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# A subset of CD163<sup>+</sup> macrophages displays mixed polarizations in discoid lupus skin

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## Abstract

**Introduction:** Lesional skin of patients with discoid lupus erythematosus (DLE) contains macrophages, whose polarization has yet to be investigated. To test our hypothesis that M1 macrophages would be increased in DLE skin, we examined transcriptome alterations in immune cell gene expression and macrophage features in DLE and normal skin by using gene expression and histochemical approaches.

**Methods:** Gene expression of RNA from DLE lesional and normal control skin was compared by microarrays and quantitative real-time polymerase chain reaction (RT-PCR). Both skin groups were analyzed for CD163 expression by immunohistochemistry. Double immunofluorescence studies were performed to characterize protein expression of CD163<sup>+</sup> macrophages.

**Results:** DLE skin had twice as many upregulated genes than downregulated genes compared with normal skin. Gene set enrichment analysis comparing differentially expressed genes in DLE and normal skin with previously published gene sets associated with M1 and M2 macrophages showed strong overlap between upregulated genes in DLE skin and M1 macrophages. Quantitative RT-PCR showed that several M1 macrophage-associated genes—e.g., chemokine (C-X-C motif) ligand 10 (CXCL10), chemokine (C-C motif) ligand 5 (CCL5), and signal transducer and activator of transcription 1 (STAT1)—had amplified mRNA levels in DLE skin. CD163<sup>+</sup> macrophages were increased near the epidermal-dermal junction and perivascular areas in DLE skin compared with normal skin. However, double immunofluorescence studies of CD163<sup>+</sup> macrophages revealed minor co-expression of M1 (CXCL10, tumor necrosis factor-alpha, and CD127) and M2 (CD209 and transforming growth factor-beta) macrophage-related proteins in DLE skin.

**Conclusion:** Whereas a subset of CD163<sup>+</sup> macrophages displays mixed polarizations in DLE skin, other immune cells such as T cells can contribute to the expression of these macrophage-related genes.

**Keywords:** Discoid lupus erythematosus, Macrophage, Transcriptome, Gene expression

## Introduction

Discoid lupus erythematosus (DLE), the most common chronic cutaneous lupus subtype [1, 2], is a photosensitive and disfiguring skin disease marked by erythematous scaly papules that transition into hyper- and hypopigmented scarring plaques located most commonly on the face, scalp, and neck [3, 4]. Ten to twenty percent of patients with systemic lupus erythematosus (SLE) have diagnosed DLE [5–7], whereas up to 17 % of patients

presenting with an initial diagnosis of DLE develop SLE [1, 8, 9]. Moreover, in light of this overlap, recent studies have shown that multiple gene variations are shared by patients with SLE and patients with DLE [10, 11].

Much attention has been directed toward understanding how T and B cells are involved in cutaneous lupus. Although macrophages are the second most common inflammatory cells in DLE skin (next to T cells) [12–14], they are a relatively understudied population in DLE. Macrophages can direct T-cell differentiation and facilitate antigen presentation, thus influencing immune responses in DLE. Macrophages can be differentially activated into M1 and M2 subtypes, which have

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contrasting functions. M1 macrophages (classically activated macrophages) assume the traditional roles of phagocytes, which include targeting and clearing microbes, and depict type I immune responses. They produce pro-inflammatory reactive oxygen species and cytokines such as interleukin-12 (IL-12). M2 macrophages (alternatively activated macrophages) counteract type I immune responses with the induction of type II immune responses via secretion of IL-10, resulting in resolution of inflammation. Blood in patients with SLE demonstrated upregulation of M1 macrophage proteins such as chemokine (C-X-C motif) ligand 10 (CXCL10) [15] and downregulation of M2 macrophage proteins, including transforming growth factor-beta (TGF- $\beta$ ) [16]. However, whether this bias carries over into target tissues of patients with lupus, such as skin, is unknown.

Our objective in this study was to investigate changes in immune cell signatures in the transcriptomes of DLE lesional skin versus normal skin by microarray analysis and quantitative real-time polymerase chain reaction (qRT-PCR). Protein expression was assessed by immunohistochemistry and double immunofluorescence. Because lesional skin from patients with inflammatory skin diseases such as psoriasis [17] has been characterized by a distinctly greater presence of classically activated macrophages, we hypothesized that, compared with normal skin, DLE lesional skin would also exhibit a strong M1 macrophage polarization.

## Methods

### Patient recruitment and sample/data collection

Subjects with DLE, diagnosed by clinicopathological correlation, were recruited from the outpatient dermatology clinics at Parkland Health and Hospital System and University of Texas Southwestern (UTSW) Medical Center. Normal controls were additionally recruited from elective plastic surgery cases performed at UTSW Medical Center. DLE patients having individual lesions with a Cutaneous Lupus Activity and Severity Index (CLASI) activity score of at least 2 were included. DLE patients who had SLE, based on fulfillment of at least four American College of Rheumatology (ACR) diagnostic criteria [18] or a history of drug-induced DLE, and normal subjects with a personal history of autoimmune disease were excluded. On the basis of these screening criteria, 17 patients with DLE and 12 normal controls gave informed consent and were enrolled in the study. Medical, family, and medication histories were obtained, and clinical assessments (e.g., CLASI and Systemic Lupus Erythematosus Disease Activity Index, or SLEDAI) performed at the study visit were collected. Patient characteristics are summarized in Table 1, which is subdivided by disease group (normal and DLE) and experiments (microarray and qRT-PCR). DLE subjects underwent one

4- or 6-mm punch biopsy from active inflammatory borders of discoid lesions with individual CLASI activity scores of at least 2. Normal sun-exposed non-lesional skin was obtained as discarded specimens from cutaneous surgeries or elective plastic surgeries. Skin biopsies were placed in RNALater Solution (Ambion, Austin, TX, USA), kept at room temperature overnight, and stored at  $-80^{\circ}\text{C}$  for RNA analysis or in OCT and frozen in  $-80^{\circ}\text{C}$  for immunofluorescence experiments. This study was approved by the University of Texas Southwestern Institutional Review Board and complied with Declaration of Helsinki.

