

ORIGINAL ARTICLE

Profile of skin barrier proteins (filaggrin, claudins 1 and 4) and Th1/Th2/Th17 cytokines in adults with atopic dermatitis

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Abstract

Background Atopic dermatitis (AD) in adults and profile of skin barrier proteins and inflammatory cytokines.

Objective Evaluation of the expression of skin barrier proteins such as filaggrin, claudins 1 and 4 and of circulating inflammatory cytokines (Th1/Th2/Th17) in adults with AD.

Methods Thirty-three adult patients with AD diagnosed according to the Hanifin & Rajkacriteria, and 25 healthy controls were enrolled in the study. AD severity was measured by Eczema Area and Severity Index (EASI). Laboratory assays included immunohistochemistry analysis of skin barrier proteins, such as filaggrin, claudins 1 and 4 and interleukin-17 (IL-17) from skin samples and determination of circulating cytokine levels (IL-2, 4, 5, 6, 10, 17A, TNF and IFN- γ) by flow cytometry (Cytometric Bead Array).

Results We observed a reduced expression of filaggrin and claudin 1 in lesional skin of AD patients, when compared to controls. There was an inverse correlation of filaggrin expression and disease severity. In addition, IL-17 expression was enhanced in AD patients. Similarly, higher levels of inflammatory cytokines (IL-2, 5, 6, 10, 17A and IFN- γ) were found in AD patients.

Conclusion Our data reinforce the role of an altered skin barrier in the pathogenesis of AD. Our results show not only reduced expression of filaggrin and claudin 1 in lesional atopic skin but also inverse correlation of filaggrin expression and disease severity. Moreover, elevation of *in situ* IL-17 and of circulating interleukin levels in AD emphasize the systemic, inflammatory profile of this defective skin barrier dermatosis.

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Conflicts of interest

None declared.

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Introduction

Atopic dermatitis (AD) is a chronic, pruritic, inflammatory skin disease most common in early childhood, although up to 40% of the patients remain symptomatic throughout adulthood. AD distribution is worldwide and its prevalence varies, according to age (6–20% in children and 1–3% in adults).^{1,2} AD pathogenesis depends on multiple factors, including environmental, immune, structural and genetic components.^{3,4}

At first, researchers considered AD to be exclusively driven by a primary immunological defect (the so-called ‘inside-outside’ theory of an intrinsic inflammatory disease); however, recent studies on the skin barrier role in the development of AD corroborate with an ‘outside-inside’ hypothesis, in which AD is seen as an inherited disease with skin barrier abnormalities.⁵

The barrier dysfunction in AD involves two main components: (i) Decreased generation of filaggrin (FLG) products that precipitate both structural and functional skin changes, and therefore lead to a higher risk of eczema, asthma, and early-onset of severe, persistent AD; (ii) Tight junction (TJ) defects: Bioelectric epidermal abnormalities, indicative of a reduced level of claudin 1 (CLDN-1).⁵ Previous studies have shown that CLDN-1 deficiency in mice leads to enhanced transepidermal water loss (TEWL).⁶

Some studies suggest that outside–inside initial provocation of AD eventually leads to an outside–inside–outside vicious cycle. One downstream consequence of these barrier defects would be the generation of primary cytokines such as interleukin (IL)-1 α and IL-1 β , which are stored in large quantities in the cytosol of corneocytes. These mediators are the first step in

the cytokine cascade, a primary contributor to inflammation in AD. Sustained antigen entry through a defective barrier leading to a T helper (Th) 2-dominant infiltrate is a second cause of inflammation in AD. Accordingly, correction of the barrier abnormality alone should ameliorate both causes of inflammation in AD.⁶

A classic Th1/Th2 paradigm was previously described in AD, with increased serum IgE levels in 80% of the patients, a biphasic cytokine pattern release with predominance of Th cells expressing IL-4 and IL-13 (Th2 cytokines) in non-lesional skin during the acute phase, and a dominant Th1 profile in the chronic phase (IL-12 and IFN- γ).⁴

