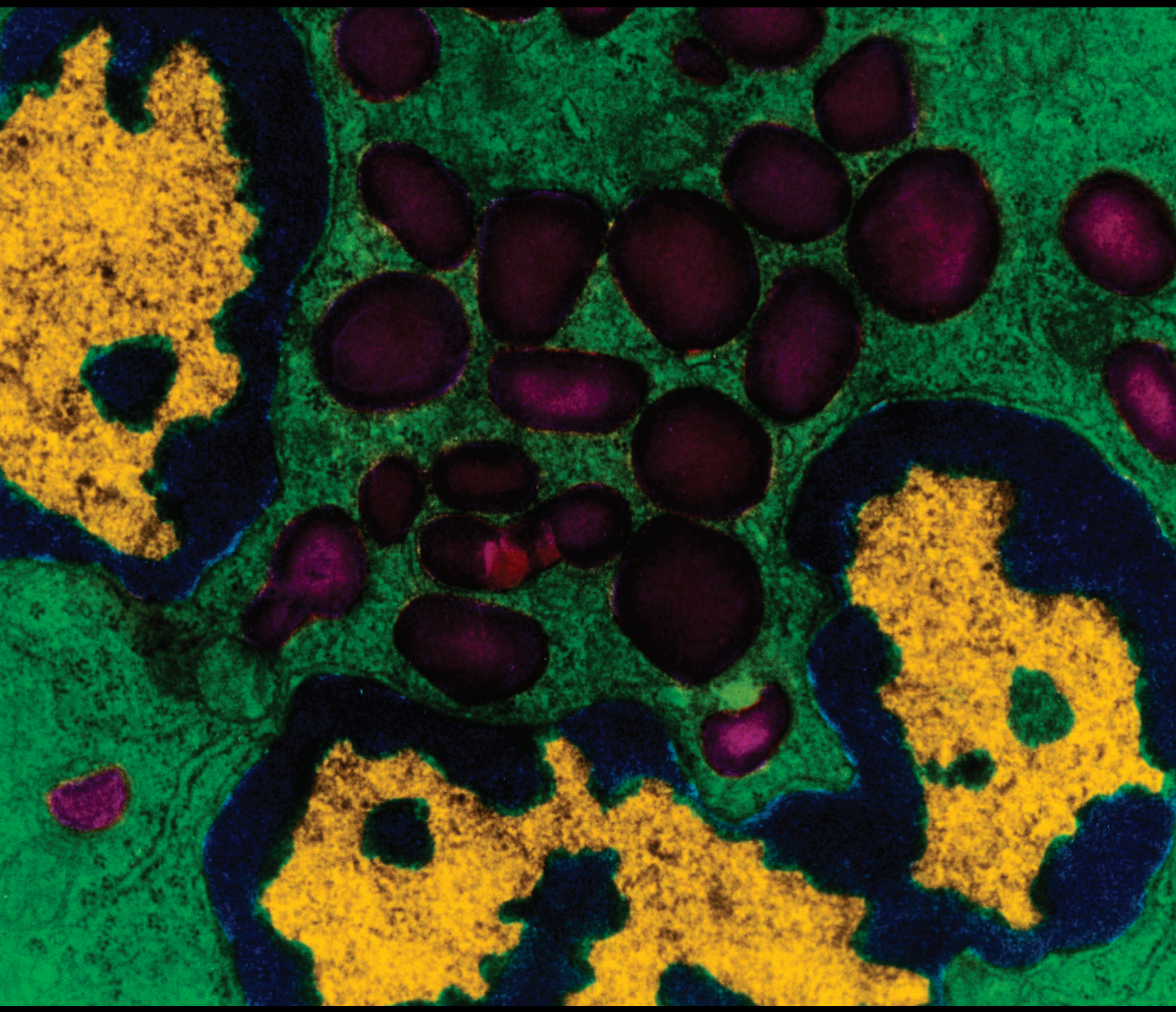
Mediators of Inflammation

Inflammatory and Immune-Mediated Cutaneous Diseases

Lead Guest Editor: Juarez A. S. Quaresma
Guest Editors: Mirian N. Sotto and Anna Balato



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Editorial Board

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Editorial

Inflammatory and Immune-Mediated Cutaneous Diseases

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The concept of tissue or compartmental immunity and its importance in the development of inflammatory or infectious diseases was introduced and gained strength following publication of the works of Engwerda and Kaye [1]. From then on, we can identify peculiar characteristics of the organ-specific or tissue-specific immune responses that include the immune responses (i) at the epithelial or mucosal barrier (skin and gastrointestinal tract), (ii) in a complex organ (liver), and (ii) in organs with immunological privilege (brain and eyes) [2–5]. Skin and mucosa are examples of epithelial tissues that have a complex organization. The in situ immune system of the tissues includes both professional immune cells and cells that under certain inflammatory or infectious conditions release substances, such as antimicrobial peptides or cytokines, that interfere with the local immune response and contribute to the host's response to pro- or anti-inflammatory stimuli. These tissues function as physical barriers and in addition to cytokines, such as tumor necrosis factor (TNF), thymic stromal lymphopoietin, IL-1, IL-6, and IL-18, their immune cells secrete catecholamines, defensins, and S100. These immune cells also secrete IL-33, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, and chemokine ligand 27 (CCL27) and are capable of expressing Toll-like receptors (TLRs) and NACHT, LRR, and PYD domain-containing protein 3 (NLRP3), which is a component of the inflammatory process mediated by IL-1. Associated with this complex network of cytokines and components of innate immunity in the skin is a subpopulation of dendritic cells, known as Langerhans cells, that are distributed in the epidermis while in the dermis are present dendritic cells corresponding to subpopulations distinct from those seen in the epidermis, such as dermal FXIIa-positive dendrocytes, plasmacytoid dendritic cells, and CD207-positive dendritic cells. This complex network of antigen-presenting cells cooperates with the various subpopulations of lymphocytes present in the skin and with other cells, such as macrophages and NK cells, for the maintenance of local and total body homeostasis [6]. Among other immune cells, NK and NKT cells, together with the M1, M2, M4, and M17 macrophages, form a network of cells and either respond immediately to the presence and entry of harmful agents or function as effector cells through their microbicidal mechanisms. The T-cell population is heterogeneous and complex, acting in response to infectious agents in spectral infectious diseases, such as leprosy and noninfectious agents in diseases such as psoriasis and atopic dermatitis. The T-cell population comprises Th1, Th2, Th17, Th9, Th22, Th25, and Treg cells [5, 6]. Many cytokines are involved in the effector response to infectious and inflammatory harmful agents and cooperate to induce a specific regenerative environment or specific regenerative processes. In the present issue, several authors discuss the role of these immune and inflammatory factors in infectious and noninfectious skin diseases. In this respect, I. Lorthois et al. discuss the role of macrophages and neutrophils in the pathogenesis of psoriasis, calling attention to the role of innate immunity and its interrelationship with tissue microenvironmental cells as one of the contributing factors to the complex inflammatory cascade observed in the disease. Studies related to the immunopathogenesis of psoriasis involving circulating lymphocytes are

discussed by J. Bartosińska et al. through the analysis of the immune response control by PD-1 expression. Later, the consequences of the systemic inflammatory response on the cardiovascular system of patients with psoriasis is discussed in the paper of S. Kaur et al. Other authors discuss the role of the complex network of inflammatory cells in the pathogenesis of urticaria and their regeneration and cutaneous involvement in inflammation-mediated diseases.

All these studies call attention to factors described recently and studied within the context of inflammatory skin diseases and their role in the evolution of these diseases. We believe that these works will give new insights into the complex dynamics of inflammatory and infectious cutaneous diseases. These concepts may serve as the basis for the development of new experimental models and may open possibilities for future investigations.

Juarez A. S. Quaresma
Mirian N. Sotto
Anna Balato

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Research Article

HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes

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Cultured primary human keratinocytes are frequently employed for studies of immunological and inflammatory responses; however, interpretation of experimental data may be complicated by donor to donor variability, the relatively short culture lifetime, and variations between passages. To standardize the *in vitro* studies on keratinocytes, we investigated the use of HaCaT cells, a long-lived, spontaneously immortalized human keratinocyte line which is able to differentiate *in vitro*, as a suitable model to follow the release of inflammatory and repair mediators in response to TNFα or IL-1β. Different treatment conditions (presence or absence of serum) and differentiation stimuli (increase in cell density as a function of time in culture and elevation of extracellular calcium) were considered. ELISA and Multiplex measurement technologies were used to monitor the production of cytokines and chemokines. Taken together, the results highlight that Ca^{2+} concentration in the medium, cell density, and presence of serum influences at different levels the release of proinflammatory mediators by HaCaT cells. Moreover, HaCaT cells maintained in low Ca^{2+} medium and 80% confluent are similar to normal keratinocytes in terms of cytokine production suggesting that HaCaT cells may be a useful model to investigate anti-inflammatory interventions/therapies on skin diseases.

1. Introduction

The skin is a continuously self-renewing organ that dynamically manages the outside-inside-outside relationships of the human body and actively participates in the host defenses [1]. Keratinocytes (KCs) represent 95% of the epidermal cells. Primarily, they play the structural and barrier function of the epidermis, but their role in the initiation and perpetuation of skin inflammatory and immunological responses, and wound repair, is also well recognized [2].

Under homeostatic conditions, KCs differentiate and mature from proliferating nucleated basal cells to the highly differentiated, nucleus-free corneocytes. Each stage of differentiation is characterized by the expression of structural proteins, such as keratins (K) and lipids [3, 4]. For example, the expression of K5 and K14 is restricted to the basal layer,

whereas K1 and K10 appear on more differentiated suprabasal cells [4], and involucrin, loricrin, and keratolinin, in the cells of the uppermost spinous layer [1]. Terminal differentiation is driven by various cytokines and growth factors, and it is typically associated with the formation of the peripheral envelopes, rich in proteins and lipids [3, 4]. The calcium (Ca^{2+}) gradient in the epidermis, increasing from the basal to the granular layer, represents one of the most important triggers of KC differentiation [4].

Resting KCs produce epidermal growth factor receptor (EGFR) ligands and vascular endothelial growth factor (VEGF), but when activated by bacterial products or by direct damage by UV light or chemicals, the expression of cytokines and chemokines changes [5, 6]. In skin diseases, such as psoriasis [7] or atopic dermatitis [8], the cytokine/chemokine network is even more complex and autocrine/paracrine loops are described [2].

Cultured human KCs are frequently employed for studies of KC functions in chronic inflammatory skin diseases [9, 10]. Primary KCs cultured *in vitro* at low Ca^{2+} concentration retain a basal phenotype, and they differentiate upon addition of $Ca^{2+} > 0.1$ mM [11, 12]. However, their use for routine monitoring of the inflammatory response of the inflamed skin presents major drawbacks. Firstly, fresh human KCs require supplementary growth factors to survive and proliferate *in vitro*; secondly, once induced to differentiate, they rapidly die and do not allow long-term investigation of the differentiation signals [13]. Moreover, donor-to-donor variability in growth characteristics and *in vitro* responses, different plating efficiencies, the short lifetime in culture, and the changes in proliferation and differentiation characteristics with increasing number of passages, complicates the interpretation of experimental data.

To minimize these problems, the spontaneously immortalized human KC cell line HaCaT from adult skin has been proposed as a model for the study of KC functions. HaCaT is a nontumorigenic monoclonal cell line, adapted to long-term growth without feed-layer or supplemented growth factors [13, 14]; it exhibits normal morphogenesis and expresses all the major surface markers and functional activities of isolated KC [14]; upon stimulation, HaCaT cells differentiate and express specific markers of differentiation, such as K14, K10, and involucrin. They can also form stratified epidermal structure [15], but they can revert, back and forth, between a differentiated and a basal state upon changes in Ca^{2+} concentration in the medium [16]; they retain the capacity to reconstitute a well-structured epidermis after transplantation *in vivo* [17].

The aim of the present study was to investigate and optimize the best conditions to use HaCaT cells as a reliable *in vitro* model to evaluate, at different stages of differentiation, the production of proinflammatory mediators, chosen among those mostly involved in skin inflammation and angiogenesis.

2. Materials and Methods

2.1. Cell Culture. HaCaT cells, spontaneously immortalized human keratinocyte line [15], were kindly provided by Cell Line Service GmbH (Eppelheim, Germany) and cultured in 5% CO_2 at 37°C in regular Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone S.P.A., Milan, Italy) containing 1.8 mM Ca^{2+}, or with DMEM (Gibco, Life Technologies, Carlsbad, CA, USA) at low concentration of Ca^{2+} (0.07 mM). Both media were supplemented with 10% heat-inactivated fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml) (Euroclone), and streptomycin (100 mg/ml) (Euroclone). For all experiments, cells were seeded at a density of 5.7×10^3 cells/cm^2 and cultured with DMEM at high or low Ca^{2+}concentration for 6 or 14 days. The samples were labeled as follows: A6, cells cultured for 6 days with low Ca^{2+} concentration (0.07 mM) and tested when 80% confluent; A14, cells cultured for 14 days with low Ca^{2+} concentration (0.07 mM) and tested when overconfluent; C6, cells cultured for 6 days with high Ca^{2+} concentration (1.8 mM) and tested when 80% confluent; and C14, cells cultured for 14 days with high Ca^{2+} concentration (1.8 mM) and tested when overconfluent. The medium was changed every 2 days. A flow chart with details of the experimental protocol is reported in Figure 1.

2.2. Isolation of Human Keratinocytes from Skin Biopsies. Primary KCs were isolated from nonlesional skin biopsies obtained from adult psoriatic patients not receiving either topical or systemic therapies for at least 6 months, or at the time of sample collection. To separate the epidermal layer from the basement membrane, the 0.4 mm punch biopsy was treated with dispase (Gibco BRL, Gaithersburg, MD, USA). After 18 h at 4°C, the epidermal sheet was separated mechanically and dissociated with TrypLE (Gibco BRL, Gaithersburg, MD, USA) for 20 min at 37°C. The obtained primary cells were then plated on 6-well tissue culture plates (Costar), precoated with coating matrix (type I collagen, Gibco BRL), cultured using a specific keratinocyte-serum-free media at low Ca^{2+} concentration (<0.07 mM), and supplemented with human keratinocyte growth factors (Gibco BRL). When the monolayer reached 60%–70% confluence, cells were split by trypsinization. For all the experiments, keratinocyte cultures between the third and fourth passages were used. Informed consent was obtained from all donors providing tissue samples, and ethical approval was obtained from the Ethics Committee of "La Sapienza" University, Rome, Italy.

2.3. Cell Proliferation Assay. The proliferation of HaCaT cells was determined at the indicated intervals using the MTT colorimetric assay as described [18]. This test is based on the ability of succinic dehydrogenase of living cells to reduce the yellow salt MTT (3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide)) (Sigma-Aldrich, St. Louis, MO, USA) to a purple-blue insoluble formazan precipitate. Experiments were performed in 96-well plates containing a final volume of 100 μl of medium/well. Cells were seeded at an initial density of 1.0×10^4 cells/cm^2, and, after 1, 6, 9, and 14 days, incubation medium was removed and replaced by 100 μl of fresh medium. Then, 10 μl of stock MTT solution (5 mg/ml in PBS) was added and plates were incubated at 37°C for 4 h. Finally, 100 μl of 10% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, St. Louis, MO, USA), in 0.01 M HCl,

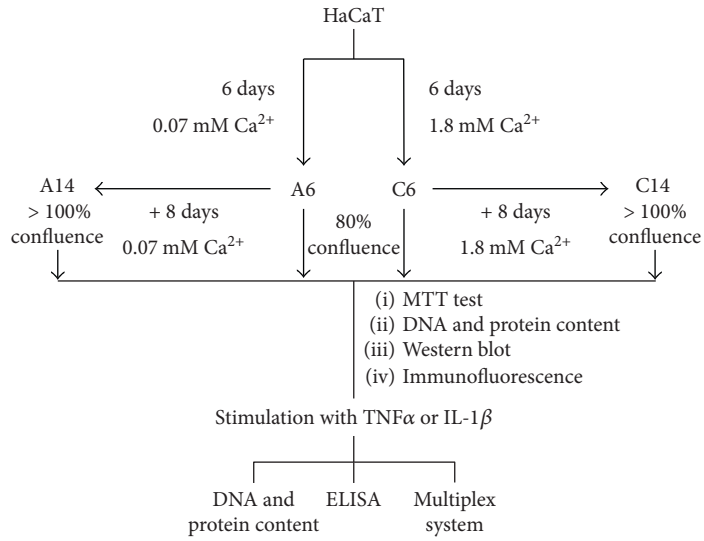

Figure 1: A flow chart with details of the experimental protocol performed on HaCaT cells.

was added to each well and the amount of formazan formed was measured at 540 nm using a Benchmark microplate reader (Bio-Rad, Richmond, CA, USA). Sixteen wells per time-point were analyzed in three independent experiments.

2.4. Cell Stimulation. For the stimulation of HaCaT cells, an amount of 1.0×10^4 cells/well in 500 μl were seeded in 24-well plate and cultured in low or high Ca^{2+} medium, with medium changes every two days. After 6 or 14 days, the medium was removed and replaced with 250 μL of medium or serum-free medium, supplemented with TNFα (10 ng/ml) or IL-1β (10 ng/ml) (PeproTech EC, Ltd., London, UK).

After different lengths of time (6, 24, or 48 hours) at 37°C, the supernatants from two wells for each different treatment and time were pooled in a single tube and frozen at −20°C to be subsequently analyzed for the presence of cytokines, chemokines, and growth factors. The cell monolayers were then washed twice with PBS, Ca^{2+}, and magnesium-free medium, dried, and stored at −20°C for the analysis of DNA content.

For the stimulation of keratinocytes from skin biopsies, $8–10 \times 10^4$ cells/cm^2 in 3 ml were seeded in 6-well plate and cultured in low Ca^{2+} medium, with medium changes every two days. When the cells reached 60%–70% confluence, the medium was removed and replaced with 3 ml of serum-free medium, supplemented with TNFα (10 ng/ml). After 48 hours of incubation at 37°C, the supernatant was collected, frozen at −80°C, and subsequently analyzed for the presence of cytokines, chemokines, and growth factor as described in the multiplex system section.

2.5. Release of Inflammatory Mediators

2.5.1. ELISA. The release of CXCL8/IL8 and VEGF from HaCaT cells was quantified by using two different high sensitivity human ELISA set (Peprotech, Rocky Hill, NJ, USA) following the method described below. Briefly, Corning 96-well EIA/RIA plates from Sigma-Aldrich (Milan, Italy) were coated with the antibodies provided, overnight at 4°C. Matrix metalloproteinase-9 (MMP-9) secretion from HaCaT cells was evaluated by a different ELISA set (RayBio® Human MMP-9 ELISA kit, Norcross, GA) using a precoated 96-well plate, supplied with the kit. In all the three cases, 300 μl of samples was transferred in duplicate into wells at room temperature for 2 hours. The results were detected by spectroscopy (signal read 450 nm, 0.1 s, by VictorTM X3) using biotinylated and streptavidin-HRP conjugate antibodies, evaluating 3,5,3,59-tetramethylbenzidine (TMB) substrate reaction. The quantification of analytes was done using an optimized standard curve supplied with the ELISA sets. The data are expressed as pg/10^6 cells. Results are mean ± SD of at least three independent cell culture experiments in duplicate.

2.5.2. Bioplex Multiplex System. Supernatants obtained from HaCaT cells and from primary keratinocytes, stimulated or not with TNFα (10 ng/ml) for 48 hours, were analyzed for the presence of chemokines, cytokines, and growth factors. Regarding chemokines, we assayed CXC chemokine ligands (CXCL1/GRO, CXCL10/IP10, CXCL12/SDF-1, and CX3CL1/fractalkine) and CC chemokine ligands (CCL2/MCP1, CCL3/MIP1a, CCL4/MIP1b, CCL5/RANTES, CCL7/MCP3, CCL11/eotaxin, and CCL22/MDC). Among cytokines, we assayed TGFα, TNFβ, IFN-α2, IFN-γ, G-CSF, GM-CSF, IL-10, IL-12p70, IL-15, and IL-33. All factors were quantified simultaneously by Bio-Plex Pro human cytokine assays according to the manufacturer's instructions (Bio-Plex Bio-Rad Laboratories, CA, USA). The analyte levels were determined using Bio-Plex array reader (Luminex, Austin, TX, USA) and the Bio-Plex manager software. The relative concentration of each analyte was obtained through the establishment of standard curves, and results are expressed as pg/10^6 cells.

2.6. Analysis of DNA Content. Analysis of DNA content was performed using the commercial kit fluorimetric "FluoReporter Blue Fluorometric DNA Quantitation Kit" (Molecular Probes, Life Technologies, Carlsbad, CA, USA) in 96-well

plates following the manufacturer's instructions. Plates with treated HaCaT cells were thawed, and 200 μL of distilled H_2O was added to each well. After three cycles of freezing-thawing, 100 μl was then transferred into a 96-well plate and used for the assay. In parallel, the standard calibration curve was made using increasing concentrations (0–1000 ng/100 μl) of DNA from calf thymus diluted in TE buffer (10 mM Tris base, 1 mM EDTA in distilled H_2O entire solution with a pH equal to 7.4). The amount of 100 μL of Hoechst dye was then added to each well containing the sample or standard, previously diluted 1 : 400 in TNE buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA with a final pH of 7.4, and 2 mM sodium azide). The emitted fluorescence was measured with microplate reader TECAN F500 (Tecan, Maennedorf, Switzerland) using excitation and emission wavelengths at 346 nm and 460 nm, respectively. The DNA content was calculated by interpolation of the absorbance of the samples on the calibration curve. The DNA content was expressed as μg DNA/10^6 cells counted, in each well, using a Burker chamber and trypan blue.

2.7. Protein Extraction and Analysis of Protein Content. For total protein extraction, HaCaT cells were cultured for 6 or 14 days with proper medium. For each time-point of the differentiation, cells were washed with cold PBS and lysed in RIPA lysis buffer (0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_2VO_4, and 8.5 μg/ml of aprotinin, in PBS), shaking for 20 min at 4°C. Samples were collected by scraper, incubated for 60 min at 4°C, and centrifuged at 12.000 rpm for 15 min at 4°C, and the supernatant was collected and frozen at −20°C until use. The soluble proteins in the extract were quantified according to the method described by Lowry et al. [19].

2.8. Western Blot Analysis. For Western blot analysis, 40 μl of total protein was separated on 7.5% SDS-PAGE gel and transferred to a polyvinylidenedifluoride transfer membrane (PVDF) (Bio-Rad, Richmond, CA, USA) for 16 h at 150 mA, using transfer buffer (25 mM TrisHCl, 190 mM glycine, 20% methanol, and 0.05% SDS). The membranes were blocked by incubation in blocking buffer (PBS containing 0.1% Tween and 5% dried nonfat milk) for 2 h and 30 min at room temperature. Then, membranes were blotted overnight at 4°C with various dilutions of primary antibodies, specifically, rabbit polyclonal IgG anti-involucrin (1 : 2500; Genetex, Irvine, CA, USA), mouse polyclonal IgG anti-cytokeratin 14 (1 : 1500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and rabbit monoclonal IgG anti-cytokeratin 10 (1 : 10,000; Genetex, Irvine, CA USA). Mouse monoclonal anti-β-actin antibody (1 : 6000; Sigma-Aldrich, St. Louis, MO, USA) was used to normalize gel loading. Blots were washed six times with PBS-0.1% Tween and incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibodies. Involucrin and K10 were detected with a donkey anti-rabbit IgG (1 : 10,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For K14 and actin, a goat anti-mouse IgG (1 : 10,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used. All blots were developed by ECL Western blotting detection LiteAblot® plus Kit Reagent (Euroclone S.P.A., Milan, Italy), following the manufacturer's protocol. Immunoreactive proteins were visualized by autoradiography on Hyperfilm ECL (GE Healthcare Life Sciences, UK). The relative intensities of band signals were quantified by digital scanning densitometry, and β-actin was used to normalize the results to protein content.

2.9. Immunofluorescence. HaCaT and primary keratinocytes, cultured on 8-well slide chambers, were washed with PBS, fixed, and permeabilized in ice-cold methanol for 5 min. First, cells were incubated in PBS containing 0.1% bovine serum albumin (BSA) for 10 min, then were incubated with primary antibodies against K10 (rabbit monoclonal IgG anti-cytokeratin 10; 1 : 200; Genetex, Irvine, CA, USA) for 60 min in PBS containing 1% BSA. The cells were subsequently incubated with secondary antibody Alexa Fluor® 488-labeled goat anti-rabbit IgG and Alexa Fluor 488-labeled goat anti-mouse IgG (1 : 1000, Invitrogen, Life Technologies, Carlsbad, CA, USA) for 30 min, respectively. The nuclei of the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA), which specifically recognizes DNA. The slides were observed by using Nikon Eclipse TE200 inverted microscope with immersion objective at 60x magnification and photographed with Nikon digital camera (Nikon, Japan).

2.10. Statistical Analysis. Data are expressed as mean ± SD of at least three experiments performed in duplicate. Data were analysed by unpaired one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Statistical analysis was done using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Extracellular Ca^{2+} and Cell Density on Proliferation and Differentiation of HaCaT Cells. To set up and validate HaCaT cells as an *in vitro* model to study the inflammatory response of human keratinocytes, two known stimuli of KC differentiation, cell density and extracellular Ca^{2+} concentration, were used. HaCaT cells were plated at the same density in low (0.07 mM) and high (1.8 mM) Ca^{2+} medium, and cell proliferation was assessed by both MTT assay and cell counts at day 6 and day 14.

In low Ca^{2+} medium, a steady increase in metabolic activity and cell number was observed over time, while in high Ca^{2+} medium, cell growth was slower. Comparing low to high Ca^{2+} medium, about 27.5% and 70.3% decrease of MTT activity and 40% and 60% decrease in cell count were seen at days 6 and 14, respectively (Figure 2(a) and Table 1S). However, no significant changes in cell morphology were observed in the different conditions. Phase contrast images showed that HaCaT cells were flat and spread out after 6 days of growth both in low (A6) or high (C6) Ca^{2+} medium when they were at 80% confluence; at day 14 both in low (A14) and high (C14) Ca^{2+} concentration, they became more cubical in shape with higher

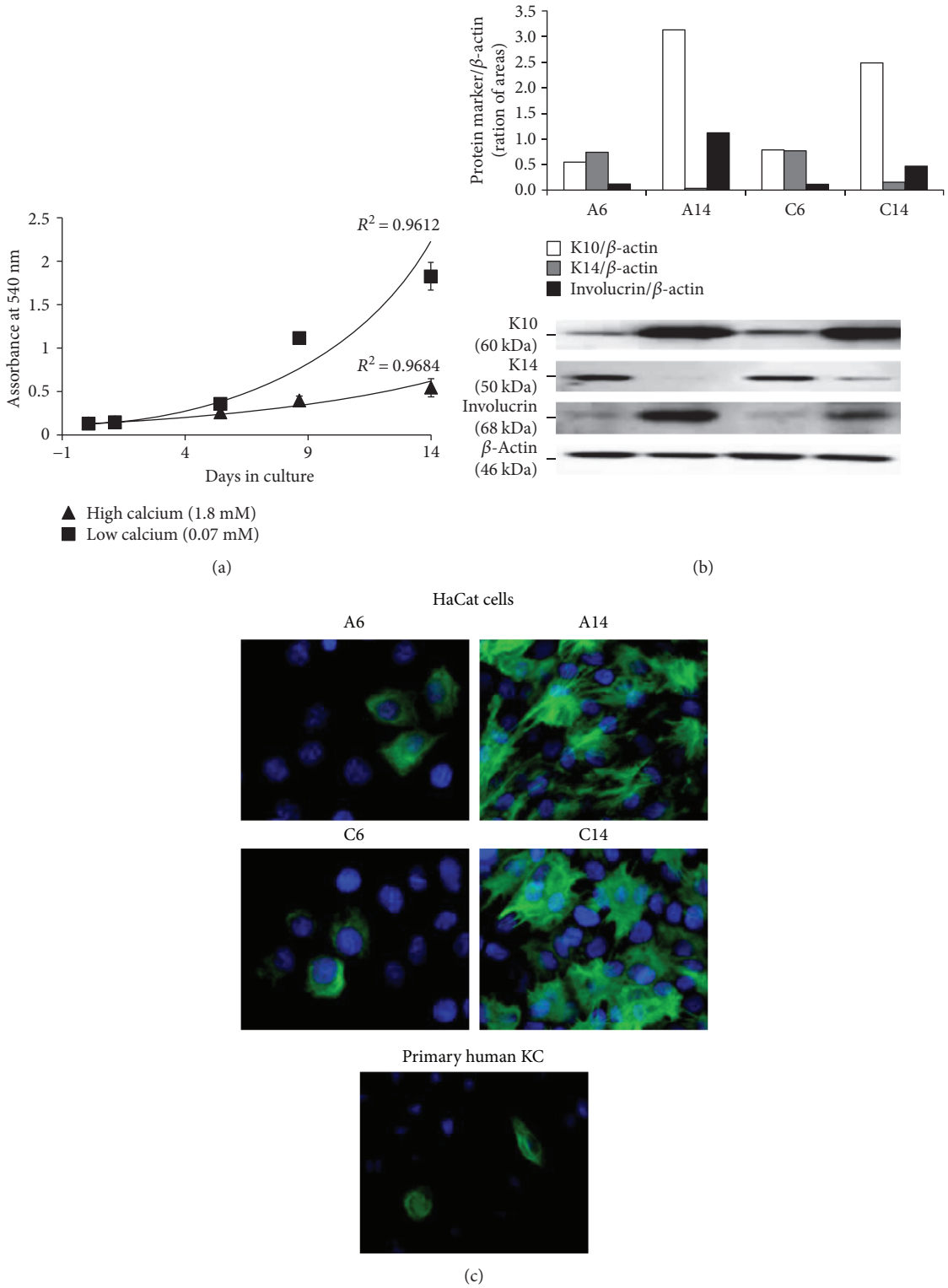

Figure 2: Proliferation and differentiation of HaCaT cells in low (0.07 mM) and high (1.8 mM) Ca^{2+}-containing medium. (a) Proliferation of HaCaT cells plated at the same density (1.0×10^4 cells/cm^2) assessed by the MTT assay at 2, 6, 9, and 14 days of incubation. Values represent mean ± SD of three independent experiments. (b) Western blot analysis of the expression of keratinocyte (KC) differentiation markers (K10, K14, and involucrin) in HaCaT cells grown in low (a) and high (c) Ca^{2+}-containing medium for 6 (A6 and C6) or 14 (A14 and C14) days. The relative intensities of band signals quantified by digital scanning densitometry are reported in the histogram; β-actin was used to normalize the results to protein content. This blot is a representative of three independent experiments. (c) Immunofluorescence staining of HaCaT cells, grown in low (A) and high (C) Ca^{2+}-containing medium for 6 (A6 and C6) and 14 (A14 and C14) days, and of primary human KC, grown in low Ca^{2+}-containing medium, with anti-K10 antibodies (magnification: 60x).

cell-to-cell packing and stratification (Figure S1). As reported in Table 1S, no significant changes were observed in the DNA and protein content in HaCaT cells grown in medium with different Ca^{2+} concentrations at the 6th or 14th day.

The expression of three classical KC differentiation markers (K14, K10, and involucrin) in response to changes in cell density and extracellular Ca^{2+} levels was assessed by Western blot analysis. Physiologically, the expression of K14 is restricted to the epidermal basal layer, while the presence of K10 and involucrin indicates a more differentiated phenotype.

As shown in Figure 2(b), the expression of K14 was higher in HaCaT cells at 80% confluence (day 6) than in overconfluent cells (day 14). At the same time, the levels of K10 and involucrin increased from day 6 to day 14, confirming the role of cell density on differentiation. Comparing day 6 to day 14, the fold increase in low Ca^{2+} medium was more evident for involucrin than for K10 (10.4-fold versus 5.7-fold, resp.), whereas in high Ca^{2+} medium, the increase was 4.7-fold versus 3.2-fold, respectively.

Regarding the effect of Ca^{2+}, at day 6, HaCaT cells showed similar levels of K14, K10, or involucrin in A6 and C6 culture conditions, suggesting that Ca^{2+} concentration did not significantly influence their expression at 80% cell confluence (Figure 2(b)). Unexpectedly, at day 14, the levels of both K10 and involucrin were slightly lower in overconfluent HaCaT cells grown in high compared to low Ca^{2+} medium (Figure 2(b)). This result could be a consequence of their lower density (Table 1S).

The acquisition of HaCat cell-differentiated phenotype was confirmed by immunofluorescence experiments (Figure 2(c)). The expression of the differentiation marker K10 increased significantly in HaCaT cells at day 14 compared to day 6 with almost 100% cells being positive. In addition, again, no differences in K10 levels were seen between HaCaT cells grown in low or high Ca^{2+} medium both in 80% confluent or overconfluent cells. Control samples without primary antibodies were negative, confirming specificity (data not shown).

The pattern of K10 expression was also evaluated on primary human keratinocytes grown in low Ca^{2+} and serum-free medium. Interestingly, as shown in Figure 2(c), K10 expression on human primary keratinocytes paralleled HaCaT cells grown for 6 days in low Ca^{2+} medium (A6).

3.2. Release of Inflammatory Mediators from HaCaT Cells. HaCaT cells grown in different culture conditions described above were then utilized to evaluate the production of a series of bioactive molecules, known to be released in the skin during inflammation or repair, both in basal conditions and upon proinflammatory stimulation.

In a first set of experiments, we focused on three main mediators: CXCL8/IL8, VEGF, and MMP-9, which are crucial for inflammatory cell recruitment, angiogenesis, and matrix remodeling, respectively. TNFα and IL-1β were used as stimuli. Influence of serum in the medium was also verified.

The supernatants were obtained from HaCaT cells plated at the same density in low or high Ca^{2+} medium and treated, after 6 or 14 days of culture, with 10 ng/ml TNFα or IL-1β for 6 or 24 h, as indicated, in absence (Figure 2(a)) or presence of serum (Figure 2(b)). The supernatants were recovered to measure CXCL8/IL8, VEGF, and MMP-9 by ELISA tests. The results are reported in Figure 3 and Figure S2.

Compared to basal levels, the stimulation with TNFα in the absence of serum induced a significant increase of CXCL8/IL8 and MMP-9, but not of VEGF, both in low and high Ca^{2+} medium at day 6 (Figure 3(a)). Conversely, the presence of serum during TNFα treatment at day 6 that did not elicit appreciable effects on CXCL8/IL8 release lowered the TNFα-induced MMP-9 release and increased both basal and stimulated levels of VEGF (Figure 3(b)). The concentration fold increase, following TNFα stimulation, was serum independent.

Overall, it appears that the ability of HaCaT cells of releasing these mediators decreased appreciably at day 14, compared to day 6, and this is independent on the Ca^{2+} concentration or the presence of serum (Figure 3). Of note, the MMP-9 amount released at day 6 and day 14 is higher in the absence than in the presence of serum, both in basal condition and upon stimulation.

Similar profiles were observed when cells were stimulated with IL-1β (10 ng/ml) (Figure S2). Higher levels of CXCL8/IL8, VEGF, and MMP-9 were seen at day 6, compared to day 14, independently of serum or Ca^{2+} concentration. In the presence of serum, unstimulated cells produced higher levels of mediators than without serum, and, except for IL8, the increase of VEGF or MMP-9 triggered by IL-1β was not significant.

To extend these observations, different chemokines and cytokines were studied by magnetic bead suspension array using the Bio-Plex Pro technology. The experiments were conducted in serum-free medium to reduce the interferences described in the previous paragraph. As shown in Figures 4(a) and 4(b), the stimulation of HaCaT cells with TNFα (10 ng/ml) for 48 h resulted in the upregulation of secretion of almost all the 18 mediators tested in the different culture conditions, although the level of stimulation varied among them. In particular, 6 days of culture in high Ca^{2+} concentration seem to represent the best combination for optimal production of the majority of mediators, except for CCL4/MIP1b, INFα2, INFγ, and G-CSF. Of note, CCL7/MCP3 seemed to be the only cytokine strongly upregulated by TNFα independently of the day of culture and Ca^{2+} concentration, whereas CCL22/MDC was the only cytokine for which overconfluency, and not Ca^{2+} levels, contributed to its production. On the contrary, Ca^{2+} concentration, but not the days in culture, seemed to be particularly relevant for TNFα-induced release of GM-CSF. No measurable values for CXCL12/SDF-1, CX3CL1/fractalkine, or IL-33 were obtained from any samples (data not shown).

