

Th2 Cytokines Act on S100/A11 to Downregulate Keratinocyte Differentiation

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Atopic dermatitis (AD) is an inflammatory skin disease associated with frequent skin infection and impaired skin barrier function. Recent studies indicate that increased Th2 cytokine expression contributes to reduction in antimicrobial peptides and reduced filaggrin (FLG) expression, however, the mechanisms leading to this effect is unknown. Using proteomics, we found the S100 calcium-binding protein A11 (S100/A11) to be significantly downregulated in the presence of IL-4 and IL-13. Culturing keratinocytes with increased calcium concentrations significantly induced S100/A11 expression. This corresponded with an increase in human β -defensin (HBD)-3 and FLG expression. Interference of S100/A11 expression, by siRNA, inhibited induction of HBD-3 and FLG. Furthermore p21, a cyclin-dependent kinase inhibitor downstream of S100/A11, was required for calcium-mediated induction of HBD-3 and FLG. Importantly, transduction of p21-recombinant protein into keratinocytes prevented IL-4/IL-13-mediated inhibition of FLG and HBD-3 expression. S100/A11 and p21 gene expression was also found to be significantly lower in acute and chronic AD skin. This study demonstrates an important role for S100/A11 and p21 in regulating skin barrier integrity and the innate immune response.

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that affects nearly 17% of children, and can significantly compromise their quality of life (Leung *et al.*, 2004; Zuberbier *et al.*, 2006). Management of this illness is complicated by frequent skin infections, including a propensity toward *Staphylococcus aureus* infection, eczema herpeticum, and eczema vaccinatum. Recent genetic studies have highlighted the importance of skin barrier defects as loss-of-function (null) mutations in skin barrier genes encoding filaggrin (FLG) are associated with severe AD and development of asthma in AD patients (Morar *et al.*, 2006; Palmer *et al.*, 2006; Weidinger *et al.*, 2006; Sandilands *et al.*, 2007). These observations are consistent with animal model studies of AD demonstrating that a defective skin barrier enhances allergen sensitization, leading to systemic allergic responses such as increased IgE levels and airway hyper-reactivity (Spergel *et al.*, 1998). Of note, the FLG-null mutation is observed in less than one-third of the general AD population

and most patients outgrow AD (Leung *et al.*, 2004; Palmer *et al.*, 2006). Since most AD patients have a defective skin barrier, which responds to anti-inflammatory therapy, this suggests there are non-genetic mechanisms, which modulate skin FLG expression (Sugiura *et al.*, 2005; Cork *et al.*, 2006). Indeed, we have recently observed that skin FLG expression can be downregulated by the Th2 cytokines, IL-4 and IL-13 (Howell *et al.*, 2007a).

The increased susceptibility of these patients to recurrent bacterial and viral skin infections has been linked to an acquired deficiency of antimicrobial peptides in the epidermis of AD (Ong *et al.*, 2002; Howell *et al.*, 2006a,b). Acute AD skin is characterized by the over-expression of interleukin (IL)-4 and IL-13 (Hamid *et al.*, 1994; Homey *et al.*, 2006). These Th2 cytokines are known not only to induce allergic responses, but to downregulate innate immune response genes in the skin (Nomura *et al.*, 2003a,b). The mechanism by which IL-4 and IL-13 inhibits FLG and human β -defensin expression (HBD) in keratinocytes is unknown. Our current study used a hypothesis-free proteomics approach to identify novel proteins involved in the effects of IL-4 and IL-13 on human keratinocyte differentiation. Our data identify the S100 calcium-binding protein A11 (S100/A11) as a target in Th2 cytokine-mediated inhibition of FLG and the antimicrobial peptides, HBD-3, expression in AD.

RESULTS

Proteomic identification of proteins in keratinocytes modulated by IL-4 and IL-13

Proteomics was used to investigate the effect of IL-4 and IL-13 stimulation on protein expression in the HaCaT keratinocyte

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Abbreviations: AD, atopic dermatitis; CyDye, cyanin dye; FLG, filaggrin; NHK, normal human keratinocytes; RT-PCR, reverse transcriptase-PCR; SCCA, squamous cell carcinoma antigen; siRNA, small interfering RNA

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cell line. Initial protein targets were identified using two-dimensional gel electrophoresis that incorporates differential fluorescent labeling (Unlu *et al.*, 1997). Overall, 2,627 spots were detected on the gel; 722 of which were excluded due to extremely low intensity, having a high slope (that is, dust particles), or being on the peripheral edges of the gel. Based on the initial gels, we targeted protein spots with an approximately twofold absolute difference between the two samples. Comparison of the two cell states was performed in triplicate and the percentage of protein spots with an absolute value of approximately twofold difference was consistent among the three gels.

