicate, that cytokines in the circulation does not necessarily correspond the molecular processes taking place in the LE target organ, for example skin.

Interestingly, other investigators described that in the skin affected by systemic sclerosis, approximately 1/3 of the infiltrating cells express IL-16 [18]. In comparison, in the current study we found that approximately 2/3 of the infiltrating cells in CLE carry this molecule, while proportion in DM, PC and HC was about 1/3, similar to that described systemic sclerosis[18]. The predominant sources of IL-16 are T cells, but also eosinophils, DCs, mast cells, macrophages and monocytes can also produce IL-16[12, 13]. IL-16 is generated as a precursor molecule, and when cleaved by caspase-3, two molecules with different functions are generated: N-terminal pro-IL-16 and C-terminal secreted/mature IL-16. Pro-IL-16 molecule functions as a regulator of T cell growth, and a secreted mature IL-16 functions as a CD4 and/or CD9 ligand and facilitate cell motility and activation[12, 13]. It is known, that majority of infiltrating cells in the skin are T-cells, and as our results indicate, they carry the IL-16 molecule. We suggest that IL-16 could function as a chemoattractant in the CLE lesions. Also, intranuclear expression of IL-16 is known to impede cell cycle progression and could possibly negatively affect cell growth and regeneration within the skin lesions[19].

Multiple cytokines have been found to be involved and mediate local inflammatory responses in CLE including type I and III IFNs, TNF- α , IL-1 β , HMBG1, as well as the Th17 pathway along with IL-21[14–17]. Our analysis confirmed that IFN regulated protein (IRP) network is the most abundant protein pathway

activated in the lesions of both CLE and DM. While this prominence of the IRP network was similar between the two diseases, CLE lesions appeared to have higher expression and diversity of the proteins within IFN-pathway.

The transporters associated with antigen processing (TAP-1 and TAP-2) are of importance for normal expression of MHC class I and presentation of intracellular peptides, while TAPBP is a catalyst molecule in the binding of antigen. Defective function of this pathway is implicated in type I autoimmune diabetes mellitus[20]. High expression of these molecules in both CLE and DM suggest that autoantigen presentation could be occurring intracutaneously in inflammatory infiltrates and could be of interest in further investigation.

Aminoacyl-tRNA synthetases (ARS) is another abundant molecular group detected in the lesions of both CLE and DM, but not in the control skin. The detected ARS included tryptophanyl (WARS) and threonyl (TARS) as the most abundant. WARS has been demonstrated to have additional functions in the immune system, including regulation of IFN- γ production and this could be of importance in both CLE and DM[21].

Detection of complement components (C1q, C3 and C4) and immunoglobulins (Ig) at the dermo-epidermal junction (the so-called lupus band) has been used in CLE diagnostics in many decades using direct immunofluorescence technique[22]. The test has been criticized for its limited specificity and sensitivity[23]. Deposition of MAC within the CLE and lupus nephritis inflammatory infiltrates has been reported earlier [24, 25]. Also, detection of MAC within the endothelium and perifascicular, usually atrophic, muscle fibers is utilized as a distinct diagnostic feature for DM [10]. Proteomic findings indicate that activation and deposition of complement, including membrane attack complex (MAC) is more abundant in CLE cutaneous inflammatory foci, than DM, where intramuscular MAC activation seems to play more important role[10].

The limitations of this study include limited amount of individuals included per each group (5 cases per group) that were run in the proteomic analysis, however the actual findings were confirmed in higher number of cases (CLE (n = 13) and DM (n = 7), and both PC (n = 5) and HC (n = 5)). We acknowledge that less stable proteins could possibly been destroyed during the biopsy handling and those lower abundant could be missed by mass-spectrometry analysis, since IL-16 was the only cytokine that was abundantly detected by this technique.