### Microarray analysis

RNA from nine DLE lesional and eight normal skin sections was extracted by using RNeasy Lipid Tissue Mini kit (Qiagen, Hilden, Germany). RNA quality was evaluated with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified by spectrophotometry. Biotinylated RNA was amplified by using the TotalPrep RNA amplification kit (Ambion) and hybridized with Illumina Sentrix Expression Beadchips, Human HT-12v4 (Illumina, San Diego, CA, USA). Each array contains more than 31,000 probed genes. Microarray data have been submitted to the public repository, Gene Expression Omnibus.

### Reverse transcription and qRT-PCR

Skin RNA from 17 patients with DLE and 12 normal controls was reverse-transcribed into cDNA by using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). cDNA of selected genes using forward and reverse primers (Additional file 1: Table S1) was amplified with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in accordance with the instructions of the manufacturer. qRT-PCR under the following conditions (3 minutes at  $95^{\circ}\text{C}$ , then 40 cycles of 20 seconds at  $95^{\circ}\text{C}$ , 1 minute at  $55^{\circ}\text{C}$ , and 30 seconds at  $72^{\circ}\text{C}$ ) was performed in a CFX96 qRT-PCR machine (Bio-Rad Laboratories). Cycle threshold ( $C_T$ ) values were standardized to the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and converted to fold change by using the  $2^{-\Delta\Delta C_T}$  formula [19].

### Immunohistochemistry analysis

A subset of DLE lesional ( $n = 5$ ) and normal skin ( $n = 6$ ) biopsies were bisected and transferred in 10 % formalin. Four-micron sections were deparaffinized in xylene and rehydrated in graded alcohols to distilled water. Endogenous peroxidase activity was quenched for 10 minutes at room temperature by using 0.3 %  $\text{H}_2\text{O}_2$  and 0.1 % sodium azide. For epitope retrieval, slides were placed in 1 mM EDTA, pH 8.5, for 30 minutes in a steamer and then cooled for 10 minutes. Slides were incubated with mouse monoclonal anti-CD163

**Table 1** Subject demographic data

|  | Normal (microarray) | DLE (microarray) | Normal (qRT-PCR) | DLE (qRT-PCR)    |
|--|---------------------|------------------|------------------|------------------|
| Number   | 8                   | 9                | 12               | 17               |
| Age at visit in years, median (range)                          | 47.0 (40.9–54.0)    | 40.0 (21.5–56.5) | 48.0 (39.3–66.4) | 40.2 (21.5–59.9) |
| Gender, male/female  | 1/7                 | 1/8              | 3/9              | 4/13             |
| Ethnicity, N (%)   |                     |                  |                  |                  |
| African-American   | 0 (0)               | 7 (77.8)         | 0 (0)            | 12 (70.6)        |
| Caucasian  | 8 (100)             | 0 (0)            | 12 (100)         | 4 (23.5)         |
| Hispanic   | 0 (0)               | 2 (22.2)         | 0 (0)            | 1 (11.1)         |
| CLASI activity score, median (range)                           | N/A                 | 6 (2–20)         | N/A              | 7 (2–21)         |
| CLASI damage score, median (range)                             | N/A                 | 14 (6–20)        | N/A              | 10 (0–20)        |
| SLEDAI score, median (range)                                   | N/A                 | 0 (0–0)          | N/A              | 0 (0–4)          |
| Disease duration in years, median (range) <sup>a</sup>         | N/A                 | 2.8 (0–16.3)     | N/A              | 1.5 (0–16.3)     |
| Lupus medications, N (%)                                       |                     |                  |                  |                  |
| Topical/intralesional corticosteroids/topical immunomodulators | N/A                 | 5 (55.6)         | N/A              | 12 (70.6)        |
| Hydroxychloroquine   | N/A                 | 4 (44.4)         | N/A              | 8 (47.0)         |
| Quinacrine   | N/A                 | 0 (0)            | N/A              | 1 (5.9)          |
| Methotrexate   | N/A                 | 0 (0)            | N/A              | 1 (5.9)          |
| None   | N/A                 | 2 (22.2)         | N/A              | 4 (23.5)         |
| ACR SLE criteria, N (%) <sup>b</sup>                           |                     |                  |                  |                  |
| Discoid rash   | N/A                 | 9 (100)          | N/A              | 17 (100)         |
| Photosensitivity   | N/A                 | 7 (77.8)         | N/A              | 12 (70.6)        |
| Oral ulcers  | N/A                 | 2 (22.2)         | N/A              | 3 (17.6)         |
| Hematological disorder   | N/A                 | 1 (11.1)         | N/A              | 1 (11.1)         |
| Anti-nuclear antibody <sup>c</sup>                             | N/A                 | 3 (33.3)         | N/A              | 3 (17.6)         |
| Number of ACR SLE criteria, median (range)                     | N/A                 | 3 (1–3)          | N/A              | 2 (1–3)          |

Abbreviations: *ACR* American College of Rheumatology, *CLASI* cutaneous lupus disease area and severity index, *DLE* discoid lupus erythematosus, *N/A* not applicable, *qRT-PCR* quantitative real-time polymerase chain reaction, *SLE* systemic lupus erythematosus, *SLEDAI* systemic lupus erythematosus disease and activity index

<sup>a</sup>Disease duration was not available for two patients with DLE

<sup>b</sup>No patients with DLE had malar rash, arthritis, serositis, renal disorder, neurologic disorder, or immunological disorder