Protein spots were picked from the gel, digested with trypsin, and subjected to peptide mass fingerprinting for protein identification. The spots chosen for excision were based on spot volume/intensity, consistency of fold change, position on the gel and proximity to other 'picked' spots (that is, to avoid overlap). Preparative gels were stained with the total protein stain Deep Purple to assess the presence of any mass shifts between labeled and unlabeled protein and to determine the feasibility of mass spectrometry-based identification. This was performed since mass shifts can occur with low molecular weight proteins and cyanine dye (CyDye) expression does not always correspond with protein abundance, respectively (Hrebicek *et al.*, 2007). Based on this selection criteria, nine protein spots were picked for identification using mass spectrometry (Figure 1a and b). Within this set of 9 spots, six resulted in statistically significant ($P < 0.01$) identifications based on a low probability value that the identified protein is a random event. Of these, a total of five unique proteins were identified from six spots on the gel (Table 1). These proteins were sorted based on the percentage of mass spectrometry "visible" sequence coverage. This is different than the total sequence coverage, in that it does not include tryptic peptides below 500 Da or above 5,000 Da as these are the mass cutoffs for data acquisition. Most low- (<500 Da) and high- (>5,000 Da) mass peptides are difficult or impossible to detect at the abundances found in gel spots due to matrix clusters dominating the low mass region of the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight spectra and a drastic drop off of sensitivity for higher masses.

IL-4 and IL-13 downregulate S100/A11

Due to the high percentage of mass spectrometry coverage (Table 1), and previous literature suggesting an important role for the S100 protein family in epidermal function (Nomura *et al.*, 2003b), we further investigated S100/A11, a member of the S100 family that has not been previously implicated in AD or cytokine modulation. Using real-time reverse transcriptase-PCR (RT-PCR) and immunoblotting, we rigorously investigated the modulation of S100/A11 gene and protein expression, respectively, by IL-4 and IL-13. HaCaT cells stimulated with IL-4 and IL-13 expressed significantly lower levels of S100/A11 mRNA (1.82 ± 0.17 ng S100/A11 per nanogram of glyceraldehyde-3-phosphate dehydrogenase (GAPDH); $P < 0.001$) as compared with cells treated with media alone (3.93 ± 0.23). Furthermore, treatment with high