In conclusion, using a novel technique laser capture microdissection combined with hypothesis free mass-spectrometry investigation we identified that IL-16 is abundant and the only detectable cytokine in inflammatory foci of CLE lesions. We confirmed high expression of IL-16 by IHC, and also detected focal expression of caspase-3, the enzyme that cleaves IL-16 into its active forms. Abundant deposition of components of classical complement activation pathway is another feature of CLE, while abundant expression of IFN regulated proteins is a characteristic of both CLE and DM. Our findings could be useful in diagnosing and differentiating CLE and DM in difficult cases, if validated clinically. These observations offer novel information on molecules involved in disease mechanisms and propose a novel pathway to be explored in search for CLE therapeutic targets.

Abbreviations

CLE – cutaneous lupus erythematosus

DM - dermatomyositis

SLE – systemic lupus erythematosus

IL - interleukin

PC – CLE patient control skin

HC – healthy control

IHC – immunohistochemistry

C - complement

MAC – membrane attack complex

IQR – interquartile ratio

CLASI - Cutaneous Lupus Disease Area and Severity Index

CDASI - Cutaneous Dermatomyositis Disease Area and Severity Index

TAP-1 – transporter associated with Antigen Processing 1

TAP-2 - transporter associated with Antigen Processing 2

TAPBP TAP binding protein

ARS - aminoacyl-tRNA synthetases

IFN - interferon

IRP - interferon regulated proteins

CNS - central nervous system

M - mean

SD – standard deviation

Declarations

Ethics approval and consent to participate

Study was approved by the Swedish Ethical Review Agency and study was conducted according the declaration of Helsinki. Written informed consent was granted by all study participants.

Availability of data and materials

The datasets generated and analysed during the current study are not publicly available due to ethical permit which does not allow data sharing to the third part, but are available from the corresponding author on reasonable request, for non-commercial purposes.

Competing interests

The authors declare no competing interests.

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Authors contributions

TBN, IEL, MWH, ES, VO - conceived the study. KPS, IEL, ES, VO - recruited patients, collected database and biological material, AM, JSL, AH, TSM, CMC, BM, VO – performed experiments. TBN, AM, JSL, AH, TSM, CMC, BM, ES, VO interpreted and analyzed the data. VO and AH drafted manuscript. All authors participated in manuscript revision and its final approval.

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Tables

Table 1. Characteristics of the cohort

	CLE	DM	Healthy controls	CLE uninvolved
	n=13	n=7	n=5	n=5
Cases included in proteomics	5	5	5	0
Age, M (SD)	59 (18)	52 (12)	66 (21)	
Sex, female	13	5	3	
CLE/SLE	13/10	-		
DM/amyopathic DM	-	6/1		
Organ involvement at inclusion				
Active rash	13	7		
Active arthritis	5	0		
Active nephritis	2	0		
Active CNS	2	0		
Active myositis	0	6		
Paramalignant disease	0	3		
Cutaneous activity indices				
CLASI (available in 10) M(SD)	8.8 (4.3)	-		
CDASI (available in 6), M(SD)	-	17 (4.7)		

CNS -central nervous system; CLASI Cutaneous Lupus Disease Area and Severity Index, CDASI- Cutaneous Dermatomyositis Disease Area and Severity Index, M – mean, SD – standard deviation.

Table 2. Comparison of the most abundantly expressed proteins in the inflammatory foci of CLE and DM skin lesions:

Numbers of proteins	CLE/HC	DM/HC	CLE/DM
Interleukin-16 (IL-16)(x fold)	12	1	12
IFN regulated and associated proteins, high in both LE and DM (x fold)			
IFIT1	71	57	1.2
IFIT3	70	46	1.3
DDX58	48	33	1.5
ISG15	48	30	1.6
OAS2	36	21	1.7
MX1	31	16	2
MX2	29	14	2
STAT1	29	16	1.9
OAS3	28	33	0.83
EIF2AK2	23	15	1.5
IFI44L	18	18	1
IFIT2	13	11	1.2
DDX21	6	6.5	1
IFI16	6	4.5	1.1
ADAR	7.3	5	1.4
IFN-regulated and associated proteins, high in LE, but low in DM (x fold)			
MNDA	31	low*	31
PTPN6	24	low*	24
DDX60	18	low*	18
<hr/>			
STAT2	14	low*	3.8
ZC3HAV1	14	low*	7
OASL	14	low*	14
IFI44	10	low*	1.6
IFIT5	8	low*	2
OAS1	8	low*	8
Antigen processing and transport (x fold)			
TAP1	18.5	10	1.9
TAP2	24	19	1.3
TAPBP	6	5.4	1.1
Aminoacyl-tRNA synthases and other enzymes (x fold)			
WARS	90	55	1.6
TARS	13	10	1.3
TYMP	9.4	9	1
ACLY	28	9	3
GOT1	8	8	1

