Journal of Autoimmunity 101 (2019) 86–93

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

The identification of CCL18 as biomarker of disease activity in localized scleroderma

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ARTICLE INFO

Keywords:
Localized scleroderma
Morphea
Eosinophilic fasciitis
Shullman syndrome
CCL18
Pulmonary and activation-regulated chemokine (PARC)
Biomarker
Skin serum
Cytokine
Chemokine

ABSTRACT

Background: Localized Scleroderma (LoS) encompasses a group of idiopathic skin conditions characterized by (sub)cutaneous inflammation and subsequent development of fibrosis. Currently, lack of accurate tools enabling disease activity assessment leads to suboptimal treatment approaches.
Objective: To investigate serum concentrations of cytokines and chemokines implicated in inflammation and angiogenesis in LoS and explore their potential to be utilized as biomarker of disease activity. Additionally, to investigate the implication of potential biomarkers in disease pathogenesis.
Methods: A 39-plex Luminex immuno-assay was performed in serum samples of 74 LoS and 22 Healthy Controls. The relation between a validated clinical measure of disease activity (mLoSSI) and serum analytes was investigated. Additionally, gene and protein expression were investigated in circulating cells and skin biopsies.
Results: From the total of 39, 10 analytes (CCL18, CXCL9, CXCL10, CXCL13, TNFRII, Galectin-9, TIE-1, sVCAM, IL-18, CCL19) were elevated in LoS serum. Cluster analysis of serum samples revealed CCL18 as most important analyte to discriminate between active and inactive disease. At individual patient level, CCL18 serum levels correlated strongest with mLoSSI-scores ($r_s = 0.4604$, $P < 0.0001$) and in longitudinal measures CCL18 concentrations normalised with declining disease activity upon treatment initiation. Additionally, CCL18 was elevated in LoS serum, and not in (juvenile) dermatomyositis or spinal muscular atrophy. Importantly, CCL18 gene and protein expression was increased at the inflammatory border of cutaneous LoS lesions, with normal expression in unaffected skin and circulating immune cells.
Conclusion: CCL18 is specific for disease activity in LoS thereby providing relevance as a biomarker for this debilitating disease.

1. Introduction

Localized Scleroderma (LoS), also known as morphea, encompasses a group of idiopathic skin conditions which are characterized by development of cutaneous and subcutaneous fibrosis. Early clinical manifestations are characterized by the presence of inflammation and the expansion of sclerotic lesions [1,2]. The goal of anti-inflammatory treatment is to slow down disease progression and prevent the development of irreversible sequalae, such as cutaneous and subcutaneous atrophy. Early and accurate disease activity assessment is vital for prompt treatment initiation. However, currently available clinical scoring tools and imaging modalities fail to accurately assess disease activity in large proportions of patients. Specifically, deep involvement is notoriously difficult to monitor and most likely leads to debilitating

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https://doi.org/10.1016/j.jaut.2019.04.008
Received 17 February 2019; Received in revised form 6 April 2019; Accepted 8 April 2019
Available online 18 April 2019
0896-8411/

Abbreviations

AUC	Area Under the Curve
cDNA	complementary DNA
CCL	Chemokine (C−C motif) ligand
CT	Cycle Treshold
EF	Eosinophilic Fasciitis
HKG	Housekeeping Gene
IL	InterLeukin
IQR	InterQuartile Range
JDM	Juvenile DermatoMyositis
LoS	Localized Scleroderma
LoSDI	Localized Scleroderma Damage Index
LoSCAT	Localized Scleroderma Cutaneous Assessment Tool
mLoSSI	modified Localized Scleroderma Skin Severity Index
PBMC	Peripheral Blood Mononuclear Cells
PLS-DA	Partial Least Squares Discriminant Analysis
ROC	Receiver Operator Characteristics
RT-PCR	Real time PCR
R_s	Spearman rank correlation coefficients
SMA	Spinal Muscular Atrophy
SSc	Systemic Sclerosis
VIP-Score	Variable Importance on Projection Score

sequelae, such as contractures, immobility of joints and severe atrophy of affected limbs [3,4]. The lack of available tools underscores the need for biomarkers of disease activity in LoS. Ultimately, identification of additional tools to determine disease activity should aid caregivers to individualize and optimize treatment regimen.

The exact driving mechanisms behind LoS pathogenesis remain to be elucidated. However, extensive extracellular matrix formation and autoimmune dysfunction are thought to be key pathogenic processes [2,5,6]. Likewise, these mechanisms are considered crucial in systemic sclerosis (SSc) pathogenesis. The similarities between LoS and SSc have led to many theories about their relatedness and most theories for LoS pathogenesis are therefore deduced from SSc research [7]. In SSc, endothelial dysfunction and immune dysregulation have been shown to precede collagen deposition [8,9]. We therefore questioned whether signals in inflammation and angiogenesis could be regarded early pathophysiological mechanisms responsible for progression of the disease. Here, we investigated serum concentrations of cytokines and chemokines involved in angiogenesis and inflammation in LoS and investigate their potential as biomarker of disease activity in LoS.

2. Material and methods

2.1. Participants/patients

Eligible patients were ≥4 years old and visited the outpatient clinics of dermatology or rheumatology of the University Medical Centre (UMC) Utrecht or Radboud UMC, Nijmegen, The Netherlands. All patients or legal guardians signed informed consent previous to study participation. The study was conducted in accordance with principles of the Declaration of Helsinki and approved by the Institutional Review Board of the UMC Utrecht (NL13046.091.06).

2.2. Disease/subtype classification & clinical assessments

Patients were classified as described by Laxer and Zulian [10], with the addition of eosinophilic fasciitis (EF) as a distinct subtype. Diagnosis of EF was established by clinical picture of fascia involvement and demonstration of eosinophils at the fascia in histological investigation of deep skin biopsies.