3.3. Release of Inflammatory Mediators from Primary Human Keratinocytes. The release of inflammatory mediators was also measured in primary nonlesional epidermal KCs obtained from psoriatic patients. These cells were grown in low Ca^{2+} medium and, when tested at 60–70% confluence, showed K10 expression comparable to A6 HaCaT cells (Figure 2(c)). Regarding the production of cytokines/chemokines, normal

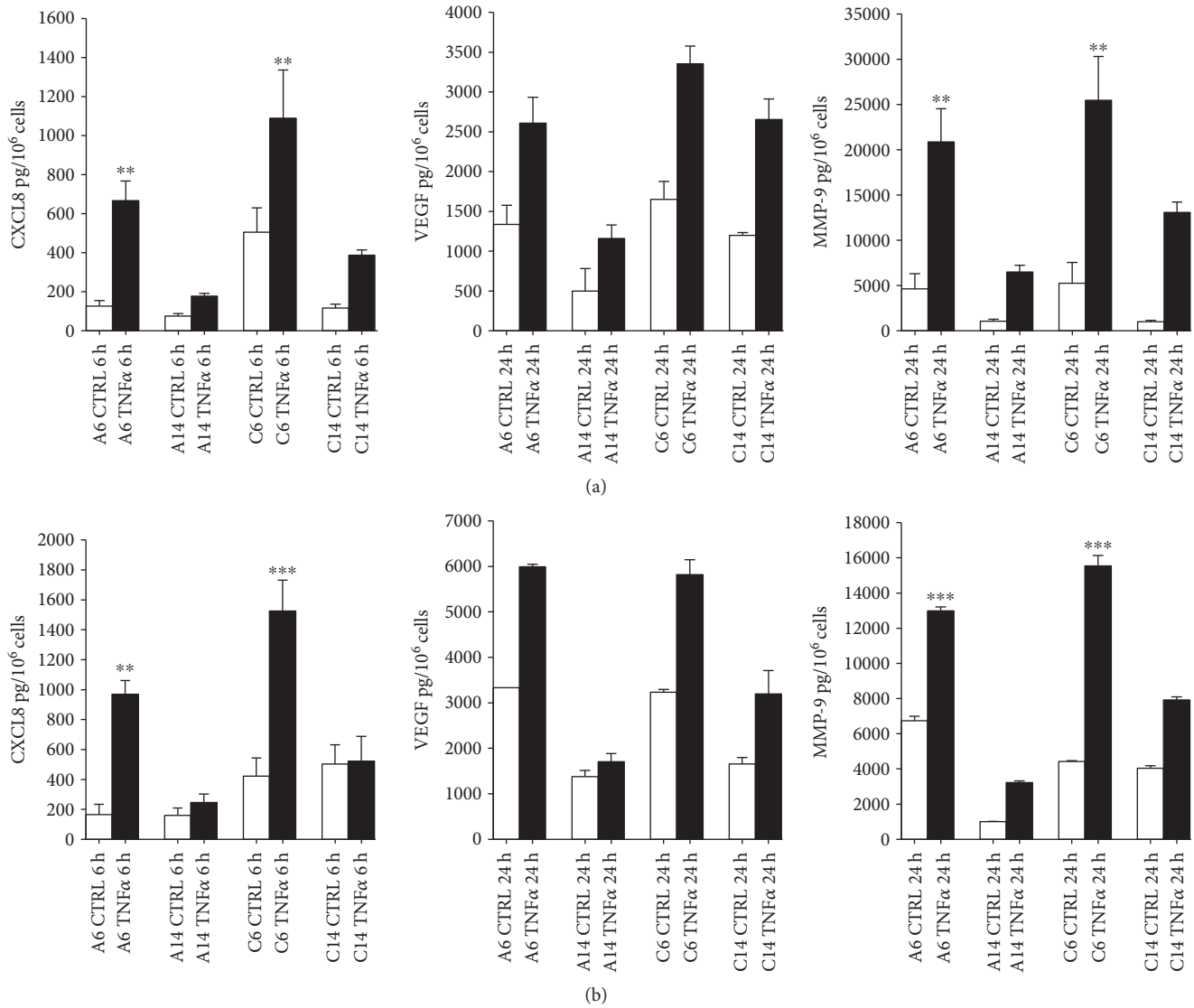

Figure 3: *In vitro* release of CXCL8/IL8, VEGF, and MMP-9 from HaCaT cells stimulated by TNFα during cell differentiation. The amount of CXCL8/IL8, VEGF, and MMP-9 was measured by ELISA in the supernatants of HaCaT cells plated at the same density (1.0×10^4 cells/cm^2), grown in low (a) and high (C) Ca^{2+}-containing medium for 6 (A6 and C6) or 14 (A14 and C14) days (white bars), and treated with 10 ng/ml TNFα for 6 or 24 hours (black bars), as indicated, in the absence (a) or the presence (b) of serum. The data are expressed as pg/10^6 cells, and values are the mean ± SD of at least three independent experiments in duplicate.

KCs secreted basal level of all the molecules analyzed and the treatment with TNFα induced a further increase of almost all of them (Figures 5(a) and 5(b)). All the CXC chemokine family members appeared to be highly upregulated, reaching a value for the CXCL8/IL8 more than 20,000 pg/10^6 cells (Figure 5(a)). Among the CC chemokines, only CCL2 showed approximately fivefold increase above controls. Of note, high concentration of growth factors, as TGFα and GM-CSF, was detected in medium of primary KCs stimulated with TNFα (Figure 5(b)).

4. Discussion

Keratinocytes are active players in epidermal repair and in the skin's immune defense through the secretion of growth factors, cytokines, and chemokines. To facilitate and standardize the *in vitro* studies on KCs, we investigated the use of HaCaT cells as a suitable model to follow the release of cutaneous inflammatory and repair mediators in response to TNFα or IL-1β, and in relation to different culture conditions and differentiation levels.

HaCaT cells are a long-lived, spontaneously immortalized human KC line, which exhibit basal cell properties and display substantial changes in response to two well-established *in vitro* prodifferentiating agents: the increase in cell density, as a function of time in culture, and extracellular Ca^{2+} concentration.

The switch from low to high extracellular Ca^{2+} concentration is considered not only a major regulator of the KC differentiation, but also of their proliferation both *in vitro* and *in vivo*. A Ca^{2+} gradient within the epidermis promotes the sequential differentiation of KC from the basal layer to

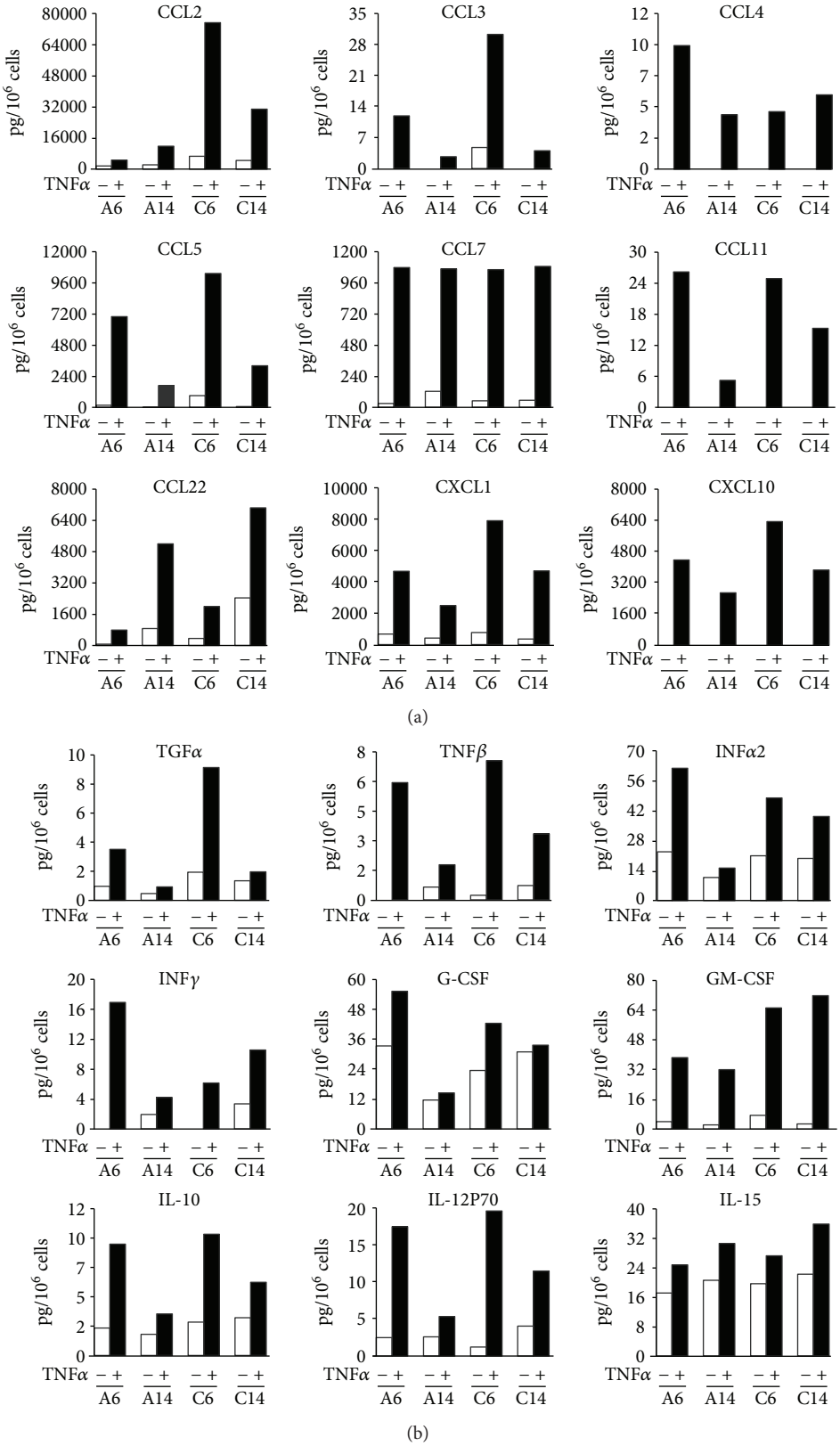

Figure 4: Bioplex multiplex system analysis of supernatants from HaCaT cells stimulated by TNFα during cell differentiation. The amount of chemokines (a), cytokines, and growth factors (b) was measured by Multiplex system technology in the supernatants of HaCaT cells plated at 1.0×10^4 cells/cm^2, grown in low (a) and high (c) Ca^{2+}-containing medium for 6 (A6 and C6) and 14 (A14 and C14) days (white bars), and treated with 10 ng/ml TNFα for 48 hours (black bars) in a serum-free medium. The data are expressed as pg/10^6 cells.

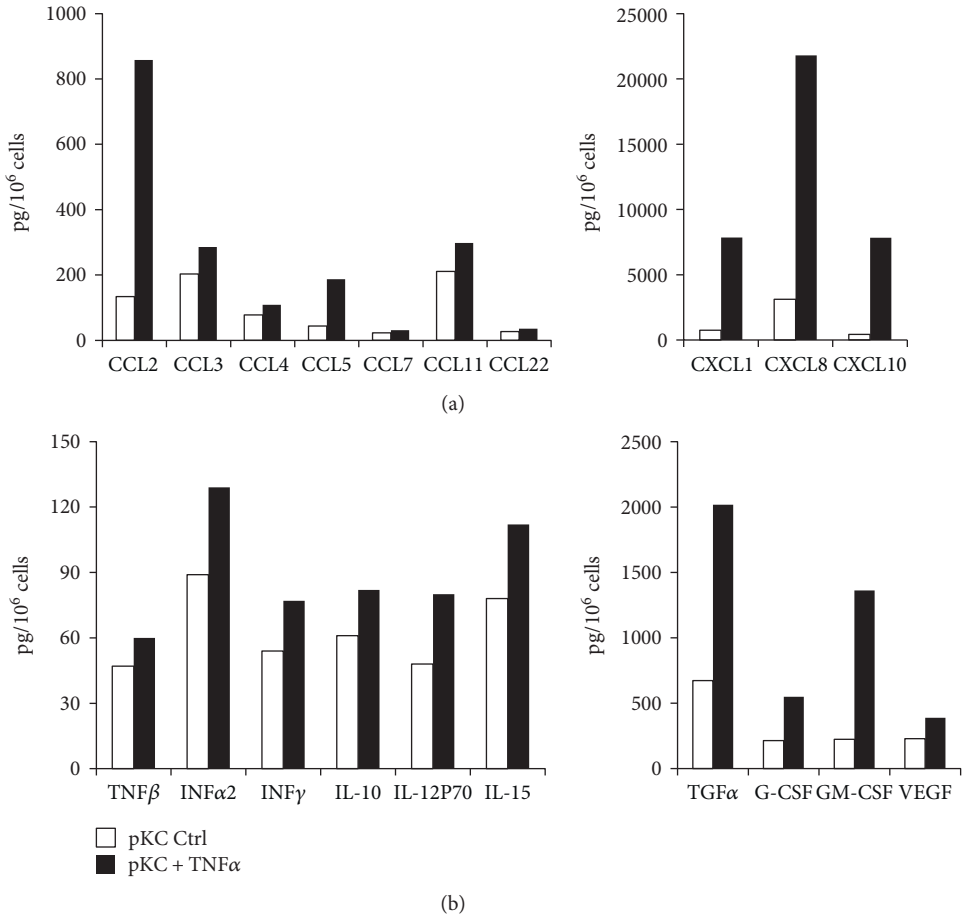

Figure 5: *In vitro* release of cytokines, chemokines, and growth factors from primary adult human keratinocytes stimulated by TNFα. The amount of chemokines (a), of cytokines, and of growth factors (b) was measured by Multiplex system technology in the supernatants of primary adult human keratinocytes (pKC) plated at the same density ($8–10 \times 10^4$ cells/cm^2) and grown in low Ca^{2+}-containing medium (white bars). The treatment with 10 ng/ml TNFα was carried out for 48 hours in a serum-free medium when cells were 60–70% confluent (black bars). The data are expressed as pg/10^6 cells.

the stratum corneum. Moreover, Ca^{2+} regulates the formation of desmosomes, adherent junctions, and tight junctions. The latter maintain cell-cell adhesion and play an important role in intracellular signaling that regulate Ca^{2+} levels and cell cycle arrest, critical for the differentiation process [12].

With this in mind, we first tested the differentiation potential of HaCaT cells in response to the increase in cell density and in extracellular Ca^{2+} concentration, by evaluating the expression of three markers of differentiation, namely K14, whose expression is restricted to the basal layer, K10, and involucrin that indicate a more differentiated phenotype. Our data (Figure 1 and Table 1S) confirm and extend previous observations that the increase in cell density and extracellular Ca^{2+} concentration favors HaCaT differentiation [14–17]. HaCaT cells remain in the basal state (high levels of K14) when maintained in low Ca^{2+} conditions and less than 80% confluent (A6), while they begin to differentiate expressing high levels of K10 and involucrin after a long-term culture (A14), that is when, although maintained in low Ca^{2+} medium, they are overconfluent. Different from previous observations, we noticed that HaCaT cells maintain their basal phenotype also when the cells are cultured in high Ca^{2+} conditions, but at less than 80% confluence. This observation suggests that, although extracellular $Ca^{2+} > 0.1$ mM appears to be a major regulator of HaCaT differentiation, the role of cell density is also relevant and it might have been underestimated in previous *in vitro* studies.

Moreover, in our study, the Western blot analysis of K10 and involucrin expression showed that when HaCaT cells are maintained in high Ca^{2+} condition, the overconfluence promotes a slight decrease of both K10 and involucrin levels and a weak increase of K14. Although this observation may be a consequence of the lower density of C14 with respect to A14, these data are in agreement with the changes of Ca^{2+} and confluence-dependent expression of K1, a biomarker of more differentiated cells [20], but not with the changes reported for the transglutaminase biomarker [17]. Altogether, this emphasizes the importance of a careful monitoring of differentiation marker expression during long-term culture of HaCaT cells and of standardization of the culture conditions.

The effect of the Ca^{2+} switch on the proliferation of HaCaT cells is poorly investigated. Our viability assays and cell counts highlight that the extracellular Ca^{2+} concentration

strongly influences the proliferative ability of HaCaT cells. Up to day 2 of culture, no differences in the proliferation rate of HaCaT cells grown in low or high Ca^{2+} medium were seen, whereas a difference in the growth rate was evident in low Ca^{2+} medium starting from day 6, which became significantly higher at day 14. These findings are at variance of those showing a progressive, time-dependent increase of HaCaT proliferation in high Ca^{2+} medium [14]. A possible explanation could be the higher extracellular Ca^{2+} concentration used in the present study (1.8 mM) compared to 1.2 mM in [14]. In fact, it has been demonstrated that only an increase in intracellular Ca^{2+} concentration above 1.5 mM results in a reduced growth rate [21].

In the second part of this study, HaCaT cells, cultured in low or high Ca^{2+} medium for different lengths of time, were used to investigate the release of CXCL8/IL8, VEGF, and MMP-9 in response to two proinflammatory stimuli, TNFα, and IL-1β. These cytokines were chosen for their predominant role in the pathogenesis of skin inflammation, since they both regulate genes previously shown to be specifically overexpressed in psoriasis [10, 22–24]. Our results show that the release of CXCL8/IL8, VEGF, and MMP-9 was higher in HaCaT cell basal state (A6-C6) compared to more differentiated cells (A14-C14). Ca^{2+} concentrations slightly enhanced the secretion of these mediators, especially when IL-1β was used as proinflammatory stimulus; this effect could be partially explained by the activation of the Ca^{2+} responsive promoter of activator protein (AP)-1 [25].

The presence of serum reduced the TNFα-induced release of MMP-9 and enhanced the VEGF secretion, both at basal and stimulated levels. As previously reported, growth factors derived from fetal calf serum may influence a variety of parameters involved in KC proliferation, differentiation, and wound healing processes [26–28]. Based on these results, we strongly suggest that serum should not be used when the proinflammatory mediators, VEGF, or MMP-9, are assayed. This should be extended also to the assays using normal human KC.

Several papers demonstrated that TNFα in KC regulates different genes involved in inflammation and angiogenesis, such as IL-1, ICAM-1, VEGF [29, 30], TGF-β, chemokines (CCL20, CCL27, CCL5, CCL2, CXCL10, and CXCL11), and members of the CXCL8 family, including CXCL1, CXCL2, and CXCL3 [10, 22, 23]. Therefore, using the Multiplex technology, we evaluated the effect of Ca^{2+} concentration and cellular density on the secretion of different cytokines, chemokines, and growth factors in HaCaT cell line. All the analyzed parameters were upregulated to a great extent by TNFα at day 6 in comparison to day 14; only CCL22/MDC seemed to be preferentially released in more differentiated HaCaT cells. CCL4, INFα2, INFγ, and G-CSF had a higher release in low Ca^{2+} condition, whereas, on the opposite, in high Ca^{2+} concentration, a comparable or higher secretion of CCL2, CCL3, CCL5, CCL7, CCL11, CCL22, CXCL1, CXCL10, TGFα, TNF-β, IL-10, and IL-15 was seen. To summarize, both long-term culture and Ca^{2+} concentration in the medium affect the ability of HaCaT cells to release chemokines and growth factors.

Finally, to validate HaCaT cells as a reliable model to dissect the inflammatory/repair response of human KCs, we measured the release of inflammatory mediators in primary nonlesional epidermal KCs obtained from psoriatic patients. These cells were assayed in low Ca^{2+}medium and in the absence of serum.

The levels of the CXC family of chemokines, induced by TNFα on human KCs (CXCL1, CXCL8, and CXCL10) correlated relatively well to the values released by HaCaT cells in low Ca^{2+}, considering the fold induction. Similar results were obtained on growth factors released (TGFα, G-CSF, GM-CSF, and VEGF). Not so close was the correlation with the CC family of chemokines some of which (CCL4, CCL5, CCL7, and CCL11) were highly released in A6 and C6 HaCaT cells, but quite low in normal human KCs; CCL2 was higher in C6 HaCaT as in normal KCs, whereas CCL3 was similar. Our results also show that HaCaT cells especially at day 14 of culture produce more CCL22/MDC than normal human KCs, thus confirming previously published data [31, 32]. This is not the first time that a differential release of chemokines is described in primary human KCs or HaCaT cells stimulated with exogenous stimuli. Data in the literature reported that CXCL10 and IL8 were released to a similar extent by both cell types, while CXCL9 and CCL20 were more efficiently produced by primary human KCs [33].

In conclusion, this study is the first report where several variables, all together, including the influence of Ca^{2+} concentration, the cell density, the differentiation state, and the presence of serum, were considered as factors that may influence release of proinflammatory mediators by KCs. Our results support the use of HaCaT cell line, under carefully optimized *in vitro* condition, as a reliable model, with respect to normal KCs, to screen for new anti-inflammatory compounds for skin diseases. Indeed, HaCaT cell line has been successfully used for studying those pathologies in which skin keratinocytes are involved, such as infectious diseases or tumors, or as *in vitro* carcinogenesis model of human skin [34]. By modulating Ca^{2+} concentration in culture medium and maintaining 80% confluence, HaCaT cells are in the conditions of producing cytokines at medium/low levels (A6 condition) or at medium/high levels (C6 condition) as expected in highly activated KCs from skin lesions. This *in vitro* system has the advantage of being reproducible and reliable and definitively less invasive and with less variability than KC from skin biopsies. However, one of the drawbacks is the inability of reproducing the skin complexity and cellular heterogeneity in basal or inflammatory conditions. 2D-3D culture systems have been proposed which mimic the differentiation process [35]. It cannot be excluded that in a relatively short time we may have available 3D culture systems in which several cell lineages KC, immune cells, and fibroblasts will be cocultured to ensure a better reproduction of the skin microenvironment.

Abbreviations

CCL: C-C motif chemokine ligand
CXCL: C-X-C motif chemokine ligand
CX3CL: C-X3-C motif chemokine ligand

G-CSF: Granulocyte-colony stimulating factor
GM-CSF: Granulocyte-macrophage colony-stimulating factor
IFN: Interferon
IL: Interleukin
K: Keratin
KC: Keratinocyte
MMP-9: Matrix metalloproteinase-9
TGFα: Transforming growth factor alpha
TNFα: Tumor necrosis factor alpha
TNFβ: Tumor necrosis factor beta
VEGF: Vascular endothelial growth factor.

Disclosure

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Conflicts of Interest

The authors state no conflict of interest.

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Supplementary Materials

Supplementary 1. Table 1S: Cell recovery and protein content of HaCaT cells grown in low (A) or high (C) Ca^{2+} medium for 6 (A6, C6) or 14 (A14, C14) days.
Supplementary 2. Figure S1: Changes in HaCaT cell morphology during cell differentiation.
Supplementary 3. Figure S2: In vitro release of CXCL8/IL8, VEGF, and MMP-9 from HaCat cells stimulated by IL-1β during cell differentiation.

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Review Article

Contribution of *In Vivo* and Organotypic 3D Models to Understanding the Role of Macrophages and Neutrophils in the Pathogenesis of Psoriasis

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Psoriasis, a common chronic immune-mediated skin disease, is histologically characterized by a rapid keratinocyte turnover and differentiation defects. Key insights favor the idea that T cells are not the only key actors involved in the inflammatory process. Innate immune cells, more precisely neutrophils and macrophages, provide specific signals involved in the initiation and the maintenance of the pathogenesis. Current data from animal models and, to a lesser extent, three-dimensional *in vitro* models have confirmed the interest in leaning towards other immune cell types as a potential new cellular target for the treatment of the disease. Although these models do not mimic the complex phenotype nor all human features of psoriasis, their development is necessary and essential to better understand reciprocal interactions between skin cells and innate immune cells and to emphasize the crucial importance of the local lesional microenvironment. In this review, through the use of *in vivo* and 3D organotypic models, we aim to shed light on the crosstalk between epithelial and immune components and to discuss the role of secreted inflammatory molecules in the development of this chronic skin disease.

1. Introduction

Psoriasis is a chronic autoimmune disease that affects 2-3% of the world's population, characterized by hyperproliferation and abnormal differentiation of epidermal keratinocytes [1–6]. Psoriatic skin lesions are also characterized by increased permeability of lymphatic capillaries, increased blood flow, and angioproliferation [7–10]. Eighty percent of patients suffer from mild to moderate forms of the disease, while 20% of patients develop moderate to severe psoriasis, affecting more than 5% of their body surface area [11]. It is also known that patients with plaque psoriasis have an increased risk of inflammatory diseases affecting noncutaneous sites—including psoriatic arthritis, cardiovascular disease, and inflammatory bowel disease—associated with common pathophysiological mechanisms. These comorbidities are multifactorial and in many cases related to inflammation, induced by close pathogenic mechanisms related to cytokine dysregulation.

PSORS1 is the major susceptibility locus for psoriasis vulgaris and lies within an approximately 300 kb segment of the major histocompatibility complex on chromosome 6p21.3 [12–14]. Several studies have indicated *HLA-Cw6* as the primary *PSORS1* risk allele within the candidate region, coherent with the fact that MHC class I molecules play an important role in the function of $CD8^+$ T cells [15, 16].

More than 32 PSORS have been identified, containing genes involved in inflammatory metabolic pathways and epidermal proliferation as well as skin barrier function, but have not demonstrated their complete involvement in

pathology. Also, the variations in the number of copies of a gene may be involved in the pathology. For example, beta defensins, antimicrobial peptides involved in innate immunity, are a good example of a gene known to be associated with psoriasis. Of the 8 defensins, the hBD-2, hBD-3, and hBD-4 proteins encoded, respectively, by DEFB2, DEFB3, and DEFB4 were linked to keratinocyte stimulation via proinflammatory interleukins 8, 18, and 20 [17]. The eight defensin genes are linked on two different chromosomes, chromosome 20 as well as chromosome 8p23.1. Most of the defensin genes encoded on chromosome 8p23.1 have longer gene repeat units, which are highly variable in copy number. Several studies have attributed a relationship between psoriasis and the number of gene copies of these defensins [18]. In 2012, a meta-GWAS (genome-wide association studies), which aims to identify SNPs (single nucleotide polymorphisms) in DNA associated with a clinically defined disease (phenotype) by comparing the allele frequency of each SNP between a group of individuals with psoriasis versus healthy patients, confirmed 21 SNPs, and identified 15 new SNPs [19].

The current research tends to demonstrate that the process is initiated by an inflammatory immune reaction against autoantigens of the skin, in which dendritic cells, T lymphocytes, macrophages, and neutrophils play a pivotal role. Dendritic cells, antigen-presenting cells, are present in greater numbers in psoriatic lesions. Dendritic cells of lymphoid origin, such as plasmacytoid dendritic cells, would be involved in initiating lesions [20], recognizing autoantigens, and causing IFN-α secretion by these cells [21]. This would follow the activation of innate immunity cells, such as neutrophils or macrophages, and adaptive immune cells, such as T lymphocytes. Persistent activation of these cells would lead to the chronicization of psoriatic lesions, such as a vicious circle of inflammation [2, 22, 23].

Resident macrophages and dendritic cells are among the cells most involved in the "sensing" of danger signals. The activation of macrophages via the secretion of proinflammatory cytokines, such as IL-6 and TNF-α, but also of chemokines, such as CXCL8 (also known as IL-8), CCL5, CXCL1, and CXCL2, promotes the recruitment of inflammatory cells, like neutrophils [24]. Multiple signals are likely to trigger, via interaction with their receptor(s), a secretion of chemokines, thus attracting neutrophils to the inflammatory site. Macrophages and neutrophils may act as T lymphocyte-dependent effectors, as they are present at the site of inflammation even before a specific immune response has developed.

It is therefore evident that monocytes, macrophages, and neutrophils have a particular function in the early phases of inflammation, and their role in driving and maintaining this inflammatory process in the pathogenesis of psoriasis must be clarified. Here, we aim to discuss the specific role of innate immune cells, such as neutrophils and macrophages, in the initiation and the sustainability of chronic inflammatory skin diseases, such as psoriasis, through the use of organotypic models and mouse models. These models allow a better understanding of cellular and molecular mechanisms with the aim of identifying new potential therapeutic targets.

2. Macrophages

Monocytes can differentiate to become tissue-resident macrophages or dendritic cells. Macrophages are phagocytic cells [25] within the dermis, important for tissue homeostasis and the regulation of lymphocyte activation and proliferation [26]. Some macrophages are long-term tissue residents and play an important role in controlling the repair [27, 28] and regeneration of skin tissue [29]. Inflammatory macrophages, on the other hand, participate in the innate immune response and play a dual role in the immune system as phagocytes and antigen-presenting cells capable of activating T lymphocytes (Figure 1).

As early as the 1980s, some evidence of increased macrophage activity in psoriasis [30] highlights their key role in inducing psoriasis-like skin disease.

In 2010, Fuentes-Duculan and colleagues observed that a subpopulation of CD163-positive macrophages was found mostly in psoriatic lesions. The CD163 marker, a scavenger receptor intervening in the elimination of the hemoglobin-haptoglobin complex, expressed both on the surface of the mature tissue macrophages and on blood monocytes, is in normal human skin, a marker more assimilated to the so-called "alternative" macrophages or M2 [31]. However, CD163-positive macrophages also express IFN-γ-regulated genes (STAT1, CXCL9), and IFN-γ is known to be a "type 1 cytokine." These results suggest a great phenotypic plasticity of the macrophages in responding to their environment and acquiring new properties and new markers, while preserving their original characteristics.

Other macrophage markers such as RFD7 [32], CD68 [33, 34], CD107 [35, 36], MARCO [37], Stabilin-1 [38], and MS-1 [39, 40] are not generally expressed at the same time on their surface, but their expression fluctuates according to their location in the skin compartments. For example, $CD68^+$ cells coexpress CD163 in the upper reticular dermis while they do not colocalize with CD163 near the dermoepidermal junction. It has also been shown that these markers coexpress to some extent CD11c, a marker of myeloid dendritic cells, but CD163 has the weakest coexpression with CD11c, an ideal candidate for labeling macrophages in psoriasis [31]. The cutaneous macrophages of lesional plaques probably do not have a single phenotype, M1, or M2 but rather have a mixed, microenvironment-dependent phenotype, associated with the different roles they can play in a context of chronic inflammation.

Egawa et al. have also demonstrated that $CCR2^+$ monocytes recruited at inflammatory sites had the potential to acquire an M2 phenotype in response to IL-4, thus exerting an anti-inflammatory function [41], while the expression of CCR2 (MCP-1/CCL2 protein receptor) of the peripheral monocytes of patients with psoriasis or atopic dermatitis was increased compared to that of healthy patients [42]. It has also been demonstrated that macrophages M1 or M2 have the ability to be repolarized by the cytokines of Th2 or Th1 lymphocytes, respectively [43].

The hypomorphic PL/J CD18 murine model is characterized by reduced expression (2–16% of the wild-type levels) of the common integrin 2 (CD11/CD18) chain,

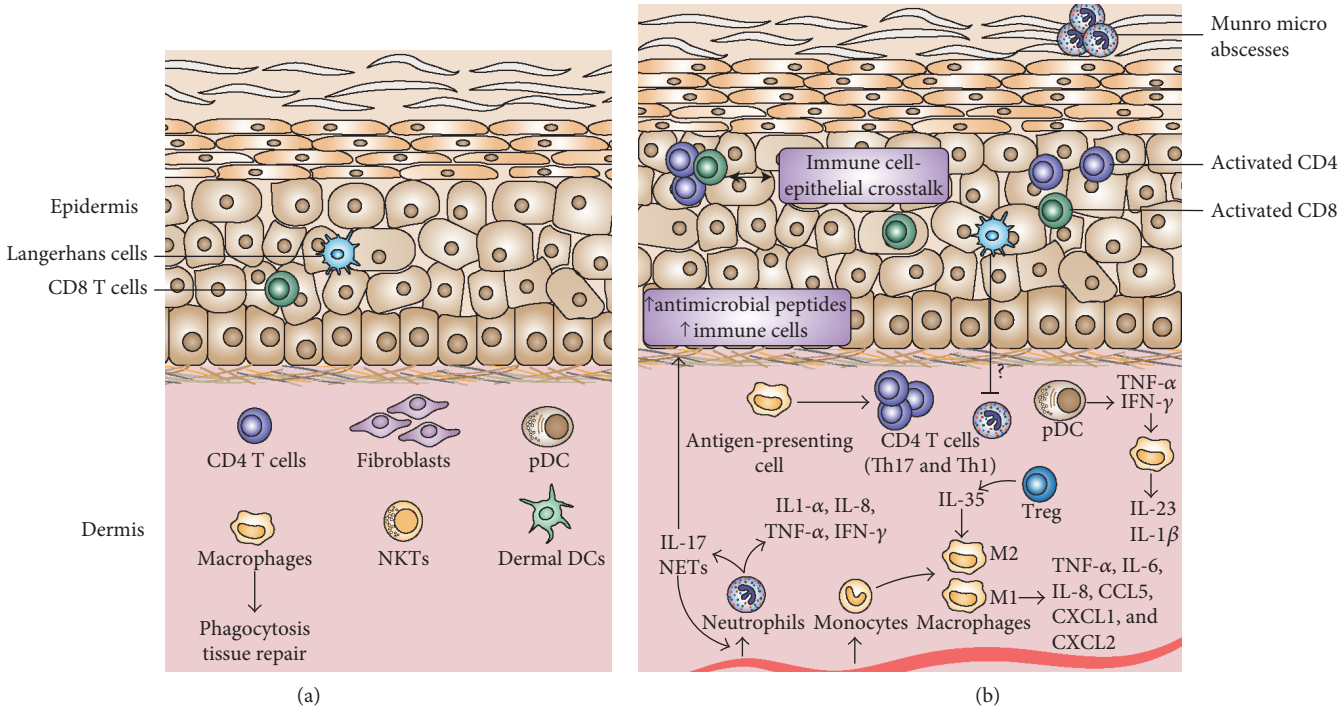

Figure 1: Location and function of macrophages and neutrophils in healthy (a) and psoriatic (b) skin. Langerhans cells and CD8 T cells are present in healthy epidermis, while CD4 T cells, as well as macrophages, NK T cells, dermal dendritic cells (DCs), and plasmacytoid dendritic cells (pDCs), are found in the dermis mainly composed by fibroblasts and extracellular matrix. Immune cell infiltrates are present in the psoriatic epidermis, consisting of activated CD4 and CD8 T cells and clusters of Munro's abscess in the *stratum corneum*. In the dermis, the activation of cells of both innate and adaptive promotes an inflammatory response. Neutrophils and macrophages (differentiated from monocytes) infiltrate the dermis and secrete proinflammatory cytokines. The crosstalk between skin cells and immune cells leads to a complex inflammatory response and contributes to the development of a pathological epithelial phenotype. NET: neutrophil extracellular trap; Treg: regulatory T cell; M: macrophage.

leukocyte adhesion molecules required for cell-cell contacts. Transgenic mice generally develop psoriasiform skin inflammation: erythema, scaling, abnormal keratinocyte proliferation/differentiation, subcorneal microabscesses, and increased inflammatory infiltrate. The authors reported that the activation of cutaneous macrophages by recombinant MCP-1 or LPS alone was not sufficient to produce chronic psoriasis-like inflammation as observed *in vivo* in human skin lesions [44]. Nevertheless, the combined injection of recombinant murine MCP-1 and TNF-α in nonlesional skin area of hypomorphic CD18 mice led, respectively, to the accumulation and activation of macrophages, unlike CD4-positive T cells. Activated macrophages would secrete more TNF-α, responsible for sustained activation of macrophages, thus causing a positive feedback loop. T cells, mast cells, and endothelial cells would participate directly in the chronicization of lesions via TNF-α secretion. Finally, depletion of skin macrophages in this murine model via the injection of clodronate liposomes (specific depletion of macrophages) and the neutralization of a single cytokine, TNF-α, attenuates the severity of skin inflammation, emphasizing the importance of macrophages in the psoriasis physiopathology.