* - detected as 5 or fewer peptides, therefore regarded as unreliable result.

Table 3. Intralesional expression of complement components, regulators and receptors was higher in CLE than in DM

Proteins	CLE/HC	DM/HC	CLE/DM
C1R	9	1	9
C3	1.2	0.6	2
C4BPA	5.5	2	3
C5	4.2	1.3	7
C6	22	low*	22
C7	18	low*	18
C8A	6	low*	12
C8B	4.5	low *	9
C8G	2	low*	2.5
CFHR1	2.4	low*	2.4
CFHR2	9	1	9
CFHR5	14	low *	14
ITGB2	6	0.6	10

CLE – cutaneous lupus erythematosus, DM- dermatomyositis, C-complement, C4BPA- complement factor 4 binding protein alpha chain, CFHR – complement factor H related protein, ITGB2 – integrin B2. * - detected as 5 or fewer peptides, therefore regarded as unreliable result.

Figures

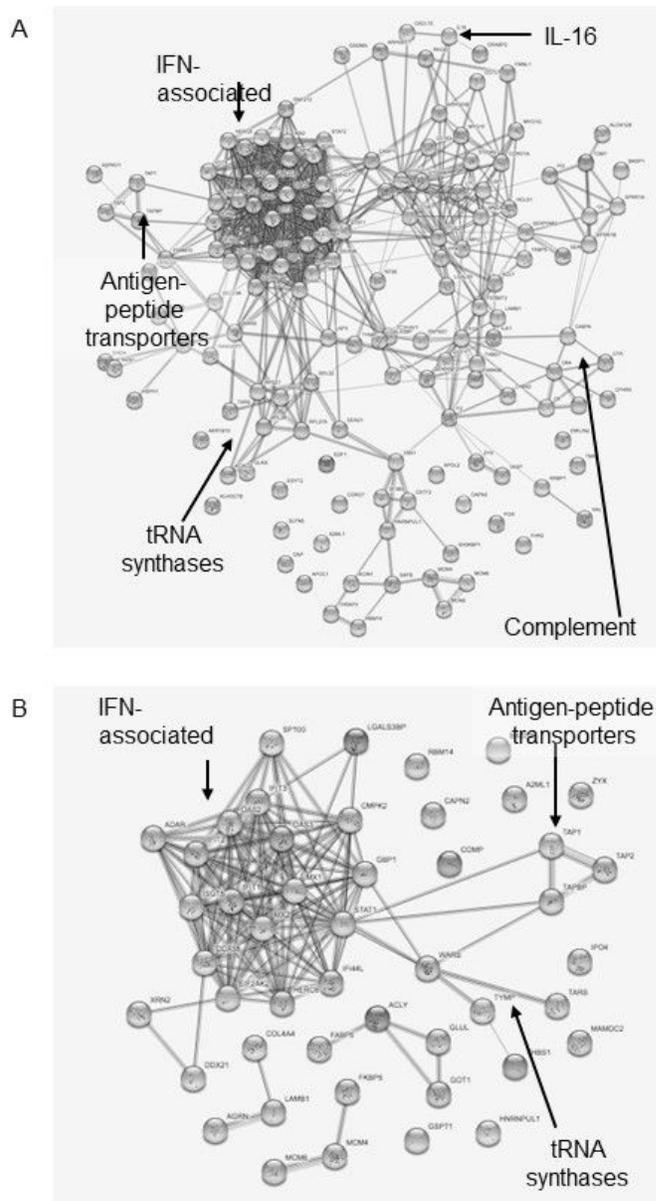


Figure 1. Niewold et al

Figure 1

The protein network of the upregulated proteins (5 fold or more) in cutaneous lupus erythematosus (CLE) vs healthy controls (A) and dermatomyositis (DM) vs controls (B), analysis performed by string-db.org on-line platform. The significant protein networks are indicated by arrows.