<sup>c</sup>Positive anti-nuclear antibody (ANA) test was determined by history of ANA titers of at least 1:160, as determined by indirect immunofluorescence, or positive enzyme-linked immunosorbent assay

antibody (Neomarkers/Thermo Fisher Scientific, Waltham, MA, USA) or isotype control for 50 minutes at 25 °C [20]. After phosphate-buffered saline (PBS) rinse, horseradish peroxidase-conjugated goat anti-rabbit horseradish peroxidase-conjugated IgG antibody (Leica Novocastra, Wetzlar, Germany) [21] was added for 45 minutes at 25 °C [20]. Finally, the slides were immersed for 8 minutes in 25 °C diaminobenzidine (Invitrogen, Carlsbad, CA, USA), enhanced with 0.5 % copper sulfate in PBS for 1–3 minutes at 25 °C, counterstained in hematoxylin, and dehydrated in graded alcohols and xylene. Cell counts were performed by two independent evaluators (BC and GH) on the most representative sections in the epidermal-dermal junction, perifollicular, and perivascular areas of the biopsies by using ImageJ [22]. Cell counts were averaged and divided by surface area assessed.

### Immunofluorescence

Six-micron frozen DLE (n = 6–8) and normal (n = 4–5) skin sections fixed in acetone were blocked in 5 % normal goat serum in PBS with 0.3 % Triton X-100 for 1 hour. Slides were then incubated with rabbit anti-human primary antibodies—CD127 (Abcam, Cambridge, MA, USA), CD209 (Abcam), CXCL10 (PeproTech, Rocky Hill, NJ, USA), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Novus, Littleton, CO, USA), and TGF- $\beta$  (Novus)—overnight followed by PBS washes. Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Life Technologies, Carlsbad, CA, USA) were added for 30 minutes followed by PBS washes, except for TGF- $\beta$  and TNF- $\alpha$  antibody-stained slides, whose signals were amplified by using the TSA Biotin kit in accordance with the instructions of the manufacturer (PerkinElmer, Waltham, MA, USA). Mouse anti-human CD163 antibody (Novus) or mouse

anti-human CD3 antibody (eBioscience, Inc., San Diego, CA, USA) was added for 1 hour, followed by PBS washes. Slides were incubated with Alexa Fluor 594-conjugated goat anti-mouse antibodies (Life Technologies) for 30 minutes and washed with PBS. Slides were coverslipped with Vectashield mounting medium and analyzed. Images were acquired with an Olympus BX60 fluorescent microscope (Olympus, Tokyo, Japan).

### Statistical analysis

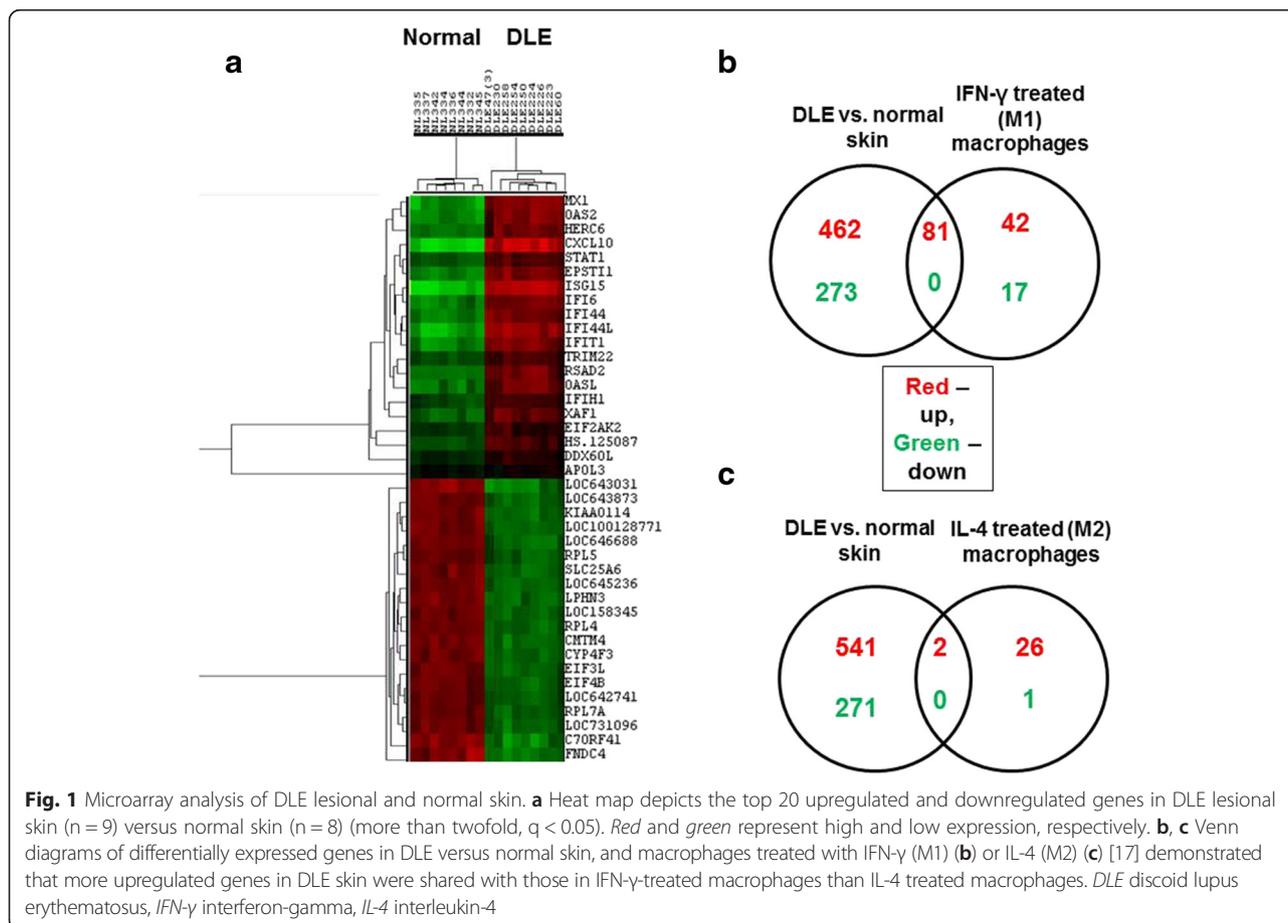
Based on 31,000 probed genes on the Illumina Human HT-12v4 Expression BeadChip, a sample size of eight for each study group was used and this had predicted power to detect twofold gene expression difference with more than 80 % power at a 5 % false-positive rate assuming a standard deviation of 0.65 [23]. Microarray data were quantum-normalized and background-subtracted by using Bead Studio Software version 3.2.2 and analyzed on GeneSpring GX (Agilent Technologies, Santa Clara, CA, USA). To connect genes of interest with existing biological pathways, Ingenuity Pathway Analysis (Ingenuity System Inc., Redwood City, CA, USA) was used to identify networks ordered by *P* values. Significance Analysis of Microarrays (SAM) [24] helped