A specific deletion of the keratinocyte NF-κB kinase inhibitor (IKK2) in mice results in an inflammatory and hyperproliferative cutaneous phenotype. The treatment of transgenic mice with a TNF-neutralizing antibody abolished the inflammatory phenotype and therefore improved the skin phenotype. The injection of clodronate liposomes in this murine model restored the expression of early and late keratinocyte differentiation markers and reduced the number of granulocytes and T cells present, highlighting the importance of macrophages in the accumulation of granulocytes and T cells in inflammatory skin areas [45]. Moreover, a K14-Cre-IKK2$^{fl/fl}$ murine model showed the upward expression of gene coding for proteins regulated by IFN-γ. The presence of IFN-γ receptor may accelerate the onset of the psoriasis-like inflammatory skin disease in K14-Cre-IKK2$^{fl/fl}$ mice but is not essential for it to develop. The authors demonstrated that the migration of macrophages to the lesional skin areas and their subsequent activation was a necessary key feature for the development of psoriatic inflammation. The activated macrophages were then able to initiate and maintain psoriasiform skin inflammation. These investigations were also commented in the *Journal of Clinical Investigation* by Clark and Kupper in 2006 [46].

The topical application model of imiquimod (IMQ), a 7/8 TLR (toll-like receptor) agonist widely used in mice, activated immune cells, such as macrophages and plasmacytoid dendritic cells. The mice displayed a hyperplastic cutaneous epithelial-squamous phenotype similar to human psoriasis. Imiquimod-treated mice, KO for CX3CR1 (fractalkine/CX3CL1 receptor), developed minor inflammation

compared to WT mice. In fact, the production of IL-12, IL-23, IL-17A, IL-22, IL-6, IL-1, TNF-α, and IL-36 cytokines was decreased in these mice. The macrophages of CX3CR1$^{-/-}$ mice expressed CCR2, unlike WT mice treated with IMQ, and attenuated the inflammation generated, in part, by Th1/Th17 lymphocytes, following functional changes [47]. The authors hypothesized that CCR2 could partially compensate for the loss of CX3CR1 by directing the migration of resident macrophages. Surprisingly, CCR2$^{-/-}$ mice exhibited an exacerbation of inflammation despite altered recruitment of inflammatory monocytes to cutaneous sites. CX3CR1$^{-/-}$ mice expressed fewer M1 macrophage markers compared to WT mice, suggesting that decreasing the number of M1 macrophages would contribute to decreased inflammation resulting from CX3CR1 deficiency. Finally, a transfer of macrophages from WT mice to CX3CR1$^{-/-}$ mice normalized psoriasiform type inflammation induced by IMQ, emphasizing the importance of macrophages in the regulation of psoriatic inflammation.

The KC-Tie2-overexpressing mice developed a cutaneous psoriasiform phenotype. These mice spontaneously developed characteristic hallmarks of human psoriasis, including acanthosis (hyperplasia of the epidermis), increases in dermal CD4$^+$ T cells, infiltrating epidermal CD8$^+$ T cells, dermal dendritic cells and macrophages, and increased expression of cytokines and chemokines associated with psoriasis (IFN-γ, TNF-α, IL-1α, IL-6, IL-12, IL-22, IL-23, and IL-17). Cathelicidin, β-defensin, and S100A8/A9 were also upregulated in the hyperproliferative skin [48].

The administration of clodronate liposomes in the skin of transgenic KC-Tie2 mice resulted in the elimination of F4/80$^+$ macrophages, CD11b$^+$ myeloid cells, and CD11C$^+$ dendritic cells. The eradication of these cells would result in the disappearance of acanthosis, a decrease in the number of T cells, and a significant reduction in the production of TNF-α, IL-23, IL-1, IL-6, and S1008/9, stressing the importance of myeloid cells and their cytokines in maintaining the pathology [49].

The use of murine models, in particular the topical application of imiquimod in mice, makes it possible to mimic the dominance of monocytes, neutrophils, and dendritic cells derived from monocytes at an early lesion stage, and later, the decreased number of neutrophils and monocytes and the parallel increase in the number of dermal macrophages and Langerhans cells [50], which are then depleted. Langerhans cells are found mainly in the spinous layer of the epidermis. It is estimated that they constitute 2 to 4% of the epidermal cell population [51]. Derived from the bone marrow, they are transported by the blood to the epidermis [52]. After capturing the outer antigens, they migrate to the lymph nodes where they will initiate a specific immune response by displaying these antigens to the T lymphocytes. Langerhans cells are therefore antigen-presenting cells [52]. Similarly, in the murine DKO model, whose Jun and JunB transcription factors were deleted, resulting in psoriasiform type inflammation, the increase in the number of Langerhans cells (LCs) by proliferation followed by their subsequent decrease would reproduce the presence of proliferative Langerhans cells in a human lesional context [53]. In this model, the authors indicated that LCs would exert an immunoregulatory role by increasing the expression of IL-10 and PD-L1. Without LCs, the absence of regulatory signals would result in increased skin inflammation in these mice. The depletion of LCs did not alter the number of regulatory T cells in the skin, thus excluding the possibility that a reduced number of regulatory T cells could be responsible for worsening of the pathology. In addition, genetic depletion of LCs during the inflammatory phase in mice treated with imiquimod caused increased neutrophil infiltration and extension of pustular plaques, suggesting an anti-inflammatory role for LCs during psoriatic inflammation [50].

Recently, Leite Dantas et al. focused on the contribution of macrophages and T cells in the development of psoriasiform inflammation in a transgenic mouse model ihTNFtg (doxycycline- [Dox-] inducible human TNF-transgenic mouse line). In this murine model, the authors found that only macrophages (M1 and M2), Th1, and Treg were present in large quantities. While depletion of macrophages greatly reduced the development of the disease, Treg depletion increased the infiltration of macrophages into psoriatic inflammatory areas, contributing to the worsening of the pathology. Adoptive transfer of Treg in RAG-1-deficient mice, without either mature B or T lymphocytes, or immunocompetent mice induced the opposite effect, attenuation of symptoms. Thus, Tregs would limit migration of macrophages to injured areas, thereby reducing the harmful effect on tissue of macrophages in these transgenic mice [54].

Recently, IL-35, produced by regulatory T cells, demonstrated immunosuppressive effects in mouse models of psoriasis. Indeed, IL-35 may reduce the local infiltration of macrophages by reducing the levels of cutaneous expression of the macrophages M1 while conversely increasing M2 macrophages. Also, IL-35 may regulate the production of proinflammatory CD4$^+$ T cell cytokines and may decrease local lymphocytic infiltration of Th17 cells in K14-VEGF-A-Tg mice and in mouse models of imiquimod-induced psoriasis [55]. IL-37, expressed in macrophages, epithelial cells, and effector-memory cells, likewise demonstrated an immunosuppressive role in K14-VEGF-A transgenic mice by downregulating the production of proinflammatory cytokines, such as CXCL8, IL-6, and S100A7 [56]. IL-37 acted as a negative feedback inhibitor of inflammatory responses as the reduction of IL-37 protein synthesis in PBMCs with specific siRNA increased the production of several proinflammatory mediators [57] (Table 1).

3. Neutrophils

Polymorphonuclear neutrophils (PMNs), or leukocytes, are phagocytic cells characterized by a segmented lobular nucleus and cytoplasmic granules filled with degradation enzymes. PMNs are the most abundant circulating white blood cells and are the first type of cells involved in acute inflammatory reactions to bacterial infections [58]. These phagocytes ingest the microbes and release reactive oxygen species, antimicrobial peptides, proteases, and neutrophil extracellular traps [59, 60].

Table 1: The role of macrophages and neutrophils in different mouse models of psoriasis or other immune diseases.

In vivo models	Mouse model	Phenotype	Histopathological hallmarks	Role of macrophages	Role of neutrophils
Schon et al. [61]	Flaky skin	Psoriasiform skin lesions	Prominent infiltrate of neutrophils, and microabscesses within the hyperproliferative epidermis, hyperkeratosis, mixed inflammatory immune infiltrate	x	Yes
Wang et al. [44]	Hypomorphic PL/J CD18	Psoriasis-like skin inflammation	Abnormal keratinocyte proliferation/ differentiation, subcorneal microabscesses, increased inflammatory infiltrate	Yes	Not causal for the maintenance of the skin inflammation
Stratis et al. [45]	K14-Cre-IKK2fl/fl	Inflammatory and hyperproliferative cutaneous phenotype	Hyperplastic epidermis with loss of the granular layer, focal parakeratosis, infiltration of the dermis with macrophages, T cells, mast cells, granulocytes and microabscesses	Yes	Not required for the development of the disease
Ward et al. [49]	KC-Tie2	Cutaneous psoriasiform phenotype	Acanthosis, increased CD4-positive T cells, epidermal CD8-positive T cells, dermal dendritic cells and macrophages	Yes	x
Sumida et al. [72]	Topical application model of imiquimod	Hyperplastic cutaneous epithelial-squamous phenotype	Epidermal proliferation, abnormal differentiation, epidermal accumulation of neutrophils in microabcesses, neoangiogenesis and infiltrates of immune cells	x	Yes
Keijsers et al. [69]	Leukotriene application Tape-stripping	Skin inflammation *in vivo* associated with the histopathology of psoriasis	Epidermal proliferation, influx of polymorphonuclear cells in the epidermis and dermis, followed by a mononuclear cell infiltrate	x	Yes
Leite Dantas et al. [54]	Doxycycline-inducible human TNFα–transgenic mouse	Inflammatory arthritis and psoriasis-like phenotype	Hyperproliferation and aberrant activation of keratinocytes, infiltration with Th1, Treg lymphocytes and macrophages	Yes	x
Morimura et al. [47]	Topical application model of imiquimod in CX3CR1-deficient mouse	Hyperplastic cutaneous epithelial-squamous phenotype	Epidermal proliferation, abnormal differentiation, epidermal accumulation of neutrophils in microabcesses, neoangiogenesis and infiltrates of immune cells	Yes	x
Zhang et al. [55]	K14-VEGF-A-transgenic mouse	Psoriasis-like chronic inflammatory skin disease	Epidermal hyperplasia, impaired epidermal differentiation, accumulation of dermal CD4 lymphocytes and epidermal CD8 lymphocytes	Yes	x

Neutrophils infiltrate psoriatic lesions early from blood vessels within the dermis and form microabscesses, called Munro abscesses in humans, due to their accumulation in the form of microbial clusters in the thickened and parakeratotic stratum corneum [61] (Figure 1). Neutrophils accumulate in the skin, attracted by a gradient of chemotactic factors, which may be small induced secreted cytokines, such as IL-8, NAP-2, and NAP-3; membrane lipid derivatives such as leukotriene and platelet-activating factor (PAF); or substances of bacterial origin (LPS) [62–65].

Schon et al. noted that neutrophil depletion in the flaky skin (fsn)/fsn mutant mouse model of psoriasis-like lesion development contributed to a decrease in epidermal thickness, neutrophilic infiltrate, epidermal microabscesses, and the number of CD3-positive T cells, thus to an improvement of psoriasiform skin lesions [61].

Extravasation of neutrophils was made possible by the binding of integrin $\alpha_M\beta_2$ (CD11b/CD18) to ICAM-1 (CD54) of endothelial cells. ICAM-1 would be expressed de novo on the surface of hyperproliferative psoriatic keratinocytes, thus contributing to the migration of neutrophils expressing $\alpha_M\beta_2$ to the epidermis [61].

Some neutrophil-derived T cell attractants, such as *defensin-1*, *defensin-2*, or CAP37/azurocidin [66], may explain the reduction in the number of tissue T lymphocytes upon *in vivo* depletion of neutrophils in SCID mice. However, the influx of neutrophils into the epidermal compartment via chemoattractants seems to follow the influx of lymphocytes.

Moreover, the depletion of neutrophils in patients with moderate to severe generalized pustular psoriasis via an extracorporeal circulation therapy that selectively eliminates elevated myeloid lineage leukocytes resulted in a decrease in erythroderma, pustules, and edema up to 10 weeks after therapy [67].

Neutrophils would represent a major source of IL-17 [68–70], via the formation of extracellular traps [71], whose production is defined as the ultimate stage in a process of neutrophil polymorphonuclear activation. The topical application of leukotriene B4 (LTB4), found in high concentrations in psoriatic lesions, is a chemoattractant for neutrophils, eosinophils, monocytes, macrophages, mast cells, dendritic cells, and effector T cells. LTB4 induced a rapid influx of polymorphonuclear cells into the epidermis and dermis, followed by an infiltrate of mononuclear cells [69]. Moreover, a recent study has demonstrated that LTB4 receptor 1 (BLT1) and CXCR2 promoted the recruitment of neutrophils at psoriatic lesional sites and that these cells would secrete IL-1β, perpetuating psoriatic inflammation [72] (Table 1).

Moreover, the release of this mediator, IL-17, by NETosis amplified the accumulation of neutrophils [73] by increasing the expression of CXCL1, CXCL2, and IL-8. IL-17 increased the expression of antimicrobial peptides—β-defensin-2 (HBD-2), S100A7, S100A8, S100A9, and LL37—by keratinocytes [74–76]. These antimicrobial peptides can stimulate immune cell infiltration, and NET-derived DNA-LL37 nucleic acid complexes promoted IFN-α secretion of plasmacytoid dendritic cells [21]. IFN-α and TNF-α would stimulate the influx of inflammatory dendritic cells and macrophages, which would produce cytokines, including IL-23 and IL-1β, in the presence of IFN-γ.

Blocking IL-17A via a neutralizing antibody (secukinumab) reduced hyperkeratosis, acanthosis, and hyperproliferation, significantly decreased the levels of gene expression of chemokines derived from keratinocytes, such as CXCL1 (GRO) and CXCL8 (IL-8), and triggered near-total elimination of IL-17-positive epidermal neutrophils. The authors suggested that the inhibition of IL-17A would indirectly block the influx of neutrophils due to the lack of keratinocyte response to IL-17A [70].

Furthermore, the specific deletion of the A-chain of IL-17 receptor in mice contributed to the delay and attenuation of psoriatic inflammation in mice treated with imiquimod but did not prevent its development. KO mice for IL-17RA showed a delay and alteration of peripheral neutrophils at the site of injury. The authors hypothesized that IL-6, strongly expressed in KO mice for IL-17RA and treated with imiquimod, may play a role in the development of pathology in the absence of IL-17RA [77]. Nevertheless, there is no doubt that compensatory mechanisms ensure the attraction of neutrophils to the inflammatory site in the absence of the IL-17RA signaling pathway.

The importance of IL-6 has also been demonstrated in a murine model in which IL-17A and GFP are coexpressed in keratinocytes, resulting in the formation of psoriatic-like lesions. A blockage of IL-6 signaling would reduce the pathology in these mice by reducing the formation of neutrophil microabscesses in the epidermis and reducing the number of myeloperoxidase-positive cells [78].

In 2011, Garcia-Romo et al. reported that NETs would produce a greater amount of LL37 in response to PMA and IFN-γ in systemic lupus erythematosus [79]. The secretion of LL37 facilitated the uptake and recognition of DNA by plasmacytoid dendritic cells. The antimicrobial peptide LL37 was also overexpressed in the lesional psoriatic skins and would participate in the activation of cells of innate immunity [80].

Neutrophils participate in the secretion of inflammatory mediators, including IL-1α, IL-8, TNF-α, and IFN-γ cytokines [81–84]. The interaction of neutrophils and fibroblasts may increase the secretion of IL-8 [85]. In addition, IL-12, expressed on the surface of mononuclear cells, such as epidermal neutrophils, is overexpressed in psoriatic lesions. Although fibroblasts do not secrete IL-12, the fibroblast-neutrophil interaction upregulates IL-12 secretion, highlighting the importance and necessity of considering cooperation between the different cells to better understand these interactions. Furthermore, IL-12 promoted the survival and growth of Th1 cells, as well as their differentiation, and inhibited the formation of Th2 cells.

The formation of neutrophil-containing microabscesses would be dependent on IL-1R1. It has been demonstrated in the imiquimod-induced murine model that the signaling of IL-1 via IL-1R1 regulated constitutive and induced chemokine expression in response to imiquimod, involved in the *in vivo* recruitment of neutrophils. However, the deletion of IL-1R1 did not block the formation of microabscesses,

implying that other cytokines are involved in their formation [86]. CEACAM-1 expression in superficial keratinocytes found in psoriatic lesions would also contribute to the persistence of neutrophils and to the underlying inflammation in psoriatic patients [87].

Aldara cream modifies the immune response by stimulating the body's defenses that fight certain types of skin affections. The topical application of Aldara in mice deficient in IL-17A, IL-17F, or IL-22 drastically reduced the severity of psoriasis. However, $\gamma\delta$ T cell populations and innate RORγt-positive lymphocytes produced large amounts of these inflammatory cytokines and were necessary and sufficient for the formation of psoriatic lesion plaques in this murine model. A reverse RORγt agonist, developed by Janssen, has demonstrated its efficacy in murine models for psoriasis and inflammatory arthritis. The blockade of the Th17 differentiation led to the decrease of the production of IL-17A by the memory T cells and reduced the production of IL-17A and IL-22 by the cells NKT and $\gamma\delta$ [88].

Furthermore, the combined action of mannan-activated macrophages and IL-17A from T cells provoked the infiltration of neutrophils into skin compartments, leading to histopathological features [89].

IL-17F would induce the secretion of IL-8 by the keratinocytes and would favor the infiltration of neutrophils into the dermis. On the other hand, the blocking of neutrophil infiltration by an anti-IL-8 antibody underlined the importance of the IL-17F/IL-8 axis in the pathophysiology of psoriasis [90].

At last, a treatment with ustekinumab (human monoclonal antibody directed against IL-12 and IL-23p40) or infliximab (monoclonal chimeric antibody directed against TNF), both used to treat psoriasis, in severe psoriatic patients appeared to decrease the activity of neutrophils and monocytes. Indeed, the expression of CD62L, a molecule of cell adhesion, is restored in patients receiving biological therapy, while expressions of CD11b (also called integrin alpha M) and CD66b, another adhesion molecule, were decreased after treatment. Also, the ratio of activated $CD14^{high}$ monocytes was normalized in patients receiving therapy [91], stressing once again the importance of these immune cells in psoriasis.

In vivo models have demonstrated the complex but evident interrelationship between different immune cells at the level of psoriatic lesions and provide a better understanding of the influence that cells have on each other and the possible modulating effect of cytokines and chemokines on the functioning of neighboring cells in the local microenvironment (Figure 1).

4. 3D Organotypic Skin Models

At present, few organotypic models emphasize the importance of macrophages or neutrophils in psoriasis, but the enthusiasm for such models could lead us to make new discoveries in the years to come.

Some models already demonstrated the role of T cells in the pathogenesis of psoriasis. In the 1990s, transplants of human psoriatic skins in immunodeficient mice [92] and injected with autologous T cells, from either peripheral blood or the lesion site, indicated that only the latter was able to maintain the psoriatic phenotype in the grafted mice [93]. In 2010, to counter the limitations associated with such models, Guerrero-Aspizua et al. isolated both skin cells—keratinocytes and fibroblasts—and peripheral blood from psoriatic patients and reproduced cutaneous equivalents by bioengineering [94]. These equivalents were then grafted onto immunodeficient mice. The authors indicated that intraepidermal injection of activated human immunocytes induced the formation of psoriatic lesion in the skin model of xenotransplantation. The authors observed that the combination of factors secreted by Th1 cells and cytokines derived from Th17 cells was essential for the complete development of a psoriatic phenotype, emphasizing the importance of T cells in the pathology.

In 2002, Del Rio and colleagues performed a long-term follow-up of gene-transferred bioengineered artificial human skin based on a fibroblast-containing fibrin dermal substrate orthotopically grafted onto mice [95]. This preclinical approach, considered more clinically relevant and better predictive models of drug efficacy, will certainly identify new therapeutic targets for psoriasis. Moreover, the topical application of nanosomes containing siRNAs inhibiting the expression of hBD-2 in such a mouse model improved the cutaneous phenotype and reduced the number and the size of blood vessels in the dermal compartment [96].

Modeling psoriatic inflammation requires paying close attention to the immune component. Some organotypic 3D models, with no immune component, attempt to mimic psoriatic inflammation. In our lab, we have already demonstrated that the generation of skin equivalents from human psoriatic fibroblasts and keratinocytes produced by the self-assembly method displayed major hallmarks of psoriasis [97]. Also, although the addition of a cytokine cocktail [94, 98–100], or a single protein [101], to mimic psoriatic inflammation has demonstrated some histopathological aspects of psoriasis, it is unlikely that all mediators released by immune cells will be generated with a mixture of a few inflammatory cytokines. Although essential, animal models cannot reflect the etiology of psoriasis or represent the human complexity associated with pathology.

An alternative to animal experimentation is the development of equivalent three-dimensional models generated from human skin cells. These organotypic models, which are rapidly expanding, make it possible, by adding one or more cutaneous components, to dissect the specific role of each cell type present. Conversely, current models, usually with only one immune component, cannot summarize all the cellular and molecular interactions occurring *in vivo* due to the absence of other components, which are certainly important to the general pathophysiology of psoriasis. Moreover, deletion of an element (macrophages by clodronate liposomes, e.g.) very rarely results in a complete reversion of the phenotype, thus implying that other constituents are involved in the pathology development.

Thus far, too few *in vitro* three-dimensional models including the main cellular components involved in the pathophysiology of autoimmune diseases, such as psoriasis or atopic dermatitis, are currently being studied to further

Table 2: Observed features in 3D organotypic skin models.

(a)

3D *in vitro* models	Components	Support	Observed features
Dezutter-Dambuyant et al. [102]	Fibroblasts + keratinocytes + endothelialized cells + CD34-positive cells	Solid scaffold of bovine collagen, chitosan and chondroitin 4-6 sulfate	Differentiation of interstitial dendritic dells
Bechetoille et al. [104]	Fibroblasts + dermal macrophages derived from monocytes	Solid scaffold of bovine collagen, chitosan and chondroitin 4-6 sulfate	Display phagocytosis and remain responsive to LPS
Chau et al. [103]	Fibroblasts + keratinocytes + dendritic cells	Nondegradable microfibre scaffolds and a cell-laden gel	Able to migrate and remain responsive to stimulation with skin sensitizers
Pageon et al. [106]	Fibroblasts + keratinocytes + monocytes	AGE-modified collagen lattices	Differentiation of CD14+ monocytes into dendritic cells and macrophages

(b) 3D models for psoriasis (without immune cells).

3D *in vitro* models	Components	Support	Observed features
Barker et al. [111]	Fibroblasts + keratinocytes isolated from human lesional skin	Collagen gels	Psoriasis-like phenotype
Tjabringa et al. [98]	Healthy keratinocytes + de-epidermized dermis + cytokines (IL-1α, TNF-α, IL-6 and IL-22)	De-epidermized dermis	Psoriasis-like phenotype
Jean et al. [97]	Psoriatic fibroblasts + psoriatic keratinocytes	Fibroblast-derived dermal matrix	Psoriasis-like phenotype
van den Bogaard et al. [105]	Healthy keratinocytes + activated CD4-positive T cells and Th1/Th17-polarized T cells	Decellularized deepidermized dermis	Psoriasis-like phenotype

understand the role of the microenvironment in maintaining inflammation. The influence of the components on the modulation of the soluble and nonsoluble factors constituting the local lesional microenvironment can be apprehended by the generation of 3D models. However, the difficulty of incorporating immune cells into skin models, along with the complexity involved in modeling a three-dimensional environment necessary and sufficient to maintain cell viability and to respect the anatomical arrangement of cells, represents a major challenge.

In 2006, Dezutter-Dambuyant et al. optimized the development of a reconstructed skin model by incorporating hematopoietic progenitor cells in an endothelialized skin equivalent. The team demonstrated that the differentiation of dendritic cell precursors into Langerhans cells depends on the state of differentiation of keratinocyte cells. In this case, the differentiation program of the Langerhans cells started only if keratinocytes were differentiated. Interaction between fibroblasts and keratinocytes in a three-dimensional skin model supported the regulation of their own differentiation and also that of anatomically close cells [102].

The encapsulation of dendritic cells in an agarose and fibronectin gel, compartmentalized between a layer of fibroblasts and keratinocytes and treated with dichlorobenzene for 24 hours, makes it possible to study the cellular interactions and mechanisms of skin sensitization [103]. In addition to maintaining their viability and horizontal and vertical migration, dendritic cells appeared to maintain an immature phenotype in the presence of fibronectin, expressed higher levels of endocytic receptors, and had a greater potential to induce T cell activation.

In 2011, Bechetoille et al. developed a dermal equivalent model of bovine collagen, chitosan, chondroitin sulfate, fibroblasts, and dermal macrophages derived from monocytes [104]. Macrophages, with the "classical" fusiform morphology, expressed CD14, CD163, and DC-SIGN/CD209 markers and produced large amounts of IL-10 in response to LPS, but little TNF. While LPS stimulated immune responses by interacting with the CD14 membrane receptor and induced the secretion of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, a stimulation of LPS macrophages in this *in vitro* model promoted their anti-inflammatory activity.

In 2014, van den Bogaard et. al developed a three-dimensional healthy *in vitro* skin substitute model in which they injected allogeneic healthy T lymphocytes into the dermal compartment. The authors demonstrated that the polarization of T cells towards a Th1 or Th17 phenotype, then injected in skin substitutes, induced the expression of molecular markers associated with psoriasis, although no hyperproliferation or acanthosis was observed. The inflammatory phenotype thus developed is similar to the psoriatic phenotype observed in human lesional plaques [105].

In another context, it has also been demonstrated that the generation of glycated cutaneous equivalents promoted the differentiation of monocytic cells towards a macrophage or dendritic cell phenotype. It would be interesting to characterize the influence of these differentiated cells on the modulation of the secretory profile in glycated substitutes [106] (Table 2).

5. Future Directions

The aim of this review was to analyze the importance of immune cells, and more particularly leukocyte cells, as neutrophils and macrophages in the pathophysiology of psoriasis. The emphasis is often on T cells, yet the influence of other immune cells on lymphocytes and keratinocytes must be better characterized. The specific roles of each cell types must be dissected to better understand the cellular hierarchy established in psoriasis. We also discussed the need to develop new *in vitro* models with a three-dimensional microenvironment and appropriate and relevant cellular components to better mimic the pathology and improve our understanding of it. Besides, it would also be advantageous to integrate other types of immune cells, such as dendritic cells, into human skin three-dimensional models, given their role in the presentation of antigens to T lymphocytes. Although there is a great temptation to develop a more complete model including immune, cutaneous, and endothelial cells to better reflect the actors involved in inflammation, it is often difficult to know if an observed effect is due to a single cell type or an interaction between anatomically close cells.

The development of complex three-dimensional models is an important issue for research in pharmacology. Today, most of the available models reproduce only very partially the *in vivo* situation because their architecture does not consider the complexity of the tissue interfaces and the vascular perfusion. Recently, some companies have begun to develop organs or tissues as relevant tools to repetitively assess the pharmacological action of drugs. For instance, Organovo, based in the United States, commercialized its first liver model in 2014, which incorporates hepatocytes, stellate cells, and endothelial cells, printed in a matrix. This model would be more discriminating than 2D cultures and would make it possible to mimic a patient's response to a drug [107]. It would be wise to focus on these *in vitro* models, given the ethical and financial constraints associated with the use of animal models.

Recently, new antipsoriatic therapies have emerged, such as apremilast (Otezla, Celgene), a phosphodiesterase 4 (PDE4) inhibitor, which is also the first oral anti-inflammatory treatment for psoriasis in more than 20 years. PDE4 inhibition would also be a potential target for systemic sclerosis, as its blockage decreased dermal fibrosis through the downregulation of profibrotic mediators from M2 macrophages [108].

Other promising drugs are currently being tested in phase III in the US, specifically targeting the inhibition of phospholipase A2 (PLA2), which controls the biosynthesis of inflammatory mediators, such as leukotrienes and prostaglandins. Other biological agents, such as etanercept (Enbrel, Amgen), infliximab (Remicade, Merck & Co./Janssen Biotech), and adalimumab (Humira, AbbVie), which are tumor necrosis factor (TNF) antagonists, are more commonly administered, although some side effects are frequently observed in patients (>10%): viral infection, dyspnea, migraine, and nausea. Since 2009, biological agents against psoriasis targeting IL-12, IL-17, and IL-23, cytokines that play a key role in inflammatory and immune responses, have been licensed on the market. Ustekinumab was the first drug specifically designed to suppress inflammation by targeting the signaling pathway of the cytokine family of interleukin-12 (IL-12) and interleukin-23 (IL-23).

Another therapeutic approach, the encapsulation of antipsoriatic agents in nanoparticles [109, 110], is currently under study with the aim of improving the efficacy, safety, and compliance of potential agents.

Advances in psoriasis research continue to lead to new therapeutic strategies that promise better management of this complex disease in the future. For sure, development of *in vitro* models and mouse models will help to revolutionize the care of psoriasis in the years to come.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this review.

Authors' Contributions

Isabelle Lorthois wrote the manuscript, which has been corrected and revised by Daniel Asselineau, Nathalie Seyler, and Roxane Pouliot.

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Review Article

Advances of Stem Cell Therapeutics in Cutaneous Wound Healing and Regeneration

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Cutaneous wound healing is a complex multiple phase process, which overlaps each other, where several growth factors, cytokines, chemokines, and various cells interact in a well-orchestrated manner. However, an imbalance in any of these phases and factors may lead to disruption in harmony of normal wound healing process, resulting in transformation towards chronic nonhealing wounds and abnormal scar formation. Although various therapeutic interventions are available to treat chronic wounds, current wound-care has met with limited success. Progenitor stem cells possess potential therapeutic ability to overcome limitations of the present treatments as it offers accelerated wound repair with tissue regeneration. A substantial number of stem cell therapies for cutaneous wounds are currently under development as a result of encouraging preliminary findings in both preclinical and clinical studies. However, the mechanisms by which these stem cells contribute to the healing process have yet to be elucidated. In this review, we emphasize on the major treatment modalities currently available for the treatment of the wound, role of various interstitial stem cells and exogenous adult stem cells in cutaneous wound healing, and possible mechanisms involved in the healing process.

1. Introduction

Skin, the largest organ of the body, has multiple important functions, such as acts as a barrier to foreign pathogens, regulates body temperature, supplies sensation, and prevents dehydration of the body [1]. An open wound could be defined as a type of injury in which the skin is torn, cut, or punctured resulting in disruption of normal anatomic structure and function [2]. Normal wound healing process is composed of a well-orchestrated process of cell migration, proliferation, and extracellular matrix deposition undergoing three overlapping but distinct phases of inflammation, proliferation, and maturation [3] and is a critical survival factor for an individual. Disruption of the cellular and molecular signals in conditions such as diabetes, infection, or radiation exposure may result in an inefficient healing. The skin wound might be of different nature and varies from surgical to accidental lacerations, burns, pressure ulcers, diabetic ulcers, and venous ulcers. In current medical practice, chronic cutaneous wound healing often demands a major, long-term medical attention and consumes a substantial amount of expenses [4]. The cost of treatment related to wounds and associated complications exceed $20 billion annually in the US [5]. For example, a diabetic foot ulcer typically costs around $50,000 to treat due to its refractory nature and continuous care [6]. Thus, enormous effort has been invested in developing innovative and efficient therapies to improve wound healing.

Current wound care has limited success and is very expensive. Thus, the approach of regenerative medicine has emerged as an alternative to improve the outcome of healing and has potential in reducing continuous economic burden. Regenerative therapy mainly focuses on stem cells that have the ability to self-renew and differentiate into multiple cell types and is crucial for physiologic tissue renewal and for regeneration after injury. As the understanding of stem cell biology grows through basic research, including preclinical

models, stem cell-based therapies are increasingly evident in translational medicine. Current review emphasizes the understanding of the role of different endogenous and adult stem cells in cutaneous wound repair.

2. Events in Normal Wound Healing Process

The skin consists of three layers such as epidermis, dermis, and hypodermis. The epidermis, most outer layer, consists of multilayered epithelium extending from the basement membrane, which separates the dermis to the air. It is devoid of extracellular matrix (ECM) except the basement membrane. The basement membrane contains progenitor cells, which undergo continuous self-renewal and differentiate into keratinocytes. The keratinocytes migrate towards the surface of the skin where they eventually undergo terminal differentiation and maturation [3]. These keratinocytes form a keratinized layer of dead cells at the skin surface, which provides the main barrier [7]. The dermis is the thickest of the three layers of skin, which is present just below the epidermis. The dermis is a connective tissue comprised of fibroblasts, ECM, vascular endothelial cells, and skin appendages (hair follicles, sweat glands) [7]. Fibroblasts secrete molecules like collagen and elastin, which provide mechanical strength and elasticity to the skin. The hypodermis underneath the dermis is composed of adipose tissue, which provides insulation and cushioning between the skin and other skeletal structures, like bone and muscle [7]. Cutaneous wound healing process is imperative to restore a skin defect and to regain lost integrity, tensile strength, and barrier function of the skin [8]. Cutaneous wound repair is a multifaceted process involving inflammation, proliferation, and tissue remodeling [9].

2.1. Inflammation. The wound healing process starts with coagulation and fibrin clot formation called hemostasis. Platelets from damaged cutaneous blood vessels are exposed to ECM upon injury and damage. Fibrin binds to monocytes and neutrophils through integrin CD11b/CD18 receptor and participates in the inflammatory phase. Fibrin also binds to endothelial and fibroblast cells via $\alpha_v\beta_3$ integrin [10] and stimulates angiogenesis. Platelets and mast cells release diffusible factors, such as tumor necrosis factor- (TNF-) α and platelet-derived growth factor (PDGF), and exert inflammatory response [11]. Local inflammatory agents, such as activated complement and histamine, cause redness and swelling. This matrix is rapidly invaded by neutrophils, followed by monocytes, and other immunocompetent cells to remove dead tissues and control infection. Polymorphonuclear cells (PMNs) are the first inflammatory cells to arrive at the site of a cutaneous wound in large numbers between 24–48 hours [12]. Several growth factors and cytokines, such as interleukin (IL)-8, PDGF, and growth-related oncogene (GRO)-α/CXCL1 chemokine (C-X-C motif) ligand, are involved in drawing PMNs to a wound bed [9]. These PMNs are the major source of proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α, and exert cascades of inflammatory reactions and prevent infection (Figure 1(b)). PMNs are removed by macrophages through apoptosis, called PMN debridement via slough eschar [9, 12]. Monocytes come to the wound bed after PMN and transform into macrophages, which are abundant during day 2 and 3 but remain there for weeks. Different factors, such as macrophage chemoattractant protein- (MCP-) 1, macrophage inflammatory protein- (MIP-) 1α, vascular endothelial growth factor (VEGF), PDGF, and transforming growth factor- (TGF-) β, attract monocytes to the wound bed, and activated macrophages secrete IL-1α, IL-1β, IL-6, and TNF-α to perpetuate inflammatory reactions [9]. This inflammatory phase lasts for the first 4 days in normal wound healing process [13]. Besides eliminating microbes and debris, these inflammatory cells also initiate repair and mediate angiogenesis as the wound exits its inflammatory phase.

2.2. Tissue Remodeling. Inflammatory cells promote the recruitment and proliferation of fibroblasts, vascular endothelial cells, and keratinocytes during the proliferative phase [14]. Approximately 4 days after injury, the provisional ECM begins to be replaced by the granulation tissue (GT). GT is composed of fibroblasts, collagen, blood vessels, and macrophages. Fibroblasts are one of the most important cell types in the wound healing process. Several matrix metalloproteinases (MMPs), such as MMP-1, -2, and -3, play important roles in migration of fibroblasts into the provisional wound matrix. Fibroblasts secrete collagen, increased the amount of deposited collagens, especially collagen-I, and enhance cross-linking, which resulted in an increase in mechanical strength of the wound. Collagen production begins approximately 3 to 5 days after tissue injury and is stimulated by a number of growth factors, including PDGF, TGF-β, epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, and fibroblast growth factor- (FGF-) 2 [9]. Fibroblasts differentiate into myofibroblasts, which promotes wound contraction and results in reduction of the wound area.