At study visit, information regarding demographics and disease history were collected. Extent of skin involvement was assessed by the Localized Scleroderma Cutaneous Assessment Tool (LoSCAT). LoSCAT is a composite of the modified Localized Scleroderma Skin Severity Index [11] (mLoSSI), capturing disease activity features, and Localized Scleroderma Skin Damage Index [12] (LoSDI), capturing disease damage features.

For control groups, patients affected by (juvenile) dermatomyositis ((J)DM)) and spinal muscular athrophy (SMA), and healthy controls (HCs) were included. Patients with DM were included if they met the Bohan and Peter criteria for definite or probable JDM [13]. Paediatric and adult patients with SMA were diagnosed by genetic confirmation of a homozygous loss of function of the survival motor neuron 1 gene [14]. Adult healthy volunteers were included as healthy controls.

2.3. Serum collection, storage and cytokine measurements

Blood was collected in a Vacutainer® SST II tube (BD catalogue no. 367985) and serum was isolated after centrifugation at 1500g for 10 min at room temperature and stored at −80 °C prior to use. Serum concentrations were evaluated for 39 chemokines and cytokines implicated in angiogenesis and inflammation via Luminex (Details in supplemental Material and Methods).

2.4. Peripheral mononuclear blood cells (PBMCs) isolation

Lithium-heparin (BD catalogue no. 367880) tubes were collected and PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque [15]. Isolated PBMCs were lysed (RLT-PLUS) and stored at −30 °C prior to use.

2.5. Gene expression in circulating immune cells

Detailed description in supplemental Material and Methods.

2.6. Skin samples

For selected adult patients, three skin biopsies were taken from: the inflammatory border (I) and the sclerotic centre (II) of affected skin and from unaffected skin (III). In addition, control samples were acquired from abdominal corrective surgery leftover material. Skin samples were fresh frozen in Tissue Tec OCT compound (Miles Scientific, Naperville, USA) at −80 °C prior to use.

2.7. Gene expression in skin samples

Detailed description in supplemental Material and Methods.

2.8. Immunohistochemistry of skin samples

Detailed description in supplemental Material and Methods.

2.9. Statistical analyses

Descriptive statistics including medians [interquartile range (IQR)] for continuous variables and percentages for categorical data were used. The Mann–Whitney test was used to compare continuous variables between patients and controls. Log-transformed cytokine values were subjected to unsupervised hierarchical clustering (Euclidian distance measure and the Ward's linkage method). A partial least squared – discriminant analysis (PLS-DA) was performed to investigate clustering of active (mLoSSI > 5) and inactive (mLoSSI ≤ 5) samples. In addition, importance of analytes ('loadings') for the visualized clustering was

depicted in a loadings-plot and quantified in a variable importance on projection (VIP)-score. Clustering analyses were performed in the ClustVis server (http://biit.cs.ut.ee/clustvis/) or the MetaboAnalyst server (http://biit.cs.ut.ee/clustvis). The performance of analytes as biomarkers to detect the disease activity was assessed by receiver operating characteristics (ROC) curves and reported as Areas Under the Curves (AUC) with 95% confidence interval and P-values. Ideal cut-off concentration to discriminate between active and inactive disease was calculated by Youden's Index. Additionally, cut-off concentrations were defined as above 2 standard deviations of the mean serum concentrations of HCs for every analyte and frequency of elevated concentrations were calculated. Spearman's rank correlation coefficient was used to explore relations among different cytokines or chemokines, and cytokines or chemokines and clinical outcome measures. Correlations were reported by Spearman's rho (r_s) and p-values. The Wilcoxon signed-rank test was performed to compare longitudinal serum concentration measures. The Kruskal-Wallis with Dunn's multiple comparisons post-hoc test was performed when comparing continuous variables between 3 or more groups. A raw p-value of 0.05 or less was regarded statistically significant. For the Luminex assay, a Bonferroni correction ($P = 0.0017$) was assessed to account for multiple comparisons (39) within assay. All analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA) and SPSS Statistics v.25 (IBM, Armonk, NY, U.S.A.).

3. Results

3.1. Demographics and clinical information

For this study, 74 unique LoS patients and 22 healthy controls (HCs) were included. The vast majority (80%, n = 59) had adult-onset disease and the median age at study participation was 45 years [IQR, 23–60] (Table 1). Over half of the patients were female (n = 43, 58%). The majority of patients were affected by the linear (n = 27, 36%), EF (n = 20, 27%) or generalized subtype (n = 15, 20%). The median mLoSSI and LoSDI were 13 [IQR, 6–23] and 7 [IQR, 4–15], respectively. At inclusion, 47 patients (63%) were systemic treatment naïve. The remaining patients received a combination of MTX and SCS (n = 7, 9%), MTX monotherapy (n = 12, 16%), SCS monotherapy (n = 6, 8%) or a miscellaneous treatment (n = 2, 3%). Longitudinal serum samples were collected for 19 patients. All 74 patients, plus the longitudinal samples (n = 19), were used for the Luminex immunoassay. Additional experiments, such as gene expression investigation in circulating immune cells and skin samples or immunohistochemistry staining were performed in selected groups of patients from the Luminex cohort (Sample overview: Supplemental Fig. 1). Supplemental Table 1 contains a detailed description of the different groups of patients included for every experiment.