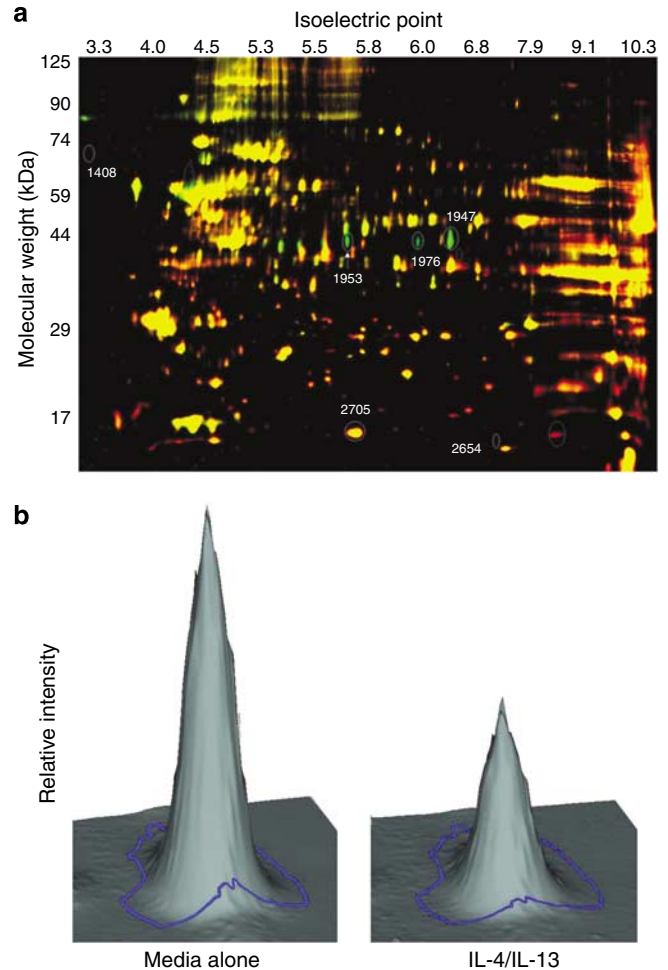


Figure 1. Proteomic analysis of keratinocytes treated with IL-4 and IL-13. (a) Proteomics. Analytical two-dimensional gels showing proteins upregulated (green) or downregulated (red) by IL-4 and IL-13 in the HaCaT keratinocyte cell line. Spot numbers compared to the "master column" in Table 1. (b) Histogram of the S100/A11 spot on the analytical gel. Spot no. 2,705 was excised from the two-dimensional gel and digested with trypsin. The digest solution was spotted with matrix and analyzed by mass spectrometry.

CaCl_2 (10 mM) significantly induced S100/A11 expression (10.04 ± 0.28 ; $P < 0.001$) in HaCaT cells as compared with lower CaCl_2 (1.5 mM) (3.93 ± 0.23). This induction was significantly inhibited by IL-4 and IL-13 (2.27 ± 0.37) (Figure 2a). This observation was confirmed at the protein level using immunoblotting (Figure 2b). HaCaT cells are an immortalized keratinocyte cell line; therefore we also investigated normal primary human keratinocytes (NHK). Similar to HaCaT cells, high CaCl_2 (1.3 mM) significantly ($P < 0.001$) induced S100/A11 gene expression in NHK (Figure 2c). This induction was similarly inhibited by IL-4 and IL-13 treatment.

Deficiency of S100/A11 in acute and chronic AD skin

To determine whether levels of S100/A11 mirrored known cytokine changes found in human allergic skin disease, we examined skin biopsies from acute AD (previously reported to be associated with increased IL-4 and IL-13) versus chronic

Table 1. Mass spectrometry data for all the significant spots with greater than twofold change on the preparative gel

Accession	Mass	Score ¹	Protein	Master ²	Volume ratio (Cy3/Cy5)	% MS "visible" peptides ³
gi 5032057	1,1847	62	S100 calcium-binding protein A11	2,705	-2.0	90
gi 7245516	9,968	105	pinLC8	2,654	-2.0	85
gi 239552	44,564	212	Squamous cell carcinoma antigen	1,947	4.5	65
gi 2118384	45,044	147	Squamous cell carcinoma antigen-2 (leupin)	1,953	5.1	60
gi 239552	44,564	138	Squamous cell carcinoma antigen	1,976	4.2	45
gi 62896815	53,580	86	Heat-shock 70-kDa protein	1,408	4.7	25

Cy, cyanin; MS, mass spectrometry.

¹Mascot search engine score based on probability implementation of the MOWSE algorithm.

²Corresponds to spot numbers in Figure 1.

³Percentage of identified peptides between 600 and 4,000 Da.