However, recent findings in AD and psoriasis changed the exclusive Th1/Th2 paradigm with the discovery of Th17 T cells that synthesize IL-17 and IL-22. Many Th1 diseases have a strong IL-17 signal; in psoriasis, increased number of Th17 cells and enhanced expression of IL-17 contribute to neutrophil chemotaxis and increased expression of antimicrobial peptides.⁷ The precise role of Th17 T cells in AD, however, remains unclear. There is some evidence in animal models of the association of FLG deficiency with Th17-dominated skin inflammation, development of eczematous skin lesions with age and sensitization upon contact with proteins.^{8,9}

Defective skin barrier proteins of atopic individuals may interfere with *in situ* or circulating IL-17. A better understanding of the components of AD pathogenesis and their possible interactions may generate better clues for specific targets in AD therapy. Therefore, the aim of this study was to evaluate the expression of skin barrier proteins (filaggrin, claudins 1 and 4), and of inflammatory cytokines (Th1/Th2/Th17) in adults with AD.

Methods

Subjects

Thirty-three AD patients were selected (23 males and 10 females, age 18–48 years) and diagnosed according to the Hanifin & Rajka diagnostic criteria.¹⁰ All patients were from the Outpatient Clinic, Department of Dermatology, University of Sao Paulo Medical School. AD severity followed EASI score (range 0–72; 10 mild, 15 moderate and 8 severe).¹¹ None of the patients were treated with corticosteroids (topical or systemic) or immunosuppressants for at least 4 weeks (Table 1).

Twenty-five healthy controls (HC) were also selected (13 males and 12 females, age 23–69 years), without history of skin diseases. All individuals read the informed consent and agreed to participate. The study was approved by the local Ethics Committee. Tissue specimens were obtained by a 4-mm punch biopsy under local anaesthesia.

Immunohistochemistry

Immunohistochemistry was performed on 4-micra paraffin-embedded samples, as described elsewhere.¹² Table 1 displays the chosen antibody specifications, dilution and detection system.

Quantification of immunolabelled cells

Immunohistochemical stained specimens were scanned using the digital slide scanner Pannoramic Scan - 3DHitech. For IL-17, three dermal areas (each field = 605 μm $\times$ 380 μm) under $\times 200$ magnification were chosen. For filaggrin and claudins, an epidermal section was chosen under $\times 200$ magnification. Photographs were analysed utilizing Image-Pro Plus version 4.5.0.29 (Media Cybernetics, Inc, Bethesda, MD, USA) for Windows.¹³

Determination of circulating cytokine levels by flow cytometry

Circulating levels of IL-2, 4, 5, 6, 10, 17A, TNF and IFN- γ cytokines were established by flow cytometry utilizing a Human Enhanced Sensitivity Flex Set - Cytometric Bead Array, CBA, (BD Biosciences, CA, USA). The assay was performed following manufacturer's instructions. Briefly, sera samples and cytokine patterns from the set were incubated with capture beads, and recovered with specific antibodies for the respective cytokines. Samples were incubated with Phycoerythrin (PE)-conjugated antibodies. Acquisition of samples was performed with a flow cytometer (LSRFortessa-BD Biosciences), and the results were obtained by BD FCAPArray3.0 (BD Biosciences). Minimum levels of detection were as follows: IL-2: 88.9 fg mL⁻¹; IL-4: 144.4 fg mL⁻¹; IL-5: 67.8 fg mL⁻¹; IL-6: 68.4 fg mL⁻¹; IL-10: 13.7 fg mL⁻¹; IL-17A: 26.1 fg mL⁻¹; TNF: 67.3 fg mL⁻¹; IFN γ : 66.7 fg mL⁻¹.

Statistical analysis

Mann–Whitney and Kruskal–Wallis non-parametric tests were utilized to compare two or three sets of data respectively. Correlation between data was established using Spearman non-parametric

Table 1 Antibody specifications, dilution and detection system utilized in the study

Antibody	Company	Dilution	Detection system
CLDN-1	Zymed [®] – Invitrogen Corporation, Carlsbad, CA, USA code 51 -9000	1/400	LSAB + System-HRP, code K0690, Dako, Carpinteria, CA, USA
CLDN-4	NeoMarkers [®] Fremont, CA, USA cat. RB - 9266 - PO	1/200	LSAB + System-HRP, code K0690, Dako, Carpinteria, CA, USA
FLG	Novocastra [®] Newcastle, UK code NCL –filaggrin	1/150	LSAB + System-HRP, code K0690, Dako, Carpinteria, CA, USA
IL-17	R&D Systems, Inc. Minneapolis, MN, USA code AF- 317- NA	1/40	LSAB + System-HRP, code K0690, Dako, Carpinteria, CA, USA