identify genes with statistically significant differences; *q* values, defined as the lowest false discovery rate at which genes were called significant by SAM analysis [25], were less than 0.05. Gene set enrichment analysis was performed to determine whether differentially expressed genes were over-represented in referenced gene lists [26], specifically those from macrophages incubated with either interferon-gamma (IFN- $\gamma$ ) (M1 macrophages) or IL-4 (M2 macrophages) [17]. Heat maps and clustering analyses were performed by using Cluster and Treeview software [27]. For qRT-PCR and immunohistochemistry data, Mann-Whitney *U* test was used to compare the two study groups.

## Results

### Transcriptomes of DLE lesional skin were enriched with M1 macrophage-related genes

From a total of 21,628 genes, whose expression was either “present” or “marginal” in at least 75 % of all samples, microarray analysis of DLE lesional skin (*n* = 9) versus normal sun-exposed skin (*n* = 8) revealed 543 up-regulated and 273 downregulated genes in DLE skin (more than twofold, *q* < 0.05) (Fig. 1a and Additional file 1: Table S2). Pathway analysis showed that the two



most significant signaling cascades included genes involved in antigen presentation and cytotoxic T cell-mediated apoptosis. Because IFN- $\gamma$  was the top upstream regulator gene, these findings supported a prominent role of cell-mediated immunity in DLE pathogenesis. Specifically, we noted several upregulated genes in DLE skin that may be expressed by different cells associated with cell-mediated immunity (e.g., T<sub>H</sub>1 cells: CXCR3, signal transducer and activator of transcription 1 (STAT1), and STAT4; CD8<sup>+</sup> cells: granzymes A, B, K, and perforin; and macrophages: CD68, CD163, and CXCL10) (highlighted in Additional file 1: Table S2).

We then focused on analyzing expression of macrophage-related genes in DLE versus normal skin to investigate macrophage polarization (M1 or M2) in DLE skin. We first generated Venn diagrams to identify common genes in our lists of differentially expressed genes in DLE and normal skin and previously published sets of differentially expressed genes in macrophages before and after treatment with IFN- $\gamma$  (M1 macrophages) [17]. Eighty-one genes were commonly upregulated in both gene sets, but none was mutually downregulated (Fig. 1b and Additional file 1: Table S3). Differentially expressed genes in DLE and normal skin and macrophages before and after treatment with IL-4 (M2 macrophages) [17] were also compared. Only two genes were enhanced in both gene sets, and none was commonly decreased (Fig. 1c and Additional file 1: Table S3). Gene set enrichment analysis showed that upregulated genes in DLE skin showed significant overlap with those in M1 macrophages (normalized enrichment score = 1.25,  $P = 0.02$ ) but not with those in M2 macrophages (Table 2). Thus, both analyses showed that DLE skin was significantly enriched with M1 macrophage-related genes.

#### qRT-PCR analysis confirms upregulation of multiple M1 macrophage-related genes in DLE skin

Several macrophage-related genes were selected for confirmatory qRT-PCR studies on DLE lesional ( $n = 17$ ) and normal sun-exposed ( $n = 12$ ) skin. CD163 (2.83 fold change (FC)) and CD68 (4.98 FC), which are both markers used to identify macrophages, had significantly higher levels in DLE skin than normal skin ( $P < 0.0001$ )

(Fig. 2a, b). Multiple M1 macrophage genes—e.g., CD127 (8.01 FC,  $P < 0.0001$ ), TNF- $\alpha$  (1.92 FC,  $P = 0.047$ ), CXCL10 (45.91 FC,  $P < 0.0001$ ), STAT1 (11.96 FC,  $P < 0.0001$ ), CCL5 (25.85 FC,  $P < 0.0001$ ), CD86, and Mx1—were also significantly upregulated in DLE skin compared with normal skin (Fig. 2c–g and Additional file 1: Table S4). IFN- $\gamma$  and IL-12, two cytokines associated with M1 macrophages, were higher in DLE skin but did not reach statistical significance (Additional file 1: Table S4). With the exception of TGF- $\beta$  (2.42 FC,  $P = 0.0002$ ), which was significantly higher in DLE skin than normal skin, multiple M2 macrophage genes—e.g., CD206 (0.72 FC,  $P = 0.32$ ), CD209 (1.10 FC,  $P = 0.97$ ), arginase-1 (1.25 FC,  $P = 0.19$ ), FOLR2, and IL-10—were not differentially expressed in DLE and normal skin (Fig. 2h–k and Additional file 1: Table S4). We also selected type I interferon-inducible genes (e.g., Mx1, ISG15, and Ly6E) and genes associated with T<sub>H</sub>1 cells (e.g., CXCR3) and CD8<sup>+</sup> T cells (e.g., CD8 and granzyme B) for qRT-PCR analysis. These were significantly elevated in DLE skin versus normal skin ( $P < 0.0001$ ) (Additional file 1: Table S4).