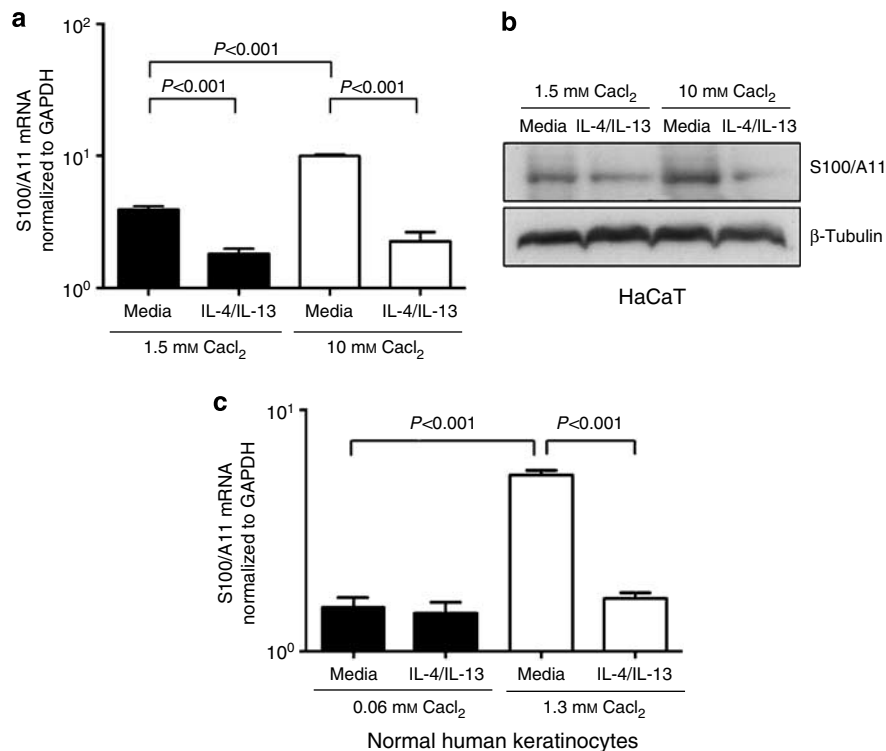


Figure 2. Calcium-induced S100/A11 expression is inhibited by IL-4 and IL-13. Keratinocytes were cultured in Ca²⁺-rich environments in the presence and absence of IL-4 (100 ng ml⁻¹) and IL-13 (100 ng ml⁻¹). S100/A11 expression was evaluated using real-time RT-PCR and western blot technology in HaCaT cells ((a) and (b), respectively) and real time RT-PCR in NHK (c). Columns and error bars represent mean ± SEM.

AD (associated with combination of Th1 and Th2 cytokines) and psoriasis (associated with increased Th1 cytokines). Using real-time RT-PCR, we found that S100/A11 gene expression is significantly decreased in acute AD (0.58 ± 0.05 ng S100/A11 per nanogram of GAPDH; $P < 0.001$) and chronic AD (1.14 ± 0.20 ; $P < 0.001$), as compared to skin from normal subjects (6.68 ± 0.51) or psoriasis patients (6.86 ± 0.46) (Figure 3a). This was confirmed at the protein level using immunostaining (Figure 3b). The composite data for S100/A11 immunostaining is shown in Figure 3c.

Role for S100/A11 in keratinocyte differentiation and host defense

S100 proteins play an important role in calcium-dependent cellular processes (Unlu *et al.*, 1997). We therefore investigated the role of S100/A11 in calcium-driven keratinocyte differentiation and the induction of the antimicrobial peptide HBD-3. In HaCaT cells, we demonstrate that increased CaCl₂ (10 mM) induces the expression of both HBD-3 (5.19 ± 0.49 ng HBD-3 per nanogram of GAPDH; $P < 0.001$) and FLG (0.015 ± 0.002 ng FLG per nanogram of GAPDH; $P < 0.001$) as compared to low CaCl₂ (1.5 mM) (HBD-3:

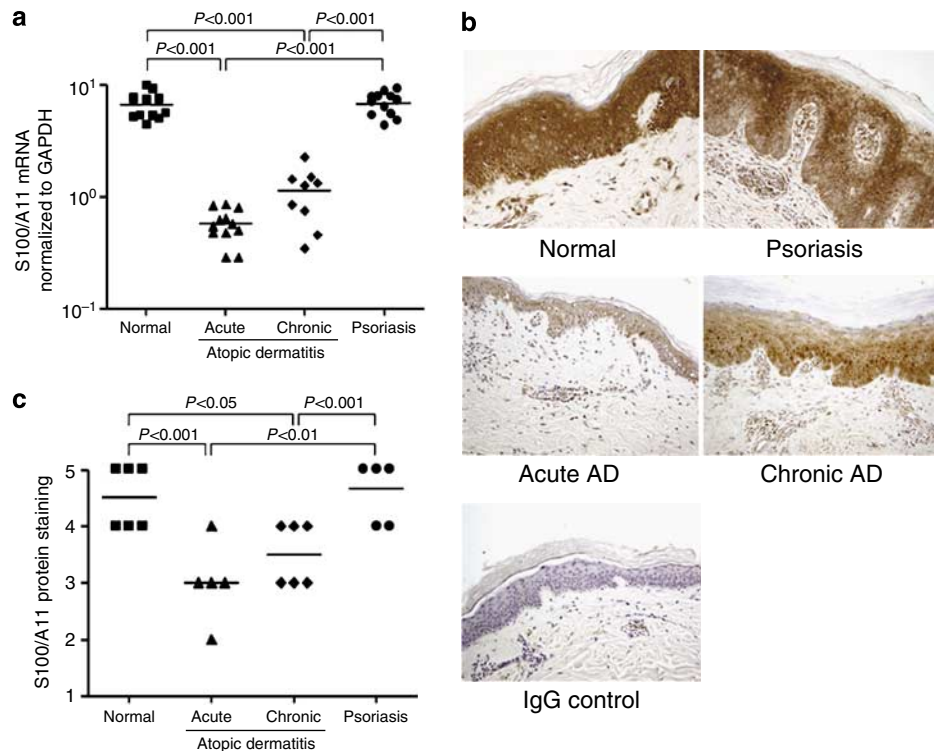


Figure 3. S100/A11 deficiency in AD skin. (a) RNA was isolated from the skin of normal subjects ($n=11$), AD patients with acute lesions ($n=12$), AD patients with chronic lesions ($n=9$), or psoriasis ($n=12$). S100/A11 gene expression was evaluated using real-time RT-PCR. (b) Representative skin sections from normal subjects ($n=6$), AD patients with acute lesions ($n=6$), AD patients with chronic lesions ($n=6$), or psoriasis ($n=5$) immuno-stained for S100/A11 are shown. Images were collected at original magnification $\times 40$. (c) The intensity of immunostaining was graded visually on a scale from 0 (no staining) to 5 (the most intense staining).

0.43 ± 0.05 ; FLG: 0.002 ± 0.0003). This induction was significantly ($P<0.001$) inhibited by the presence of IL-4 and IL-13 (Figure 4a). Similar results were observed in the more physiologically relevant NHKs (Figure 4b). Using western blots, we confirmed that IL-4 and IL-13 down-regulate FLG and the precursor, pro-FLG (Figure 4c).

To determine whether S100/A11 is the key mediator of calcium induced HBD-3 and keratinocyte differentiation, we used small interfering RNA (siRNA) to inhibit S100/A11 gene expression (Figure 5a). HaCaT cells were transfected with either control siRNA or S100/A11 siRNA, followed by incubation with low (1.5 mM) and high (10 mM) CaCl_2 . Calcium-mediated induction of HBD-3 and FLG was significantly inhibited in HaCaT cells transfected with S100/A11 siRNA (HBD-3: 0.29 ± 0.08 ng HBD-3 per ng GAPDH; $P<0.001$; FLG: 0.002 ± 0.001 ng FLG per ng GAPDH; $P<0.001$) as compared with cells transfected with control siRNA (HBD-3: 5.14 ± 0.52 ng HBD-3 per nanogram of GAPDH; FLG: 0.016 ± 0.001 ng FLG per nanogram of GAPDH) (Figure 5b). Since the expression of loricrin and involucrin have previously been shown to be inhibited by Th2 cytokines (Kim *et al.*, 2007b), we also examined expression of these two epidermal skin barrier proteins. Interestingly, loricrin and involucrin expression were not significantly modulated by the inhibition of S100/A11 (data not shown), suggesting that S100/A11 did not have a global effect on epidermal differentiation.

Requirement for p21 in cell differentiation and host defense

p21 is a downstream target of calcium and S100/A11. Furthermore, it is transcriptionally activated to induce growth arrest by halting the cell cycle in the G1 phase (Hrebicek *et al.*, 2007). Importantly, we found that p21 gene expression is significantly decreased in acute AD (0.08 ± 0.02 ng p21 per nanogram of GAPDH; $P<0.001$) and to a lesser extent chronic AD (1.13 ± 0.34) as compared to normal (5.96 ± 0.76) and psoriasis (5.67 ± 0.51) skin biopsies (Figure 6a). These data support a physiologic link between S100 and p21 in AD. To study this phenomenon in more molecular detail, we treated keratinocytes with high CaCl_2 (10 mM) and found that they were unable to induce p21 protein expression in the presence of IL-4 and IL-13 (Figure 6b). Therefore, we investigated whether p21 is an integral part of the calcium-mediated induction of HBD-3 and FLG expression.