2.3. Proliferation. Neovascularization also occurs in concert with the help of invaded capillaries, recruited vascular endothelial cells, and endothelial progenitor cells to support the newly formed tissue and to transport circulatory cells to the wound [4]. Endothelial cell migration is initiated on day 2 of postwounding and stimulated by VEGF, FGF, angiopoietin, and TGF-β. Several MMPs including MMP-1, MMP-2, MMP-9, MMP-19, and membrane associated MT-MMPs play crucial a role in various aspects of angiogenesis. Deposition of GT mediates reepithelialization to the provisional wound bed. Keratinocytes migrate from the wound edges and proliferate on the surface of the GT [13]. For the progression of wound healing, bidirectional interactions between keratinocytes and fibroblasts are necessary by creating a paracrine loop [9, 15]. Occurrence of GT usually observed between 5 to 20 days of postwounding [7]. In the maturation phase, the wound becomes reepithelialized and the dermis regains most of its tensile strength. After complete wound closure, tissue remodeling takes place below the epidermis

and may take up to a year or longer to complete [3]. In adults, a mature, nonerythematous flat linear scar formation is the hallmark of an ideal wound healing [16].

3. Acute and Chronic Wounds

Acute cutaneous wounds resulted from a trauma, which undergo a repair process and lead to a benign scar when the repair process is orderly and timely [2]. Failure of this process may lead to an undesirable scar or a nonhealing wound due to the extended wound area or the depth exceeds the patient's ability to heal (Figure 1). The ability to heal diminishes in different pathological conditions. Patients with chronic wounds (most notably diabetic foot ulcers) have underlying conditions, such as high blood sugar level and obesity, that impair wound healing. Pressure ulcers and venous ulcers are also some of the most common forms of chronic wounds. Chronic wounds are frequently linked to old age [17] and correlates with a poor reservoir of fully functional stem cells [18–20]. It is also linked with the age-related decreased strength and elasticity of skin and decreased blood flow to the extremities due to sedentary lifestyle and smoking [7]. Several studies suggest that psychological stress have a negative impact on wound healing [21, 22].

4. Current Treatments for Wound Healing

To achieve a complete healing of the wound, an appropriate wound care is critical, and standard treatment modalities are used to improve the wound bed. Therapy for chronic wounds mainly focuses on the identification and correction of the precipitating and perpetuating factors. This approach includes the use of antibiotics for accompanying cellulitis, revascularization of ischemic limbs, and compression devices for venous ulcers and rigorous off-loading for decubitus (pressure) ulcers [23, 24]. Despite the advancement in current wound care, chronic wounds do not heal or heal very slowly in the majority of the cases. Therefore, in recent years, efforts have been made to develop more and more advanced treatment strategies such as application of growth factors and cytokines [25], skin grafting [26, 27], and hyperbaric oxygen (HBO2) therapy [28].

4.1. Growth Factors and Cytokines. Therapeutic effects of various growth factors and cytokines were tested in the clinical management of nonhealing wounds. Among these growth factors, PDGF, VEGF, bFGF, and granulocyte-macrophage colony stimulating factor (GM-CSF) were tested extensively [29]. PDGF-BB was the most popular and approved by the Food and Drug Administration (FDA) for the treatment of diabetic neuropathic ulcers of the foot in the United States of America. However, later, the FDA announced the malignancy risk associated with this product [29, 30]. Hence, the journey of finding appropriate therapeutic growth factor for chronic wounds still continues.

4.2. Skin Graft. Efforts have also been devoted into tissue engineering in making appropriate skin grafts to heal refractory wounds successfully. The skin is the first tissue, which was successfully engineered in the laboratory for clinical application. There were two approaches to develop bioengineered skin, matrix-based product, where biodegradable matrix was used and the cell-based products, where cells were used for the application. There are bioengineered skin constructs, which are currently available and approved for clinical practice for the treatment of diabetic neuropathic ulcers. A bilayer living skin construct is also approved for venous and diabetic ulcers. Integra® is the first commercially available engineered skin substitute used for deep burn wound. Cross-linked collagen and chondroitin-6-sulfate copolymer are mixed together to form the dermal matrix. A silicon sheet is used which acts as a temporary epidermal layer [26]. Alloderm® is another skin substitute, specifically a dermal substitute, used for both wound repair and reconstructive surgery. This dermal substitute is made up of human cadaver dermis and used successfully for a full-thickness burn. Alloderm has reduced angiogenic components due to the risk of graft rejection [27]. Epicel™ is an example of a cultured autologous epidermis made up of human keratinocytes and used as an epidermal substitute for burned wounds, acute wounds, and chronic wounds [27]. Although Epicel has a little risk of rejection for large area wound coverage, this graft has limitation due to its short half-life and fragile nature. Instead of having novelty, artificially engineered skin is having certain disadvantages. In order to apply onto a patient, a skin biopsy not only takes several weeks to be expanded into sufficient cultured epidermis but also the product is very costly [26].

4.3. Hyperbaric Oxygen Therapy. Oxygen therapy under pressure also called as hyperbaric oxygen (HBO2) has been tried to improve wound healing for the last forty years with limited clinical benefits. HBO2 uses in wound healing on the basis of the fact that oxygen under certain pressure when applied to wounds can stimulate angiogenesis, promote fibroblast proliferation, and enhance immune function. There are very few evidences from clinical studies that demonstrate the efficacy of HBO2 therapy in any kind of foot ulcers or refractory wounds [28]. However, the application of HBO2 is currently not in clinical practice because this therapy could lead to significant side effects including myopia, oxygen toxicity in the brain leading to seizures, and pneumothorax [31].

Hence, approximately 50% of the patients with chronic ulcers do not heal when their ulcers were previously resistant to conventional therapy [32]. It is more and more evident from the wound healing experience of the last decade that more radical steps, such as stem cell therapy, need to be taken to propel the treatment of chronic wounds in a direction that will not only take care the external complexities of the wound but also will act on multiple modalities of wound healing systemically. For example, adult stem cells, which are multipotent and angiogenic, might be a suitable candidate for this purpose. Additionally, these stem cells can also be used as a vehicle for gene therapy, such as VEGF, and PDGF-BB [33, 34], which will add an extra dimension in treating chronic wounds such as diabetic ulcer. However, selection of a suitable cell type as a clinical candidate for wound healing therapy would be a great challenge.

5. Role of Stem Cells in Wound Repair (Endogenous and Exogenous)

The epithelium of the skin has a remarkable ability of self-renewal over the lifetime and also produces daughter cells that differentiate into one or multiple lineages. Cutaneous wound healing is the natural response but in case of severe conditions such as burn or diabetes, the repair process is insufficient to achieve an effective cure. In these chronic conditions, the result is neither aesthetically nor functionally perfect with the loss of epidermal appendages and the generation of connective tissue scar. Although epidermal stem cells in the basal layer, as an endogenous source of stem cells, can regenerate skin, but these cells are not sufficient to provide perfect repair after deep and extensive skin damage. Thus, exogenous supply of stem cells in traumatic conditions may be one of the novel therapeutic strategies to achieve perfect skin repair.

6. Endogenous Stem Cells

6.1. Hair Follicle and Interfollicular Epidermal Stem Cells. Three major compartments of the epidermis, such as interfollicular epidermis, sebaceous gland, and hair follicle, are capable of self-renewal (Figure 1). Among these compartments, interfollicular epidermis and sebaceous glands undergo constant self-renewal, whereas hair follicles undergo cycles of phases such as resting, growth, and involution [35]. In physiological condition, these compartments of the epidermis are rejuvenated by the differentiation of their own stem cells. However, during injury, these epidermal compartments are capable of repopulating one another [36, 37]. In case of full-thickness wounds, where the hair follicle is obliterated, wound healing occurs slowly from the wound edge; whereas, in case of partial thickness, wound healing is accelerated and relies on reepithelialization with the migration of cells from the hair follicle and sebaceous gland [38, 39]. The hair follicle bulge to epidermal stem cells in partial thickness wound regeneration, which is transient and bulge-derived cells are replaced eventually by interfollicular epidermal stem cell progeny as the injury is recovered or stress is relieved [37, 40]. Although bulge epidermal stem cells are not essential for wound closure [39], these cells significantly expedite closure in the early stages of wound healing [41]. Thus, hair follicle and its connective tissue sheath are attractive targets for the development of regenerative therapies due to its accessibility and richness of stem cells.

6.2. Endothelial Progenitor Cells. Endothelial progenitor cells play an important role in wound healing process via angiogenesis and facilitate wound closure. These progenitor cells might be tissue resident or originate from the bone marrow. Bone marrow-derived endothelial progenitor cells home to the site of cutaneous injury in response to hypoxia-inducible factor (HIF)-1-induced stromal cell-derived factor (SDF)-1 in hypoxic milieu [42, 43]. However, these phenomena are impaired in pathophysiological conditions such as diabetes and with the age [44]. Thus, diabetic wound fails to heal, especially with the increasing age. Hence, appropriate exogenous stem cell transplantation might be an alternative strategy to cure chronic wounds. It is also believed that resident endothelial progenitor cells in the skin also contribute to wound neovascularization through angiogenesis [45]. Isolated tissue resident endothelial progenitor cells contribute to angiogenesis by differentiating into blood vessels upon transplantation [46].

7. Cell-Based Therapy for Wounds

Human stem cells may offer considerable opportunities providing both undifferentiated and differentiated cells for gene therapy, drug discovery, and regenerative medicine [47]. In addition, stem cells could be transduced ex vivo and manipulated cells reintroduced into the host. Manipulated stem cells could also offer new therapeutic approaches for specific diseases conditions. Wound repair is a complex process and is influenced by numerous secreted factors, including cytokines, chemokines, and growth factors. In theory, application of stem cells to wounds is advantageous over administration of a single agent because stem cells have a unique feature of interacting with wound environment and modulate their activity to release multiple factors, which may facilitate wound healing process (Figure 1). Stem cells can also potentially serve as a source of cells for providing skin substitutes in applications for tissue engineering. Thus, the selection of a suitable stem cell is a challenge in order to achieve a desirable efficacy in wound healing. Embryonic stem cells could be the most favorable over adult stem cells for the repair and regeneration of skin tissues due to their capacity of self-renewal and unlimited supply of differentiated keratinocytes or keratinocyte progenitors for treating cutaneous injuries. However, embryonic stem cell-related research has raised difficult ethical issues and has evoked a great public interest and controversy.

Moreover, embryonic stem cells have a potential to generate tumors. The development of therapies using stem cells in the context of injury and wound healing has primarily relied on adult stem cells. Adult stem cells derived from the bone marrow, peripheral blood, umbilical cord blood, or adipose tissue with their limited capacity of self-renewal and proliferation would be more acceptable for therapeutic application in human skin tissues. Thus, an immense amount of research is going on to prove the efficacy and mechanisms of action of these stem cells for skin regeneration. There are already some encouraging results from human studies using multipotent adult stem cells as therapeutic agents for tissue repair [44, 48–50]. Endogenous stem cell populations are thought to play an important role in different aspects of skin wound healing including inflammation, reepithelialization, neovascularization, and tissue remodeling [51]. However, in pathological conditions, it has been observed that administration of exogenous adult stem cell accelerated wound healing through various mechanisms such as acceleration of reepithelialization, stimulation of neovascularization in a paracrine manner, or directly differentiating into various cell types such as keratinocyte, fibrocytes, endothelial cells, and pericytes (Table 1).

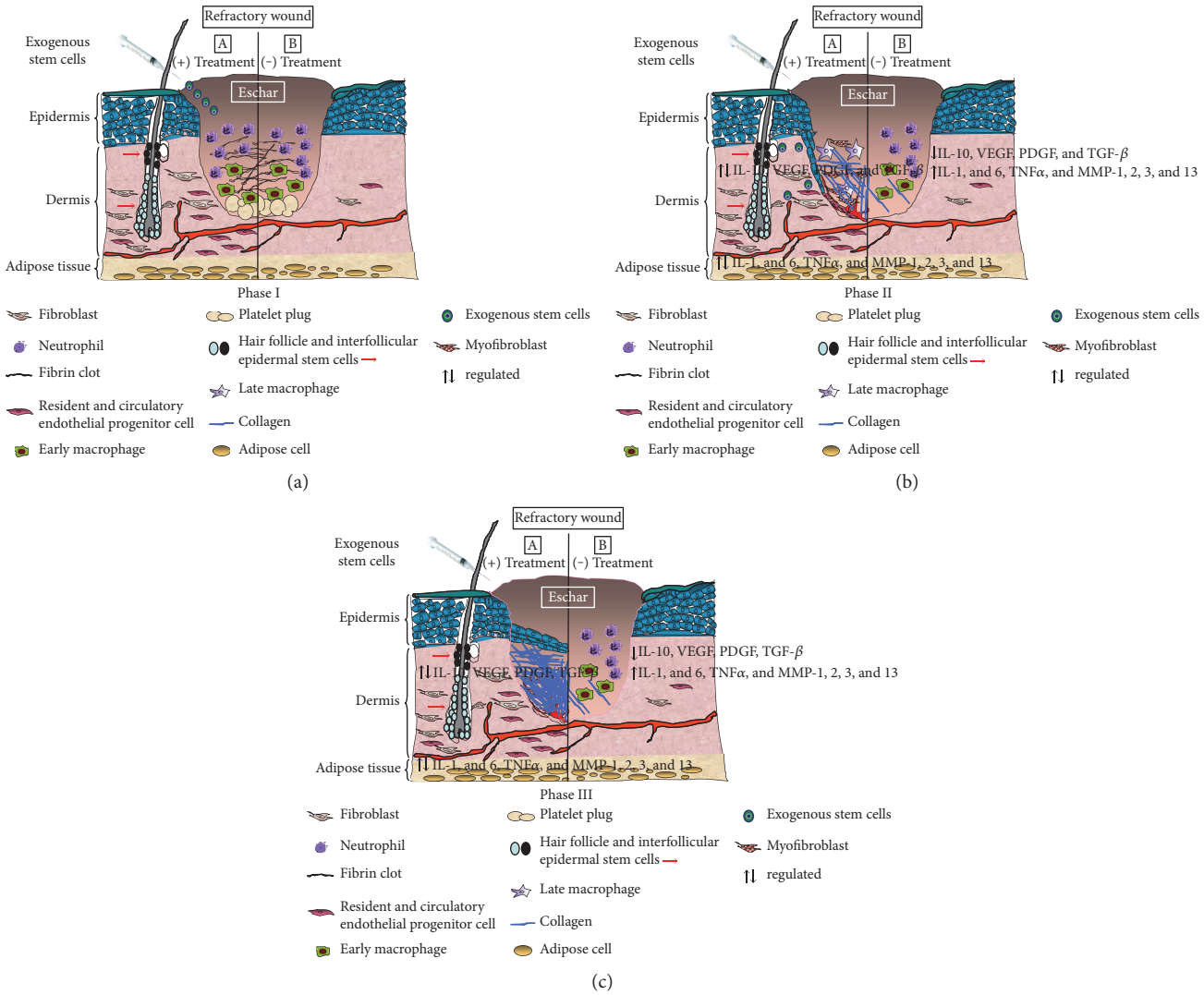

Figure 1: Graphical presentation of stem cell-mediated effect on refractory wound healing process. (a) Phase I: in inflammatory phase, the wound bed contains a large number of neutrophils, early phase macrophages, platelet plugs, and fibrin clots. Initiation of healing process occurs at this phase. (b) Phase II, A: systemic or local administration of stem cell homed to the wound bed. Exogenous stem cells mobilize host resident stem cells to take part in the healing process in GT formation by facilitating angiogenesis. Exogenous stem cells also directly take part in this healing process. The surrounding mobilized fibroblasts also differentiate into myofibroblasts and with collagen deposition facilitate reepithelialization process. B: in the absence of stem cell therapy, inflammatory cells such as neutrophils and macrophages still remain within the wound bed and impaired recruitment of endogenous stem cells occurs, which mediate an imbalance in the orchestrated harmony. GT formation is hindered due to the lack of angiogenesis, myofibroblast differentiation, collagen deposition, and reepithelialization. (c) Phase III: stem cell therapy generates scar tissue within the wound by replacing the provisional matrix. However, without stem cell therapy, refractory condition remains. The wound bed remains enriched with inflammatory cells and their proinflammatory secretory products. IL: interleukin; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor beta; MMP: matrix metalloproteinase.

7.1. Embryonic Stem Cells. Embryonic stem cells (ESCs) are pluripotent in nature which reside within the blastocyst. These cells have a potential to differentiate into any of the three primary germ layers namely endoderm, mesoderm, or ectoderm [52]. Embryonic stem cells can be differentiated into keratinocytes in presence of selected medium containing specific growth factors. These keratinocytes are capable of forming multilayered epidermis in culture, making them a key cell type for bioengineered skin [53]. However, the use of embryonic stem cells remains controversial, as ethical concerns exist regarding the harvest of cells from live embryos. Moreover, the potential for immune rejection and teratoma formation remains as other concerns. Hence, focus has been redirected towards adult stem cells as an alternative source with potential to apply in various disease conditions.

7.2. Induced Pluripotent Stem Cells. Induced pluripotent stem cells (iPSCs) are the multipotent cells with self-renewal properties, which are engineered from differentiated adult somatic

Table 1: Overview of stem cell-based therapies for cutaneous wound management *in vivo* and their mechanism of action.

Wound model	Type of cell therapy	Regenerative mechanisms	Reference
Excisional wound splinting model/Balb/C mice	BM-MSC	Recruitment of macrophages and endothelial lineages by paracrine factors	[124]
Excisional wound splinting model/diabetic mice	BM-MSC	Differentiation and angiogenesis	[129]
Excisional wound splinting model/C57BL/6 J mice	GMSC	Immunomodulation, M2 macrophage polarization	[118]
Full-thickness excisional wound/STZ-induced diabetic rat	ASC	Differentiation and vasculogenesis	[130]
Full-thickness excisional wound/STZ-induced diabetic mice	Human blood-derived CD34+ cells	Vasculogenesis	[103]
Limb ischemia and wounding/STZ-induced diabetic mice	Human fetal aorta-derived CD133+ cells	Paracrine stimulation of angiogenesis and activation of Wnt signaling	[105]
Full-thickness excisional wound in NOD/SCID mice	Nanofiber-expanded cord blood-derived CD34+ cells	Fibroblast proliferation and enhancement of collagen deposition and decreased MMP expression	[101]
Full-thickness excisional wound in NOD/SCID mice	Nanofiber-expanded cord blood-derived CD34+ cells	Immunomodulation, angiogenesis	[102]

BM-MSC: bone marrow-derived mesenchymal stem cells; GMSC: human gingiva-derived MSC; STZ: streptozotocin; ASC: adipose-derived stem cells.

cells, such as fibroblasts and keratinocytes, using transcription factors (e.g., Oct-3/4, Sox2, c-Myc, and KLF4) [54–56]. Unlike ESCs, iPSCs not only eliminate ethical issues but also reduce the chances of immune rejection while using it therapeutically [57]. A negligible immune response was also observed in iPSCs derived from human skin fibroblasts [58]. The unique reprogramming of iPSC technology made it possible to generate genetically diverse patient-specific cell lines from genetic skin disorders or chronic wounds which have tremendous potential for disease modeling and drug screening [59, 60]. During the last decade, significant progress has been made in the differentiation of the mouse, human iPSCs in to dermal stem cells and hair follicle lineages [58, 61], mesenchymal cells with the potential of forming dermal papilla [62], fibroblasts [63], melanocytes [64], keratinocytes [65, 66], among others. The multipotent capacity with limited immunoreactivity of iPSCs makes them a prospective agent for treating chronic skin disorders and unresolved wounds [67]. iPSCs generated from patients also could be modified and have the potential for cell therapy that have been shown in several studies as a proof of concept [66, 68]. However, application of iPSCs in human patients need further extensive analyses for safety and reliability of the reprogramming technology due to the risk of teratogenicity, mutagenesis, among others [69]. iPSCs can also provide a foundation for modeling a complex human organ like skin tissue due to their ability to be differentiated into multiple cell types in the body, and their unlimited growth potential was also demonstrated in various *in vivo* models [70, 71]. iPSCs therefore hold a great promise in the field of wound repair and regenerative medicine.

7.3. Mesenchymal Stem Cells. Mesenchymal stromal cells, also known as mesenchymal stem cells (MSCs), are adult stem cells capable of self-renewal and multipotential differentiation [72, 73]. MSCs can be obtained from the bone marrow and other tissues such as adipose tissue, nerve tissue, umbilical cord blood, and dermis with phenotypic heterogeneity [74–79]. In regenerative medicine, unlike embryonic stem cells, the use of mesenchymal stem cells could avoid ethical issues. Also, allogeneic MSC transplantation may induce little immunoreactivity to the host [80, 81]. Thus, MSCs have received considerable attention for modulating wound repair [82]. MSCs have been tested for skin repair and regeneration in various acute and chronic skin injuries like acute incisional and excisional wounds, diabetic skin ulcers, radiation, and thermal burns [76, 83, 84]. Inflammation and oxidative stress generated during wound healing not only attract bone marrow-derived mesenchymal stem cells at the wound area and conducive to self-renewal and proliferation [85] but also support wound healing through differentiation and the promotion of blood vessel formation. MSC therapy has shown enhanced wound healing through increased angiogenesis, reepithelialization, and tissue granulation. In clinical settings, MSC also showed a great promise in treating refractory wounds. In clinical studies, after MSC treatment, patients showed improvement of their wounds within days following administration, characterized by a decrease in wound size, an increase in the vascularity of the dermis, and increased dermal thickness of the wound bed [48, 86]. Additionally, coadministration of MSC at the wound site along with an autologous graft composed of autologous skin fibroblasts on biodegradable collagen membranes also decreased wound size and increased vascularity and dermal thickness in chronic diabetic foot ulcers [84]. All these findings from preclinical and clinical studies demonstrated that MSCs can contribute to wound repair and may be a resource for regenerative therapy.

7.4. Adipose-Derived Stem Cells. Adipose-derived stem cells (ASCs) are the precursor cells that are present within the stromal-vascular fraction of an enzymatically digested fat

tissue. Minimal invasive nature of tissue harvest has made these stem cells more attractive for regenerative medicine. ASCs are multipotent in nature and can be differentiated into different lineages such as bone, fat, cartilage, and muscle [75, 87]. ASCs can be characterized while in culture dish as $CD73^+/CD90^+/CD105^+/CD44^+/CD45^-/CD31^-$ cells, which can be distinguished from the bone marrow-derived MSCs by their expressions of CD36 and negative for CD106 molecules on their cell surface [88]. Although both of these cell types share surface markers, biologically they are different in terms of proliferation rate and differentiation, cytokine secretion, and chemokine expressions [89–91]. Thus, ASCs and MSCs may contribute to the wound healing differently. The capability of ASCs to secrete growth factors, to differentiate into multiple cell types, and to promote angiogenesis renders them a viable skin substitute [92, 93]. The ability of ASCs for soft tissue reconstruction makes them attractive for wound healing [94].

7.5. Hematopoietic Stem Cells. The possible role of hematopoietic stem cells (HSC) in skin regeneration is evident in many occasions. HSC can be isolated from the bone marrow (BM), umbilical cord blood, and peripheral blood by using its surface markers. In several occasions, skin "chimerism" (identification of epithelial cells of donor genotype) has been observed after clinical HSC transplantations such as BM or peripheral blood mononuclear cells (PBMC) [95–97]. The findings of donor-derived contribution of HSC to epithelial lineages in the host offer the broad-spectrum plasticity of HSC and indicate the possibility of skin regeneration by transplantation of HSC in chronic wound disorders. In a murine excisional wound model, a significant number of differentiated green fluorescent protein (GFP) positive cells were found in the hair follicles, sebaceous glands, and epidermis in host skin 21 days after transplantation of syngeneic GFP + bone marrow cells [48]. Additionally, a study has also shown that the differentiation potential of human umbilical cord blood stem cells into keratinocytes *in vitro* [98]. Apart from plasticity, the role of HSC in angiogenesis is also evident in myocardial infarction model, which is important and may be ascribable for the perfect and functional repair of skin tissue [99]. An emerging concept, epithelial and mesenchymal cell interaction is supposed to be a vital phenomenon in keratinocyte proliferation and differentiation, might play a crucial role in cutaneous wound healing and reepithelialization [4, 92]. The expression of CD34 and CD133 cells in dermal fibroblast and follicular matrix during embryogenesis provides an indication for the role of HSC in the molecular control of epithelial-mesenchymal cell interactions [100].

Peripheral blood, fetal aorta, and umbilical cord blood are also enriched with stem and progenitor cells, which express CD34 and CD133 markers. These cells are also multipotent and have shown a neovascularization potential in preclinical ischemic models [33, 34]. In preclinical wound healing models, we and others reported that CD34+ or CD133+ cells accelerate wound closure. We have demonstrated the wound healing ability of nanofiber-expanded cord blood-derived CD34+ cells in a mouse excisional wound model and an *in vitro* cellular model. We have shown that after systemic administration, these stem cells reached to the wound bed and facilitated wound healing. Our study revealed that nanofiber-expanded cord blood-derived CD34+ cell therapy accelerates wound healing by inhibiting several matrix metalloproteinases at the wound bed which prevents collagen degradation and increased the abundance of collagen components, procollagen1A1 at the wound bed [101]. Unlike previous experiments, we demonstrated for the first time that nanofiber-expanded cord blood-derived CD34+ stem cells accelerated wound closure by secreting collagen and thereby positively contributed to extracellular matrix [101], indicating that CD34+ stem cell treatment is having a potential to treat the refractory wounds resulting from diabetes or traumatic skin injuries. We further extended this work to explore the regulation of inflammatory response by CD34+ cell therapy using the same mouse wound model. Overall, our study demonstrated that CD34+ cell therapy mediated suppression of prolonged inflammation, positively contributed to increased angiogenesis, and accelerated wound closure compared to nontreated wounds [102]. These data provided a valuable information regarding the benefits of CD34+ stem cell-mediated wound healing and cell therapetic mechanism behind accelerated wound closure. In another study, treatment with human CD34+ peripheral blood mononuclear cells also accelerates healing of full-thickness skin wounds in diabetic mice by accelerated revascularization and epidermal healing [103]. A similar observation was also found in a report where cord blood-derived CD34+ cell treatment accelerated diabetic wound closure by stimulating keratinocytes, fibroblast proliferation, and neovascularization in a paracrine manner [104]. In another study, human fetal aorta-derived CD133+ progenitor cells and their conditioned medium treatment accelerated healing in ischemic diabetic ulcer by stimulating angiogenesis with activation of the Wnt signaling pathway in the host [105]. These findings indicate that blood-derived progenitors may have a therapeutic potential in the treatment of skin lesions in complex pathological conditions such as diabetes.

8. Mechanisms of Stem Cell-Mediated Wound Healing

8.1. Immunomodulation, Resolution of Inflammation, and Fibrosis. An imbalance in regulation of inflammation at the wound bed leads to defective healing. Sustained unresolved inflammation leads to chronic wound. Prolonged inflammation even leads to fibrotic scar formation. In chronic wounds, unresolved inflammation leads to increased protease activity and deregulated fibroblast activity, which resulted in decreased collagen deposition and ECM formation. Hence, resolution of inflammation is a big challenge in diabetic wound healing. In recent years, several studies have demonstrated the immunomodulatory function of cultured adult stem cells in laboratory conditions obtained from various sources like the umbilical cord blood, amniotic fluid, and bone marrow. Thus, allogeneic stem cell therapy induces immunomodulation in the wound bed and facilitates wound healing by resolving inflammation, as well as helping in reducing scar formation [106].

A substantial number of studies have demonstrated that treatment of MSCs has significant immunomodulatory effects during wound healing and in other inflammatory conditions [107]. This immunomodulatory effect on the host not only makes them a suitable candidate for allogeneic transplantation [108] but also makes them an attractive cell therapeutic agent to treat chronic wounds [106]. Studies have demonstrated that MSCs obtained from various sources such as the umbilical cord and bone marrow showed an anti-inflammatory effect in rat cutaneous wound and *in vitro* fibroblast model. MSC treatment demonstrated a significantly lower number of inflammatory cells and proinflammatory cytokines such as IL-1 and TNF-α with an increased level of IL-10 at the cutaneous wound bed in a rat model [109]. In addition, when murine BM-MSC were cocultured with human fibroblasts, the mRNA levels of intercellular adhesion molecule 1 (ICAM1) has decreased [110]. These studies suggest the potential of MSC in attenuating wound inflammation and inducing healing in chronic inflammatory stage.

Allogeneic transplantation of cord blood and cord blood-derived stem cells is also regarded as less immunogenic [111]. Our results demonstrated that nanofiber-expanded cord blood-derived CD34+ cells might have an immunomodulatory effect *in vitro* and *in vivo* wound healing models [102]. Systemically transplanted CD34+ cells accelerated wound closure, which was correlated with decreased inflammatory activity at the wound bed characterized by reduced inflammatory gene expression such as, IL-1β, TNF-α, IL-6, and NOS2A. At the same time, expression of anti-inflammatory molecule IL-10 was significantly increased indicating that CD34+ cell therapy has the potential to control the inflammation during wound healing process. To further elucidate the mechanism, we showed that CD34+ cells secrete IL-10, an anti-inflammatory molecule, and suppress NF-κB activation in a human primary fibroblast cell model. In the similar *in vitro* model, when human primary fibroblasts were cocultured with CD34+ cells in presence of inflammatory stimulus with TNF-α, NF-κB activation was significantly decreased by upregulation of IL-10 [102]. Sustained or unresolved inflammation prevents wound healing by inhibiting angiogenesis and catabolizes extracellular matrix in the wound bed. Our results suggest that nanofiber-expanded cord blood-derived CD34+ cell therapy might be a potential candidate to treat chronic wounds to resolve inflammation in a timely manner that will facilitate further angiogenesis and ECM formation for accelerated healing. Other studies have also suggested that ASCs also modulate the immune system and downregulate the inflammation by releasing growth factors critical for healing which are described in the reviews [112].

Antimicrobial activity is critical for wound clearance from infection. Studies have shown that MSCs have antimicrobial activities, which may also be helpful for chronic wound resolution. Antimicrobial activities of MSCs were shown directly by the secretion of antimicrobial factors such as LL-37 [113]. In another study, it was shown that MSCs could secrete immune-modulating factors, which will upregulate bacterial killing and phagocytosis by immune cells [114].

Tissue resident and peripheral macrophages play a significant role in initiation of inflammation after injury and resolving inflammation in a timely manner during the healing process. Macrophages shift gears between proinflammatory M1 and anti-inflammatory alternatively activated M2 states. Studies have shown in various models that MSCs may influence macrophage M1/M2 polarization after contact with macrophage [115–117]. M2 polarized macrophages play an important role in the resolution of inflammation and clearance of dead cells from the wound environment for accelerated healing. In a mouse wound healing model, it was shown that the human gingiva-derived MSC treatment *in vivo* promoted an M2 macrophage polarization, which was correlated well with anti-inflammatory wound environment and accelerated cutaneous wound healing [118]. The ability of MSCs to resolve inflammation might be useful in treating chronic unresolved wounds.

Moreover, dysregulated fibrosis or scarring is caused by excessive deposition of ECM. Inflammation largely regulates fibrosis process. The immunomodulatory activity of MSCs might regulate fibrosis and therefore anti-inflammatory activity of MSCs reduces the scar formation. In an *in vivo* murine wounding model, it showed that BM-MSCs attenuated development in skin fibrosis [119].

8.2. Differentiation. In several studies, it has shown that adult stem cells are multipotential and able to contribute to wound healing by differentiating into several tissue lineages starting from the inflammatory cells to myofibroblasts. During the inflammatory phase, HSCs from the bone marrow undergoes myelopoiesis with the help of MSC and supply the leukocytes to the wound region [92]. Also, tissue resident stem cells undergo differentiation in response to various stimuli during the wound healing process [120, 121]. From the regenerative perspective, differentiation is one of the key phenomenon by which exogenously applied adult stem cells exert therapeutic efficacy in cutaneous wound models. MSC is one of the most widely studied in this regard. Several studies have demonstrated that transplanted MSCs can differentiate into epidermal keratinocytes, endothelial cells, and pericytes directly participating in the structural repair of a wound. MSC transplantation led to accelerated cutaneous wound closure in both normal and diabetic mice, where MSCs express keratinocyte-specific markers suggesting their role to promote wound healing by differentiation [76]. Similarly, another study demonstrated that MSCs also transdifferentiate into keratinocytes, endothelial cells, and pericytes in cutaneous wounds after intravenous injection in mice [122]. Adipose-derived stromal cells are also capable of differentiating into epithelial, endothelial, and fibroblast lineages *in vivo* when applied to wounds by means of a seeded scaffold [92]. A similar kind of low-level transdifferentiation phenomenon was also observed in human skin after HSC transplantation where the mesodermal origin of HSC contributes to epithelial lineage in the host [95, 96].

8.3. Angiogenesis. Optimal repair and restoration of functional vasculature is crucial to achieve ideal healing of wounds. The process of neovascularization primarily

accomplishes revascularization of the wound bed. Neovascularization is achieved through two independent processes called angiogenesis and vasculogenesis, which lead to the development of functional microvascular networks. Traditionally, vasculogenesis is the de novo synthesis of new vessels by endothelial progenitor cells whereas angiogenesis is the development of new vessels from existing capillaries [123]. In a refractory wound-like diabetic wound, wound revascularization is affected due to an imbalance in the release of soluble mediators and improper function of endogenous progenitor and stem cells which is essential for ideal wound healing, leading to a hindered and orchestrated healing process. Several other and our own published studies [101, 102] have demonstrated that transplantation of adult stem or progenitor cells contribute to angiogenesis or vasculogenesis through directly differentiating into cell types essential for blood vessel formation or by stimulating endogenous mediators or cells in a paracrine manner. Studies have found that MSC expresses high levels of vascular endothelial growth factor (VEGF) and angiopoeitin-1, which indicate that the MSC-mediated accelerated wound healing is due to the release of proangiogenic factors and induction of angiogenesis [76]. Additionally, paracrine signaling and the release of soluble factors (e.g., VEGF) by MSC are found to promote angiogenesis at the wound bed after cutaneous injury in normal and diabetic mice [124, 125]. Another interesting hypothesis about MSC is that these cells may also act as pericytes, which stabilizes the blood vessel formation [126]. Future studies will further bolster this claim. However, the role of MSC in promoting angiogenesis is firmly evident by several instances in normal and refractory wound healing models [127, 128].

In other stem cells like ASC, it was noticed that ASC treatment also promotes angiogenesis and accelerates wound healing by producing VEGF [93]. Thus, induction of angiogenesis to accelerate wound healing by stem cell therapy is not only evident in MSC and ASC but is also found in several other types of stem and progenitor cells [103, 105]. Thus, increased angiogenesis by stem cell therapy not only supports GT formation but also supplies nutrients and clear the apoptotic cells from the wound bed and helps in wound resolution.

9. Conclusions and Future Directions

It is evident that stem cells have a tremendous potential for cutaneous tissue regeneration, as these cells not only can regenerate lost tissue but also promote wound repair through paracrine manner. Several cell types, such as embryonic stem cells, iPSCs, mesenchymal stem cells, resident tissue stem cells, epithelial stem cells, adipose-derived stem cells, and hematopoietic stem cells, are currently under intense investigation. Recent data on autologous MSC therapy in cutaneous repair showed a great promise as a therapeutic agent in clinical practice. Despite rapid progress in evaluating the efficacy of MSC transplantation for wound healing, several questions still need to be addressed. Using specific markers to characterize and isolate a distinct pool of MSC, which is homogeneous and functional, is one of the key points to be addressed. Further studies are necessary to characterize the niche of MSC, which helps MSCs to be effective in the wound healing process. Further investigation on experimental and clinical application of stem cells in wound healing is necessary to identify the ideal source of stem cells and the most efficacious mode of cell delivery.