3.2. Identification of elevated cytokines and chemokines in localized scleroderma

The following cytokines and chemokines, implicated in angionesis and inflammation, were investigated in serum by Luminex Immunoassay: IL-18, TWEAK, OSM, CCL2, CCL4, CCL17, CCL18, CCL19, CCL22, CCL27, CXCL9, CXCL10, CXCL13, VEGF, TNFRII, SPARC, sVEGFR-1, PIGF, Gal-1, Gal-3, Gal-9, sP-selectin, sE-selectin, Thrombomodulin, Endoglin, OSF-2, Angiopoietin-1, Angiopoietin-2, TIE-1, TIE-2, PDGFBB, YKL-40, PAI-1, sICAM-1, sVCAM, C5a, Fetuin, Fibronectin and THBS-1.

Median serum concentrations were calculated for LoS (n = 74) and HCs (N = 22) and the following analytes were significantly elevated in LoS: CCL18, CXCL9, CXCL10, CXCL13, TNFRII, Galectin-9, TIE-1, sVCAM, IL-18, CCL19, sICAM, and YKL-40 (Table 2, individual plots Supplemental Fig. 1). Ten out of 12 analytes remained significantly elevated after Bonferroni correction for 39 comparisons and were further investigated for biomarker potential. Median serum concentrations for all 39 analytes are depicted in Supplemental Table 2.

3.3. What clinical phenotype is associated with high levels of serum concentrations of chemokines and cytokines?

First, unsupervised hierarchical clustering analyses of all patients (n = 74) and HCs (n = 22) was performed based on the serum concentrations of ten significantly different analytes. Two large clusters could be identified: one consisted almost exclusively of LoS patients ('Localized Scleroderma Cluster', n = 56, containing 54 LoS samples) and the other cluster was comprised of nearly all HCs and the remainder of LoS patients ('Normal-like cluster', n = 40, containing 20 LoS samples) (Fig. 1).

The LoS patients that ended up in the 'Localized scleroderma-cluster' (n = 54) and 'Normal-like cluster' (n = 20) were compared for several characteristics to determine the clinical phenotype that drove the observed clustering. The patients that clustered together in the 'Localized Scleroderma Cluster' had significantly higher disease activity scores (median mLoSSI = 16 [8–25]), compared to the LoS patients that ended up in the 'normal-like cluster' (median mLoSSI = 8 [4–12]) (P 0.0047). Other investigated characteristics, such as treatment received or age at participation, did not differ significantly between the two groups (Supplemental Table 3).

3.4. CCL18 was identified as the most specific biomarker for disease activity in LoS

Unsupervised hierarchical clustering revealed a cluster of LoS patients with more active disease that could clearly be distinguished from HCs. To further investigate the relation between the serum cytokine

Table 1
Cohort characteristics.

Biological Group	HC	LoS
No.	22	74
Age, Median [IQR]	43 [27–56]	45 [23–60]
Sex, No. (%)		
Female	18 (82%)	43 (58%)
Male	4 (18%)	31 (42%)
Subtype		
Circum. Sup.	n/a	8 (11%)
Linear	n/a	27 (36%)
Deep	n/a	4 (5%)
Generalized	n/a	15 (20%)
Eosinophilic Fasciitis	n/a	20 (27%)
Disease Onset, No. (%)		
Adult-Onset	n/a	59 (80%)
Paediatric-Onset	n/a	15 (20%)
Clinical Scores, Median [IQR]		
Disease Activity		
mLoSSI	n/a	13 [6–23]
PGA-A	n/a	16 [3–32]
Disease Damage		
LoSDI	n/a	7 [4–15]
PGA-D	n/a	17 [8–29]
Treatment, No. (%)		
Naïve	22 (100%)	47 (63%)
MTX and SCS	n/a	7 (9%)
MTX monotherapy	n/a	12 (16%)
SCS monotherapy	n/a	6 (8%)
Miscellaneous	n/a	2 (3%)
Antinuclear Antibodies	n/a	23 (43%)[a]

HC, Healthy Control; *LoS*, Localized Scleroderma; *Circum. Sup.*, Circumscribed Superficial Subtype; *mLoSSI*, modified Localized Scleroderma Skin Severity Index; *LoSDI*, Localized Scleroderma Damage Index; *PGA-A*, Physician's Global Assessment of disease activity; *PGA-D*, Physician's Global Assessment of disease damage; *MTX*, Methotrexate; *SCS*, Systemic Corticosteroids.

[a] Antinuclear Antibody test results were available for 54 patients.

Table 2
Ten cytokines and chemokines were elevated in localized scleroderma serum samples compared to healthy controls.

Analyte	Unit	Healthy Control, N = 22		Localized Scleroderma, N = 74		P-value
		Median	IQR	Median	IQR	
CCL18	pg/ml	7158	2772–14 069	20 902	8497–32 040	< 0.0001[a]
CXCL9	pg/ml	15.63	15.63–15.63	64.28	15.63–232.0	< 0.0001[a]
CXCL10	pg/ml	153.3	110.2–223.8	412.5	235.8–762.2	< 0.0001[a]
CXCL13	pg/ml	0.56	0.56–5.92	11.02	1.74–40.15	< 0.0001[a]
TNFRII	pg/ml	1515	1129–1999	2226	1793–2901	< 0.0001[a]
Gal-9	pg/ml	2497	2160–3024	3825	2793–5678	< 0.0001[a]
TIE-1	pg/ml	3468	1272–5538	8983	6296–13 237	< 0.0001[a]
sVCAM	ng/ml	1830	1494–2012	2232	1969–2642	< 0.0001[a]
IL-18	pg/ml	54.45	28.71–101.9	115.9	78.16–176.7	0.0002[a]
CCL19	pg/ml	104.9	67.51–139.50	144	118.5–240.6	0.0010[a]
sICAM	ng/ml	207.94	169.73–258.45	278.24	212.06–357.67	0.0039
YKL-40	ng/ml	50.17	42.06–67.54	67.03	47.32–102.80	0.037

P-values were calculated by Mann-Whitney test.
[a] Comparisons that remained significant after Bonferroni Correction 0.0013 (0.05/39 = 0.0013).