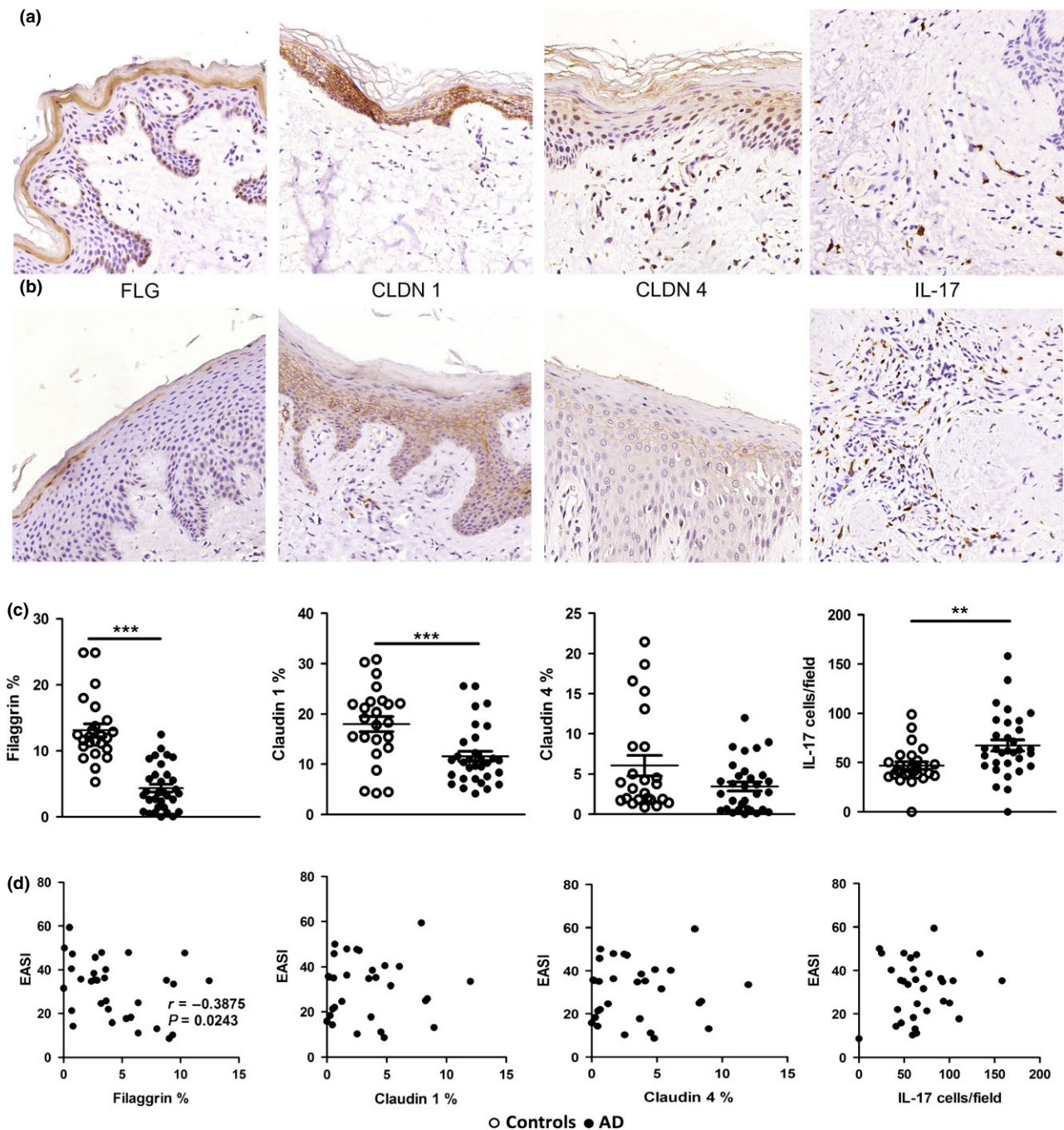


Figure 1 Expression of FLG, CLDN1, CLDN4 and IL-17 in skin specimens from adults with AD tested by immunohistochemistry. (a) Photograph of a skin specimen from a healthy control: FLG, CLDN1 and 4, IL-17 expression (×200). (b) Photograph of a skin specimen from an AD patient: reduced expression of FLG, CLDN1 and 4, and enhanced expression of IL-17 (×200). (c) Expression of FLG (area %), CLDN1 (area %), CLDN4 (area %) and IL-17 (cells/field) in the skin of the healthy control group (HC, $n = 33$), compared to AD patients (AD, $n = 25$). (d) Correlation between disease severity (EASI) and skin barrier proteins and IL-17 in the AD group. Line represents arithmetic mean of skin barrier proteins (area %) and IL-17 (cells/field) in skin specimens. ** $P \leq 0.01$ and *** $P \leq 0.001$.

metric correlation test. A difference between groups was considered statistically significant at $P \leq 0.05$.

Results

Expression of FLG, CLDN1, CLDN4 and IL-17 in skin specimens from adults with AD

Figure 1a shows skin barrier protein and IL-17 expression in skin specimens from HC. In Fig. 1b, we demonstrate the expression of skin barrier proteins and IL-17 in skin specimens from AD individuals. There was a reduced expression of FLG and CLDN1, and an enhanced IL-17 expression in our AD patients, when compared to controls. There was no significant difference in CLDN-4 expression in AD skin. Interestingly, we found an inverse correlation with FLG and disease severity, as shown in Fig. 1d. There was also a positive correlation between the expression of CLDN1 and 4 (data not shown).

Cytokines circulating levels of Th1/Th2/Th17 in adults with AD