#### DLE lesional skin contained higher numbers of CD163<sup>+</sup> macrophages than normal skin

Samples of DLE lesional skin ( $n = 5$ ) and normal sun-exposed skin ( $n = 6$ ) were immunostained with CD163, a scavenger receptor that is highly specific for macrophages in the skin [28] (Fig. 3a, b). DLE skin had significantly higher concentrations of CD163<sup>+</sup> cells at the epidermal-dermal junction (median: 87.8 cells/mm<sup>2</sup> versus 25.3 cells/mm<sup>2</sup>;  $P = 0.004$ ) and perivascular areas (median: 128.9 cells/mm<sup>2</sup> versus 48.6 cells/mm<sup>2</sup>;  $P = 0.03$ ) than normal skin. DLE skin showed higher amounts of CD163<sup>+</sup> cells in perifollicular areas compared with normal skin (median: 50.0 cells/mm<sup>2</sup> versus 10.0 cells/mm<sup>2</sup>), but this difference did not reach statistical significance ( $P = 0.26$ ) (Fig. 3c–e).

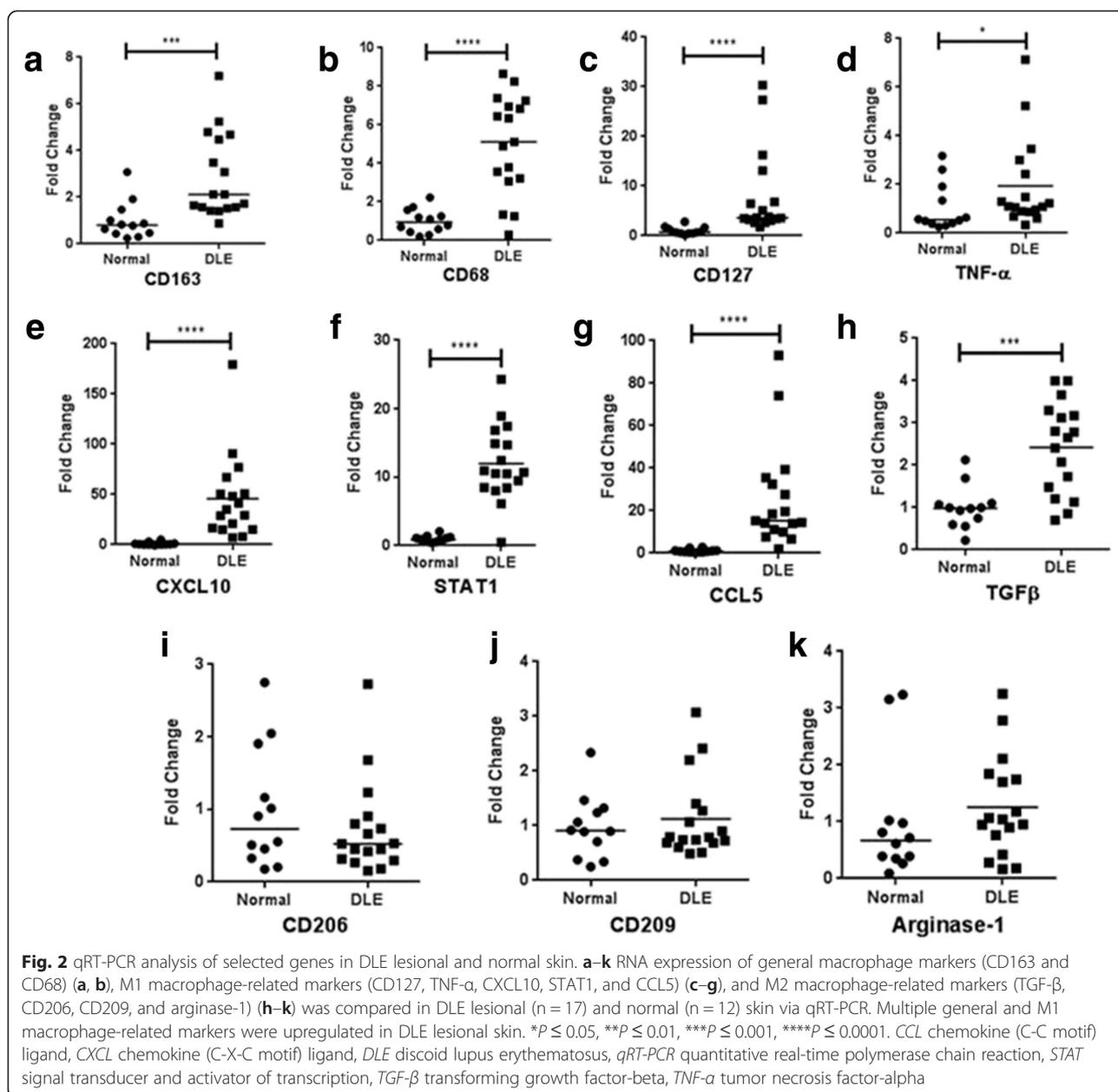
#### A minority of CD163<sup>+</sup> macrophages showed co-staining with M1- and M2-related proteins in DLE skin

To determine whether CD163<sup>+</sup> macrophages predominantly expressed M1 or M2 macrophage-related proteins or both, double immunostaining of CD163 and selected

**Table 2** Gene set enrichment analysis of differentially expressed genes in DLE lesional and normal skin

| Name  | Size | ES    | NES   | <i>P</i> value | FDR q value |
|---|------|-------|-------|----------------|-------------|
| IFN- $\gamma$ -stimulated (M1) macrophages upregulated  | 113  | 0.92  | 1.25  | 0.02           | 0.02        |
| IFN- $\gamma$ stimulated (M1) macrophages downregulated | 16   | -0.55 | -1.24 | 0.25           | 0.25        |
| IL-4 stimulated (M2) macrophages upregulated            | 26   | 0.39  | 0.97  | 0.49           | 0.49        |
| IL-4 stimulated (M2) macrophages downregulated          | 1    | 0.95  | 1.11  | 0.27           | 0.27        |