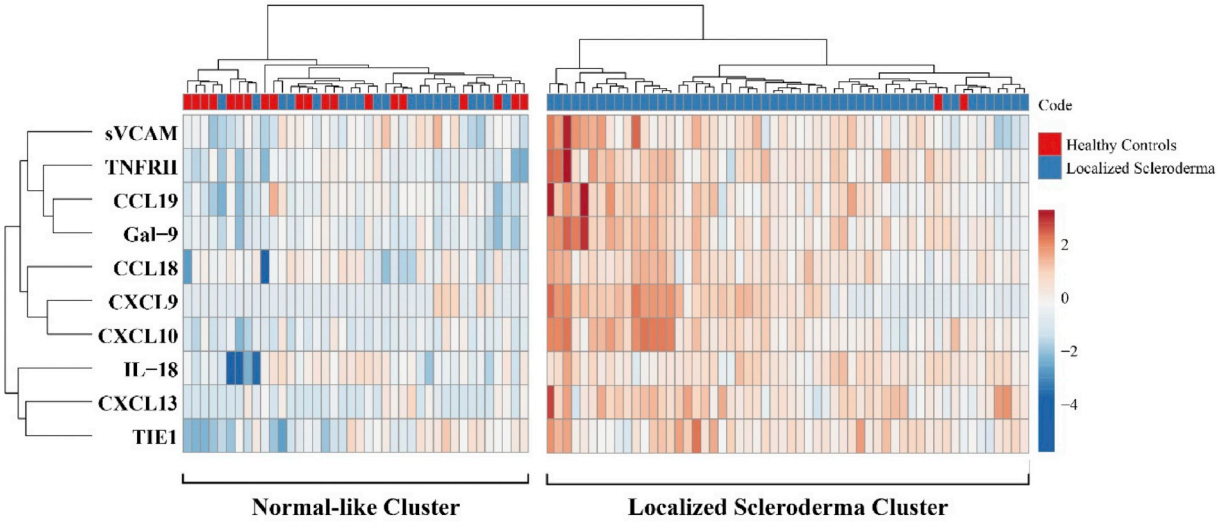

Fig. 1. Unsupervised hierarchical clustering and heatmap of localized scleroderma (LoS) and healthy control (HC) samples based on serum cytokine and chemokine concentrations. Two large clusters could be identified: one consisted almost exclusively of LoS patients ('Localized Scleroderma Cluster', n = 56, containing 54 LoS samples) and the other cluster was comprised of nearly all HCs and the remainder of LoS patients ('Normal-like cluster', n = 40, containing 20 LoS samples). Depicted are the transformed serum concentrations values (see Materials and Methods) from LoS patients and HCs. Unit variance scaling is applied to rows. Heatmap colours represent the serum concentrations in a color-coded way: blue (low) to red (high). Clustering was performed with ClustVis (http://biit.csut.ee/clustvis/) using Euclidean distance and Ward linkage and is depicted as dendrograms for columns and rows. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

concentrations and disease activity, all LoS serum samples (n = 93; including longitudinal samples n = 19) were classified as active (mLoSSI > 5, n = 67) or inactive (mLoSSI ≤ 5, n = 26) disease.

First, clustering of active and inactive samples was explored in a PLS-DA including all 39 analytes. Separation of samples is displayed in Fig. 2a. CCL18, CXCL13, CXCL9, CXCL10 and TIE-1 were the most important analytes (loadings) for the visualized clustering (Fig. 2b). The variable importance on projection (VIP)-score, which quantifies the contribution of an analyte in the visualized model, demonstrated that CCL18 was the most important analyte for the separation of patients with active and disease (Fig. 2c). This made CCL18 the most attractive analyte to investigate into more detail as biomarker of disease activity in LoS.

The potential of CCL18 to function as a biomarker of disease activity was supported by spearman rank correlation coefficients (r_s) between mLoSSI-scores and serum concentrations: CCL18 demonstrated the strongest correlation to mLoSSI ($r_s = 0.4604$, $P < 0.0001$) (Fig. 3a), followed by CXCL9 ($r_s = 0.4546$, $P < 0.0001$) and CXCL10 ($r_s = 0.4389$, $P < 0.0001$) (Supplemental Table 4 contains all analytes).

Next, a cut-off concentration was defined as above 2 standard deviations of the mean serum concentrations of HCs for every analyte. The frequency of elevated serum concentrations was calculated in the groups with active and inactive disease. Of all analytes, CCL18 demonstrated the greatest difference (33%) between the 2 groups: 52% of the active (35 of 67) and only 19% of the inactive samples (5 out of 26) had elevated CCL18 values (Fig. 3b). (Supplemental Table 5 contains all analytes).

Lastly, CCL18 demonstrated the highest AUC in ROC-analysis for the detection of active versus inactive disease (AUC = 0.754, confidence interval, 0.643–0.864, P-value < 0.001) (Fig. 3c). With an ideal cut-off concentration set at 11 800.00 pg/ml (Material and Methods), CCL18 serum concentrations had a sensitivity of 73% and a specificity of 75% to capture disease activity. (Supplemental Table 6 contains all analytes).