Human umbilical cord blood is rich in stem and progenitor cells and is easily accessible for blood collection. The regenerative potential of CD34+ and CD133+ cell therapy in cutaneous tissue repair obtained from peripheral or umbilical cord blood opens up a possibility of providing cheap and affordable care for refractory wounds. Thus, a suitable technology like ex vivo expansion technique would be useful to get a large number of stem cells for the clinical application. Our group and others have shown promising results in expansion of umbilical cord blood-derived stem cells. The expanded stem cells were characterized (CD34+) and have shown their multipotential and angiogenic capabilities in preclinical ischemic models as well as in murine cutaneous wound models [33, 34, 101, 102] where CD34+ stem cell therapy accelerated wound closure by resolving inflammation with concurrent inhibition of MMP expressions. Additionally, these cells also can be manipulated *in vitro* with proangiogenic factors like VEGF and PDGF, which are efficacious in improving ischemia-related complications in preclinical peripheral and cardiac ischemic models. Thus, looking at their angiogenic and anti-inflammatory potential, this pool of stem cells may have a very promising future in treating refractory wounds. Moreover, nanofiber-expanded stem cells coupled with the innovative biotechnologies may open a new direction for plastic and reconstructive surgeons. Finally, the use of stem cells to induce cutaneous tissue regeneration holds a great promise for modern regenerative medicine.

Conflicts of Interest

No competing financial interests exist.

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Research Article

Suppressed Programmed Death 1 Expression on $CD4^+$ and $CD8^+$ T Cells in Psoriatic Patients

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Psoriasis is a chronic inflammatory disease mediated by T cell immunity. Programmed death 1 (PD-1), a coinhibitory receptor, plays an important role in immune regulation and maintaining peripheral tolerance. The aim of the study was to compare the expression of PD-1 on the peripheral T cells between psoriatic patients and healthy controls. The study included 75 psoriatic patients and 52 healthy volunteers. The percentages and absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$, $CD4^+PD\text{-}1^+$, and $CD8^+PD\text{-}1^+$ T cells were analyzed using flow cytometry. The absolute numbers and percentages of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells were significantly decreased in the psoriatic patients in comparison with the control group. No significant correlations were found between the absolute numbers and percentages of $CD4^+PD\text{-}1^+$ or $CD8^+PD\text{-}1^+$ T cells and clinical characteristics of psoriasis. Decreased PD-1 expression on the T cells may be responsible for impaired negative regulation of immune response in psoriasis pathogenesis.

1. Introduction

Psoriasis is a chronic inflammatory disease with the substantial involvement of T lymphocytes, where already activated T cells in the blood and skin are bound to cause cutaneous inflammation and keratinocyte hyperproliferation [1]. This pathological process has been explained before, but the role of certain regulatory mechanisms responsible for the immune tolerance in this disease needs to be further researched.

Various mechanisms, either contact dependent or related to secretion of some soluble factors, are employed to suppress immune responses. A number of surface molecules on T cells which regulate their state of activation have been identified.

The activation of T cells requires at least two signals: the first signal is an interaction between the T cell receptor (TCR) and major histocompatibility complex (MHC) on the antigen-presenting cell (APC), and the second signal is costimulation which could be stimulatory or inhibitory. One of the most important coinhibitory signals is an interaction between the programmed death 1 (PD-1), a molecule belonging to the CD28 family which is expressed on the T cells, B cells, macrophages, and PD-L1 (PD-ligand 1) or PD-L2, on the APC. Binding of ligands to PD-1 leads to downregulation of T cell activity [2–4].

PD-1, a checkpoint inhibitor, is vital for the immune regulation and tolerance [4]. Its blockage is known to provoke a shift of the cellular reactivity towards the proinflammatory response. The role of PD-1 expression in psoriasis pathogenesis has not been entirely explained so far. Therefore, we have made an attempt to determine the expression of PD-1 on $CD4^+$ and $CD8^+$ T cells from the peripheral blood of psoriatic patients. It is likely that the absence of negative costimulation from PD-1 is responsible

for continuous T cell activation and sustained skin inflammation in psoriasis, which may also contribute to the systemic nature of the disease.

2. Materials and Methods

2.1. The Study Group. The study group consisted of 75 psoriatic patients hospitalized in the Department of Dermatology, Venereology and Pediatric Dermatology Medical University of Lublin, Poland. The inclusion criteria were as follows: the duration of psoriasis for at least one year, active psoriatic skin lesions, and age at least 18 years. The exclusion criteria were as follows: cardiovascular, cerebrovascular, hematologic, hepatic or renal disease, neoplasm, chronic viral infections, erythrodermic, pustular or guttate psoriasis, addiction to drugs, and systemic antipsoriatic treatment.

The control group included 52 healthy volunteers, age- and gender-matched to the psoriatic group.

Informed consent was obtained from all the participants, and the study was approved by the Local Ethics Committee at the Medical University of Lublin (KE-0254/81/2015).

2.2. Assessment of Psoriasis Severity. The severity of psoriasis was assessed with PASI (Psoriasis Area and Severity Index), BSA (Body Surface Area), IGA (Investigator Global Assessment), and DLQI (Dermatology Life Quality Index). Psoriatic fingernail plate changes were assessed using NAPSI 80 (Nail Psoriasis Severity Index 80). We also analyzed the duration and the age of psoriasis onset.

2.3. Flow Cytometry Analysis of Peripheral Blood Mononuclear Cell Populations and Expression of PD-1. Flow cytometry analysis was performed in the Experimental Hematooncology Department at the Medical University of Lublin, Poland, with professionally trained and experienced staff in such analyses in patients with lymphoproliferative diseases.

Eight mL of peripheral venous blood from the psoriatic patients and healthy volunteers were collected into anticoagulated tubes. We isolated mononuclear cells using density gradient centrifugation on Ficoll-Hypaque (Biochrom AG, Berlin, Germany). Interphase cells were removed, washed twice in phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} and resuspended in RPMI 1640 containing 2% human albumin. The viability of obtained PBMCs was always >95%, as determined by trypan blue staining. Viable cells were quantified in a Neubauer chamber. 5×10^5 cells were incubated for 20 min. at room temperature with fluorochrome-labeled monoclonal antibodies (Mabs): anti-CD3-PerCP (Becton Dickinson), anti-CD4-FITC (Becton Dickinson), anti-CD8-PE (Becton Dickinson), and anti-PD-1-APC (clone MIH4) (Becton Dickinson). Approximately 100,000 stained cells in each sample were analyzed by flow cytometry using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). Unstained cells were used as a negative control (for each patient). Data analysis was accomplished by using FACS Diva 8.0.

For each person, lymphocytes were identified and gated from PBMCs by setting appropriate forward and side scatter parameters. Next, 3 steps of gating were applied in flow cytometry analysis (Figure 1 shows it for one exemplary patient with psoriasis). The first dot plot showed $CD3^+$ cells selected from all lymphocytes, and as a result, we obtained percentages of $CD3^+$ cells of all lymphocytes. The two gates on the second step of the cytometry were selected separately: one gate $CD4^+$ T cells and the other gate $CD8^+$ T cells, resulting in percentages of $CD4^+$ and $CD8^+$ T cells, respectively, for all $CD3^+$ cells. The two gates on the third step of the cytometry were selected separately: one gate $CD4^+PD\text{-}1^+$ T cells and the other gate $CD8^+PD\text{-}1^+$ T cells, resulting in percentages of $CD4^+PD\text{-}1^+$ T cells and $CD8^+PD\text{-}1^+$ T cells for $CD4^+$ and $CD8^+$ T cells, respectively (Figure 2 shows 2 exemplary persons: one patient with psoriasis and one healthy volunteer).

Then, for each person, we calculated the absolute number of $CD3^+$ cells, multiplying the total number of lymphocytes by percentages of $CD3^+$ (the result from the first step of gating). Then, we calculated the absolute numbers of $CD4^+$ and $CD8^+$ T cells, multiplying the absolute number of $CD3^+$ cells (calculated above) by percentages of $CD4^+$ and $CD8^+$ T cells, respectively (the results from the second step of gating). At the end, we calculated the absolute numbers of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells, multiplying the absolute numbers of $CD4^+$ and $CD8^+$ T cells, respectively (calculated above) by percentages of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells, respectively (the results from the third step of gating).

2.4. Statistical Analysis. Data of both absolute numbers (cells/μL) and percentages of PBMCs and PD-1 expression were statistically analyzed in the Institute of Statistics and Demography, Warsaw School of Economics, Poland, using SPSS and STATISTICA softwares.

We used a *t*-test to compare age and stochastic independence and χ^2 test to compare gender between the psoriatic patients and the control group.

We analyzed the clinical data of the psoriatic patients. Mean values (M) and standard deviations (SD) were estimated for continuous variables or absolute numbers (n) and relative numbers (%) of occurrence of items for categorical variables.

Comparisons of number and percentages of T cells and expression of PD-1 between the psoriatic patients and the control group were performed using logistic regression models. We estimated odds ratios of psoriasis occurrence versus control group (psoriasis—yes versus psoriasis—no), with independent variables: the absolute numbers (the first models) and percentages of PBMCs and PD-1 expression (the second models). In order to interpret the logistic regression analyses' results, we calculated OR-1 and expressed them in %. As independent variables are continuous, we obtained an average percentage change in risk of psoriasis (in plus or in minus) if independent variable increased about 1 unit (1 cell/μL in the first model or 1 percentage in the second model).

We used the Pearson correlation coefficient (r) to investigate mutual correlations of the number and percentages of PD-1 expression between $CD4^+$ and $CD8^+$ T cells, as well

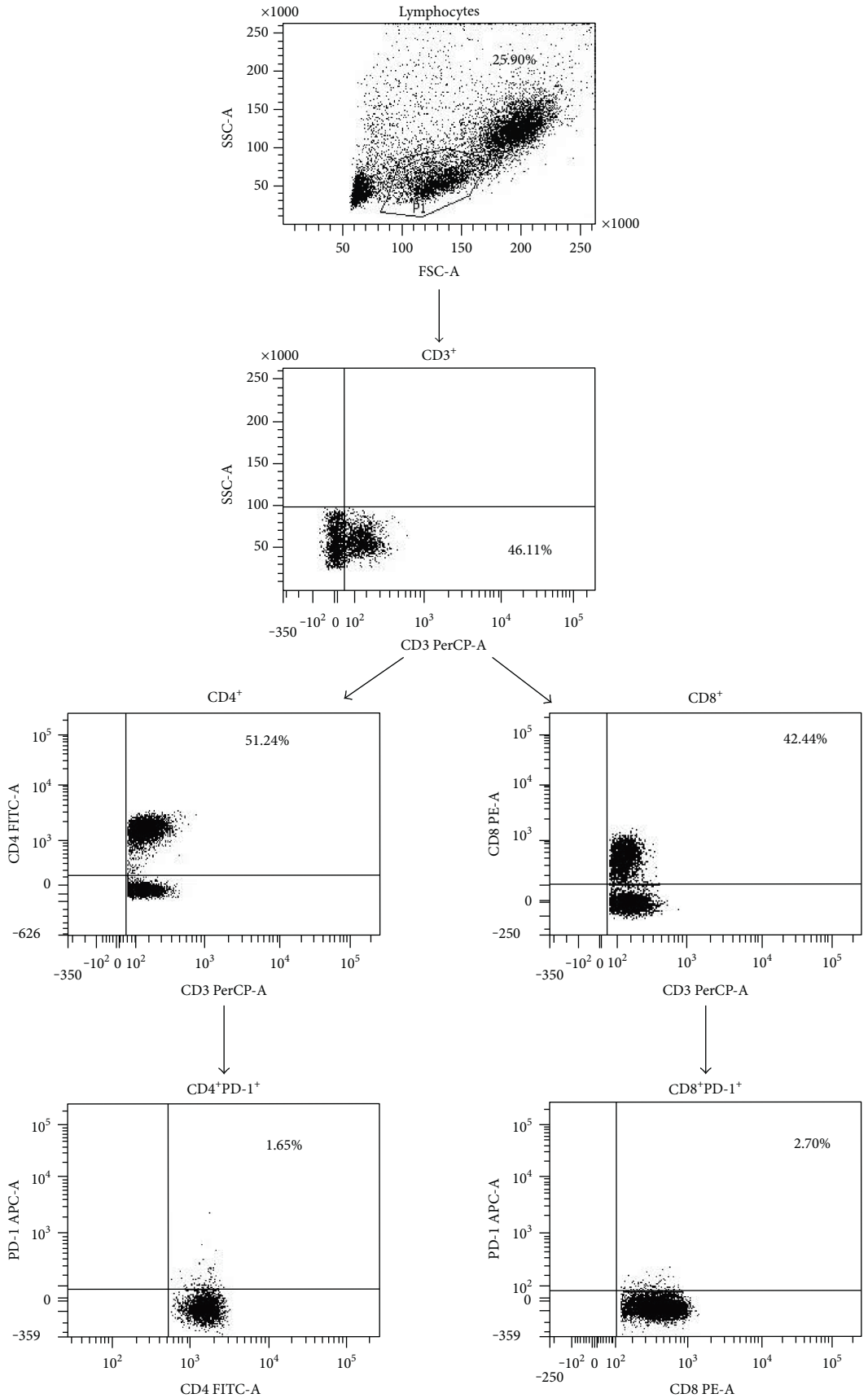

Figure 1: Full gating strategy for flow cytometry analysis of PD-1 expression on T cells from a psoriatic patient.

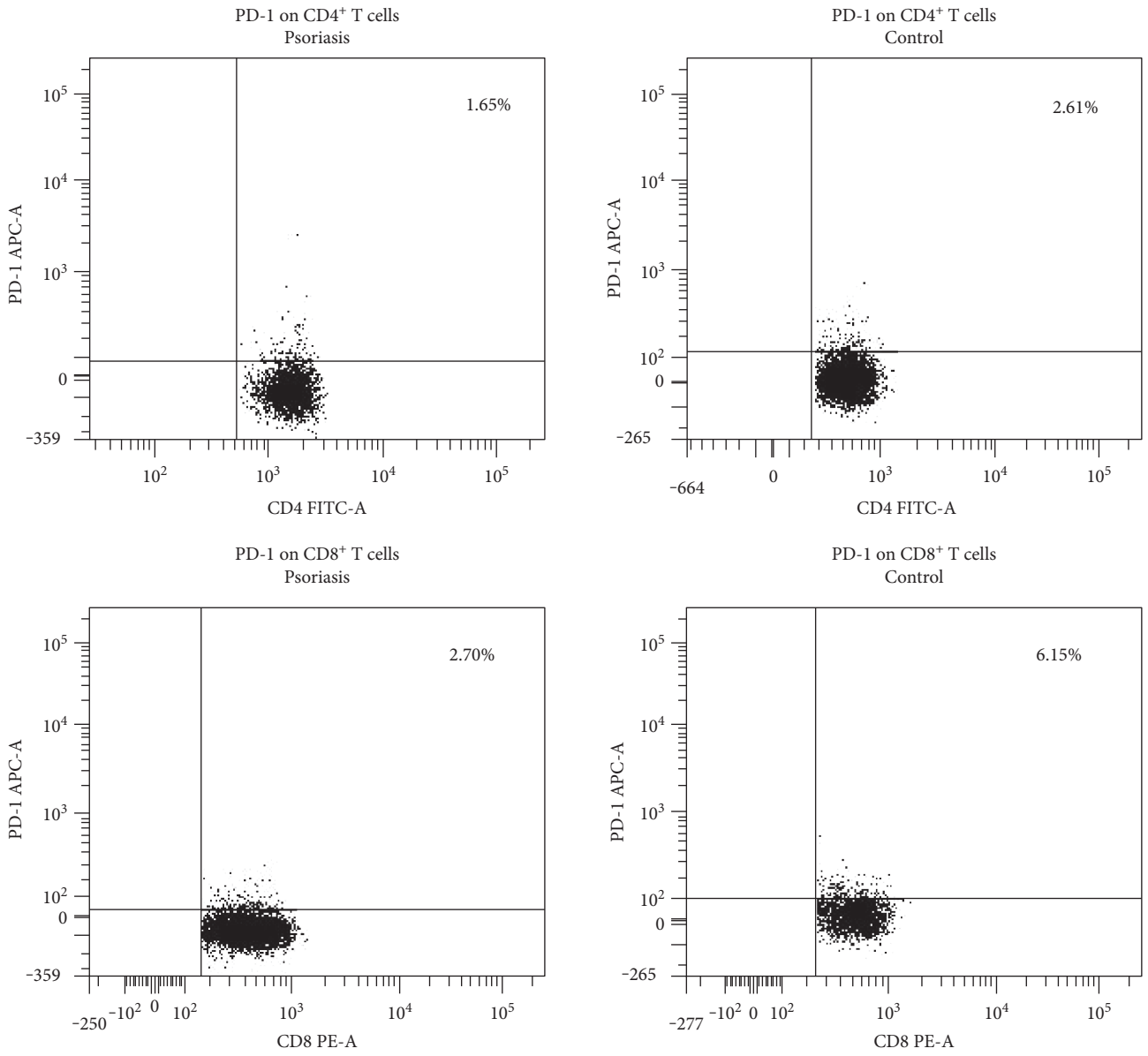

Figure 2: Representative flow cytometry analysis of PD-1 expression on $CD4^+$ and $CD8^+$ T cells from psoriatic patients and healthy controls.

as correlations of the number and percentages of T cells and PD-1 expression with clinical data in psoriatic patients.

On the basis of the central limit theorem, it is commonly assumed in practice that parameters' estimators of sample size over 60 (even 30) sample units are asymptotically normal distributed.

In statistical tests, we agreed to a probability up to 0.05 in making an error in rejection of true null hypothesis, which assumes no dependence between variables.

3. Results

3.1. Characteristics of the Study Group. The psoriatic patients and the control group did not significantly differ in age ($t = 0.233$, $p = 0.785$) and sex ($\chi^2 = 0.381$, $p = 0.537$). Age and sex of the psoriatic patients compared to the control group, as well as patients' clinical data for psoriasis duration and severity, were presented in Table 1.

3.2. Comparison of the PBMC Population Distribution and PD-1 Expression between the Psoriatic Patients and Healthy Controls. Table 2 presents the distribution of PMBC populations and PD-1 expression in the psoriatic patients compared to the control group and the logistic regression analysis results of the psoriasis' odds ratios compared to the healthy controls.

The absolute numbers and percentages of the $CD3^+$ and $CD8^+$ cells were significantly increased in the psoriatic patients in comparison with the control group. The estimated odds of psoriasis increased by 0.1% if the number of $CD3^+$ increased by 1 cell/μL and by 0.4% if the number of $CD8^+$ increased by 1 cell/μL, on average. The estimated odds of psoriasis increased by 5.1% if percentages of $CD3^+$ cells

Table 1: Clinical data of psoriatic patients.

Characteristics	Psoriasis patients ($N = 75$)	Healthy control ($N = 52$)
Age (years), $M \pm SD$	47.1 ± 14.6	48.7 ± 15.2
Sex (male), *n*, %	65, 84.7	43, 82.7
Duration of psoriasis (years), $M \pm SD$	20.0 ± 13.6	
Age of psoriasis onset (years), $M \pm SD$	27.1 ± 14.6	
Positive family history of psoriasis, *n*, %	28, 37.3	
PASI, $M \pm SD$	14.0 ± 9.4	
BSA (%), $M \pm SD$	24.0 ± 17.8	
IGA, *n*, %		
Mild	17, 22.7	
Moderate	38, 50.7	
Severe	16, 21.3	
Very severe	4, 5.3	
NAPSI 80, $M \pm SD$	18.5 ± 16.5	
DLQI, $M \pm SD$	14.0 ± 7.8	

PASI: Psoriasis Area and Severity Index; BSA: Body Surface Area; IGA: Investigator Global Assessment; DLQI: Dermatology Life Quality Index; NAPSI 80: Nail Psoriasis Severity Index 80; M: mean value; SD: standard deviation.

increased by 1% and by 3.9% if percentages of $CD8^+$ increased by 1%, on average.

The absolute numbers and percentages of the $CD4^+$ T cells did not significantly differ between the psoriatic patients and the healthy controls.

The absolute numbers and percentages of the $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells were significantly decreased in the psoriatic patients in comparison with the control group. The estimated odds of psoriasis decreased by 3.3% if the number of $CD8^+PD\text{-}1^+$ increased by 1 cell/μL, by 36.9% if percentages of $CD4^+PD\text{-}1^+$ cells increased by 1%, and by 29.5% if percentages of $CD8^+PD\text{-}1^+$ cells increased by 1%, on average.

3.3. Analysis of Mutual Correlations between PD-1 Expression on $CD4^+$ and $CD8^+$ T Cells in the Psoriatic Patients. We observed a positive correlation between the absolute numbers of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells ($r = 0.534$, $p < 0.001$), as well as between the percentages of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells ($r = 0.347$, $p = 0.002$).

3.4. Analysis of Correlations between the PBMC Population Distributions and PD-1 Expression and Clinical Data in the Psoriatic Patients. We correlated the PBMC population distributions and PD-1 expression with characteristic of psoriasis, that is, duration and the age of the disease onset and psoriasis severity expressed by PASI, BSA, IGA, NAPSI, and DLQI (Table 3).

The absolute cell number of $CD3^+$ correlated negatively with PASI and NAPSI 80.

The absolute number and percentages of $CD4^+$ T cells correlated negatively with PASI and IGA, and the absolute cell number of them also correlated negatively with BSA and NAPSI 80.

The absolute number of $CD8^+$ T cells did not correlate with any clinical data, but the percentages of them correlated positively with PASI, BSA, and IGA.

The absolute number of $CD4^+PD\text{-}1^+$ T cells correlated negatively only with PASI, and the absolute number of $CD8^+PD\text{-}1^+$ T cells correlated negatively only with the age of psoriasis onset.

The percentages of $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells did not correlate with any clinical data of the psoriatic patients.

4. Discussion

The issue of the expression of negative costimulatory molecule PD-1 on the peripheral $CD4^+$ and $CD8^+$ T cells was interesting enough for us to launch an innovative research into its role in the psoriasis pathogenesis. The results of our investigations showed significantly decreased protein PD-1 expression on both $CD4^+$ and $CD8^+$ T cells and a positive correlation between the $CD4^+PD\text{-}1^+$ and $CD8^+PD\text{-}1^+$ T cells. It is well known that the peripheral blood T cells, including $CD4^+$ and $CD8^+$ T cells, are involved in psoriasis following a persistent stimulation by immunogens [5]. It has been found that in the psoriatic skin lesion the $CD4^+$ T cells accumulate mainly in the dermis, whereas the $CD8^+$ T cells are found in the epidermis [6]. Sigmundsdóttir et al. [7] observed a significantly higher frequency of $CD8^+$ T cells in the blood and their positive correlation with PASI.

According to our study, the frequency of $CD3^+$ and $CD8^+$, but not $CD4^+$ T cells, was significantly higher in the psoriatic patients than in the healthy controls. Similar to the results published by Sigmundsdóttir et al. [7], we have found a positive correlation between the percentages of $CD8^+$ T cells and PASI, but also by IGA. The increased numbers of circulating inflammatory $CD8^+$ T cells may confirm their active role in the psoriasis pathogenesis. Nevertheless, in our study, the absolute number of $CD8^+$ T cells negatively correlated with the patient's age at the age of psoriasis onset. $CD4^+$ T cells are believed to be necessary for the initiation of psoriatic skin lesions [7]; however, the exacerbation of psoriasis is observed in acquired immunodeficiency syndrome (AIDS) upon $CD4^+$ T cell depletion [8]. In our study, the negative correlations between the percentages and absolute number of $CD4^+$ T cells and severity of psoriasis measured by PASI and IGA were observed.

Although many studies on psoriasis pathogenesis have been conducted so far, the role of the PD-1/PD-L1 pathway in the disease has not been explained yet.

Considering the fact that PD-1 plays a role in a normal immune response silencing, its reduced expression may contribute to the chronicity and frequent recurrence of psoriasis. Therefore, the reduced expression of PD-1 on T cells could be a marker of the disease activity and failure of the feedback mechanism which would be able to prevent the immune overstimulation and autoimmunity.

The results of our study might suggest deregulation of immune suppression mechanisms, which may lead to

Table 2: The distribution of PBMCs and PD-1 expression in the psoriatic patients compared to the control group.

PBMC and PD-1 expression	IU	Psoriasis	Control	Logistic regression analysis (psoriasis versus control)		
		$M \pm SD$	$M \pm SD$	OR (95% CI)	OR-1 (%)	p
$CD3^+$	Cells/μL	1146.4 ± 408.5	964.1 ± 339.9	1.001 (1.000, 1.002)	0.1	0.041
	%	59.2 ± 13.0	48.2 ± 17.0	1.051 (1.024, 1.080)	5.1	<0.001
$CD4^+$	Cells/μL	687.2 ± 351.4	525.3 ± 253.2	1.001 (1.000, 1.002)	0.1	0.092
	%	59.4 ± 18.2	54.0 ± 17.5	1.017 (0.997, 1.037)	1.7	0.103
$CD8^+$	Cells/μL	349.2 ± 215.5	235.6 ± 144.7	1.004 (1.002, 1.006)	0.4	0.001
	%	31.0 ± 16.8	23.3 ± 10.1	1.039 (1.012, 1.068)	3.9	0.005
$CD4^+PD\text{-}1^+$	Cells/μL	14.1 ± 11.3	14.7 ± 9.8	0.972 (0.940, 1.004)	−2.8	0.089
	%	2.0 ± 1.2	2.9 ± 1.7	0.631 (0.475, 0.837)	−36.9	0.001
$CD8^+PD\text{-}1^+$	Cells/μL	10.2 ± 8.3	17.8 ± 19.7	0.967 (0.939, 0.996)	−3.3	0.024
	%	3.3 ± 2.1	6.6 ± 4.4	0.705, (0.602, 0.824)	−29.5	<0.001

PBMCs: peripheral blood mononuclear cells; PD-1: programmed death 1; M: mean value; SD: standard deviation; OR: odds ratio; cells/μL: number of lymphocytes/μL.

Table 3: Correlations between clinical data and the distribution of PBMCs and PD-1 expression in the psoriatic patients.

PBMC and PD-1 expression	IU	r / p	Duration of psoriasis (years)	Age of psoriasis onset (years)	PASI	BSA	IGA	NAPSI 80	DLQI
$CD3^+$	Cells/μL	r	−0.113	−0.043	−0.279	−0.177	−0.181	−0.260	−0.011
		p	0.335	0.712	0.015	0.130	0.120	0.024	0.924
	%	r	−0.036	−0.030	0.093	0.050	0.057	−0.043	0.136
		p	0.758	0.797	0.429	0.670	0.626	0.717	0.243
$CD4^+$	Cells/μL	r	−0.120	0.085	−0.373	−0.238	−0.297	−0.231	−0.106
		p	0.307	0.471	0.001	0.040	0.010	0.046	0.367
	%	r	−0.047	0.162	−0.295	−0.205	−0.291	−0.078	−0.159
		p	0.687	0.164	0.010	0.078	0.011	0.508	0.174
$CD8^+$	Cells/μL	r	0.070	−0.190	0.140	0.106	0.164	−0.084	0.147
		p	0.548	0.102	0.229	0.366	0.161	0.476	0.207
	%	r	0.116	−0.168	0.338	0.253	0.309	0.069	0.154
		p	0.320	0.150	0.003	0.029	0.007	0.559	0.186
$CD4^+PD\text{-}1^+$	Cells/μL	r	−0.117	−0.030	−0.283	−0.129	−0.148	−0.180	−0.018
		p	0.317	0.797	0.014	0.269	0.204	0.122	0.882
	%	r	−0.034	−0.120	−0.114	−0.001	−0.008	0.017	0.026
		p	0.771	0.305	0.330	0.999	0.945	0.884	0.828
$CD8^+PD\text{-}1^+$	Cells/μL	r	−0.035	−0.257	0.023	−0.036	0.090	−0.103	−0.004
		p	0.767	0.026	0.848	0.759	0.445	0.379	0.973
	%	r	−0.029	−0.053	−0.022	−0.061	0.047	−0.042	−0.061
		p	0.804	0.655	0.852	0.601	0.692	0.721	0.601

PASI: Psoriasis Area and Severity Index; BSA: Body Surface Area; IGA: Investigator Global Assessment; DLQI: Dermatology Life Quality Index; NAPSI 80: Nail Psoriasis Severity Index 80; PBMCs: peripheral blood mononuclear cells; PD-1: programmed death 1; cells/μL: number of lymphocytes/μL.

abnormal persistent T cell activation in psoriasis. Ferenczi et al. [9] found that in psoriatic patients, most of the lesional T cells expressed the three primary activation markers (CD25, CD69, and HLA-DR), whereas psoriatic blood T cells were characterized by high CD25 expression. Lymphocyte activation through analysis of CD25 and CD69 expression in psoriatic patients was also determined by Porto Ferreira et al. [10]. Higher percentages of activated ($CD25^+$ and $CD69^+$) cells were detected in both $CD4^+$ and $CD8^+$ lymphocyte subpopulations in the blood of psoriatic patients. Although the results compared to controls were significant only for the percentage of $CD25^+$ cells in the $CD8^+$ T cell subpopulation, there was a trend to increased expression of CD25 and CD69 in both $CD4^+$ and $CD8^+$ T cells. The presence of activation cells has been observed in the early stages of the disease, and even before clinically apparent lesions,

activation molecules are probably engaged in lymphocyte migration and recruitment [11, 12]. These results indicate that T cells which express activation markers are involved in the initiation and progression of psoriasis lesions [10]. Therefore, deregulated PD-1 signaling pathway might result in sustaining chronic inflammation and promotion of autoinflammatory changes.

In their quantitative real-time RT-PCR, Western blotting, and immunohistochemistry studies, Kim et al. [13] found decreased expression of PD-L1 as well as PD-L2 in psoriatic epidermis compared to the healthy controls. Interestingly, the authors compared the results not only with the normal skin but also with the skin samples collected from the patients with allergic contact dermatitis, pityriasis rosea, and lichen planus in which no decreased expressions of PD-L1 and PD-L2 were present. The authors suggested that decreased expression of PD-L1 and PD-L2 could result from impairment of the Treg function in psoriasis and it could allow continuous T cell activation. Expression of PD-L1 and PD-L2 on the endothelial cells reduces the influx of T cells into the inflamed tissues. Thus, PD-1 reduced expression on $CD4^+$ and $CD8^+$ T cells may contribute to the increased flow to the tissues and to the synthesis of cytokines. It is probable that in psoriasis PD-L1 and PD-L2 on keratinocytes may interact with PD-1 on T cells and modulate the immune response. Kim et al. [13] suggested that PD-L1 and PD-L2 could possibly be used in psoriasis treatment, that is, as a topical drug which would be able to normalize their epidermal expression.

In the group of 20 psoriatic arthritis patients, Peled et al. [14] found that the percentages of $CD3^+PD\text{-}1^+$ T cells were higher in the patients than in the healthy controls. Similar to the results of our study, the authors did not find any correlation between the level of PD-1 expressing T cells and PASI. Although in our study the absolute number and percentages of $CD4^+$ T cells correlated negatively with PASI and IGA as well as the percentages of $CD8^+$ T cells correlated positively with PASI, no correlation was observed between $CD4^+PD\text{-}1^+$ or $CD8^+$ $PD\text{-}1^+$ T cells and the clinical characteristics of psoriasis. Therefore, it might be speculated that a decreased expression of PD-1, regardless of its level, is a triggering factor of Th1 and Th17 activation.

Recently Shin et al. [15] observed decreased levels of $PD\text{-}1^+$ blood follicular helper T cells (T_{FH}) in the group of 28 psoriatic patients. The absolute number of $CXCR5^+PD\text{-}1^+$ T_{FH} cells correlated positively with the disease duration. The authors also did not find any correlation with PASI. Wang et al. [16] in the group of psoriatic patients found increased frequency and activation of T_{FH} (confirmed by the higher expression of their two important surface markers ICOS (inducible T cell costimulatory) and PD-1). Niu et al. [17] observed also higher levels of circulating $CD3^+CD4^+CXCR5^+$ cells; $CD3^+CD4^+CXCR5^+ICOS^+$, $CD3^+CD4^+CXCR5^+PD\text{-}1^+$, and $CD3^+CD4^+CXCR5^+ICOS^+PD\text{-}1^+T_{FH}$ cells; and $CD19^+IgD^+CD27^-$naive B and $CD19^+CD86^+$-activated B, but lower levels of $CD19^+IgD^+CD27^+$ preswitch and $CD19^+IgD^-CD27^+$ postswitch memory B cells compared with healthy donors. Importantly, the observed frequencies of these T cell subpopulations were very low.

PD-1 expression is known as a possible diagnostic marker in malignancy, including cutaneous carcinogenesis. Upregulated PD-1 and PD-L1 were observed in several human cancer types, including melanoma and hematological neoplasms [2, 3]. Their higher expression in skin lymphomas may be of special interest. Interestingly, the assessment of PD-1 expression may appear to be useful in differential diagnosis between the Sezary syndrome and various erythrodermic inflammatory dermatoses, including psoriasis. Çetinözman et al. [18] observed that in psoriatic erythroderma patients, the median percentages of the $PD\text{-}1^+$ T cells was 20% in comparison to 90% in the SS patients.

Moreover, the agents with anti-PD-1 action may inhibit Treg or promote the shift of Treg into Th17. PD-1 inhibitors are used in various human cancers and malignant neoplasm treatment in order to block the interaction between PD-1 and PD-L1 to increase antitumor immunity [4]. In the light of Dulos et al.'s [4] study, it is probable that augmentation of Th1 and Th17 responses during anti-PD-1 treatment is responsible for resistance to PD-1 blockade. Since IL-17 might be involved in tumor growth as a proangiogenic factor, some authors suggest a possible synergistic effect of the anti-PD-L1/Th17 axis for cancer treatment.

Interestingly, it has been observed that patients treated with nivolumab, a human anti-PD-1 antibody, may develop skin rashes, dermatitis, and psoriasiform dermatitis. Moreover, PD-1 genetic deficiency in mice ($pdcd1^{-/-}$) results in spontaneous development of arthritis, dilated cardiomyopathy, or lupus-like autoimmune disease [19, 20]. Imai et al. [20] found that in PD-1-deficient (PD-1KO) mice after imiquimod application both IL-17A and IL-22 were enhanced and resulted in increased dermal inflammation, epidermal acanthosis, and neutrophilic abscess formation. Their study also showed that PD-1 blockade by specific antibody markedly exacerbated psoriasiform dermatitis in mice.

Moreover, some recent reports present the development of severe psoriasis in patients treated with immunotherapy using PD-1 inhibitors. Chia and John [21] presented a 74-year-old man with metastatic lung cancer in whom severe psoriasis flare developed after two cycles of a PD-1 inhibitor (pembrolizumab) treatment. Psoriasis was resolved after the cessation of immunotherapy, topical corticosteroid, and phototherapy application. Similarly, Matsumura et al. [22] observed an exacerbation of psoriasis in an 87-year-old patient after two cycles of nivolumab for metastatic melanoma.

Therefore, in our study, we have also performed logistic regression analysis which has shown that estimated odds of psoriasis decreased by 36.9% if $CD4^+PD\text{-}1^+$ T cells increased by 1% and by 29.5% if $CD8^+PD\text{-}1^+$ T cells increased by 1%, compared to the control group. The finding confirms the role of negative costimulation in preventing psoriasis development and shows that patients treated with PD-1 inhibitors will require dermatological consultations and treatment.

Since disturbed regulatory mechanisms of the immune response and immune tolerance are observed in psoriasis, the assessment of the PD-1/PD-L1 pathway in the pathogenesis

of psoriasis needs in-depth investigation. Pinpointing the role of PD-1 expression in psoriasis could provide unambiguous answers to whether PD-1 triggers the onset of psoriasis, favouring its development or both.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Middermal Elastolysis: Dermal Fibroblasts Cooperate with Inflammatory Cells to the Elastolytic Disorder

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Little is known about the cause and pathophysiology of middermal elastolysis (MDE). In this condition, variable inflammatory infiltrate may be present or not together with loss of elastic fibres in the middermis that spares both papillary and lower reticular dermis. MDE may be a consequence of abnormal extracellular matrix degradation related to an imbalance between elastolytic enzymes released from inflammatory and resident cells and their naturally occurring inhibitors. However, the cause of this imbalance is still an object of investigation. In order to shed light on the role of fibroblasts in MDE, we used fibroblast cultures from MDE and control subjects to evaluate matrix metalloproteinases (MMPs) and their major inhibitor TIMP-1, which in combination with neutrophil or macrophage proteases released in inflamed areas may influence the elastolytic burden. We demonstrate that fibroblasts derived from MDE produce *in vitro* low levels of TIMP-1, the major inhibitor of MMPs. Elevated levels of MMP-2, MMP-14, and TIMP-2 capable to activate in a cooperative manner pro-MMP-2 are present in MDE tissue samples. Additionally, significant reaction for MMP-1 is present in the same MDE areas. These data all together suggest that ECM changes in MDE are due to cooperation of different cell populations (i.e., inflammatory cells and fibroblasts).

