Appendix S1.

MATERIAL AND METHODS

Study population

The study enrolled patients with LP ($n = 25$; 3 males, 22 females) and healthy individuals ($n = 25$; 3 males, 22 females) from the Dermatological Outpatient Clinic of the Hospital das Clínicas de São Paulo (HC-FMUSP). Other forms of LP, such as oral LP, lichen planopilaris, or drug-induced LP, were not included in this evaluation, nor were any associations between LP and autoimmune diseases or other skin diseases investigated. The majority of patients had at least 2 affected limbs, with papules involving up to approximately 30% of the trunk. The patients had not been treated with any type of topical or systemic corticosteroid, retinoid or immunosuppressant prior to skin biopsy and blood collection. Two patients with LP were seropositive for HCV. The median age was 42.0 ± 5.5 years (range 20–71 years) for the subjects with LP and 43.46 ± 8.46 years (range 22–65 years) for the healthy individuals. All individuals provided written informed consent, and the study protocol was approved by the institutional ethics committee of the HC-FMUSP.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood using Ficoll-Hypaque (Amersham Pharmacia Biotech, NJ, USA) gradients and resuspended in Roswell Park Memorial Institute (*RPMI*) 1640 (Invitrogen, Carlsbad, CA, USA) medium supplemented with gentamicin (10 μg/ml) and 10% AB human serum (Sigma-Aldrich, St Louis, MO, USA). The PBMCs (0.5×10^5 cells/well) were cultured in flat-bottomed microplates (Costar, Cambridge, MA, USA) at 37°C and in 5% CO_2. The PBMCs were stimulated with agonists of TLR4 (lipopolysaccharide (LPS) 1 μg/ml) or MRP8 (S100A8, 1 μg/ml; ProSpec-Tany TechnoGene Ltd, Ness-Ziona, Israel) alone, or LPS (1 μg/ml; Sigma) was added 30 min before the addition of S100A8 (1 μg/ml). CD107a PerCP-Cy5 was added at the beginning of these incubations. The agonist concentration was established in our previous assays. After 1 h of incubation, Brefeldin A (10 μg/ml; Sigma) was added, and the cultures were incubated for 5 h. As a positive control, a combination of phorbol 12-myristate 13-acetate (10 ng/ml; Sigma) and ionomycin (1 μg/ml; Sigma) was added. After incubation, the cells were labelled with LIVE/DEAD PE-Texas Red viability marker (Invitrogen), fixed with Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) and stained with the antibodies CD3-Qdot 605, CD4-Horizon V450, CD8-V500 Horizon, TNF-PE-Cy7, IL-1-beta-FITC, CD19-Horizon V500, CD16-APC-Cy7, and CD56-Alexa-fluor 700 (BD Biosciences) to evaluate $CD8^+$ T-cell or NK-cell subsets. A total of 200,000 events were acquired using a flow cytometer (LSR Fortessa, BD Biosciences, USA) and then analysed using FlowJo Software (Tree Star, Ashland, OR, USA).

Toll-like receptor signalling pathway PCR array of $TCD8^+$ cells stimulated with S100A8

$CD8^+$ T cells from the PBMCs of patients with LP ($n = 3$) and HC ($n = 3$) were sorted using a FACSAria III instrument (BD Biosciences). The $CD8^+$ T cells (0.5×10^5 cells/well) were cultured in a 96-well microplate (Costar, Cambridge, MA, USA) at 37°C and in 5% CO_2 and stimulated with MRP8 (S100A8, 1 μg/ml) for 4 h; afterwards, the cells were stored in RNAlater solution (Sigma-Aldrich) at –20°C. We assessed 84 genes related to TLR-mediated signal transduction and innate immunity using a Human Toll-Like Receptor Signalling Pathway PCR array (Qiagen). The RNA was extracted from $TCD8^+$ cells using the RNeasy Micro Kit Protocol (Qiagen, Hilden, Germany). Then, reverse transcription was performed using the RT² PreAMP cDNA Synthesis kit (Qiagen), and the cDNA equivalent was used with an RT² Profiler PCR Array plate (Qiagen) on an ABI 7500 RT-PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Data were evaluated using 7500 software version 2.0.6 (Applied Biosystems) combined with Microsoft Excel (Microsoft, Redmond, WA, USA) for statistical evaluation. ΔΔCt values were calculated by normalizing the gene expression levels of housekeeping genes and then comparing with controls. The relative expression level of each gene was expressed as fold regulation. When comparing each gene's signal intensity between S100A8-stimulated and S100A8 non-stimulated $TCD8^+$ cells in patients with LP or HC groups, the cut-off was considered ≥ 2-fold for gene induction and ≤ 2-fold for gene repression.

Real-time PCR

One-punch biopsies (6 mm) of skin lesions, primarily located on the arms and forearm, were performed under local anaesthesia, and the samples were stored in RNAlater solution (Sigma-Aldrich) at –20ºC. The frozen samples were then processed using a Tissue Ruptor (Qiagen, Valencia, CA, USA). Total RNA was also extracted from the skin biopsies using an RNeasy Plus Mini Kit (Qiagen). Reverse transcription was performed using a Reverse Transcriptase Kit (Qiagen). The primers utilized for the real-time PCR assays were as follows:

S100A8 (MRP8): 5'-GGGATGACCTGAAGAAATTGCTA-3' forward, 5'-TGTTGATATCCAACTCTTTGAACCA-3' reverse; S100A9 (MRP14): 5'-GTGCGAAAAGATCTGCAAAATTT-3' forward, 5'-GGTCCTCCATGATGTGTTCTATGA-3' reverse; and GAPDH: 5'-GAAGGTGAAGGTCGGAGT-3' forward, 5'-GAAGATGGTGATGGGATTTC-3' reverse.

Real-time qPCR was performed using an Applied Biosystems 7500 Fast system with the specific primers and SYBR Green (Applied Biosystems, Carlsbad, CA, USA), as described by Pereira et al. (17). The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in samples within the same plate were analysed to normalize the mRNA contents of the tested samples. The cycling protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The amplification results were analysed using Sequence Detection System (SDS) software (Applied Biosystems), and the normalized expression was calculated as described by Livak & Schmittgen (18).

ELISA assay for S100A8/S100A9 serum detection

An ELISA was used to detect the S100A8/S100A9 protein in sera according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

S100A8 protein detection by immunohistochemistry

One part of each skin biopsy was formalin-fixed and then embedded in paraffin and sectioned at 4 μm. The histological sections were deparaffinized in xylol baths and then rehydrated in ethanol, blocking with 3% hydrogen peroxide. The slides were incubated with the primary antibody, rabbit monoclonal anti-S100A8 (Abcam, Cambridge, MA, USA) or phosphate-buffered saline (PBS) in 1% bovine serum albumin in PBS (pH 7.4) and visualized with Permanent Red LSAB-AP chromogenic solution (Dako, CA, USA), non-immune IgG was used for negative staining control. The slides were scanned using an Aperio Scan-scope Cs (Aperio Technologies, Vista, CA) and then analysed using Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MA, USA). The total tissue distribution of

S100A8 in the stained area divided by the total area measured in the epidermis or dermis was calculated.

Statistical analysis

The data were analysed using GraphPad Prism software (version 5.0). Student's *t*-test was performed to determine significant gene expression fold changes in PCR array analysis. The alternate distribution was examined using non-parametric Mann-Whitney test for statistical comparisons between groups, and $p \leq 0.05$ was considered significant. Baseline values were subtracted from all respective stimulated values in flow cytometry analysis.