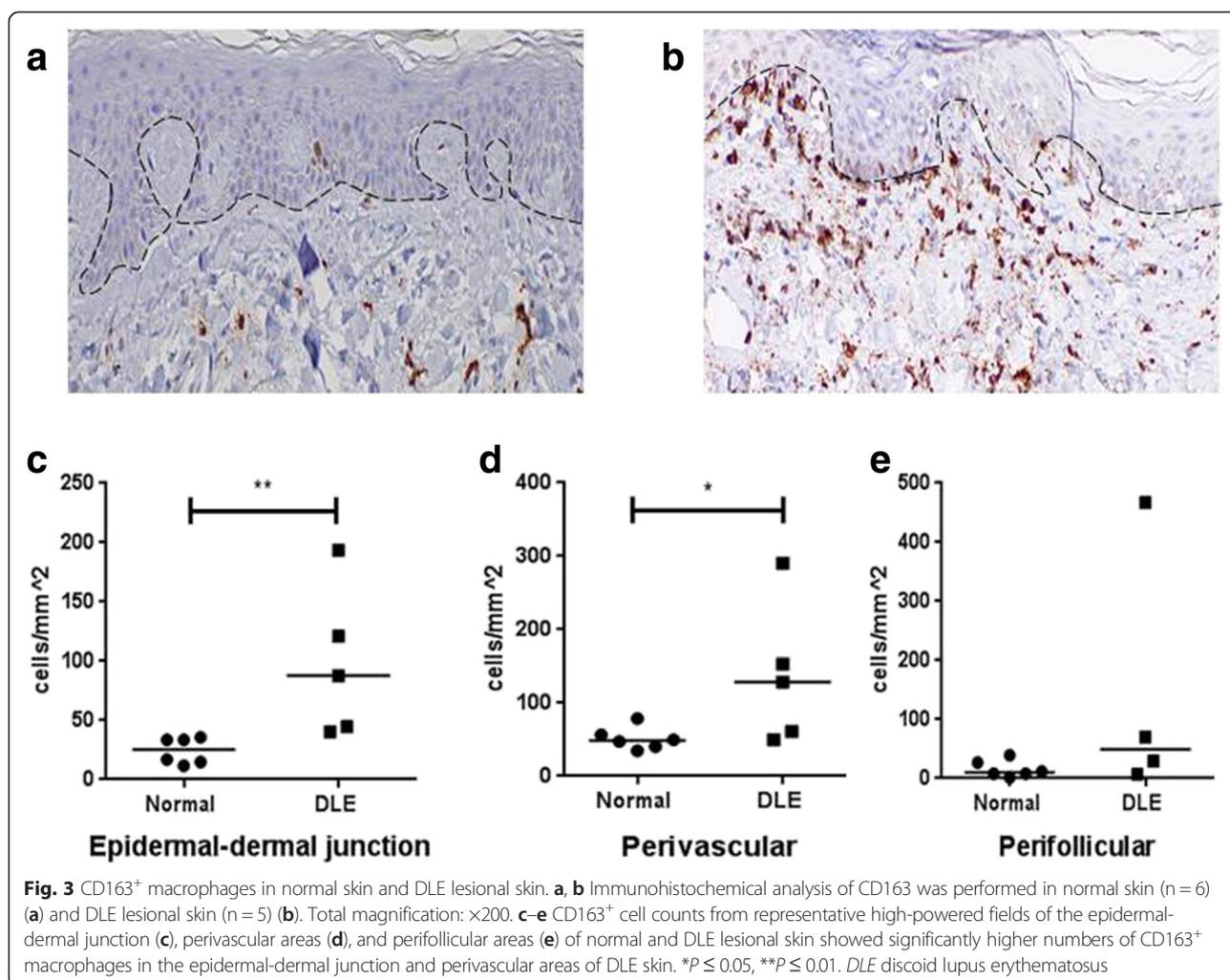
Abbreviations: DLE discoid lupus erythematosus, ES enrichment score, FDR false discovery rate, IFN- $\gamma$  interferon-gamma, IL-4 interleukin-4, NES normalized enrichment score



M1 and M2 macrophage-related proteins (CXCL10, CD127, TNF- $\alpha$ , TGF- $\beta$ , and CD209) were performed in DLE lesional ( $n = 6–8$ ) and normal ( $n = 4$  or 5) skin. DLE and normal skin both showed a minority of CD163<sup>+</sup> macrophages near the epidermal-dermal junction and in the perivascular and periadnexal areas of the dermis co-expressing CXCL10, CD127, TNF- $\alpha$ , TGF- $\beta$ , and CD209, and the greatest overlap was seen with CD209 (Fig. 4a–e). Because of this result, we postulated that other inflammatory cells such as T cells could also contribute to the upregulation of M1 macrophage-related proteins in DLE lesional skin. Co-staining of a minority of CD3<sup>+</sup> T cells with CD127 was detected in the dermis of DLE skin (Fig. 4f).

## Discussion

Transcriptome analysis of DLE lesional skin yielded strong expression of a signature of M1 macrophage-related genes. Inspection of selected M1 and M2 macrophage-related genes by qRT-PCR confirmed upregulation of multiple M1 macrophage-related genes in DLE skin. Because M1 macrophages are activated by IFN- $\gamma$  that is produced by T<sub>H</sub>1 cells and promote T<sub>H</sub>1 responses through their antimicrobial activity [29], our findings are consistent with earlier studies showing a predominant T<sub>H</sub>1 bias in DLE skin [30]. Moreover, we performed gene set enrichment analysis comparing previously published gene sets of T<sub>H</sub>1 and T<sub>H</sub>2 cells [31] with DLE and normal skin transcriptome data and