1. Introduction

Middermal elastolysis (MDE) is a rare disease of dermal connective tissue, described for the first time by Shelley and Wood in 1977, as "wrinkles due to idiopathic loss of elastic tissue of the middermis"[1]. Since its first description, approximately 80 cases have been reported in literature; however, it is believed that its true incidence is underestimated. It mostly affects Caucasian young or middle-aged women (30–50 years), rarely men (usually interested in more advanced age); familiar forms are not described [2]. The disorder exclusively involves the skin, and no extracutaneous manifestations have been documented [3].

Clinically, it may appear with patches of fine wrinkling, perifollicular papular protrusions ("peau d'orange" appearance), and inflammatory skin changes, such as reticular erythema. There are conflicting views on the nature of the different clinical variants that for many authors may represent different stages of the same disease. Thus, the histopathological changes reported above may represent in ascending order the different chronological aspects of the same disease. Erythema can appear at first, but it disappears afterwards; however, in some patients, erythematous lesion develops and persists hereafter as reticulate erythema. The true course of the disease has not yet been exactly established because only isolated cases of this rare condition reach a dermatologist's

observation. At light microscopy, MDE can be appreciated as selective loss of elastic fibres in the middermis sparing both papillary dermis and lower reticular dermis. A variable inflammatory infiltrate may be present or not according to the age of the lesion. At the present time, the aetiology is unknown and the role of sun exposure is still an object of discussion [3–5].

A role for elastolytic activities has been put forward by some authors [3–5], who consider the disease as a possible consequence of an abnormal extracellular matrix degradation, caused by a defect of elastin maturation because of a decrease of lysyl oxidase-like 2 (LOX2) activity [6] and/or by an increase of serine- or matrix metalloprotease activities (MMPs). This may result in an imbalance between these enzymes and their naturally occurring inhibitors [7, 8].

In order to shed light on the role of fibroblasts in MDE, we used *in vitro* cell cultures of fibroblast from MDE and healthy control subjects. By using different methodological approaches, we evaluated fibroblast MMPs and their major inhibitor TIMP-1, which by their own or in combination with neutrophil or macrophage serine proteases may increase the elastolytic burden in the middermis. The results we obtained *in vitro* were together with those obtained in MDE tissues in which inflammatory cells are present or not.

2. Materials and Methods

2.1. Light and Electron Microscopy. Specimens for this investigation were derived from patients whose clinical history is summarized below. For light microscopy, cutaneous tissue was fixed in 10% buffered formalin and embedded in paraffin wax. Tissue slides were further processed and stained with haematoxylin and eosin (H&E) and Giemsa-Orcein.

For transmission electron microscopy (TEM), the tissue was fixed in 2.5% glutaraldehyde in 0.1% cacodylate buffer, washed in the same buffer, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon/Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Philips EM 400.

2.2. Isolation and Culture of Human Fibroblasts from Skin Biopsy. Primary fibroblast cultures from the skin specimens obtained from MDE patients and control subjects were isolated by explant technique from de-epidermized dermal biopsies.

Tissue specimens were washed in D-MEM with antibiotics, finely minced and allowed to adhere to plastic flasks. Dermal pieces were removed from the culture dish when adherent cells were visible on the plastic surface surrounding tissue fragments. The cultures were grown in D-MEM supplemented with antibiotics, L-glutamine, and 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every other day up. At passage 4, dermal fibroblasts were characterized for standard cell surface markers, namely, Vimentin and CD90 (Thy), and cell growth was assessed by cell count. Subconfluent cultures were washed three times with D-MEM, incubated for 24 hours in serum-free medium. The supernatants were collected in test tubes and kept at −80°C until zymographic analysis was performed. Adherent cells were treated with TRI Reagent (AMBION) in order to recover DNA after cell lysis.

2.3. ELISA Assay. The quantitative determination of TIMP-1 was carried out by using samples from three different culture media of fibroblasts derived from each subjects. Determination was carried out by using "Invitrogen Hu TIMP-1 ELISA kit," according to the manufacturer's instructions.

All the samples were diluted 1 : 20 with standard diluent buffer. Measurements were performed on triplicate samples (standards and tissue samples) by using a microtiter plate reader (Victor3 1420 MultiLabel Counter—PerkinElmer; equipped with software Wallac 1420 Station) at 450 nm.

2.4. Zymography. The gelatinolytic activities related to MMP-2 and MMP-9 were visualised by gelatin zymography in the supernatant from three different culture media of fibroblasts derived from each subjects. The molecular weight markers and samples were electrophoresed under nonreducing conditions by SDS-PAGE in 10% polyacrylamide gels copolymerized with 1% gelatin (Biorad). Before loading, the amount of protein in samples was normalized by DNA content determined spectrophotometrically at A_{260} after RNA extraction with TRI Reagent (Ambion). All samples were diluted 1 : 1with "Zymogram sample buffer" (Biorad).

After electrophoresis, gels were washed vigorously twice for 15 min in 2.5% Triton X-100 to remove SDS, then incubated in 50 mM Tris/HCl, pH 7.5, 5 mM CaCl2, at 37°C overnight. Gels were stained with 0.5% Coomassie blue G250 for 3 hours. After destaining, the MMP activity was detected as clear bands against the blue background.

The same protocol was carried out to reveal an activity likely related to MMP-3 on 12% polyacrylamide gels copolymerized with 1% casein (Biorad).

An additional zymographic analysis was carried out by loading the active human MMP-2 (Abcam, ab81550, active human MMP-2 full length protein) together with our samples and molecular weight standard. This was done in order to confirm that the most relevant active band we observed in gelatin gel is related to MMP-2.

2.5. Skin Samples. For this study, different samples of skin taken from two female patients with typical features of MDE were used. In particular, tissues in which MDE changes are associated or not with inflammatory reaction were processed and utilized for preparing primary cell cultures. Control samples were taken from healthy donors who gave informed consensus for biopsies.

Briefly, patient number 1 is a 56-year-old Caucasian woman who presented with one-year history of a persistent reticulate slightly itching erythema, on upper chest, shoulders, and proximal upper limbs. Skin examination also revealed, on the back, many round areas where the skin appeared "orange peel." No other cutaneous or systemic diseases were found in the medical history. In addition, a photo-test was performed to exclude any potential photo-induced dermatoses. Skin biopsies for light and electron

microscopy were used for diagnosis and to exclude other elastolytic disorders of the skin [9].

Patient number 2 is a 40-year-old Caucasian woman who came to our clinic with a three-year history of asymptomatic, well-demarcated, skin-colored finely wrinkled patches, ranging from 1 to 4 cm in diameter, on the trunk and upper limbs, and a reticulate erythema on the chest; furthermore, perifollicular papules were noticed on the back. Over the past six months, she had noticed ring-shaped erythematous patches, sometimes confluent to delineate strange polygonal figures, located distally on the thighs and forearms; some of those had become increasingly wrinkled. Medical history revealed a mild form of fibromyalgia, and laboratory tests were normal. Two biopsy specimens were taken from affected areas on the thigh (the most recent lesions) and thorax (the older ones). Tissue samples were processed for light and electron microscopy for diagnostic use and to exclude other elastolytic disorders of the skin.

2.6. Immunohistochemical Analysis. Tissue sections (7 μm) from middermal elastolysis or healthy control skins were used for immunohistochemical analysis of MMP-3, MMP-9, MMP-2, MMP-1, TIMP-2, and MT1-MMP (MMP-14).

In this context, we used primary rabbit polyclonal antibodies against human metalloproteinase 3 (MMP-3) (1 : 100; Novus Biological, NB100–91878), metalloproteinase 9 (MMP-9) (1 : 250; Novus Biological, NBP1–57940), metalloproteinase 2 (MMP-2) (1 : 500; Novus Biological, NB200–193), metalloproteinase 14 (MT1-MMP) (1 : 100; Millipore, AB6004), and rabbit polyclonal antibodies against mouse iNOS (1 : 100, Abcam Ltd., Cambridge, UK). Additionally, we used mouse monoclonal antibody to reveal human TIMP-2 (1 : 400; Millipore MAB 3310) and metalloproteinase 1 (MMP-1) (1 : 50; Arigo Biolaboratories, ARG21506).

All the sections were pretreated with 3% hydrogen peroxide for endogenous peroxidase blocking. Antigen retrieval was performed by heating sections in a microwave oven for 20 min in 0.01 M citrate buffer, pH 6.0, and allowing to cool slowly to room temperature.

All the sections were incubated with 3% bovine serum albumin for 30 min at room temperature to block nonspecific antibody binding and then incubated with the respective primary antibodies, overnight at 4°C.

Subsequently, tissue sections were rinsed with PBS, incubated with sheep anti-rabbit IgG (diluted 1 : 200) for 30 min at room temperature followed by incubation with peroxidase-antiperoxidase complex, prepared from rabbit serum. Color development was performed using DAB as chromogen. As negative controls for the immunostaining, primary antibodies were replaced by nonimmunised rabbit serum. The M.O.M. kit was used for immunodetection of mouse monoclonal antibodies against TIMP-2 and TIMP-1 (Transduction Laboratories, Lexington, KY, USA).

2.7. Statistical Analysis. Data are expressed as means ± SD. The significance of the differences was calculated using one-way analysis of variance. A P value of less than 0.05 was considered significant.

3. Results

3.1. Microscopy. At light microscopy, tissue specimens taken form patient number 1 reveal a focal decrease of elastic fibres in the upper dermis and a band-like loss of elastic fibres in the middermis. A perivascular inflammatory infiltrate is seen around vessels of the upper dermis (Figure 1(a)). In the middermis, some histiocytes are scattered among the collagen bundles (Figures 1(a) and 1(b)) where some patchy areas of elastinolysis are appreciated. At transmission electron microscopy (TEM), no evident changes are seen in the upper dermis (Figure 1(c)), and an almost complete loss of elastic fibres is present in the middermis region (Figure 1(d)). The changes mainly affect the amorphous component of elastic tissue rather than the microfibrillar one. Elastic tissue appears irregularly aggregated, and degenerated elastic fibres are present in phagosomes of macrophages within the extracellular matrix (ECM) (Figures 1(d) and 1(h)). The latter cells with an irregular surface and an active phagocytic activity show the characteristic morphological features of M2-polarized macrophages. These pathological findings are typical changes in MDE.

Haematoxylin and eosin stains of biopsy specimens taken from patient number 2 demonstrated flattened epidermis without alterations, homogeneous papillary dermis, and areas of reticular dermal oedema (Figure 1(e)). In this patient lymphocyte and monocyte, inflammatory infiltrate is particularly evident (arrowhead). In some areas, a significant neutrophil infiltrate is seen. A band-like loss of elastic fibres in the middermis can be appreciated after elastic tissue stain (Figure 1(f)). At TEM examination (Figures 1(g) and 1(h)), the loss of elastic fibres in the middermis is apparent and the *elastophagocytosis* of damaged elastic fibres is also appreciated within macrophages (Figure 1(i)). These features are morphological characteristics of MDE.

3.2. Zymographic Analysis. In order to identify stromelysins, zymographic analysis was carried out on casein gels. By using this methodology, no appreciable bands either compatible with MMP-3 or MMP-7 molecular weights were revealed in supernatant samples of cultures derived from MDE and healthy areas (data not shown). On the other hand, zymographic analysis on gelatin gels carried out to reveal gelatinases (i.e., MMP-2 and MMP-9) resulted in the appearance of a single 72 kDa band (Figure 2(a)). This band looks more intense in fibroblasts derived from MDE areas (lane b) than those taken form healthy subjects (lane a).

According to molecular weight (~72 kDa), the band found in both MDE patients (lane b) corresponds to the inactive form (zymogen) of human MMP-2, since the active form of human MMP-2 has a relative mobility corresponding to ~59 kDa (lane c) [9].

Representative zymogram of supernatants from fibroblast cell cultures of two MDE patients and control subjects is reported in Figure 2(a), lanes d and e, respectively.

3.3. ELISA for TIMP-1. TIMP-1 reduction (of approximately 30%) was observed in MDE fibroblasts (128 ± 18 ng/ml) as

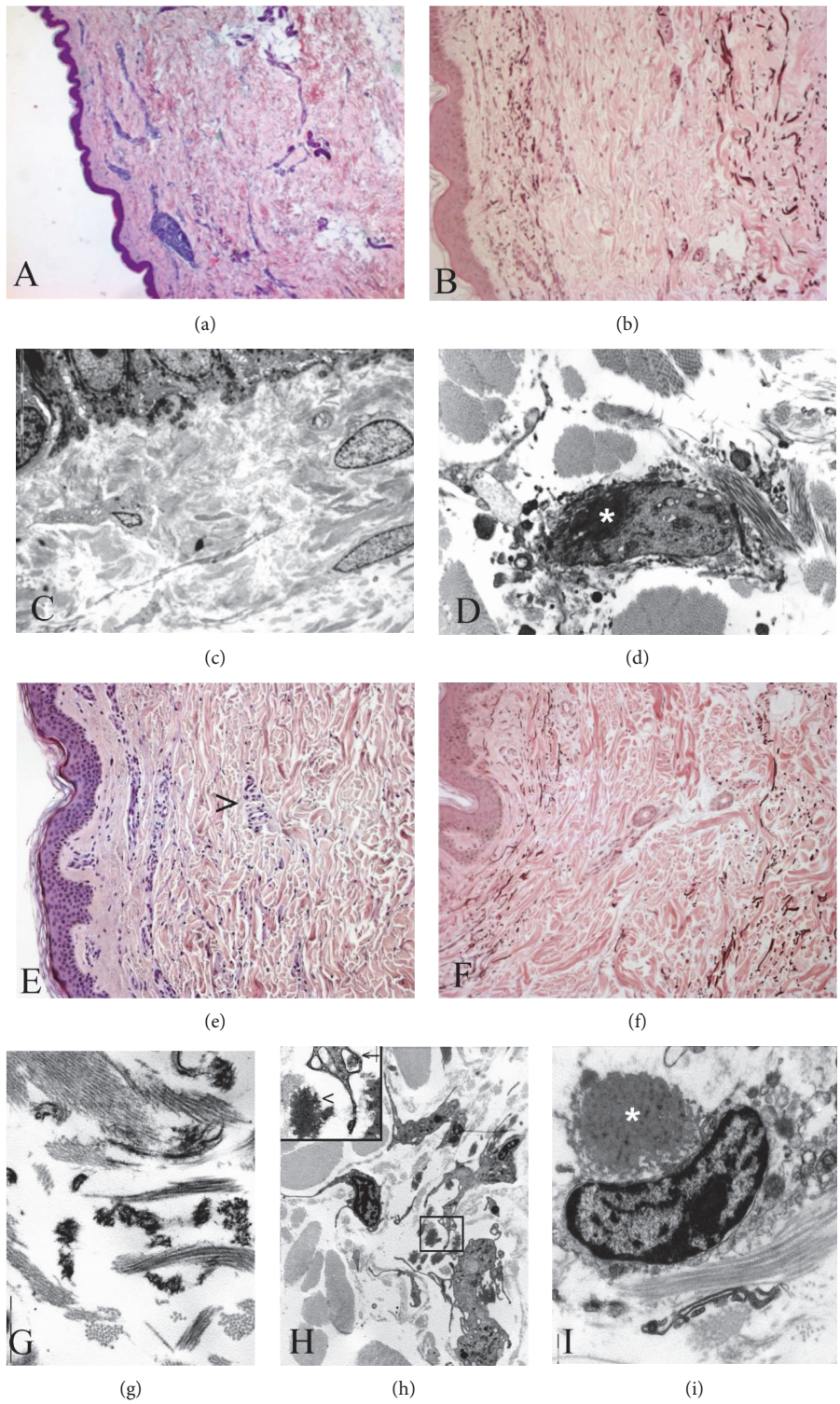


Figure 1: (a–d) Skin biopsy from patient number 1. Inflammatory infiltrates in the upper dermis (a and b) are associated with small areas of elastinolysis in the middermis (b). At TEM examination, there is an almost complete loss of elastic fibres in the middermis. No alterations are seen in the upper dermis (c). *Elastophagocytosis* (white star) by macrophages can be also appreciated (d). (a) H&E stain, ×40; (b) Giemsa-Orcein stain, ×100; (c) and (d) uranyl acetate & lead citrate, original magnification ×13.000. (e–i) Skin biopsy from patient number 2. Large inflammatory infiltrates are present in the middermis (e) where a band-like loss of elastic fibres can be appreciated (f). At TEM examination, a fragmentation of elastic fibres is evident in the middermis (g) ((h), inset arrowhead) where a lot of activated macrophages with an irregular surface are scattered within the extracellular matrix (h). Several macrophages are engaged in *elastophagocytosis* ((i), white star; (h), inset, arrow). (e) H&E stain, ×100; (b) Giemsa-Orcein stain, ×100; (g–i) uranyl acetate & lead citrate, (g) and (i) original magnification ×13.000, (h) original magnification ×7000.

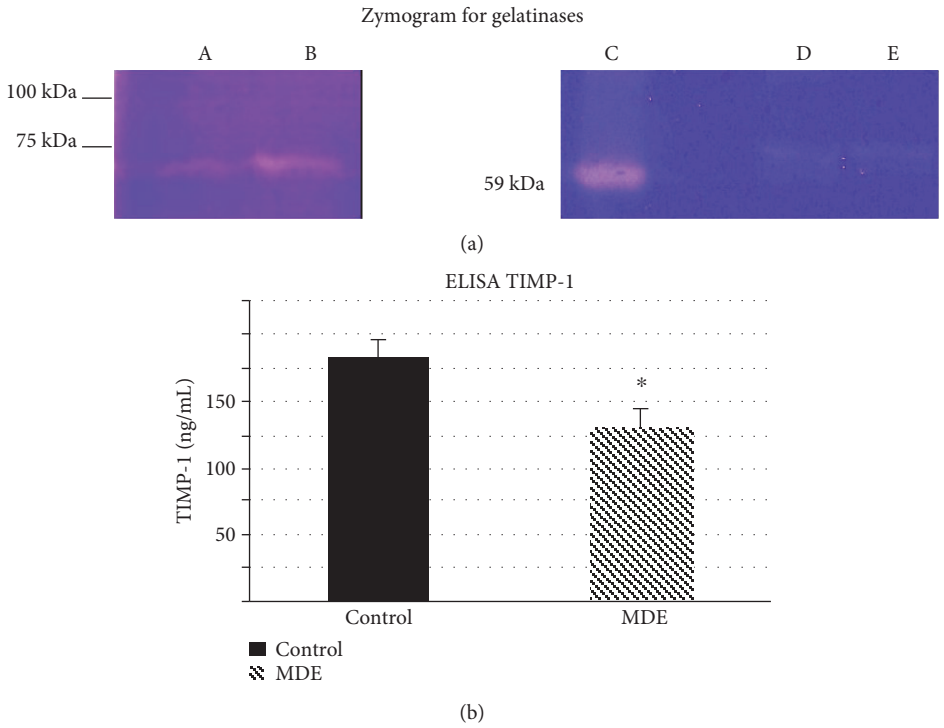


Figure 2: (a) Representative zymogram for gelatinases. (A) supernatant of fibroblast culture from a control subject; (B) supernatant of fibroblast culture from a MDE patient; (C) active form of human MMP-2 (59 kDa form), (D) and (E) are the same samples of (A) and (B), respectively. (b) Quantitative determination of TIMP-1 by ELISA. The values are mean ± SD of triplicate determinations on supernatants from three fibroblast cultures of MDE patients and three controls. $^*p < 0.05$ versus control samples.

compared with that detected in fibroblasts of control samples (183 ± 15 ng/ml) (Figure 2(b)).

3.4. Immunohistochemistry. In order to see whether destructive macrophages are present in MDE together with scavenger macrophages, we performed an immunohistochemical study to detect iNOS, a marker of M1 phenotype. A strong positive staining for iNOS has been observed in several middermis macrophages of patients with MDE (Figure 3(b)). No reaction was noticed in the middermis of control patients (Figure 3(a)). This suggests that destructive M1 macrophages (iNOS positive) together with M2 macrophages (with active phagocytosis) may be at the same time present in the skin of MDE patients.

Evident positive reactions for MMP-2 (Figure 3(d)) and for TIMP-2 (Figure 3(f)) have observed on the cell surface of middermal fibroblasts and inflammatory cells. Only weak reactions for MMP-2 (Figure 3(c)) and TIMP-2 (Figure 3(e)) have been detected on cell membranes in the middermis of control subjects. With regard to MMP-14, a diffuse reaction for this enzyme was localized in large areas of the middermis from MDE patients (Figure 3(h)). A mild immunohistochemical reaction for MMP-14 is detectable also on cell membranes in the middermis from control subjects (Figure 3(g)).

Immunostaining for MMP-3 does not show a positive reaction both in MDE and control subjects (data not shown). On the other hand, a weak staining for MMP-9 is observed on MDE epidermal keratinocytes and histiocytes. A trivial reaction for this enzyme is also present in tissue samples of healthy control subjects (data not shown). Additionally, a strong reaction for MMP-1 can be appreciated in the upper and middermis of MDE tissues by using a monoclonal antibody that recognizes full length MMP-1 (Figure 4).

4. Discussion

In this study, we demonstrate that fibroblasts, derived from MDE skins, produce *in vitro* low levels of TIMP-1, the major inhibitor of matrix metalloproteinases and elevated levels of pro-MMP-2. These findings have been confirmed by immunohistochemical analysis carried out on tissues derived from MDE and healthy subjects. Of interest, MMP-14 and TIMP-2 capable to activate on cell surface pro-MMP-2 in a cooperative manner [10] are present in the middermis. In addition, significant amount of MMP-1 is observed in MDE tissue.

The data we reported strongly suggest that middermal elastinolysis is due to cooperation of different cell populations (i.e., inflammatory cells and fibroblasts).

Actually, very little is known on the pathogenic events that lead to MDE [3]. In almost half of the cases reported, sun exposure was implicated and/or the lesions were photodistributed [2–4]. However, in our patients, there is no histological evidence of chronic sun damage or involvement of chronically sun-exposed sites. Also, the role of inflammation in this pathology is still an object of discussion [3, 9]. The first reports on this condition exclude that inflammation plays a relevant role because no inflammatory infiltrate was found

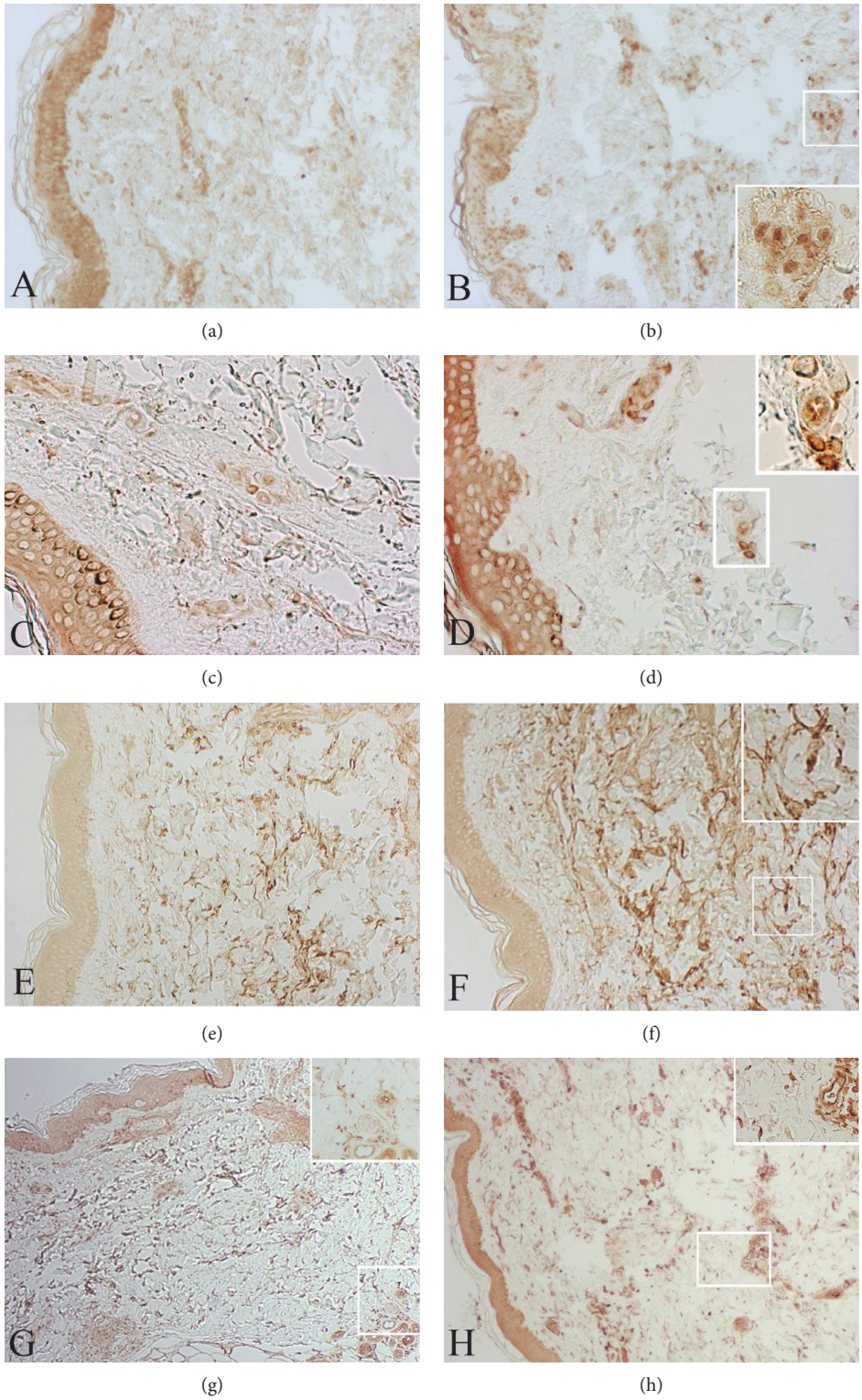
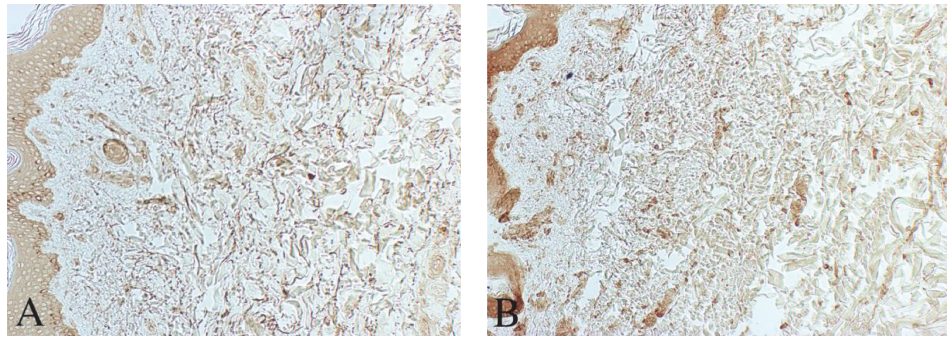


Figure 3: Skin biopsies from MDE patient (b, d, f, and h) and healthy donor (a, c, e, and g). M1 macrophages (iNOS positive) are present in MDE areas together with iNOS negative macrophages (b). No reaction is noticed in the middermis of control patients (a). A weak reaction for MMP-2 (c) and TIMP-2 (e) is detected on cell membranes in the middermis of a control subject. A positive reaction for MMP-2 (d) and TIMP-2 (f) is evident on the cell surface of middermal fibroblasts and inflammatory cells. A diffuse reaction for MMP-14 is localized in large areas of the middermis from MDE patient (h). A mild reaction for MMP-14 is present also on cell membranes in the middermis from a control subject (g). (a-b) Original magnification ×100; (c-d) original magnification ×200; (e-f) original magnification ×100; original magnification ×40.



(a) (b)

Figure 4: Representative skin samples from control subject (a) and MDE patient (b) after immunoreaction for MMP-1. As can be appreciated, a strong reaction for MMP-1 is found in the upper and middermis of the MDE tissue. Original magnification ×100.

associated with MDE changes [1]. On the contrary, recent observations suggest the possibility of an inflammatory cause in several cases [4, 11–13]. The presence of reticular erythema associated with the classical changes of MDE strongly supports this idea. Additionally, as reported in our paper, inflamed areas are characterised by the presence of activated macrophages with different phenotypes in the middermis. The data we report here suggest that MDE changes may be the result of destructive events due to a cooperation between fibroblasts and inflammatory cells. It is well known that inflammatory cells, namely, neutrophils and macrophages, can secrete destructive enzymes active against many components of ECM and can produce oxygen species capable to activate MMPs [14]. These enzyme activities falling into different classes (serine-, metallo-, and asparticproteases) can degrade ECM components by themselves, or in cooperation. In particular, neutrophil elastase or MMP-12 can promote elastolysis [14–16]. The activity of these enzymes is counteracted by naturally occurring inhibitors such as α1-proteinase inhibitor (α1-PI) and TIMP-1, respectively [17]. It has been reported that several ECM components are altered in MDE, including collagens [3, 4]. The involvement of different populations of cells in MDE lesions may be of pathogenic importance. In this regard, high levels of MMP-1 may play an additional role that contributes to the various changes that characterize MDE, by promoting proteolysis of ECM components and by recruiting neutrophils [14]. This issue deserves further investigation. As reported in this paper, we demonstrated in supernatants of MDE fibroblast cultures, higher pro-MMP-2, and lower TIMP-1 levels in comparison to those we detected in cultures of control fibroblasts. The low TIMP-1 activity together with high presence of MMP-2 that can promote proteolysis of α1-PI (the major inhibitor of serine proteases) may favour and trigger the degradation of elastic tissue by increasing the elastase burden in an inflamed tissue. In addition, active MMP-2 can participate to the development of middermal changes by activating growth factors and chemokines [18, 19]. The presence in MDE areas of molecules (MMP-14 and TIMP-2) capable to activate in a cooperative manner on cell surface pro-MMP-2 [10, 20] further support this hypothesis.

5. Conclusions

We demonstrate that fibroblasts, derived from MDE, produce low levels of TIMP-1, the major inhibitor of MMPs, and elevated levels of MMP-2. MMP-14 and TIMP-2 capable to activate in a cooperative manner pro-MMP-2 are also present in MDE areas. These data suggest that cooperation of different cell populations (i.e., inflammatory cells and fibroblasts) may result in an increased elastolytic burden that is caused of a focal loss of elastic tissue in the midreticular dermis of MDE patients.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Giovanna De Cunto and Arianna Lamberti contributed equally to this work.

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Review Article

TWEAK/Fn14 Activation Participates in Skin Inflammation

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Tumor necrosis factor- (TNF-) like weak inducer of apoptosis (TWEAK) participates in multiple biological activities via binding to its sole receptor—fibroblast growth factor-inducible 14 (Fn14). The TWEAK/Fn14 signaling pathway is activated in skin inflammation and modulates the inflammatory responses of keratinocytes by activating nuclear factor-κB signals and enhancing the production of several cytokines, including interleukins, monocyte chemotactic protein-1, RANTES (regulated on activation, normal T cell expressed and secreted), and interferon gamma-induced protein 10. Mild or transient TWEAK/Fn14 activation contributes to tissular repair and regeneration while excessive or persistent TWEAK/Fn14 signals may lead to severe inflammatory infiltration and tissue damage. TWEAK also regulates cell fate of keratinocytes, involving the function of Fn14-TNF receptor-associated factor-TNF receptor axis. By recruiting inflammatory cells, promoting cytokine production, and regulating cell fate, TWEAK/Fn14 activation plays a pivotal role in the pathogenesis of various skin disorders, such as psoriasis, atopic dermatitis, cutaneous vasculitis, human papillomavirus infection and related skin tumors, and cutaneous autoimmune diseases. Therefore, the TWEAK/Fn14 pathway may be a potential target for the development of novel therapeutics for skin inflammatory diseases.

1. Introduction

Tumor necrosis factor- (TNF-) like weak inducer of apoptosis (TWEAK) is a member of the TNF ligand superfamily and is initially described as an inducer of apoptosis in transformed cell lines [1]. TWEAK acts via binding to its sole receptor—fibroblast growth factor-inducible 14 (Fn14), the smallest member of the TNF receptor (TNFR) superfamily [2]. The specificity of TWEAK binding to Fn14 has been confirmed in multiple experiments [3]. TWEAK is broadly expressed by monocytes, dendritic cells, and natural killer (NK) cells, and macrophages/monocytes are the main source of soluble TWEAK (sTWEAK) in inflammatory tissues [4–8]. The immune organs, including the spleen, lymph nodes, and appendix, also express TWEAK [1, 4]. TWEAK has also been detected in various tumor cell lines [9–12]. Fn14 is widely expressed in various tissues including the skin, heart, brain, kidney, colon, small intestine, skeletal muscle, and pancreas [2, 13–17]. In normal tissues, the expression of TWEAK and Fn14 is relatively low. Elevated expression of TWEAK and Fn14 is usually seen in response to stress, tissue injury, or remodeling [18–20]. Optimal TWEAK-mediated activation of Fn14 promotes productive tissue responses after injury; however, excessive or persistent Fn14 upregulation and TWEAK/Fn14 activation often induce various pathological responses [21]. TWEAK/Fn14 signaling pathway participates in multiple biological activities, including the proliferation, differentiation, migration and death (apoptosis/necrosis) of cells [22–28], angiogenesis [2, 29], and inflammatory responses [2, 30].

Inflammation is one of the basic characteristics of skin disorders, especially the chronic inflammatory diseases that include psoriasis, atopic dermatitis (AD), cutaneous vasculitis, and cutaneous lupus erythematosus [31]. The occurrence of psoriasis varies according to age and geographic region, with the estimates of prevalence in adults ranged from 0.51% to 11.43% and in children from 0% to 1.37% [32]. AD is even more prevalent among people of any age. In developed countries, the incidence of AD varies in 10% to 20%, whereas it is lower but continues to increase in many developing countries [33]. Cutaneous vasculitis refers to a wide spectrum of diseases characterized by primary or

secondary blood vessel inflammation and necrosis in skin [34]. Cutaneous lupus erythematosus is a chronic autoimmune disease, with an estimated incidence of 4.2 per 100,000 people [35]. Skin infections and malignancies are also related to local abnormalities in immune and inflammatory responses [36, 37]. These disorders not only affect skin tissue but also develop extracutaneous or even systemic complications as primary inflammation exacerbates continuously. To suppress exacerbated inflammatory injuries is one of the strategies for treating skin disorders. Under skin inflammation, proinflammatory cytokines as well as chemokines are continuously released, recruiting an infiltration of immune cells. Recently, it was reported that TWEAK/Fn14 interaction increases the expression and/or secretion of various molecules involved in local inflammatory responses [38–40]. Moreover, TWEAK promotes the proinflammatory activities of other cytokines such as TNF-α, interleukin- (IL-) 1, IL-6, and interferon-γ [28, 41], which also participate in the pathogenesis of inflammatory skin diseases [28, 42, 43]. Therefore, these findings suggest a pivotal role of TWEAK/Fn14 pathway in the mechanism of cutaneous inflammation.

In this review, we update recent advances in the function of TWEAK/Fn14 signals in different skin inflammation and also highlight the potential roles of this pathway as therapeutic target in the management of various skin diseases.