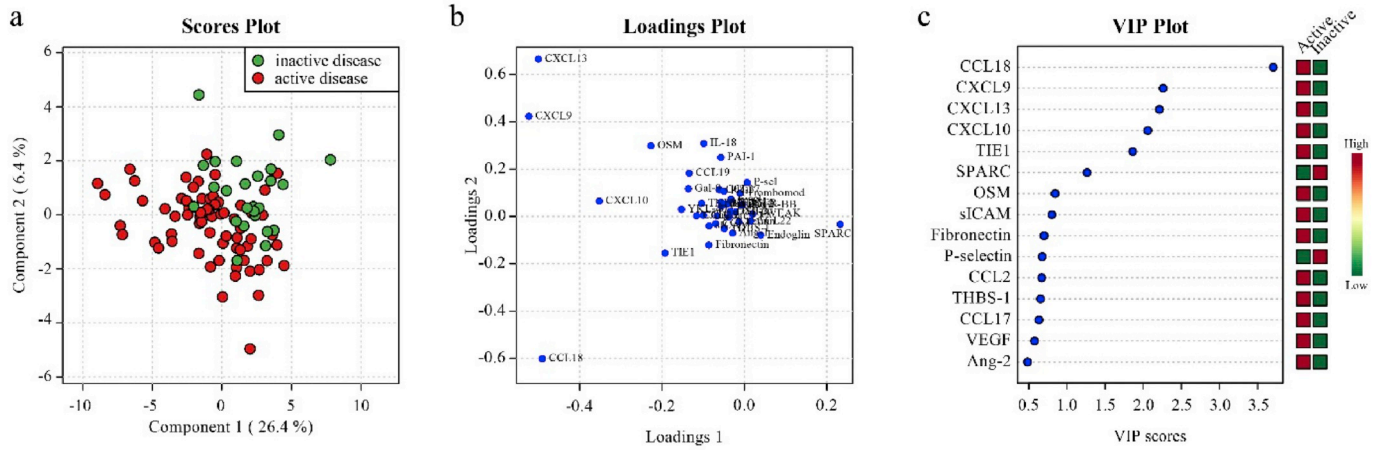

Fig. 2. CCL18 was identified as the most important analyte for the visualized clustering of active and inactive disease. A partial least squared – discriminant analysis (PLS- DA) was performed to investigate clustering of active (mLoSSI > 5) and inactive (mLoSSI ≤ 5) patients including all 39 analytes (A). Importance of each analyte ('loadings') for the visualized clustering is depicted in a loadings-plot (B) and quantified in a variable importance on projection (VIP)-score (C). The PLS-DA was performed in the MetaboAnalyst server (http://biit.cs.utee/clustvis).

3.5. Longitudinal measures CCL18 serum concentrations reflect changes in disease activity

Longitudinal samples (n = 18) for selected patients were collected at follow-up receiving treatment after being included with active disease at onset. Median serum concentrations significantly decreased from 25 313 pg/ml [14 408–67 909] at disease onset to 11 959 pg/ml [6430–21 656] after treatment initiation (P = 0.0056, Fig. 3d). In