With the use of CBA, we observed higher levels of IL-2, 5, 6, 10, 17A and IFN- γ in AD patients when compared to controls (Fig. 2). The inflammatory response in AD showed a mixed Th1/Th2/Th17 profile, with no significant correlation with decreased skin barrier protein expression. Interestingly, we did not observe any correlation between circulating cytokine levels with disease severity, and there was no significant correlation between circulating cytokine levels and the *in situ* expression of the analysed proteins (data not shown).

Discussion

Skin barrier function in AD patients is defective, with increased TEWL, and reduced capacitance. Skin barrier alterations contribute to higher susceptibility to infection (especially by *Staphylococcus aureus* – *S. aureus*) and dysfunctional immune response, consequently leading to intense, uncontrolled pruritus.^{4,14} In addition, keratinocytes release pro-inflammatory cytokines that induce the expression of adhesion molecules on vascular endothelium, facilitating the extravasation of inflammatory cells into skin tissue.⁴

A dysfunctional skin barrier is the result of reduced levels of lipids that form the stratum corneum, filaggrin or claudin defects, altered proteases or trauma from constant scratching in atopic individuals.³ Filaggrin mutations are reported in AD; recently, a study found a TJ defect in North-Americans,³ attributed to claudin 1, therefore corroborating the findings on skin of CLDN-1 knockout murine models.^{3,4}

Our AD patients from Brazil showed reduced expression of FLG and CLDN-1, corroborating results from other populations.^{3,4} We also explored the expression of an additional TJ protein (CLDN-4) in AD individuals. CLDN-4 has been associated with coeliac disease, gluten sensitivity, head and neck carcinomas and infectious diseases, but there was no significant decreased expression in AD individuals.^{15,16}

Even though the majority of patients with AD and FLG mutations outgrow their AD, there are individuals with severe AD who do not have FLG null mutations, and the opposite situation applies. This suggests that a number of additional factors are involved in the development of AD, including other gene