HaCaT cells were transfected with either control siRNA or p21 siRNA and then incubated with low (1.5 mM) *versus* high (10 mM) CaCl_2 cells incubated with p21 siRNA had significant lower levels of p21 protein expression as compared with a control siRNA (Figure 7a). Despite the increase in CaCl_2 , cells transfected with p21 siRNA failed to express HBD-3 (0.33 ± 0.07 ng HBD-3 per nanogram of GAPDH) and FLG (0.001 ± 0.001 ng FLG per nanogram of GAPDH) (Figure 7b). Since p21 is downstream of S100/A11 (Figure 7a), and also necessary for the induction of HBD-3 and FLG, we

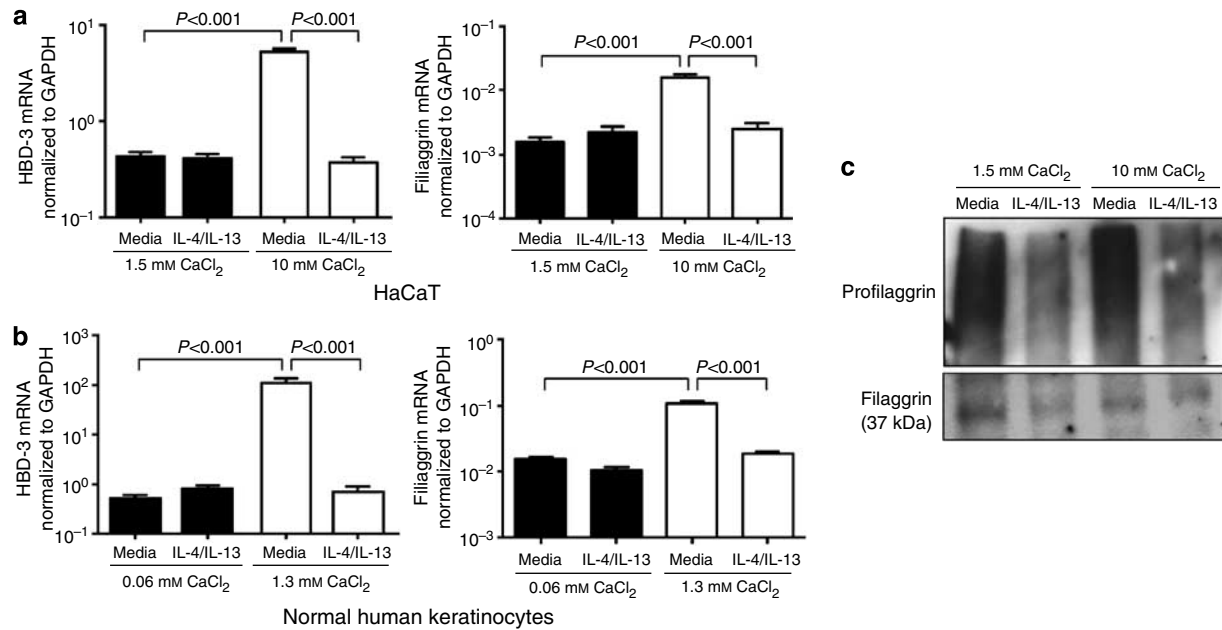


Figure 4. IL-4 and IL-13 inhibit Ca²⁺-induced HBD-3 and FLG expression. HaCaT cells (a) and NHK (b, c) were cultured in a Ca²⁺ rich environment in the presence and absence of IL-4 and IL-13. RNA (a, b) was isolated from the cells and analyzed for HBD-3 and FLG using real-time RT-PCR. Columns and error bars represent the mean $\pm$ SEM. Protein was isolated and then analyzed by Western blot analysis (c) for proFLG and FLG.