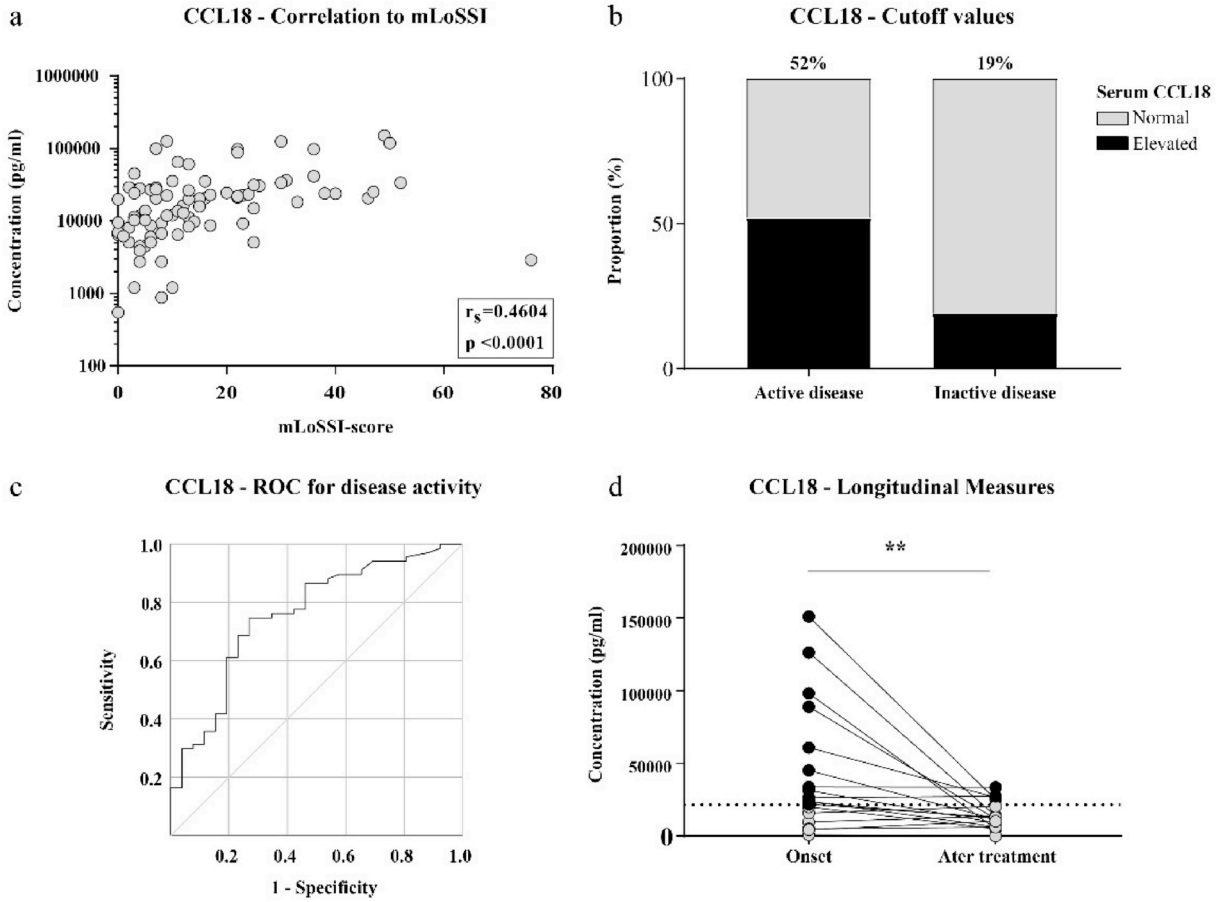

Fig. 3. CCL18 serum concentrations demonstrated superior results to function as biomarker of disease activity via three approaches: CCL18 demonstrated the highest correlation coefficient to mLoSSI scores (A); II) CCL18 values were more frequently elevated (cut-off = 21 555 pg/ml) in active (mLoSSI > 5) than in inactive (mLoSSI ≤ 5) patients (B); CCL18 serum concentrations had the highest area under the curve (AUC) to discriminate between active and inactive samples in receiver operating characteristics (ROC) – curves analyses (C). In addition, longitudinal CCL18 serum concentrations are depicted for 18 LoS patients. CCL18 serum concentrations significantly decreased in individual patients when comparing concentrations at disease onset and after treatment initiation (D). The dotted-line represents the same cut-off value as utilized in figure b. Black and grey dots represent samples above and under the set cut-off, respectively.

addition, the proportion of patients with elevated CCL18 levels decreased from 67% (12 out of 18 patients) at disease onset to 22% (4 out 18) after receiving treatment. Lastly, one patient was included with inactive disease and resampled with flaring disease; CCL18 levels increased from 549.5 pg/ml at inactive disease to 99 537.06 pg/ml during the flare. These findings demonstrate that CCL18 serum concentrations reflect disease activity changes within individual patients.

3.6. The relation between CCL18 serum concentrations and other clinical outcome measures

In this section, we briefly describe the relation between other relevant clinical variables and CCL18 serum concentrations.

3.6.1. Treatment

Longitudinal analyses demonstrated that CCL18 serum concentrations decreased after treatment initiation within individual patients. Comparison of groups with or without systemic treatment confirmed this observation; median CCL18 concentrations were 21 537 pg/ml [9380–40 343] for patients without treatment, compared to 11 893 pg/ml [6465–25 253] (p = 0.0392) for patients receiving systemic treatment (Supplemental Fig. 3a). In addition, median CCL18 concentrations decreased stepwise from 24 187 pg/ml [16 058–60 049] in the group of patients with active disease without treatment to 7223 pg/ml [4912–17 235] in the groups of patients with inactive well-controlled disease without treatment (Supplemental Fig. 3b).

3.6.2. Clinical outcome measures

Disease activity status was defined by mLoSSI throughout this paper. Similar correlations were found when correlating the Physician's Global Assessment of disease activity to CCL18 concentrations (rs = 0.5235, P < 0.0001). No correlation was present with clinical scores capturing extent of disease damage: LoSDI (r_s = 0.06596 P = 0.5439) and PGA-damage (r_s = 0.01484, P = 0.8909).

3.6.3. Disease subtypes

CCL18 concentrations were highest in the EF (median, 25 126 pg/ml [10 154–95 000]) and linear subtypes (21 093 pg/ml [8667–29 236]), followed by generalized LoS (19 820 pg/ml [6743–24 124]), Limited LoS (18 531 pg/ml [8258–55 969] and finally Deep LoS (5622 [2736–27 987] (Supplemental Fig. 4).

3.7. CCL18 is elevated in LoS and not in (juvenile) dermatomyositis (JDM) and spinal muscular atrophy (SMA)

In addition to LoS samples, CCL18 serum concentrations were investigated in 119 (juvenile) dermatomyositis ((J)DM) and 43 spinal muscular atrophy (SMA) samples to investigate the specificity of CCL18 as a biomarker for LoS and not just general inflammatory processes (Characteristics are displayed in Supplemental Table 7). The median CCL18 serum concentration was significantly increased in LoS (20 902 pg/ml [8497–32 040], P = 0.0021) and not in (J)DM (3911 pg/ml [199.9–10 558], P = 0.454) or SMA (7297 pg/ml [2773–12 555], P > 0.999) compared to HCs (7158 pg/ml [2772–14 069] (Fig. 4a). In addition, 49% of the LoS patients had elevated CCL18 serum concentrations, compared to only 7% of the (J)DM and 7% of the SMA patients (Fig. 4b).

3.8. CCL18 expression

CCL18 gene expression was investigated in circulating immune cells and full thickness skin biopsies. CCL18 is not differentially expressed in circulating immune cells of LoS patients versus HCs.

CCL18 gene expression did not significantly differ in isolated PBMCs between 58 LoS patients and 22 healthy controls (median fold change (FC) = 0.98, P = 0.6661). Moreover, no relation was present between CCL18 gene expression in PBMCs and CCL18 serum concentrations within individuals (r_s = −0.1327, P = 0.2632) (Supplemental Fig. 5).

3.9. CCL18 gene and protein expression are increased in the inflammatory border of cutaneous LoS lesions

For selected patients (N = 25), three skin biopsies were taken from: I) the inflammatory border and II) the sclerotic centre of affected skin, representing the active and inactive stage of LoS, respectively. A third biopsy was taken from unaffected skin (Fig. 5a). CCL18 gene expression was significantly increased at the inflammatory border compared to healthy control biopsies (median FC = 4.76 [2.35–10.30], P < 0.0001) (Fig. 5b), whereas CCL18 gene expression was not increased at the sclerotic centre of affected skin (median FC = 1.54 [0.625–4.665] P = 0.2491) and unaffected skin (median FC = 0.86 [0.30–1.51], P > 0.9999). Lastly, CCL18 protein expression was also increased at the inflammatory and to a lesser extent at the sclerotic centre, with minimal expression in the unaffected skin biopsy of patients and healthy controls. (Fig. 5c).