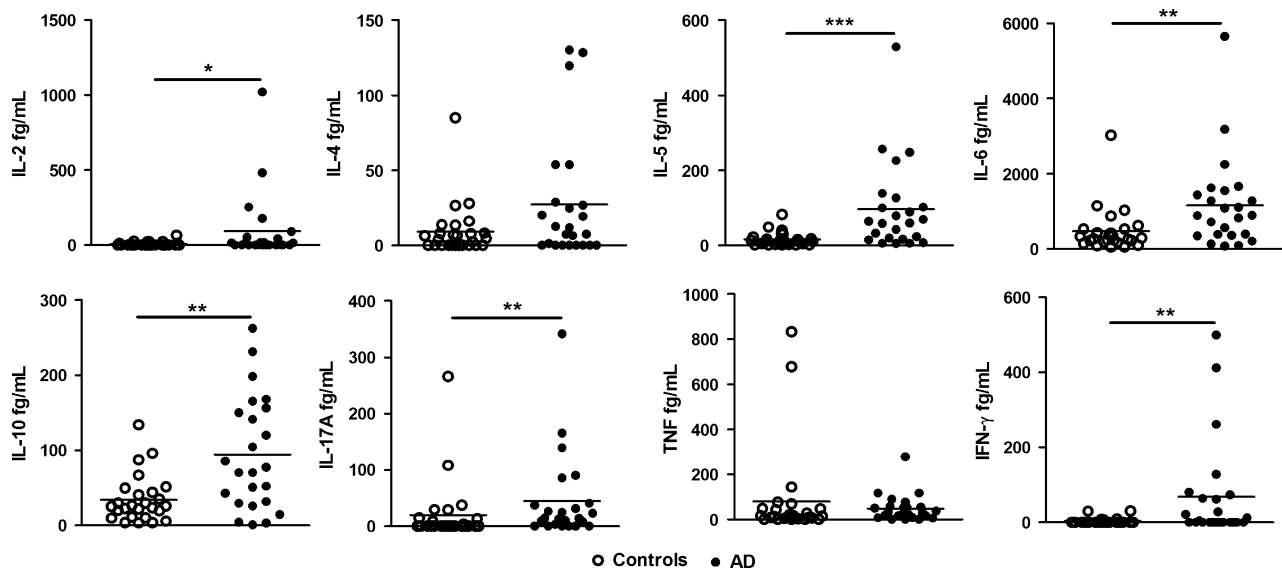


Figure 2 Circulating cytokine levels by flow cytometry. Determination of IL-2, 4, 5, 6, 10, 17A, TNF and IFN- γ serum levels from healthy controls (HC, $n = 25$), atopic dermatitis patients (AD, $n = 24$) were assessed by flow cytometry. Line represents arithmetic mean of cytokine levels expressed in fg mL⁻¹. * $P < 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

mutations and exposure to allergens and microbes. Indeed, skin barrier contributes to IgE sensitization, facilitating the inflammatory response of the atopic skin.^{17,18}

The frequent presence of *S. aureus* in atopic skin and its role in the initiation of acute flares and the perpetuation of the inflammatory chronic response remains a challenge for physicians.^{19,20} Interleukin-17 may have a high clinical impact on the pathogenesis, exacerbation and maintenance of AD. Some authors demonstrate enhanced IL-17 expression in acute, but not chronic lesions of AD skin.²¹ Other studies show an increase in Th17 cells in peripheral blood of AD patients that correlate with disease severity.²² Finally, Niebuhr *et al.*⁷ showed that α -toxins from *S. aureus* induce IL-17 production in Th17 cells from atopic individuals.

We found significantly enhanced expression of *in situ* and circulating levels of IL-17 in AD individuals. However, there was no correlation with reduced expression of FLG and CLDN-1 in AD skin. Therefore, these findings reinforce that AD pathogenesis may have different pathways, either triggered by cytokine release leading to an exacerbation in the inflammatory response, or by a reduced expression of FLG and CLDN-1 defects.

A full understanding of the complex pathogenesis of AD is necessary to discover specific biomarkers and therapeutic targets. In regard to potential AD therapy advances, studies with topical anti-inflammatory agents have shown that inflammation, especially through Th2 cytokines, reduces filaggrin expression.²³ There are reports demonstrating that IL-17A down-regulates the expression of filaggrin and genes important for cellular adhesion, which could affect epidermal barrier formation.²⁴ Therefore, interventions targeting the defective immune response, up-regulation of filaggrin expression, or protein translation process will be of significant impact in patients with barrier defects.²⁵

The reduced expression of filaggrin and claudin 1 in lesional atopic skin, and correlation of decreased filaggrin expression and disease severity strengthen the role of altered skin barrier in the pathogenesis of AD. Moreover, elevation of *in situ* interleukin-17 and of circulating interleukin levels in AD emphasize the systemic, inflammatory profile of this defective skin barrier dermatosis.

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