2. The Structural Basis of TWEAK/Fn14 Interaction

TWEAK is initially synthesized as type II transmembrane proteins of 249 amino acids and can be cleaved by furin into sTWEAK with biologic activities [44]. The C-terminal extracellular domain of TWEAK contains the receptor-binding subdomain, which is predicted to fold into a β-pleated sheet structure that forms a trimeric aggregate. The grooves between the subunits of the trimers serve as binding sites for the receptor [45]. Fn14 is a type I transmembrane protein. It has a single cysteine-rich domain in extracellular region (53 amino acids) that is necessary for TWEAK binding and a short cytoplasmic tail (28 amino acids) that possesses a single TNFR-associated factor- (TRAF-) binding site [2, 3].

The extracellular cysteine-rich domain of Fn14 contains three disulphide bonds. By analyzing the structure of Fn14, it recently revealed a highly conserved core region (Ala34–Ala69) with very few flexible side chains [46]. This region contains the residues Asp45, Lys48, and Asp62, which are particularly important for high-affinity TWEAK binding [47]. Moreover, the putative protein-protein interface in close proximity locates the side chain of Arg58, which presents a high degree of flexibility [46]. This suggests that Arg58 may act as the potential switch that opens the binding groove. Moreover, ICM-Pro algorithm (a protein structure analysis approach) was used for identifying the plausible poses of TNF ligands bound to their receptors, showing that two putative TWEAK residues, Tyr176 and Trp231, anchor TWEAK to cysteine-rich domain of Fn14 [46]. The structures of TWEAK and Fn14 molecules are diagramed in Figure 1.

3. TWEAK Enhances the Production of Cytokines in Keratinocytes and Other Skin Cells

Keratinocytes are the major component of the human epidermis. They secrete a broad spectrum of cytokines including proinflammatory cytokines, chemokines, and immunomodulatory cytokines and establish the local cytokine and chemokine milieu, which mediate multiple local and systemic consequences, such as migration of inflammatory cells, activation of immune responses, and proliferation and differentiation of keratinocytes and fibroblasts [48–50]. Keratinocytes can produce multiple cytokines such as IL-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and transforming growth factor- (TGF-) α [49, 51, 52]. Keratinocytes also synthesize the C-X-C chemokines, including interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and RANTES (regulated on activation, normal T cell expressed and secreted) [53]. KCs have the ability to regulate leukocyte influx in the skin by producing chemokines, such as chemokine (C-C motif) ligand (CCL) 2, CCL20, and chemokine (C-X-C motif) ligand (CXCL) 10, and this process is influenced by keratinocyte/T cell communication [54]. In addition, CCL20 activates memory T cells via its chemokine receptor 6 [55]. Therefore, keratinocytes and related cytokines are central in immunologic and inflammatory reactions in skin.

Other types of skin cells are also involved in cutaneous inflammation through secreting cytokines or other components. Dermal fibroblasts are activated under inflammatory condition and produce TNF-α, IL-6, and matrix metalloproteinases [56]. Fibroblasts release cytokines and growth factors that have autocrine and paracrine effects. Autocrine activity includes the TGF-β-induced synthesis and secretion of connective tissue growth factor which promotes collagen synthesis [57]. Paracrine activity affects growth and differentiation of keratinocytes by the secretion of keratinocyte growth factor, granulocyte-macrophage colony-stimulating factor, IL-6, fibroblast growth factor-10, and stromal cell-derived factor-1 [58, 59]. Infiltration of macrophages is a feature of skin inflammation. Macrophages produce TNF-α, IL-1β, IL-4, IL-17, IL-23, and other cytokines that may trigger or exacerbate inflammatory responses in skin [60]. Vascular injuries are commonly seen in skin diseases such as cutaneous lupus erythematosus, Henoch-Schönlein purpura, and urticarial vasculitis. These diseases are characterized by inflammatory reactions directed at small vessels, in which the damage to dermal microvascular endothelial cells is usually the primary event. Dermal microvascular endothelial cells can produce TNF-α, MCP-1, IL-1α, IL-1β, IL-6, and IL-8, which are fundamental in inflammation and angiogenesis [61].

Increasing evidences suggest that TWEAK significantly enhances the synthesis of cytokines in resident cells in skin tissues. TWEAK/Fn14 activation promotes the expression and/or secretion of various cytokines that are involved in inflammatory responses, including IL-6, IL-8, GM-CSF, MCP-1, and RANTES [62]. Especially, TWEAK stimulates

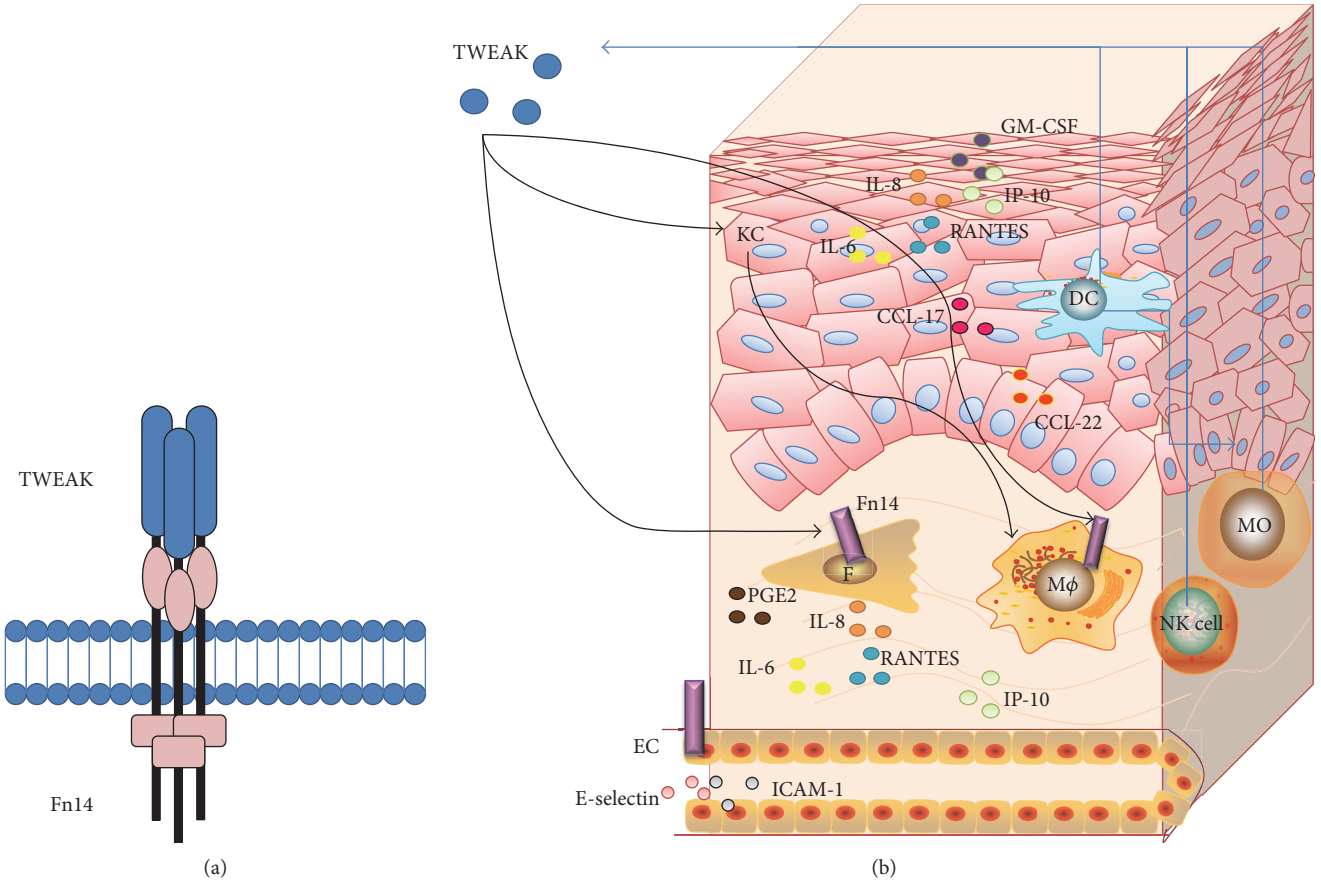

Figure 1: The diagram for TWEAK, Fn14, and relevant cytokines in skin structure. (a) The structures of TWEAK and Fn14 partners. (b) Fn14 is expressed on multiple cell types, including keratinocytes (KC), dermal fibroblasts (F), macrophages (Mϕ), and microvascular endothelial cells (EC). Intracellular TWEAK protein is expressed by monocytes (MO), dendritic cells (DC), and natural killer (NK) cells. TWEAK induces keratinocytes to express proinflammatory cytokines, such as IL-6, IL-8, RANTES, GM-CSF, IP-10, CCL17, and CCL22, which promote the migration of macrophages. TWEAK also induces the production of IL-6, IL-8, RANTES, IP-10, and PGE2 in dermal fibroblasts as well as E-selectin and ICAM-1 in microvascular endothelial cells.

keratinocytes to produce RANTES via Fn14 in a concentration-dependent manner and can be almost completely inhibited when blocking the TWEAK/Fn14 interaction with anti-Fn14 mAb [63]. TGF-β1 exhibits a synergistic effect on the TWEAK-induced RANTES production by keratinocytes [63]. TWEAK also enhances the expression of MCP-1 and IP-10 in keratinocytes [22, 64]. Moreover, TWEAK/Fn14 activation induces rapid phosphorylation of nuclear factor- (NF-) IκBα in keratinocytes [63]. TWEAK can also induce the production of CCL2, RANTES, CCL17, and CCL20 in keratinocytes, and such effect is enhanced by synergistic signals from IL-13 and IL-17, two crucial factors in the pathogenesis of AD and psoriasis [65]. Furthermore, TWEAK deficiency ameliorates chemokine expression in skin of AD, suggesting that TWEAK functions as an upstream signal molecule [65].

The proinflammatory effects of the TWEAK/Fn14 axis have been described in other cell types. TWEAK induces secretion of prostaglandin E2 (PGE2), IL-6, IL-8, RANTES, and IP-10 in dermal fibroblasts [66]. TGF-β signaling increases collagen production and Fn14 expression in cultured fibroblasts, and overexpressing Fn14 can enhance the expression of extracellular matrix genes in these cells upon TWEAK stimulation [67]. Moreover, tissular macrophages express Fn14, and TWEAK can promote the production of multiple cytokines in macrophages infiltrating injured tissues [6, 68, 69], indicating that TWEAK/Fn14 activation may also affect macrophages under skin inflammation. Upon TWEAK stimulation, keratinocytes express more CCL17 and CCL22, which contribute to the local recruitment of macrophages, and further induce inflammatory responses [70]. Furthermore, dermal microvascular endothelial cells express Fn14 [71]. TWEAK upregulates expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) and even enhances the adhesion of polymorphonuclear leukocytes to microvascular endothelial cells, leading to exacerbation of skin inflammation [71]. Obviously, the TWEAK/Fn14 pathway participates in inflammatory responses through promoting the expression of cytokines or other mediators in skin cells. The expression of TWEAK and Fn14 and their interaction in skin structure are diagramed in Figure 1.

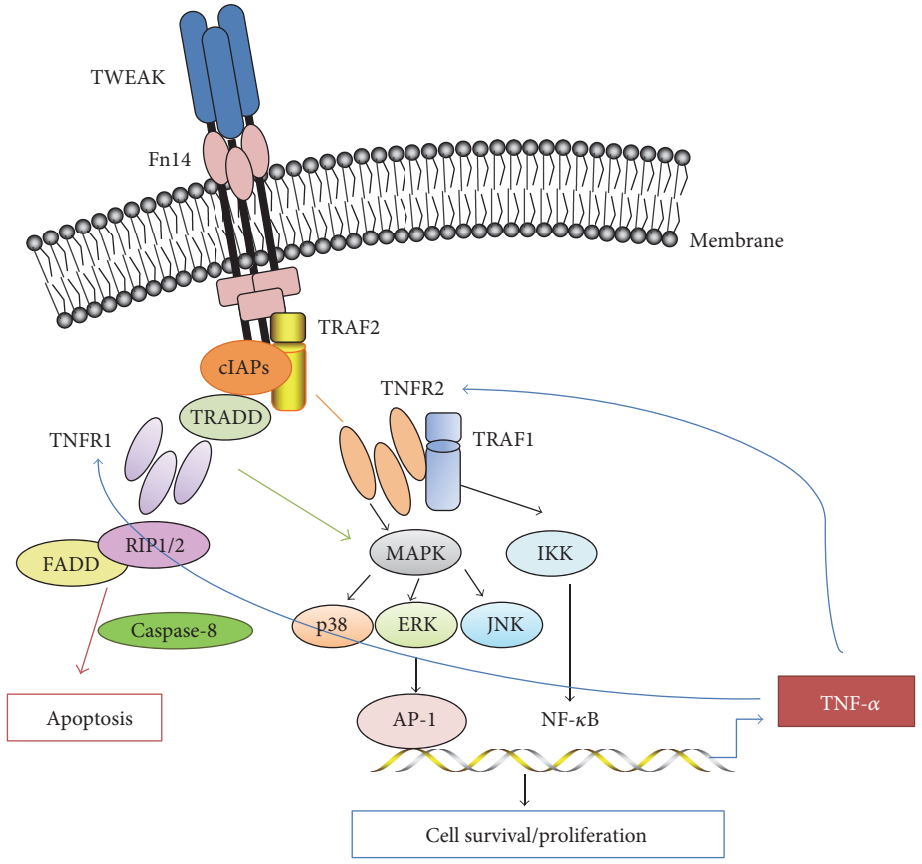

Figure 2: The diagram for the Fn14-TRAF-TNFR axis. sTWEAK binding to Fn14 recruits TRAF2 and cIAP1/2 to form cIAP-TRAF2 complex. The recruitment of TNFR1, TRADD, and FADD initiates apoptotic signaling by the recruitment and activation of caspase-8, while TNFR2 induces cell survival/antiapoptotic signals through NF-κB activation. NF-κB activation upregulates expression of multiple cellular genes that encode proinflammatory cytokines such as TNF-α. TWEAK may independently act or cooperate with TNF-α in regulating the TNFR-mediated cell fate.

4. TWEAK Regulates Cell Fate of Keratinocytes via the Fn14-TRAF2-TNFR Axis

TWEAK regulates cellular responses ranging from proliferation to cell death in a manner highly dependent on the cell type and the microenvironmental context. The biological activities of TWEAK can be mediated through two structurally distinct receptor subtypes: TNFR1 and TNFR2, with the major difference in their cytoplasmic tail. Most cells of the human body express TNFR1, while the expression of TNFR2 is much more restricted [72]. Additionally, TNFR1/TNFR2 protein ratio has been found to alter under the regulation of various cytokines [73]. Both TNFR1 and TNFR2 are expressed in the synoviocytes of patients with rheumatoid arthritis, and TNF-α stimulation downregulates TNFR1 but upregulates TNFR2 expression [74]. These findings indicate that the TNFR1 and TNFR2 expression varies under different inflammatory microenvironments, which may generate cell fate diversity.

The cytoplasmic domain of Fn14 contains a phylogenetically conserved binding motif, and TRAF1, TRAF2, TRAF3, and TRAF5 are able to bind to this site [3]. TRAF1 and TRAF2 are adaptor proteins that belong to the TRAF protein family. Both TRAF1 and TRAF2 are identified to be associated with the cytoplasmic domain of TNFR2 in a heterodimeric complex in which TRAF2 contacts the receptor directly, while TRAF1 interacts with TNFR2 indirectly through heterodimer formation with TRAF2 [75]. TNFR1 has a conserved motif in the cytoplasmic tail called the death domain. Upon activation, such death domain serves as a docking site for TRADD (TNFR1-associated death domain) and then recruits FADD (Fas-associated death domain protein) and caspase-8, forming the complex that initiates the cascade of apoptosis [76]. TRAF2 is an antiapoptotic protein and recruits the inhibitor of NF-κB kinase complex and cellular inhibitor of apoptosis proteins (cIAP) to the TNFR1 signaling complex, thus the necessity for the activation of the classical NF-κB pathway. A complex of TRAF2 with cIAP1, cIAP2, and TRAF1 has further been implicated in the inhibition of TNFR1-induced activation of caspase-8 [77]. Hence, TWEAK interacts with Fn14 in TNFR1 predominant cells that may trigger signals of apoptosis or necrosis. On the contrary, TNFR2 lacks cytoplasmic death domain. By assembling with TRAF1, TRAF2, and cIAPs, the TNFR2 signaling complex can antagonize TNFR1-induced caspase signaling and enhances cell proliferation by triggering the NF-κB pathway [78, 79]. The principle of Fn14-TRAF-TNFR axis is also diagramed in Figure 2.

Recently, we found a switch of TNFR expression profile in keratinocytes under different skin inflammations. In normal keratinocytes, TNFR1 expression is prominent, leading to cell death upon TWEAK stimulation [22, 64]. Interestingly, keratinocytes prefer to express more TNFR2 but less TNFR1 under psoriatic inflammation or HPV E6/E7 transfection, which significantly promotes cell proliferation [22, 64]. These findings are in accordance with the function of Fn14-TRAF-TNFR axis.

5. TWEAK/Fn14 Signaling Contributes to Psoriatic Inflammation

Psoriasis is one of the most common inflammatory skin diseases. Accumulation of RANTES, IL-8, IP-10, and MCP-1 is prominent in lesional skin of patients with psoriasis [22]. Moreover, both TWEAK and Fn14 are highly expressed in these lesions [22]. TWEAK can upregulate multiple proinflammatory and chemoattractive cytokines such as CCL20 and IL-19 under psoriatic inflammation [65]. The levels of phosphorylated IκB and nuclear NF-κB are also elevated, indicating activation of the NF-κB signaling pathway [22, 80]. NF-κB activation in psoriatic inflammation results in the production of multiple proinflammatory cytokines, which further mediate the trafficking and homing of T cells, monocytes, eosinophils, natural killer cells, and mast cells [81, 82]. These findings demonstrated that TWEAK/Fn14 signals are activated in psoriatic skin lesions, and downstream proinflammatory cytokines are produced consequently.

In psoriasis, the balance between the antiapoptotic and cell cycle inhibitory roles of NF-κB pathway is abnormally skewed towards the former, resulting in increased keratinocyte survival and epidermal hyperproliferation [80]. The NF-κB-activated proliferation marker Ki-67 and antiapoptotic proteins (including c-Myc, survivin, cIAP-2, and cellular FADD-like IL-1β-converting enzyme inhibitory protein) are expressed at higher levels in keratinocytes upon TWEAK stimulation, further suggesting that TWEAK signals participate in psoriatic inflammation [22, 83]. In fact, TWEAK/Fn14 interaction promotes the proliferation to apoptosis ratio of keratinocytes under psoriatic inflammation [22].

6. The Function of TWEAK/Fn14 Signals in AD

AD is a chronic inflammatory disease characterized by a relapsing form of skin inflammation, disturbance of epidermal barrier function, eczema, and spongiosis formation. Histologically, AD is featured with the infiltration of T cells, macrophages, and dendritic cells. These infiltrating cells are known to secrete a range of cytokines, including IL-8, TNF-α, RANTES, IP-10, and interferon-γ, which are upregulated in the lesional skin of patients with AD [84].

Unlike other inflammatory diseases such as psoriasis or autoimmune diseases [85, 86], the circulating level of TWEAK is not elevated in patients with AD nor do they correlate with AD severity [87]. TWEAK expression has been detected not only in lesional AD skin but also in healthy skin [87]. However, Zimmermann et al. observed that TWEAK and Fn14 are highly expressed only in lesional skin [28]. Moreover, the expression of TWEAK and Fn14 increase in the skin in experimental AD, and TWEAK deficiency limits severity of AD [65]. Conflicting results might arise from different experimental procedures. This controversy needs to be clarified in further studies.

Furthermore, a significant increase of TNF-α mRNA in keratinocytes was observed under TWEAK stimulation, and TNF-α is highly expressed in lesional skin of AD but not in healthy skin [28]. Actually, excessive apoptosis in epithelium is a key feature of AD. TWEAK can cooperate with TNF-α in the induction of keratinocyte apoptosis, contributing to the formation of AD lesions [28]. Previous studies suggested that Fn14 does not contain a "death domain," which directly triggers apoptosis [88]. TWEAK binds its receptor Fn14 on the cell membrane and results in an increase in the secretion of TNF-α, which binds to TNFR1 and triggers the extrinsic pathway of apoptosis [89, 90]. However, TWEAK also induces apoptosis or necrosis of keratinocytes without TNF-α [28, 89]. The highly expressed TWEAK and TNF-α in the lesional skin may together induce apoptosis of keratinocytes under AD inflammation. Further insight about the function of the TWEAK/Fn14 pathway in AD is expected.

7. TWEAK/Fn14 Pathway Mediates the Formation of Cutaneous Vasculitis

Cutaneous vasculitis includes a wide range of diseases that affect the blood vessels of skin and share a common pathological feature of endothelial damage and perivascular leukocyte infiltrates. Recent study showed that TWEAK and Fn14 are significantly expressed in the dermal vessel of lesional skin in patients with urticarial vasculitis but not in healthy controls [91]. Moreover, the serum TWEAK levels are correlated with the severity and the systemic involvement of urticarial vasculitis [91]. The similar pattern has been found in patients with cutaneous leukocytoclastic angiitis, Henoch-Schönlein purpura, and allergic vasculitis [91, 92].

Previously, it was found that TWEAK can induce the proliferation and migration of endothelial cells [93]. TWEAK treatment of human umbilical vein endothelial cells induced a rapid and intermittent increase in the expression levels of phosphorylated IκBα, phosphorylated ERK1/2, and phosphorylated JNK1/2 and promoted cell proliferation in a dose-dependent manner; anti-human Fn14 mAb can abrogate such effect of TWEAK on human umbilical vein endothelial cells [94]. TWEAK exhibits similar effect on human dermal microvascular endothelial cell line (HMEC-1) [92]. In a human in vitro model of the blood-brain barrier, besides regulating the proliferation of endothelial cells, TWEAK/Fn14 interaction induces production of proinflammatory cytokines (CCL2 and IL-8) and is associated with an increased permeability of the monolayer formed by these cells [95]. Moreover, TWEAK/Fn14 pathway mediates the formation of cutaneous vasculitis by upregulating vascular E-selectin and intercellular adhesion molecule-1 expression in the endothelium of blood vessels [71, 95]. It has been known that upregulated adhesion molecules are instrumental factors in triggering vascular inflammation and also the key contributors in the development of cutaneous vasculitis

[71]. TWEAK also enhances the adhesion of polymorphonuclear leukocytes to microvascular endothelial cells [71]. Therefore, TWEAK acts as a regulator of NF-κB activation and chemokine production in human endothelial cells, thus promoting leukocyte migration and vascular injury in cutaneous vasculitis.

8. TWEAK/Fn14 Interaction Plays a Role in HPV Infection and Carcinogenesis

By interacting with Fn14, TWEAK is an inducer of apoptosis of keratinocytes. However, the role of the TWEAK/Fn14 pathway in regulating the cell fate of HPV-infected keratinocytes is characterized by increased proliferation instead of apoptosis [64]. Fn14 expression increases in both HPV16-positive warts and HPV16 E6/E7-harboring keratinocytes. The TWEAK levels are also elevated in HPV16-positive warts when compared with normal skin. Moreover, the downstream proteins of TWEAK/Fn14 pathway, RANTES, and NF-κB are highly expressed in these areas. These findings indicate TWEAK/Fn14 activation in HPV16-infected keratinocytes [64]. Meanwhile, the level of cytoplasmic p18 (active subunit of caspase-8) is significantly reduced in E6/E7-transfected keratinocytes, suggesting the caspase-8 inhibition in TWEAK-induced cell proliferation [64]. E6 has the capacity to interact with caspase-8. In the case of HPV16, the full-length E6 protein is capable of directing caspase-8 degradation, thus preventing the apoptosis of infected cells [96]. E6 can also recruit active caspase-8 from the cytoplasm to the nuclei [97]. Based on these facts, we consider that TWEAK/Fn14 activation facilitates the proliferation of E6/E7-positive cells by inhibiting the caspase-8 pathway.

Moreover, HPV-infected keratinocytes in anogenital lesions are generally considered to be etiologically associated with carcinogenesis. The HPV16-induced keratinocyte immortalization has been suggested to correlate closely with epidermis-originated malignancies, such as cervical cancer and cutaneous or oropharyngeal squamous cell carcinoma [98, 99]. The tumor microenvironment contains certain factors that upregulate TWEAK expression, and Fn14 is overexpressed in the keratinocyte-originated cancers [100, 101]. However, in lesional skin of squamous cell carcinoma, expression of TWEAK varies significantly depending on the tumor differentiation levels. Strong staining was observed in the well-differentiated keratinocytes, while poor-differentiated tumor showed weak staining of TWEAK. It could be partially explained by the fact that the relative levels of TWEAK might induce different or even opposed cellular responses. Thus, TWEAK may also have a protective role in tumors [102]. These findings indicate that TWEAK/Fn14 interaction plays an important role in modulating cell fate in HPV infection and associated cancers.

9. TWEAK/Fn14 Activation Participates in Cutaneous Autoimmune Diseases

TWEAK/Fn14 activation in autoimmune diseases is strongly supported by a growing number of experimental evidences [14, 103–105]. The elevated expression of TWEAK and Fn14 in epidermis is seen in patients with cutaneous lupus erythematosus and bullous pemphigoid [8, 106]. Both TWEAK and Fn14 expressions also increase in muscles of patients with polymyositis or dermatomyositis [107]. These disorders share a similarity in inflammatory infiltration induced by chemokines that may be related to TWEAK.

Cutaneous lupus erythematosus is characterized by local activation of immune complexes or complement, autoreactive B cells and T cells and overexpression of cytokines and chemokines [108]. The TWEAK/Fn14 pathway participates in renal injuries and neuropsychiatric disease in MRL/lpr lupus-like mice [16, 38–40]. Both TWEAK and Fn14 are highly expressed in injured tissues, and their interaction induces the production of RANTES, MCP-1, and IP-10 in renal resident cells, astrocytes, endothelial cells, and other nonhematopoietic cell types [16, 38–40]. Similarly, TWEAK/Fn14 activation also exhibits effect on skin in MRL/lpr mice [14, 109]. Ultraviolet B irradiation enhances the Fn14 expression on keratinocytes in vitro and in vivo [14]. Moreover, Fn14 deficiency significantly attenuates cutaneous disease in MRL/lpr mice, as supported by the well-maintained architecture of the skin, remarkably decreased infiltration of T cells and macrophages, and less apoptotic cell in skin [14]. Furthermore, Fn14 deficiency correlates with attenuated cutaneous disease as well as reduced macrophage-derived proinflammatory chemokines (macrophage inflammatory protein-1α, CXCL1, and CXCL5) in skin of MRL/lpr mice irradiated with ultraviolet B [109]. These findings demonstrated that TWEAK/Fn14 signaling is important in the pathogenesis of cutaneous lupus erythematosus.

Systemic sclerosis affects skin tissue by chronic inflammation, vascular injuries, and excessive fibrosis. Recent study suggested that the interaction between sCD163 (marker of monocytes/macrophages) and TWEAK is associated with systemic sclerosis [110]. CD163 is a scavenger receptor that regulates inflammatory responses and may contribute to connective tissue remodeling. It has recently been demonstrated that CD163 can bind to and neutralize TWEAK [111]. The sCD163/sTWEAK ratio is significantly increased in patients with scleroderma, and higher sCD163/sTWEAK ratio correlates with greater skin involvement [110].

The TWEAK-Fn14 axis may be also involved in the pathogenesis of polymyositis or dermatomyositis [107]. Serum levels of TWEAK are lower in patients with polymyositis or dermatomyositis when compared with healthy controls and correlate negatively with serum CD163 levels in these patients. However, Fn14 expression increases in biopsied tissues of patients with polymyositis or dermatomyositis and correlates positively with muscle disease activity. Moreover, TWEAK protein expression is more detectable in biopsied muscle tissues of patients although its mRNA expression level does not differ from healthy controls.

Recently, we found that TWEAK/Fn14 signaling plays a pivotal role in the pathogenesis of bullous pemphigoid [8]. The serum levels of TWEAK are elevated in patients with bullous pemphigoid, and there is a positive correlation between serum TWEAK and anti-BP180 IgG. Both TWEAK

Table 1: The action of TWEAK in different skin diseases.

Diseases	Effect on target cells or animal models	References
Psoriasis	KC: to enhance chemokine expression and cell proliferation Murine model: to induce immune cell infiltrates in lesional skin	[22, 65]
AD	KC: to increase TNF-α expression and induce apoptosis Dermal fibroblast: to regulate chemokine expression Murine model: to induce cellular infiltrates, migration of immune cells, and chemokine expression	[28, 65]
Cutaneous vasculitis	HMEC: to regulate NF-κB activation and chemokine production Murine model: to induce endothelial damage and perivascular leukocyte infiltrates	[71, 92]
HPV infection	KC: to enhance TNFR2 expression and cell proliferation	[64]
Carcinogenesis	Various tumor cells: to induce cell proliferation or apoptosis in a cytokine-dependent way Glioma cells: to promote cell migration and invasion KC: to induce cell proliferation Vascular ECs: to upregulate FGF-2 and VEGF-A expression and to promote angiogenesis	[64, 112, 113]
Cutaneous lupus erythematosus	KC: to enhance Ro52 and proinflammatory cytokine expression and induce apoptosis Macrophage: to enhance chemoattraction and cytokine expression (including TWEAK) MRL/lpr mice: to induce chemokine production, cell infiltration, and apoptosis	[70, 109]
Systemic sclerosis	Monocytes/macrophages: to lead to greater extent of skin fibrosis or to exert as a protective role against fibrosis	[111, 114]
Polymyositis & dermatomyositis	Myoblast: to induce degradation of myosin heavy chain, to affect cell proliferation and differentiation, and to induce metabolic abnormalities Murine model: to induce muscle atrophy and interstitial fibrosis	[107, 115, 116]
Bullous pemphigoid	KC: to reduce BP180 expression and suppresses cell adhesion	[8]

and Fn14 expressions are strongly expressed in skin lesions of bullous pemphigoid. Also, TWEAK reduces BP180 expression in keratinocytes and suppresses cell adhesion, involving activation of NF-κB and extracellular signal-regulated kinase pathways. Interestingly, TWEAK upregulates sheddases such as a disintegrin and metalloproteinase 17, leading to BP180 loss in keratinocytes. Therefore, TWEAK/Fn14 activation may contribute to the pathogenesis of bullous pemphigoid. The actions of TWEAK on target cell or animal models in different skin diseases are listed in Table 1.

10. Targeting TWEAK/Fn14 Pathway as Therapeutic Approaches

The TWEAK/Fn14 pathway has become a potential therapeutic target. Growing evidences suggest that TWEAK inhibition can ameliorate inflammatory reaction and tissue damage in several animal models of autoimmune and inflammatory diseases, such as chronic autoimmune arthritis, systemic lupus erythematosus, and experimental autoimmune encephalomyelitis [104, 105, 117]. The most important TWEAK and Fn14 targeting drug formats and their molecular mode of action include anti-TWEAK antibodies, anti-Fn14 antibodies, Fn14-Fc (a fusion protein of the ectodomain of Fn14 with the Fc domain of IgG), soluble TWEAK, and Fc-TWEAK [13]. It was found that anti-TWEAK mAb can block immune complex-induced vascular damage as well as leukocyte infiltration in murine model of cutaneous reverse passive Arthus reaction and reduces expression of proinflammatory cytokines, including TNF-α and IL-6, in skin lesions [118]. Anti-TWEAK mAb (BIIB023) has been applied to patients with rheumatoid arthritis in a clinical trial, and it showed a favorable safety and tolerability profile. Moreover, the downregulation of several inflammatory biomarkers (MCP-1, IP-10, MIP-1β, and tissue inhibitor of metalloproteinase-1) was observed in these subjects [119].

TWEAK/Fn14 signals also show therapeutic potential in the management of tumors. Firstly, agents that inhibit TWEAK binding to Fn14 may have potential therapeutic utility [120]. Anti-TWEAK antibody (RG7212) blocks TWEAK-stimulated proliferation, NF-κB activation, and cytokine secretion and exhibits antitumor effect [121, 122]. Fn14-TRAIL (consists of the Fn14 extracellular domain fused to the soluble form of TNF-related apoptosis inducing ligand) also shows therapeutic potential due to its ability of inhibiting TWEAK/Fn14 signaling and promoting TRAIL signaling [123]. Furthermore, anti-Fn14 antibodies (PDL192 and BIIB036) exhibit an alternative NF-κB pathway-specific agonistic activity, but do not photocopy other activities of TWEAK [124].

11. Conclusions and Outlook

TWEAK is a multifunctional cytokine expressed on various cell types and tissues and acts via binding to its sole receptor Fn14. TWEAK/Fn14 activation contributes to various pathological processes, including cell proliferation and death, angiogenesis, carcinogenesis, and inflammation. TWEAK/Fn14 signals are involved in the pathogenesis of multiple skin diseases including inflammatory skin diseases, autoimmune

skin diseases, cutaneous vasculitis, HPV infection, and tumors. The involvement of this pathway has made it a promising therapeutic target of braking the never-ending cycle of local inflammation and tissue destruction. In a variety of the disease models, soluble TWEAK- and Fn14-specific antibodies and other drug formats have exhibited promising therapeutic effects.

However, the precise mechanism underlying the roles of TWEAK/Fn14 activation in inflammatory and autoimmune diseases, especially in cutaneous diseases, is not fully elucidated. In addition, the therapeutic effects on the inhibition or activation of TWEAK/Fn14 pathway have not been well explained. And based on the preclinical findings, we are supposed to explore the clinical value of TWEAK- or Fn14-related agents.

Conflicts of Interest

The authors declare no conflict of interests.

Acknowledgments

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Review Article

Mediators of Inflammation and Angiogenesis in Chronic Spontaneous Urticaria: Are They Potential Biomarkers of the Disease?

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In chronic spontaneous urticaria (CSU), different pathophysiological mechanisms, potentially responsible for the development of the disease, have been recently described. It is likely that the activation of skin mast cells with consequent release of histamine and other proinflammatory mediators is responsible for vasodilation in the lesional skin of CSU. However, the underlying causes of mast cell activation in the disease are largely unknown and remain to be identified. Thus, in this review, we discuss new insights in the pathogenesis of CSU, focusing on inflammation and angiogenesis. The understanding of these mechanisms will enable the identification of biomarkers useful for the diagnosis, follow-up, and management of CSU and will allow the development of novel, more specific, and patient-tailored therapies.

1. Introduction

Chronic urticaria (CU) is a common disease impacting negatively on multiple aspects of patients' lives. According to the recent guidelines, CU is defined as a disease characterized by the development of recurrent itchy wheals and/or angioedema occurring for 6 weeks or more and is divided in two major subtypes: chronic spontaneous urticaria (CSU) and inducible urticaria [1].

In the last decade, different pathophysiological mechanisms, potentially responsible for the development of CSU, have been described. It is likely that the activation of skin mast cells with consequent release of histamine and other proinflammatory mediators is responsible for vasodilation in the lesional skin of CSU [2]. However, the underlying causes of mast cell activation in the disease are largely unknown and remain to be identified. An autoimmune mechanism has been proposed, following the detection in a sizable subgroup of CSU patients of circulating anti-IgE or anti-FcεRI antibodies [3], or IgE antithyroid peroxidase (anti-TPO) [4]. This concept has led to investigate novel approaches for targeting circulating IgE in CSU [5].

However, the observation that a large part of CSU has no autoantibodies suggests that other mechanisms are probably involved in the pathogenesis of the disease and alternative therapies targeting these pathways are needed [6].

2. Role of Inflammatory Mediators in CSU

It is well known that mast cells are the primary effector cells in urticaria: their degranulation leads to the immediate release of preformed granular mediators such as histamine, tryptase, chymase, and proteases [2, 7]. Activated mast cells can also very rapidly synthesize and release prostaglandin- (PG-) D2, thromboxanes, leukotrienes (LTs), and platelet-activating factor (PAF). These mediators are responsible for the vasodilatation, increased vascular permeability, and stimulation of sensory nerve endings in the skin, leading to swelling, redness, and itchiness. Mast cells are also important sources of an array of cytokines, growth factors, and

chemokines which may amplify and perpetuate the inflammatory state of urticaria. We have to take into account that besides an IgE-mediated mechanism, mast cells may be activated by IgG-dependent triggers or by several nonimmunological agents such as compound 48/80, basic polypeptides (polylysine, polyarginine), morphine sulphate, substance P, and the anaphylatoxin C5a [2], suggesting that mast cell activation during CSU might be the consequence of several different stimuli.