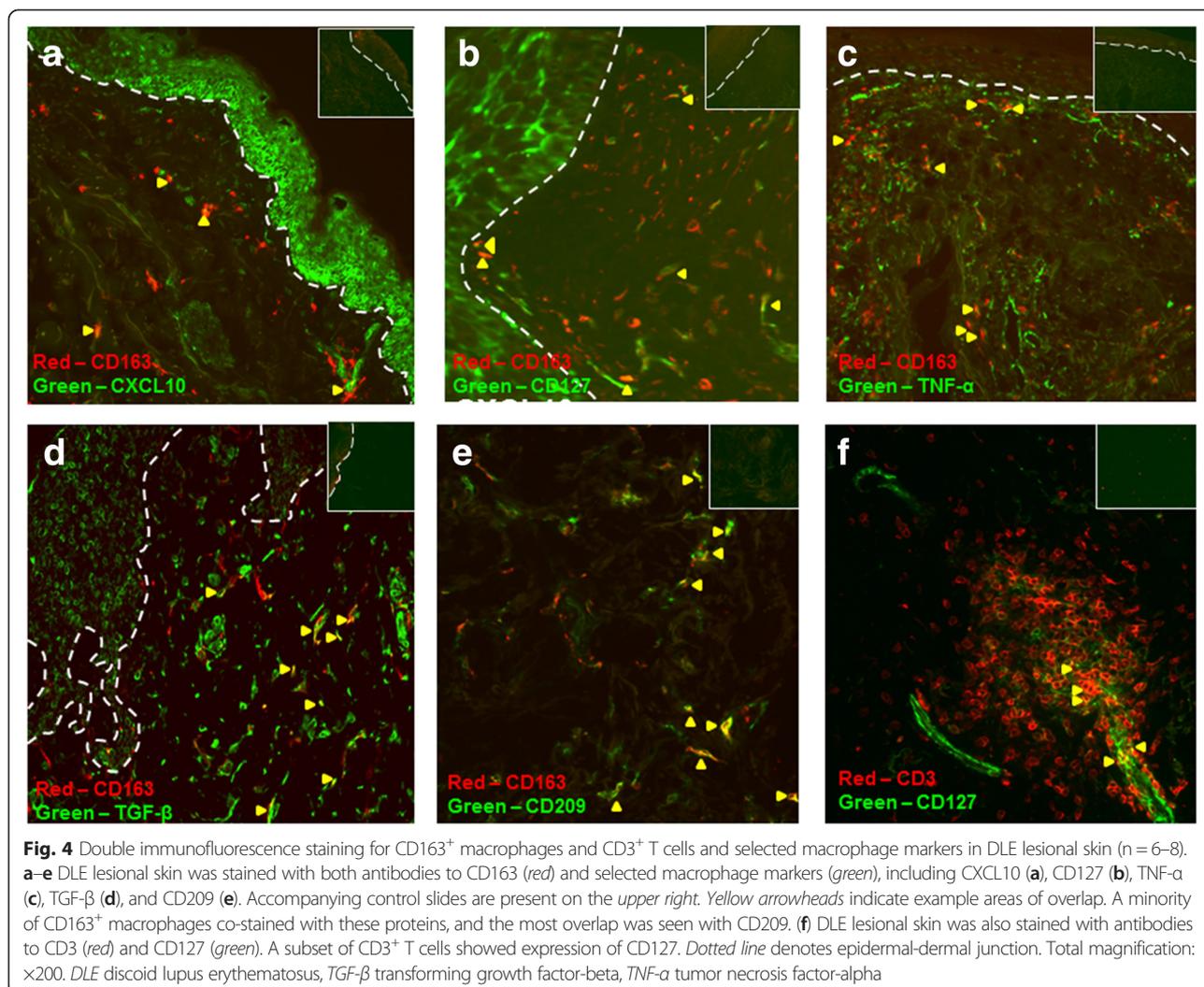


detected  $T_H1$ -biased genes being highly expressed in DLE skin ( $P = 0.01$ ) (data not shown).

Given the limitations that whole skin biopsies rather than isolated CD163<sup>+</sup> skin macrophages were studied for transcriptome analysis and that several upregulated genes may be expressed by skin cells other than macrophages, we performed double immunofluorescence staining to examine protein expression of CD163<sup>+</sup> macrophages in DLE skin. Some CD163<sup>+</sup> macrophages showed co-expression of various M1 (e.g., CD127, CXCL10, and TNF- $\alpha$ ) and M2 (CD209 and TGF- $\beta$ ) macrophage-related proteins, and the greatest overlap was seen with CD209. Although CD163 has been reported by in vitro studies to be a M2-related macrophage marker [32, 33], immunostaining studies of CD163<sup>+</sup> macrophages in peripheral tissues of different diseases have shown that they can express transcription factors specific to either M1 or M2 macrophage signaling pathways [34]. Specifically, studies on squamous cell carcinoma of the skin have reported that CD163<sup>+</sup> macrophages can produce both M1 and M2 macrophage-

related proteins [35]. The presence of different macrophage subtypes may result from the ability of macrophages to change from one subtype to another in response to various stimuli from the surrounding milieu. For instance, IL-4 can guide the transformation of M1 macrophages into M2 macrophages, which promote wound healing and angiogenesis [36]. Likewise, cytokines such as IFN- $\gamma$  produced by  $T_H1$  cells can alter the polarization of M2 macrophages to M1 macrophages that subsequently produce M1 macrophage-related cytokines and enhance their co-stimulatory molecule expression [37]. Furthermore, exogenous signals such as bacterial lipopolysaccharides can induce both M1 and M2 macrophages to produce M1 macrophage-related cytokines such as TNF- $\alpha$  and IL-1 [38].