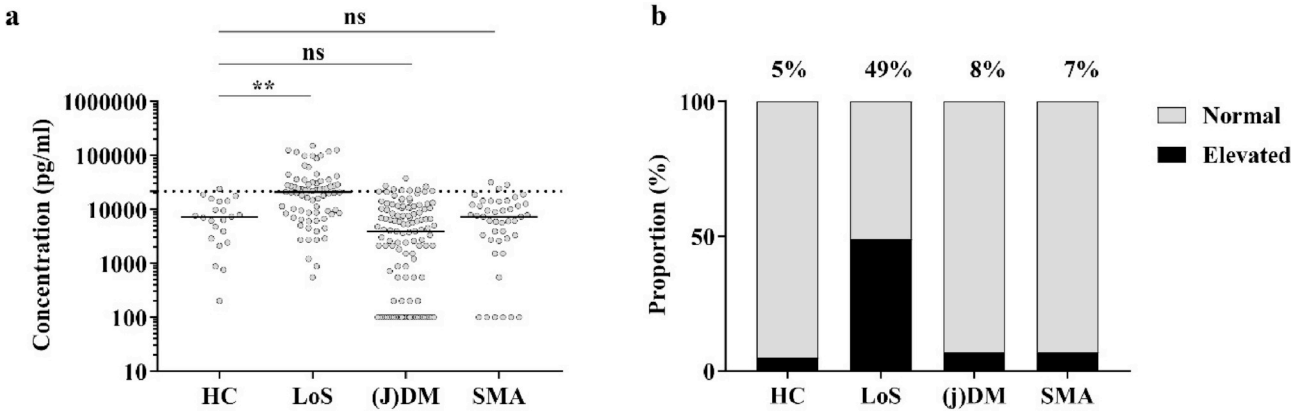

Fig. 4. The median CCL18 serum concentration was significantly increased in localized scleroderma (LoS) and not in (juvenile) dermatomyositis (JDM) or spinal muscular atrophy (SMA) compared to healthy controls (HC) (A). In addition, this specificity was also reflected in the frequency of patients with elevated serum concentrations of CCL18 (cut-off 21555 pg/ml): 49% of the LoS patients had increased CCL18 serum concentrations, compared to only 8% of the (J)DM and 7% of the SMA patients (B).

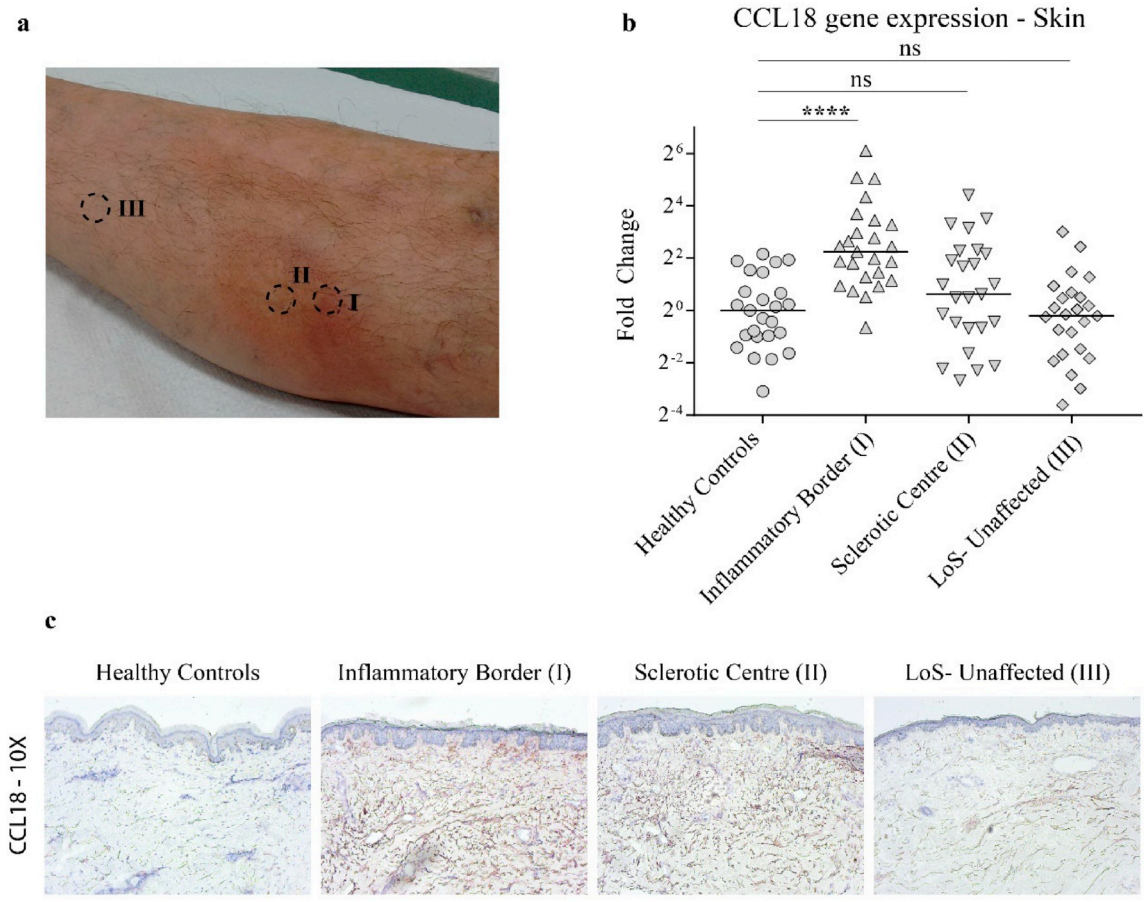

Fig. 5. Tissue gene and protein expression of CCL18. Three skin biopsies were taken from: I) the inflammatory border and II) the sclerotic centre of affected skin, representing the active and inactive stage of LoS, respectively. A third biopsy (III) was taken from unaffected skin. (A) CCL18 gene expression was significantly elevated at the inflammatory border, and not at the sclerotic centre in LoS (B). CCL18 protein expression was increased at the inflammatory border and sclerotic centre of LoS, and not at the unaffected tissue, compared to healthy controls (C).