In parallel to mast cells, basophils also appear to be involved in CSU pathogenesis. In the peripheral blood of CSU patients with high disease activity, basophils are dramatically reduced, and this may be due to their recruitment from the circulation into the skin lesions [8]. Besides their reduction in the peripheral blood of severe CSU patients, basophils also present some functional abnormalities. In fact, hyporesponsiveness of these cells to anti-IgE [9] and alteration of signal transduction pathways have been reported in at least half of the patients with active disease [9–11]. For example, one of these pathways involves the histamine-releasing factor/translationally controlled tumor protein (HRF/TCTP), a cytokine which directly induces histamine release from basophils, by signal transduction process involving Syk kinase, mimicking many of the events associated with IgE-mediated activation [11].

Interesting clues to understand the mechanisms underlying CSU come from skin biopsy specimens. Besides an increased number of mast cells, a perivascular infiltrate of CD4+ lymphocytes [12], with variable numbers of monocytes, neutrophils, eosinophils, and basophils [13, 14] was demonstrated in biopsies from urticarial wheals. The cytokine profile is characterized by an increase in IL-4, IL-5, and interferon-γ (IFN-γ), which is suggestive of a mixed Th1/Th2 response [14, 15]. Cytokines that promote a Th2 profile of inflammation such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) are increased in lesional but not in uninvolved skin, suggesting that innate pathways might play a role in the pathogenesis of CSU by activation of mast cells in the lesional skin [16]. The presence of eosinophils in biopsies from affected skin supports the view that several pathological features of CSU are in common with the allergen-induced late-phase allergic reaction [17]. Mediators released by skin mast cells following their degranulation may contribute to eosinophil recruitment, activation, and survival, leading to the perpetuation of the clinical features of urticaria. How eosinophils and their mediators may directly contribute to the development of CSU is presently unclear. However, the detection of eosinophil-derived major basic protein (MBP) in the lesional skin of CSU patients and the capacity of MBP to activate mast cells through an IgE-independent mechanism support the idea that eosinophils may directly affect mast cell degranulation with consequent amplification and perpetuation of the local inflammation in CSU [17].

Besides an increase of inflammatory mediators in the skin, several independent studies demonstrate an increase of proinflammatory cytokines in the circulation of CSU patients. IL-6, one of the main inducer of the acute-phase response of inflammation, is increased in the plasma of CSU patients and correlates with the clinical activity score of the disease. In addition, plasma IL-6 concentration is significantly lower upon spontaneous remission, suggesting that this cytokine might be a marker of disease activity [18, 19].

Among IL-1 family cytokines, the proinflammatory cytokine IL-18, initially identified as a major inducer of IFN-γ in Th1 and NK cells, was evaluated in the circulation of CSU and conflicting results have been reported [20–22]. Although the study of Tedeschi et al. [20] did not detect significant differences in IL-18 levels between the CSU and control group, our data indicate that in CSU both total and free IL-18 are increased [22]. Like in other inflammatory conditions characterized by high levels of IL-18, its soluble inhibitor IL-18 binding protein (BP), which regulates the activity of the cytokine, is also increased, in the attempt to counteract the proinflammatory effects of IL-18 [23].

Parallel to IL-1 family cytokines, a role of the IL-23/IL-17 axis and TNF-α in the pathogenesis of CSU was hypothesized [24]. High serum levels of IL-17, IL-23, and TNF-α were detected in CSU patients, and the levels of IL-23 and TNF-α, but not that of IL-17, correlated with the activity of the disease, suggesting their contribution to CSU pathogenesis and their potential role as CSU biomarkers. Recently, the role of IL-13 and periostin, involved in allergic inflammatory processes, has been investigated in CSU patients. Interestingly, while a significant increase in IL-13 was demonstrated in the CSU patients, periostin was significantly reduced in CSU patients, especially in those with severe versus mild disease [25], suggesting that the two mediators may be independently related to the pathogenesis of CSU. The inflammatory status of CSU is also supported by the results of Kaplan. In their study, blood levels of C-reactive protein (CRP), an acute-phase reactant belonging to classical short pentraxins, were found significantly higher in CSU patients compared to healthy subjects [26]. Since CRP is an acute-phase protein produced primarily in the liver under the stimulus of IL-1, TNF-α, and/or IL-6, it is conceivable that the increase of proinflammatory mediators and the consequent increase of CRP are hallmarks of an inflammatory condition of CSU patients [26]. Parallel to CRP, Kasperska-Zajac et al. have investigated the role of other members of the pentraxin family, in particular pentraxin 3 (PTX3), that is produced at the site of inflammation. The observation that PTX3 levels are increased in the plasma of CSU patients compared to healthy subjects may suggest a local inflammation due to activation of leukocytes that infiltrate the skin. Thus, the observed correlation between PTX3 and CRP in CSU patients suggests that these two pentraxins may be upregulated by the same mechanisms associated with acute-phase response [27].

On the basis of these findings, Bingham suggests that CSU is an immune-mediated inflammatory disease resulting from immunological activation events following exposure to exogenous or modified endogenous triggers in the presence of susceptibility factors [28]. Therefore, the inflammatory cascade may be a consequence of disturbances of innate and adaptive immunity [29], which leads to the recruitment of inflammatory and immune cells in the derma.

In this scenario, the chemokine signalling, mainly involved in the regulation of leukocyte trafficking [30], may

be one of the main mechanisms responsible for the recruitment of inflammatory cells in the lesional skin of CSU. Some of the chemokines involved in CSU pathogenesis might exert their effect not only by recruiting leukocytes in the tissue but also by activating mast cells in the lesional skin. For example, CCL5/RANTES, CCL2/MCP-1, and CXCL8/IL-8 are able to induce histamine and serotonin release by mast cells, suggesting their contribution to the development of urticaria by a direct effect on mast cell degranulation [31]. Besides the role of CCL5/RANTES in the recruitment of eosinophils, monocytes, and lymphocytes in the lesional skin [32], recently it has been shown that CCL5/RANTES is able to induce the migration of progenitor mast cells and their further differentiation and activation in the tissue. The effect of this chemokine on progenitor mast cells seems to be mediated by CCR5, chemokine receptor for CCL5/RANTES also expressed on progenitor mast cells [31]. Thus, since CCL5/RANTES can be produced from mast cells in the tissue and from circulating inflammatory cells following tissue infiltration, its production and its effects in the skin of CSU can persist over time contributing to the amplification and perpetuation of the inflammatory process.

Up to now, no biomarker useful for evaluation and management of patients with CSU is available. However, recent studies assessed the relevance of laboratory markers for determining the severity or predicting the evolution of disease in adult patients with CSU [33]. Among markers of activation of the extrinsic coagulation pathway, prothrombin fragment 1 + 2, D-dimer, and CRP are increased in CSU patients and seem to correlate with disease severity. In particular, the D-dimer level significantly correlates with UAS in CSU as well as in acute urticaria, suggesting its role as a marker of disease severity in both forms of urticaria [34]. Parallel to the coagulation/fibrinolysis pathways, an imbalance in pro- and anti-inflammatory adipokines in CSU patients has been also observed. In particular, according to the results of Trinh et al., lipocalin-2 (LCN2) might be used as a marker not only of disease activity but also of the clinical response to antihistamine treatment [35], suggesting new approaches to monitor disease progression and response to therapy in CSU patients. Potential disease markers have also been investigated in the presence of other comorbidities. Recently, in Korean patients with CU and metabolic syndrome (MS), a hospital-based cross-sectional study demonstrated a correlation between uncontrolled CU and the levels of C3, TNF-α and ECP. However, it is unclear whether the increased systemic inflammation is just an epiphenomenon or has any role in the pathogenesis of CU associated with MS [36].

3. Differences in ASST-Positive and ASST-Negative Subgroups of CSU

ASST is widely used in the diagnosis of CU in order to evaluate an autoimmune origin of the disease. In fact, a correlation between a positive ASST and the presence of anti-FcεRI and anti-IgE antibodies was reported. On the contrary, it is still debated whether or not this test has any prognostic value. Some studies demonstrated an increased disease activity or a longer duration of urticaria in ASST-positive versus ASST-negative patients, while others did not report significant differences between the two subgroups in terms of severity, disease duration, and quality of life scores [37–40]. Interestingly, in a study conducted by Ye et al. [41], it has been shown that ASST reactivity was a significant predictor of well-controlled CU during the 6-month stepwise treatment according to the recent guidelines. Since the reactivity of ASST is mainly due to serum factors responsible for histamine release and vasodilatation that are controlled by antihistamines, the authors hypothesized that ASST-positive patients are expected to achieve a well-controlled state within 6 months of treatment. Therefore, according to these data, the results of the ASST may be a useful parameter for predicting response to treatment and monitoring therapeutic response in patients with CU.

4. Role of Endothelium and Coagulation System in CSU

Besides the chemokine system, the endothelium plays a critical role in controlling the passage of fluid into the tissue and influencing cellular trafficking [42]. In the skin, endothelium dysfunction might increase vascular permeability, with a consequent proinflammatory response. The soluble forms of adhesion molecules, such as vascular cellular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule (ICAM-1), are widely used as biomarkers of endothelial dysfunction, and their increase in the circulation and skin biopsies [32] seems to reflect a proinflammatory endothelium phenotype in several skin diseases, including CSU [14, 32, 43, 44].

Recent studies on coagulation performed in CSU patients have produced interesting results [45]. According to these studies, the coagulation cascade seems to be activated in CSU, involving the extrinsic pathway first and the intrinsic pathway secondarily [45–47]. The detection of increased levels of factor VIIa, prothrombin fragment 1 + 2, and D-dimer in CSU suggests that, following endothelial cell activation, tissue factors are released with consequent activation of the extrinsic coagulation cascade and secondary fibrinolysis [46, 47]. Thus, these results are of particular interest when considering that thrombin can increase vascular permeability and is a potent inducer of mast cell degranulation, at least in experimental models [45]. Furthermore, the plasma levels of D-dimer, frequently elevated in patients with severe CSU, seem to decrease following treatment with omalizumab [48], suggesting a link between circulating autoantibodies, activation of coagulation, and fibrin degradation in severe CSU.

5. Role of Mediators Regulating Angiogenesis in CSU

Angiogenesis is the growth of new blood vessels from preexisting ones. It is a multistep and highly orchestrated process involving vessel sprouting, endothelial cell migration, proliferation, tube formation, and survival [49]. Under physiologic conditions, angiogenesis depends on the balance of positive and negative angiogenic mediators within the vascular

microenvironment and requires the functional activities of a number of molecules, including angiogenic factors, extracellular matrix proteins, adhesion receptors, and proteolytic enzymes [49]. Angiogenesis is also associated with pathologic conditions as direct response to tissue demands, such as chronic inflammation, fibrosis, and tumor growth [50].

Numerous inducers of angiogenesis have been identified, including members of the fibroblast growth factor (FGF) family, vascular permeability factor/vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor alpha and beta (TGF-α and TGF-β), platelet-derived growth factor (PDGF), TNF-α, hepatocyte growth factor/scatter factor (HGF/SC), granulocyte macrophage colony-stimulating factor (GM-CSF), and angiopoietin-1 and angiopoietin-2.

Among them, VEGF is the most potent direct-acting regulator of angiogenesis, and its expression is often excessive in chronic inflammatory diseases. VEGF induces proliferation, migration, and tube formation of endothelial cells. It promotes secretion of interstitial matrix metalloproteinase-1 (MMP-1) and von Willebrand factor and the expression of chemokines, as well as leukocyte adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin [51]. VEGF is also a potent survival factor for endothelial cells, and it induces in endothelial cells the expression of antiapoptotic proteins. VEGF also causes vasodilatation through the induction of the endothelial nitric oxide (NO) synthase and the subsequent increase in NO production. Therefore, VEGF acts principally on endothelial cells, even though it can influence other cell types, including hematopoietic stem cells, monocytes, and other inflammatory cells.

Recently, the presence of new blood vessels in the skin of CSU patients has been reported by Kay et al. [17]. They demonstrated that the lesional skin of CSU patients contained significantly more CD31-positive endothelial cells compared to the normal skin. Increased vascularity was also confirmed by confocal imaging using the lectin *Ulex europaeus* agglutinin 1 (UEA-1). In the same skin lesions, the increase of new vessels parallels the increased numbers of eosinophils, neutrophils, basophils, and macrophages, suggesting a direct contribution of these inflammatory cells to the formation of blood vessels. This is the first report showing angiogenesis in the skin lesions of CSU, but previous data were consistent with this observation. In fact, increased levels of VEGF have been observed in the circulation and in the tissue of CSU patients [52]. Thus, we can suggest that in CSU VEGF induces vascular leakage as well as the formation of new vessels. Since VEGF is mainly produced by inflammatory cells, we can also hypothesize that mast cells and infiltrating eosinophils and basophils present in skin lesions might contribute to the release of VEGF with consequent increase in vascular permeability and neoangiogenesis. On the other side, mast cells, eosinophils, and basophils might be a target for VEGF, leading to perpetuation and amplification of the inflammatory processes [53]. The functional activity of VEGF is tightly regulated by endogenous antiangiogenic mediators mainly produced by the degradation of extracellular matrix (ECM) components such as endostatin (ES) and thrombospondin- (TSP-) 1. Recently, we have reported increased levels of ES and TSP-1 in the sera of CSU patients, which do not correlate with the activity of the disease [54]. Thus, these antiangiogenic mediators, able to exert multiple activities during inflammation and angiogenesis, might be involved in the pathogenesis of CSU. We have to take into account that besides their antiangiogenic activities, ES and TSP-1 also exert other important roles in the skin remodelling. For example, TSP-1 destabilizes a contact between endothelial cells due to its direct effect on the cells, contributing to skin vasodilation and consequent extravasation [55] and ES, a proteolytic fragment of collagen type XVIII, acts as a vasoactive mediator due to its direct effect on endothelial cells via NO synthesis [56]. Therefore, both ES and TSP-1 might contribute to the vascular leakage in CSU, leading to the development of its clinical manifestations, such as wheals and flare formation.

Parallel to ECM fragments, some members of MMPs are increased in the circulation of CSU patients [57]. For example, MMP-9, an endopeptidase involved in ECM degradation during inflammation, tissue remodelling, and angiogenesis, is increased in the peripheral blood of CSU patients in adults and in children [58]. Some [57–59], but not all studies [60], reported an association between disease activity and plasma concentration of MMP-9. We can hypothesize that together with VEGF and ECM fragments, the increase of MMP-9 in CSU might contribute to both vascular leakage and angiogenesis, leading to the amplification and perpetuation of the inflammatory process. It is likely that MMP-9 contributes to the pathogenesis of CSU as well as that of other chronic diseases (i.e., asthma) where inflammation and tissue remodelling take place.

6. Conclusion and Prospective

In CSU, several processes such as inflammation, coagulation, and angiogenesis take place. We have discussed new insights that demonstrate the active contribution of inflammatory cells (mast cells, basophils, eosinophils, neutrophils, and lymphocytes), cytokines, growth factors, soluble adhesion molecules, ECM fragments, and MMPs in the development of CSU. Many of the abovementioned cells and mediators seem to be involved in the pathogenesis of the disease, but none of these seems to be really specific to CSU. Furthermore, some of them correlate with the urticaria activity score, but so far are not used as disease biomarkers. The understanding of the mechanisms underlying the pathogenesis of CSU will enable the identification of biomarkers useful for the diagnosis, follow-up, and management of the disease and will allow the development of novel, more specific, and patient-tailored therapies.

Conflicts of Interest

The authors declare no financial conflicts of interest.

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Review Article

Psoriasis and Cardiovascular Risk—Do Promising New Biomarkers Have Clinical Impact?

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Epidemiological studies suggest an increased prevalence of cardiovascular disease (CVD) in patients with psoriasis (PS). Therefore, emphasis has lately been laid on the necessity for clinical evaluation of the risk of CVD in these patients. The systemic inflammatory markers C-reactive protein (CRP) and interleukin- (IL-) 6, which have long been used to predict future CVD in the general population, are increased manyfold in patients with PS. Lipid abnormalities characterized by elevated triglycerides, low HDL cholesterol, and higher concentrations of LDL cholesterol and its oxidized form are also prevalent in patients. There is a need for additional laboratory markers for the assessment of cardiovascular status of patients with PS. Due to frequent comorbid overweight and obesity, biologically active compounds produced by adipocytes may have an impact on monitoring the status of the cardiovascular system of patients with PS. For this purpose, two adipokines, adiponectin and leptin, have been most extensively studied. The review focuses on some inflammatory and oxidative stress aspects in patients with PS through the analysis of the impact of prominent adipokines and oxidized low-density lipoprotein (oxLDL) to assess their eligibility for clinical practice as markers of CVD risk in patients with PS.

1. Introduction

Several lines of evidence indicate that psoriasis (PS) is associated with enhanced atherosclerosis and risk of cardiovascular disease (CVD) [1–3] such as coronary artery disease, ischemic heart disease, and myocardial infarction [4]. The results of epidemiological studies have also demonstrated that the risk to develop CVD is higher in patients with severe PS [5–9] and that the risk persists after adjusting for conventional cardiovascular risk factors such as obesity, hypertension, diabetes, and smoking [1, 8].

Central obesity, a component of metabolic syndrome (MS), is frequently encountered in patients with PS, often in association with other components of this syndrome such as insulin resistance, hypertension, and dyslipidemia [10–12]. Large epidemiological studies have demonstrated that patients with moderate to severe PS have about twice as high prevalence and incidence of obesity as compared to the general population [10, 13, 14]. According to several authors, the severity of obesity positively correlates with the Psoriasis Area and Severity Index (PASI) [15–19]. The discussion, whether obesity is a result or a cause of PS and future cardiovascular events, is still open [7, 20].

The pathogenesis of atherosclerosis followed by CVD is inflammation dependent [1, 21, 22]. Therefore, an increased prevalence of atherosclerosis in patients with PS is mostly explained by a chronic systemic inflammation [1]. However, it is still unclear whether PS itself or some other risk factors, for example genetic and lifestyle factors, are responsible for

the increased prevalence of atherosclerosis in patients with PS [8, 9].

The explanation of associations between PS and CVD is further complicated by the fact that traditional systemic antipsoriatic treatments such as acitretin, cyclosporine, and corticosteroids may contribute to the development of CVD risk factors such as hypertension, obesity, diabetes, and dyslipidemia [8] whilst anti-TNF-α treatment does not have this side effect [23].

Epidemiological studies have shown that due to comorbidities, especially CVD, the life expectancy of patients with severe PS is 3 to 5 years shorter as compared to that of nonpsoriatic controls [24–26]. Therefore, several recent studies have emphasized the necessity for additional laboratory markers for earlier diagnosis of cardiovascular complications and their follow-up [14]. Ideally, these markers should also correlate with the severity of PS estimated by PASI or body surface area (BSA). This short review focuses on some inflammatory and oxidative stress aspects of PS, considering also the potential of prominent adipokines and oxidized low-density lipoprotein (oxLDL) as markers of cardiovascular status and skin disease severity in patients with PS.

2. Search Strategy

We searched medical databases such as PubMed, Wiley Online Library, and Web of Science for English language articles published between 2003 and 2017 by entering the terms "psoriasis," "cardiovascular disease," "obesity," "body mass index (BMI)," "interleukins," "adipokines," "C-reactive protein," and "oxidized LDL." The inclusion criteria for this review comprised clinical studies and reviews, which focused on the associations between the search terms. The reference lists of articles were searched for additional publications. Potentially relevant studies were printed out and read in full text to be included in this review.

3. Inflammatory Background of Psoriasis and Obesity

The underlying mechanisms that link PS and atherosclerosis with the subsequent CVD are not well understood; however, several lines of evidence suggest that a chronic inflammation with the involvement of humoral and cellular immunity may contribute to both diseases [27, 28]. Nowadays, PS is interpreted as the outcome of an inappropriate immunocyte (mainly T cell)-based activation event to an unknown antigen [29]. During inflammation, the communication between epidermal keratinocytes, dermal vascular cells, and immunocytes, including activated antigen presenting cells and T memory/effector cells, is driven by cytokines, chemokines, adhesion molecules, and receptors [29–31]. Pathogenetic T cells are mainly represented by Th17 and Th1 lymphocytes [2, 32–34]. Accordingly, elevated serum levels of Th1-derived cytokines such as interferon- (IFN-) γ, tumor necrosis factor- (TNF-) α, and interleukin- (IL-) 2 have been detected in the sera of patients [32, 35–37]. IFN-γ and TNF-α not only induce keratinocytes to produce several other cytokines, for example IL-6 and IL-8 [2], but also increase the expression of intercellular adhesion molecule- (ICAM-) 1 that promotes the infiltration of T cells and other inflammatory cells [32].

Similarly to PS, subclinical inflammation induced by obesity is characterized by increased production of inflammatory cytokines IL-6 and TNF-α and higher C-reactive protein (CRP) levels [38–40]. There are also some other coinciding pathological pathways between PS and obesity, for example, both disorders are associated with systemic high-grade oxidative stress (OxS) and the related pathophysiological outcomes, including the promotion of LDL oxidation [11, 41, 42]. PS combined with obesity/MS is likely to produce a higher degree of systemic inflammation and OxS [43], promoting endothelial dysfunction and the formation of atherosclerotic plaques. These pathological events may activate or intensify preexisting atherosclerosis and result in the development of significant CVD [8, 44].

4. CRP and Cytokines

Previous studies have demonstrated that PS and obesity share common inflammatory mediators such as CRP and IL-6. Both IL-6 and CRP, the latter measured by highly sensitive assay (hsCRP), have been found to be associated with subclinical atherosclerosis [4, 45] and, therefore, have a predictive value for future cardiovascular events [45]. Both these markers are overexpressed in psoriasis [27, 46–49], including in patients without overweight/obesity or other traditional CVD risk factors such as hypercholesterolemia, hypertension, and diabetes [26, 50]. The findings of the studies included in this review comparing the levels of inflammatory markers and adipokines in patients with PS and nonpsoriatic controls are summarized in Table 1. The association between the levels of CRP and IL-6 with the severity of psoriatic inflammation is confirmed by the significant decrease of IL-6 levels during both light and systemic antipsoriatic therapy with corticosteroids and methotrexate [39, 51, 52]. During infliximab monotherapy, inflammatory markers decreased in concordance with the decrease of PASI [53]. Therefore, several authors are in agreement that PS may be an independent CVD risk factor [3, 47]. Thus, the levels of these inflammatory markers should be measured regularly in patients with PS as well as other chronic inflammatory diseases to discover an enhanced risk of developing CVD. An increase in hsCRP also reflects metabolic disorders, including insulin resistance and adiposity [45], which are common in patients with PS. Using hsCRP and IL-6 for the assessment of PS activity is questionable. To date, no single biomarker has been found to correlate with PASI consistently. For example, the positive correlations of PASI with CRP and IL-6 were demonstrated by Coimbra et al. [52, 54, 55], whilst others found no correlation between PASI and the levels of CRP or IL-6 [49, 56] (Table 1). In our previous work, we found a linear correlation between PASI and IL-6 [57], but not in the group of patients investigated a year before [50].

The suppression of systemic inflammation by means of antipsoriatic therapy could decrease the risk of CVD that should be accompanied by the decrease in CRP and IL-6 levels. According to Coimbra et al. [55], after 12-week

Table 1: Selected hematological parameters in patients with psoriasis as compared to nonpsoriatic controls and their relation to the Psoriasis Area and Severity Index (PASI).

Author, year [ref.]	Number of patients/controls	Mean/median PASI or BSA	P	Correlation to PASI
C-reactive protein				
Chodorowska et al., 2004 [51]	175/30	29.2	$P < 0.001\uparrow$	ND
Coimbra et al., 2010 [52]	73/38	18.0	$P < 0.001\uparrow$	In correlation with PASI
Takahashi et al., 2014 [49]	97/79	10.6/9.2 (male/female)	$P < 0.05\uparrow$	Not associated with PASI
Vachatova et al., 2016 [44]	74/65	Median 15.3	$P < 0.001\uparrow$	ND
Interleukin-6				
Coimbra et al., 2010 [55]	66/37	18.8	$P < 0.01\uparrow$	In correlation with PASI
Deeva et al., 2010 [56]	35/10	8.7	$P < 0.0001\uparrow$	Not associated with PASI
Kaur et al., 2012 [57]	58/58	9.5	$P < 0.001\uparrow$	In correlation with PASI
Adiponectin				
Coimbra et al., 2009 [54]	56/37	19.2	$P = 0.001\downarrow$	ND
Gerdes et al., 2011 [73]	79/80	12.2	$P = 0.0094\uparrow$	Not associated with PASI
Kaur et al., 2011 [50]	60/44	10.1	NS (all patients)	ND
Li et al., 2014 [37]	122/134	Median BSA 2.9%*	$P < 0.001\downarrow$	ND
Baran et al., 2015 [43]	49/16	16.5	$P = 0.004\downarrow$	Not associated with PASI
Vachatova et al., 2016 [44]	74/65	Median 15.3	NS	ND
LEPTIN				
Gerdes et al., 2011 [73]	79/80	Median 12.2	NS	Not associated with PASI
Kaur et al., 2011 [50]	60/48	10.1	NS (all patients)	Not associated with PASI
Li et al., 2014 [37]	122/134	Median BSA 2.9%*	NS	ND
Baran et al., 2015 [43]	49/16	16.5	$P = 0.002\downarrow$	Not associated with PASI
Vachatova et al., 2016 [44]	74/65	Median 15.3	$P < 0.01\uparrow$	ND
Coimbra et al., 2010 [55]	66/37	18.8	$P < 0.001\uparrow$	ND

PASI: Psoriasis Area and Severity Index. (the interpretation of PASI score differed between the studies. In most studies, the score below 10 was interpreted as mild disease, between 10 and 20 as moderate, and above 20 as severe disease.) BSA: body surface area; ↑: increased in patients; ↓: decreased in patients; NS: not significant; ND: not done; *mild disease.

phototherapy with narrow-band UVB (NBUVB) and psoralen + UVA (PUVA), CRP in patients decreased significantly ($P \leq 0.01$) compared to that of the pretreatment value while topical therapy did not influence significantly the blood levels of inflammatory markers [58]. The authors also reported a decrease in the levels of several inflammatory cytokines involved in the pathogenesis of PS (IL-22, IL-17, IL-23, IL-8, TNF-α, and vascular endothelial growth factor) after 12-week phototherapy [59]. The decrease of CRP after treatment is an evidence of decreased systemic low-grade inflammation implicated in atherosclerosis.

5. Adiponectin and Leptin

After the discovery that adipose tissue is not a simple storage of energy but metabolically an extremely active tissue, adipocyte-derived bioactive compounds termed adipokines gained enormous attention. Through the release of adipokines, adipose tissue regulates food intake, energy expenditure, insulin sensitivity, and inflammation [38, 60]. The pattern of secretion of adipokines mirrors adipose tissue function, and this pattern is important to establish the individual risk of developing metabolic and cardiovascular comorbidities of obesity [61]. Mainly two adipose tissue mass-dependent adipokines—adiponectin (ADIPO) and leptin—have attracted the attention of scientists as possible mediators of CVD risk associated with obesity [62, 63].

There are several reasons why ADIPO and leptin may serve as diagnostic or prognostic biomarkers of CVD. ADIPO has insulin-sensitizing, anti-inflammatory, and atheroprotective effects [37, 62]. Decreased circulating levels of ADIPO have been associated with higher levels of inflammatory cytokines and increased OxS in well-known risk factor of CVD such as obesity [64]. Reduced levels of ADIPO are associated with insulin resistance and several vascular adverse events, including impaired endothelium-dependent vasodilatation, impaired ischaemia-induced neovascularization, and diastolic heart failure [65–68]. ADIPO levels inversely correlate with serum CRP in obese subjects, in diabetics and in those affected by coronary artery disease [69].

Leptin, having structural and functional similarities with proinflammatory cytokines IL-6 and IL-12 [38], exerts proinflammatory and proatherogenic effect through mechanisms involving endothelial cell activation and thrombogenesis

[70]. The effects of leptin also include the accumulation of reactive oxygen species (ROS) in endothelial cells and stimulation of vascular smooth muscle cell proliferation, acceleration of vascular calcification, and enhancement of platelet adhesiveness [71]. CVD risk factors (central obesity, insulin resistance, and dyslipidemia) are associated with decreased ADIPO and increased leptin levels [37, 44].

While there are conflicting reports, most of the recent studies have demonstrated significantly decreased ADIPO or its high molecular form levels in patients with PS as compared to those of the healthy controls [37, 43, 72], especially in patients with moderate and severe disease [54] (Table 1). According to Baran et al. [43], ADIPO levels in patients with PS rose with the increase in disease activity, expressed by PASI score. In patients with normal body weight, an increase in ADIPO concentration has been reported [44, 50, 73]. It might be possible that the increase in ADIPO levels in high-risk population with advanced atherosclerosis or other chronic inflammatory conditions is part of a compensatory mechanism to limit further endothelial damage [74, 75]. Consequently, in patients with chronic inflammatory diseases, the upregulation of expression, synthesis, and release of ADIPO may occur due to increased systemic inflammation, accompanied by higher levels of TNF-α and other cytokines. ADIPO has been shown to have a counter-regulatory action on TNF-α production [38]. Obesity may counteract this compensatory mechanism because the raised levels of TNF-α and IL-6 in obesity suppress ADIPO production by adipose tissue [76, 77]. Thus, ADIPO mediates protective effects in obesity-related metabolic and vascular diseases, presumably by its anti-inflammatory action. As ADIPO levels are mainly associated with body weight and decrease in obesity, this attractive marker might be used for the assessment of general CVD risk in obese and overweight individuals to follow their progression to impaired glycose tolerance and CVD.

Leptin has an important role in the central regulation of food intake and energy expenditure, with low leptin levels driving increased food intake and reduced energy expenditure and increased leptin levels promoting decreased food intake and increased energy expenditure [78]. Leptin concentrations increase with the increase in body mass and inflammatory activity [77]. Conditions associated with the release of proinflammatory IL-6 and TNF-α are known to increase leptin synthesis [71]. In the cardiovascular system, leptin actions are potentially proatherogenic, prothrombotic, and angiogenic [79–81]. Interestingly, in obese individuals who have elevated leptin levels, only the anorectic effect of leptin is impaired, whereas other effects are maintained, a phenomenon known as selective leptin resistance [78, 81]. Thus, hyperleptinemia contributes to atherogenesis in these patients. Hyperleptinemia is associated with the impairment of NO-dependent vasorelaxation, an increase in OxS as well as in the level of endothelin (a potent vasoconstrictor) [81]. All these features are markers of endothelial dysfunction, the early stage in atherogenesis. Leptin and the CVD risk marker CRP have been found to be independently associated [71].

In several studies, plasma leptin levels in patients with PS have been observed to be higher than those of the healthy controls [13, 82, 83]. However, some other studies have reported that plasma leptin levels in patients were not different from the controls [37, 73]. There are several potential explanations for these differing results. Most of the studies had a relatively small number of patients with highly different PASI and body mass index (BMI) values. In addition, different measurement methods were used. According to Coimbra et al. [72], the increase in leptin levels in patients with PS is usually associated with overweight and obesity, not with PS severity. Considering that leptin has been shown to promote important links in the pathogenesis of PS such as cytokine secretion, keratinocytes proliferation, and angiogenesis [72], more studies are needed to characterize the relationship between leptin (or ADIPO) levels and PS. However, due to their multiple roles in inflammation, insulin resistance, diabetes, atherosclerosis and obesity, and possibility of other forms of adipokine-resistance in addition to selective leptin resistance in appetite regulation, adipokines have not yet been approved as prognostic markers of CVD in the general population [84, 85].

Whether adipokines could be used as biomarkers of comorbid CVD in patients with PS is not known. There are currently limited data concerning the changes in adipokine levels after treatment. ADIPO levels increased after 12-week phototherapy in comparison to their levels before treatment ($P \leq 0.01$) which was demonstrated by Coimbra et al. [55, 58].

6. Dyslipidemia and OxLDL

The principal CVD risk factors are hypertension and dyslipidemia; the latter is characterized by elevated levels of total cholesterol, low-density lipoprotein (LDL) and triglycerides, and lower levels of high-density lipoproteins (HDL) [86]. These changes are found consistently in patients with PS [47, 54, 58, 87]. Inflammatory mediators, including adipocyte-derived cytokines such as TNF-α, IL-6, and leptin, are known to induce dyslipidemia [7]. Therefore, proinflammatory activities observed in PS may initiate abnormalities in plasma lipid/lipoprotein levels [88]. As PS is frequently associated with obesity, the excess adipose tissue might further contribute to atherogenic dyslipidemia [7]. In fact, several studies have shown the common features of atherogenic dyslipidemia with increased blood levels of total cholesterol, triglycerides, LDL, and apolipoprotein A and low HDL and apolipoprotein B levels in patients with PS [7, 89].

In conditions of OxS that accompanies a chronic inflammatory disease, including PS, excess LDL is oxidatively modified. Oxidative modification of LDL is one of the earliest events in the pathogenesis of atherosclerosis [90], circulating levels of oxLDL and reflecting the intensity of oxLDL formation in the vascular wall [91] that can be followed by the rupture of atherosclerotic plaque and subsequent thrombosis [54]. OxLDL is a proinflammatory chemoattractant agent for macrophages and T lymphocytes and cytotoxic for endothelial cells and stimulates the release of soluble inflammatory molecules [92]. Thus, OxLDL, which contains hundreds of different oxidized lipid molecules, can be considered as a hallmark of hyperlipidemia and atherosclerosis [92].

OxLDL levels are not frequently estimated in patients with PS. A few studies have demonstrated increased levels of oxLDL in patients with a significant association with BMI [50, 54]. Significant association between circulating oxLDL and BMI was also found [54]. OxLDL binds to β_2-glycoproteins (β_2-GPI) to form oxLDL-β_2-GPI complexes in the intima, and these complexes are released into circulation [90]. Although the roles of oxLDL-β_2-GPI and their antibodies are still controversial, it has been postulated that β_2-GPI binds oxLDL to neutralize its proinflammatory and proatherogenic effect [93]. The occurrence of oxLDL-β_2-GPI has been connected to the chronic inflammation of the vasculature and OxS, and these complexes have been found in patients with systemic autoimmune disorders such as systemic lupus erythematosus, systemic sclerosis, and type 2 diabetes mellitus [90, 94, 95], but also in patients with PS [50]. In addition, the skin of patients with PS has shown positive oxLDL staining compared to sex- and age-matched healthy volunteers, whereas there was no staining in nonlesional skin samples from the patients [96]. The study of Coimbra et al. has demonstrated that topical treatment with calcipotriol or betamethasone dipropionate, or a combination of the two had no effect on oxLDL plasma level [58]. OxLDL levels in patients, who were treated with 12-week phototherapy, have improved but remained higher as compared to healthy controls [58]. In patients with PS, regular lipid screening is important, especially when treated with drugs causing hyperlipidemia such as acitretin and cyclosporine [23]. Therefore, the assay for the estimation of the level of oxLDL in patients with PS would be of value, especially in obese patients, but it has not yet been approved as a biochemical prognostic marker for atherosclerotic disease.

7. Conclusions

So far, no laboratory markers have been employed to evaluate psoriasis activity, although such a marker would be highly valuable to monitor the effect of therapy and predict recurrences. Psoriasis and obesity are both characterized by overexpression of key proinflammatory cytokines, hsCRP and IL-6, which have been accepted as classical markers of CVD risk and are therefore valuable markers for the assessment of the cardiovascular status of patients with PS. Although the amount of scientific information concerning the levels of adipokines is steadily increasing, further studies are warranted to address the role of adipokines as clinical biomarkers. Additional research is required to establish the perspective for oxLDL as a biomarker of increased cardiovascular risk.

Conflicts of Interest

The authors declare no competing interests regarding the publication of this paper.

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