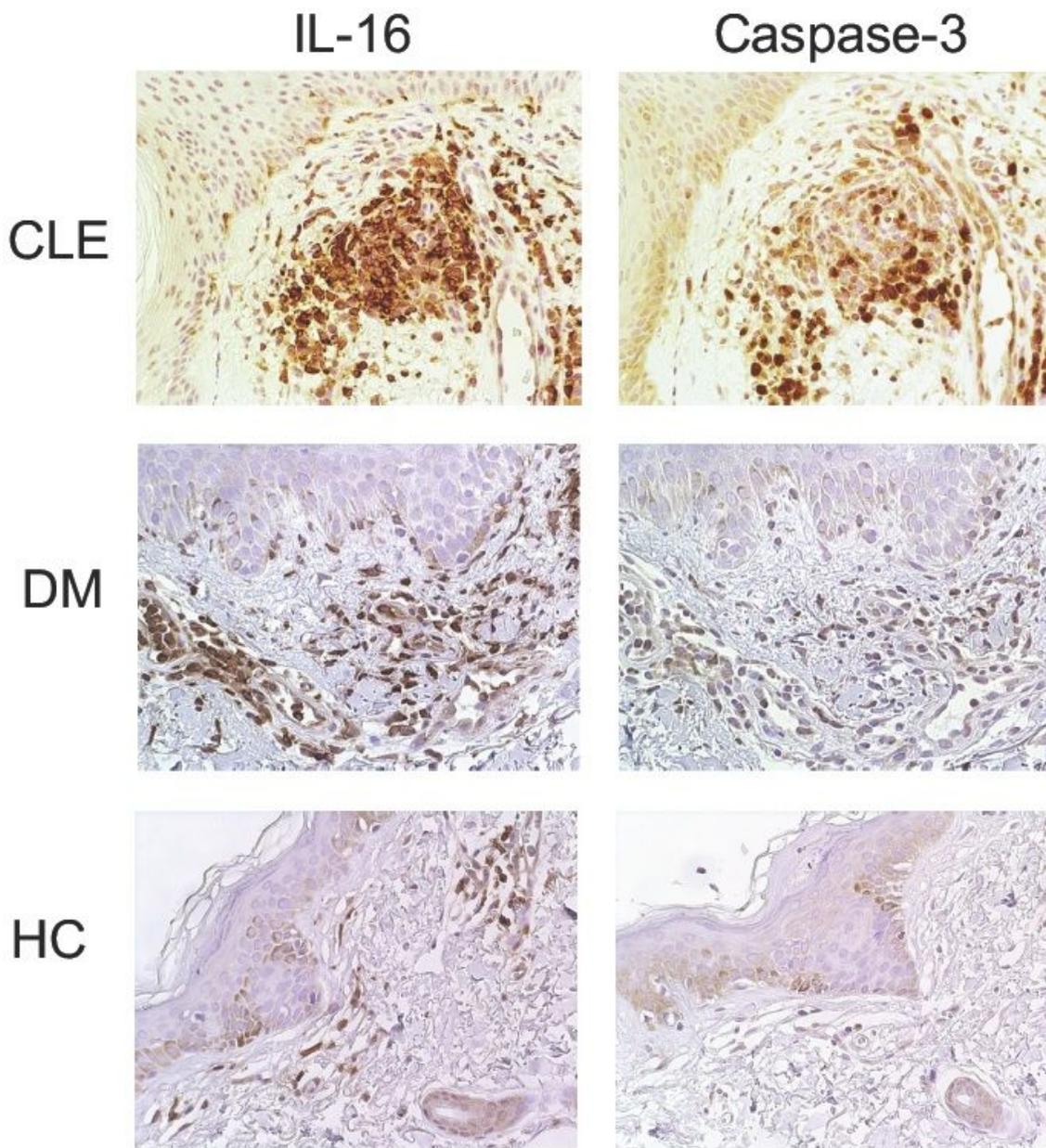


Figure 2. Niewold et al

Figure 2

Representative microphotographs of cutaneous expression of IL-16 and caspase-3 as detected by immunohistochemistry in skin biopsies of cutaneous lupus erythematosus (CLE), dermatomyositis (DM) and healthy controls (HC)(20x magnification).

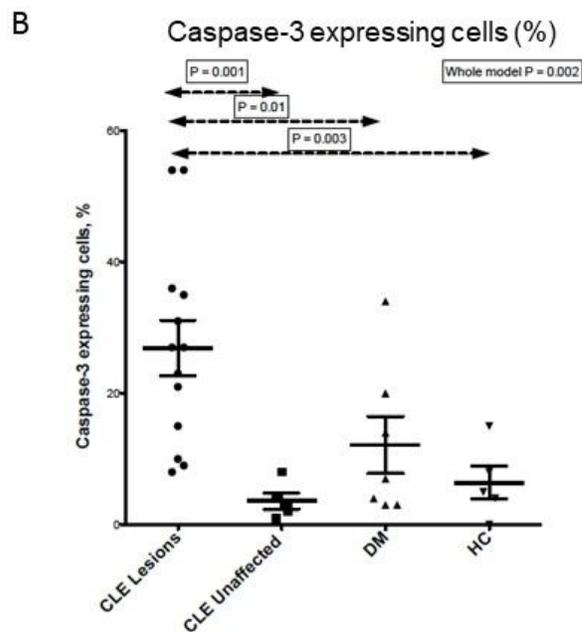
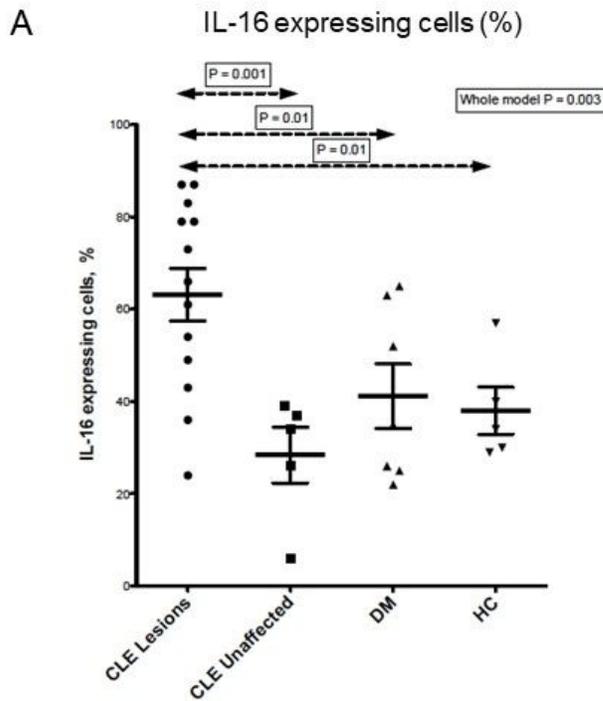


Figure 3. Niewold et al

Figure 3

Semiquantitative analysis of IL-16 (A) and caspase-3 (B) expression in cutaneous lupus erythematosus (CLE), dermatomyositis (DM), CLE uninvolved and HC skin. Statistical analysis (non-parametric Wilcoxon test) was performed using estimated proportion (%) of positive vs total amount of infiltrating or present cells per high power field (HPF) at x25 magnification. Mann-Whitney test was used for comparisons between each group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AdFigure1caL16inCLEandDMNiewoldetal20201012.pdf](#)
- [FinaladditionaltablesNiewoldetal20210110.docx](#)