The disease phase at the time of DLE skin biopsy may have impacted the immunostaining characterization of CD163<sup>+</sup> macrophages. In the acute phase, DLE lesions present clinically as erythematous scaly papules and plaques and microscopically have interface dermatitis, scattered apoptotic keratinocytes, mucin deposition, and



perivascular and periadnexal inflammatory infiltrates [39]. DLE lesions later evolve into hypopigmented scarred plaques with peripheral hyperpigmentation in the chronic phase, and their histopathology displays epidermal atrophy, mild interface dermatitis, loss of hair follicles, and dermal sclerosis [39]. M2 macrophages up-regulate IL-10, which has anti-inflammatory properties. TGF- $\beta$  is a pro-fibrotic cytokine produced mainly by M2c macrophages [40] and may contribute to the scarring process that is seen in the latter stages of DLE. Furthermore, TGF- $\beta$  can have anti-inflammatory effects by downregulating production of pro-inflammatory cytokines such as TNF- $\alpha$  [41] and maintaining peripheral tolerance by limiting the expansion of self-reactive T cells [42]. Because many of these biopsied discoid lesions had clinical features of both acute and chronic DLE, we hypothesize that these lesions may be in a transitional phase between acute and chronic DLE, and this could explain the heterogeneity of macrophage subtypes. Further projects that compare protein expression in acute

and chronic discoid skin lesions will be needed to test whether macrophages strongly favor one polarization over another in the two phases of DLE.

Macrophage bias in peripheral organs other than skin has been investigated in murine models with lupus nephritis. In lupus-prone MRL-lpr mice, transient ischemia/reperfusion injury resulted in early-onset lupus nephritis with an accompanying increase in M1 macrophages compared with M2 macrophages [43]. However, in other lupus murine models, heterogeneous populations of macrophages that did not show a distinct M1 or M2 bias were described in chronic lupus nephritis kidneys [44]. Because human lupus nephritis and DLE have similar features of acute inflammation and chronic scarring, comparisons of macrophages in these two end-organ diseases in lupus could be made to better understand disease pathogenesis in lupus.

Our double immunofluorescence data also showed that other cells, particularly T cells, contribute to enhanced levels of M1 macrophage-related proteins. A

minority of CD3<sup>+</sup> T cells showed co-expression of CD127, an M1 macrophage-related protein, in DLE lesional skin. T cells have been described to express other M1 macrophage-related proteins, including IFN- $\gamma$  [30]. Moreover, given that a T<sub>H</sub>1 cell bias has been observed in DLE skin in our and other [30] data and that T<sub>H</sub>1 cells can activate classically activated macrophages through their secretion of IFN- $\gamma$ , T<sub>H</sub>1 cells likely contribute to the M1 macrophage gene signature in DLE skin. Additionally, CD8<sup>+</sup> T cells, which also produce IFN- $\gamma$  and activate macrophages, have been observed in greater numbers in DLE skin [12, 45, 46]. Thus, the cross-talking between T<sub>H</sub>1 cells, CD8<sup>+</sup> T cells, and macrophages implies that cell-mediated immunity can play a significant role in the pathogenesis of DLE. Further studies examining their interactions and impact on evolution of DLE will be planned. As DLE skin is enriched with multiple types of immune cells [13, 46], our qRT-PCR data demonstrated that DLE skin upregulates cell surface markers expressed by dendritic cells (CD86) and monocytes (CD14), which can also express M1 macrophage-related proteins (e.g., CXCL10 [47, 48] and ISG15 [49]).

## Conclusions

Whereas transcriptome analysis suggested an M1 macrophage gene signature in DLE skin, immunohistochemical studies of DLE skin uncovered a subset of CD163<sup>+</sup> macrophages expressing both M1 and M2 macrophage-related proteins. The diversity of macrophage subtypes may be due to their gene expression plasticity and a mixture of acute and chronic phases in the DLE biopsy skin specimens. Moreover, other immune cells, such as CD3<sup>+</sup> T cells, infiltrating into DLE skin can contribute to the M1 macrophage gene signature in DLE skin. Further exploration into the multiple immune cells expressing M1 macrophage-related proteins and their interactions with each other will help further explain the evolution of DLE.

## Additional file

**Additional file 1: Table S1.** Forward and reverse primers for selected genes. **Table S2.** Differentially expressed genes in DLE lesional (n = 9) and normal (n = 8) skin from microarray analysis (more than twofold change, q value < 0.05). **Table S3.** List of commonly differentially expressed genes in DLE and normal skin, and IFN- $\gamma$  treated (M1) and IL-4 treated (M2) macrophages. **Table S4.** qRT-PCR analysis of selected genes in DLE lesional (n = 17) and normal (n = 12) skin. DLE discoid lupus erythematosus, IFN- $\gamma$  interferon-gamma, qRT-PCR quantitative real-time polymerase chain reaction. (DOCX 165 kb)

## Abbreviations

ACR: American College of Rheumatology; CCL: Chemokine (C-C motif) ligand; CLASI: Cutaneous Lupus Activity and Severity Index; CSF: Colony-stimulating factor; CXCL: Chemokine (C-X-C motif) ligand; DLE: Discoid lupus erythematosus; IFN: Interferon; IL: Interleukin; qRT-PCR: Quantitative real-time polymerase chain reaction; SAM: Significance Analysis of Microarrays;

SLE: Systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; STAT: Signal transducer and activator of transcription; TGF: Transforming growth factor; TNF: Tumor necrosis factor; UTSW: University of Texas Southwestern.

## Competing interests

BC is an investigator for Daavlin Corporation (Bryan, OH, USA). The other authors declare that they have no competing interests.

## Authors' contributions

BC conceived the study design, supervised the overall conduct of study, participated in subject recruitment and data analysis, and drafted and critically revised the manuscript. LT performed the microarray, qRT-PCR, and immunofluorescence experiments; carried out data analyses; and critically revised the manuscript. GH provided critique to study design, participated in the immunohistochemistry analyses, and critically revised the manuscript. NT participated in subject recruitment and data analysis and critically revised the manuscript. SZ participated in the statistical analysis and critically revised the manuscript. DK, NO, and CM provided critique to study design and interpretation of data and critically revised the manuscript. All authors read and approved the final manuscript.

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