4. Discussion

The current lack of accurate and reliable tools to determine disease activity is leading to undesired treatment delays and suboptimal treatment approaches in LoS patients, which propelled us to investigate novel biomarkers for this debilitating condition. By screening 39 inflammatory mediators for their relevance in LoS, we found elevated levels of CCL18, CCL19, TIE-1, CXCL13, IL-18, TNFRII, Galectin-9, CXCL9, CXCL10 and sVCAM. Of these elevated analytes, CXCL9 and CXCL10 have recently described as potential biomarkers of disease activity in LoS [716–18]. Increased levels of sVCAM have been described without a clear relation to disease activity [19].

Cluster analyses of the 10 elevated inflammatory markers revealed that disease activity status, defined by mLoSSI, was the most important clinical feature that correlated with circulating levels. After the identification of the association between serum cytokine levels and disease activity status, we demonstrated that CCL18 was the most important analyte able to separate patients with active from inactive disease. The potential of CCL18 to function as biomarker of disease activity was confirmed by ROC-analyses: CCL18 demonstrated the highest AUC to discriminate between patients with active and inactive LoS. In addition, CCL18 had superior correlation coefficient to mLoSSI, and its level normalised in patients effectively treated with immunosuppressive therapies. Based upon these observations and the finding that increased CCL18 levels were specific for LoS, and not in JDM or SMA, we propose CCL18 as a novel biomarker for disease activity in LoS.

CCL18 was originally termed pulmonary and activation-regulated chemokine (PARC) [20] and is known to exhibit chemotactic activity, via the recently discovered receptor CCR8 [21] toward multiple immune cells [2022–25]. In addition to the immunomodulatory effect, CCL18 also stimulates the production of collagen in pulmonary and cutaneous fibroblasts in vivo, and in murine models CCL18 overexpression induces pulmonary fibrosis [26]. In line with experimental studies, high levels of CCL18 are observed in multiple inflammatory and fibrotic conditions [2728]. In patients with idiopathic pulmonary fibrosis, increased levels of serum CCL18 correlated with high collagen accumulation and T-lymphocyte infiltration in the lung [29]. Furthermore CCL18 also correlated with clinical outcome measures associated with a poor outcome such as lung function impairment and decline, and risk of future acute exacerbations and mortality at 6 months [30]. Likewise, CCL18 serum concentrations have been demonstrated to be increased in SSc in 35% of the patients and CCL18 levels predicted progression of lung fibrosis, lung function decline and mortality at 5 and 10 years [3132]. Besides pulmonary fibrotic conditions, CCL18 has been demonstrated to be increased in diseases characterized by T and B lymphocyte dysfunction such as Sjogren syndrome, giant cell arteritis, rheumatoid arthritis and bullous pemphigoid [20]. We demonstrated that CCL18 gene and protein expression is increased at the inflammatory border of cutaneous LoS lesions, with normal gene expression in unaffected skin and circulating immune cells. These observations suggest that the source of CCL18 is the inflammatory skin of LoS patients. How increased CCL18 levels are elicited may shed light on the pathogenesis of LoS but remains to be investigated.

We present superior biomarker characteristics for CCL18 over CXCL 9 and CXCL10, two recently discovered biomarker candidates for LoS [716–18]. However, presented differences are marginal and studies in

larger populations and containing more robust longitudinal data should demonstrate which serum cytokine, or combination of cytokines aids best in clinical decision making.

For the current study we have utilized mLoSSI as outcome measure to define disease activity. However, mLoSSI is limited in several aspects: 1) it only captures superficial components of disease activity (erythema and thickening of the skin). This potentially leads to misclassification of disease activity status in patients with active and progressive deep LoS and EF; 2) Skin thickening, one of the mLoSSI-items, may be irreversible and consequentially mLoSSI-scores do not always accurately reflect disease activity status. This latter especially holds true for EF; a recent study by our group describes irreversible skin fibrosis in large proportions of patients affected by this subtype [33]; 3) No studies have investigated ideal cut-off values for mLoSSI to define inactive, minimally active or active disease. These limitations underscore the dire need of additional and accurate biomarkers for disease activity in LoS, but also create heterogeneity in the groups in this study. Secondly, the size of our cohort is limited, which restricts us to compare distinct molecular patterns between subtypes. Lastly, our study results warrant replication studies.

Clinical evaluation of LoS has demonstrated to be challenging with suboptimal treatment approaches as a result. In this study, we identified CCL18 as a specific biomarker for disease activity in LoS patients. Importantly, CCL18 serum concentrations will not replace but complement clinical assessment by experienced health care professionals. However, values below the set cut-off most likely reflect absence of residual inflammation, enabling the tapering of immunosuppressive medication, whereas rising levels may predict relapses and therefore indicate the need for intensification of treatment.

5. Conclusion

In this study we show that CCL18 is identified as a specific biomarker for disease activity in LoS which may aid therapeutic strategies. We propose that CCL18 is produced locally in inflamed skin. The understanding of the underlying pathways of this local production might lead to better insights in the disease pathogenesis and treatment of this debilitating disease.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.04.008.

Funding sources

This article has no funding source.

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