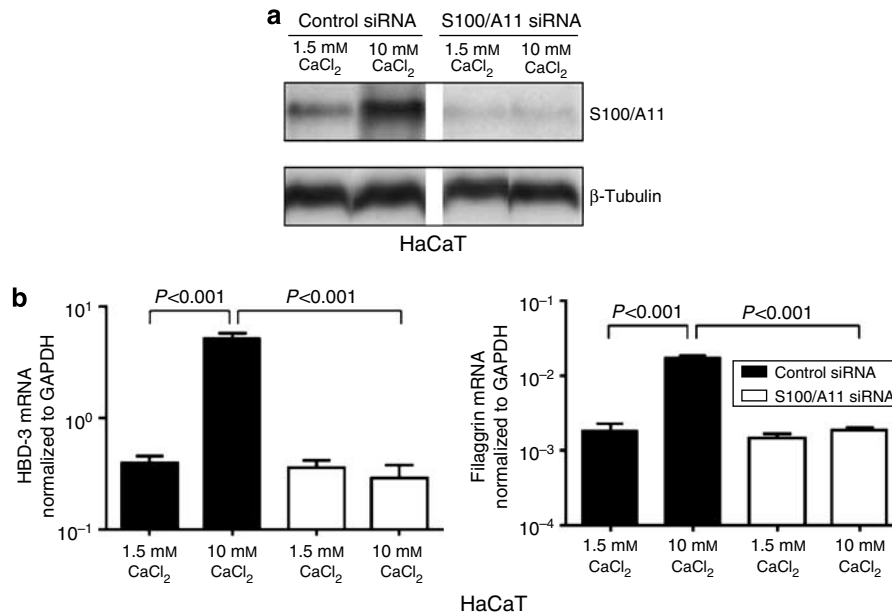


Figure 5. S100/A11 is required for calcium-induced HBD-3 and FLG expression. Keratinocytes were cultured in Ca²⁺-rich environments in the presence and absence of S100/A11 siRNA. (a) S100/A11 protein expression was evaluated using western blot. Results of a representative experiment ($n = 4$) are shown. (b) HBD-3 and FLG gene expression were analyzed by real time RT-PCR. Columns and error bars represent mean $\pm$ SEM.

investigated whether cells over expressing p21 protein could induce HBD-3 and FLG in the presence of IL-4 and IL-13. A recombinant p21 protein was delivered to HaCaT cells using a BioTrek protein delivery kit (Figure 8a). Cells were then cultured in the presence and absence of IL-4 and IL-13 for 24 hours. Delivery of p21 recombinant protein to keratinocytes

significantly induced HBD-3 (7.15 ± 1.20 ng HBD-3 per nanogram of GAPDH; $P < 0.001$) and FLG (0.017 ± 0.002 ng FLG per nanogram of GAPDH) expression as compared to cells, which had a mock protein (HBD-3; 0.40 ± 0.09 , FLG; 0.001 ± 0.001). In Figure 5b, we show that IL-4 and IL-13 are able to inhibit the induction of HBD-3 and FLG. However,

delivery of p21 recombinant protein to HaCaT cells prevented the ability of IL-4 and IL-13 to inhibit HBD-3 and FLG expression (Figure 8b).

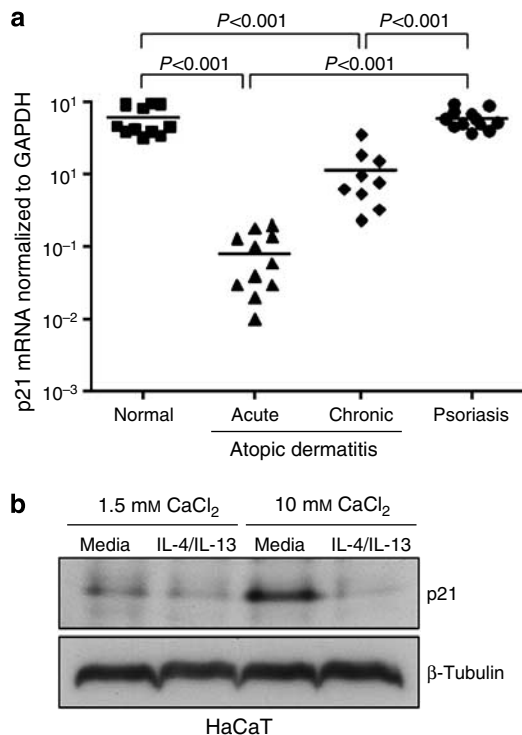


Figure 6. p21 is induced by calcium, but deficient in AD skin. (a) RNA was isolated from skin biopsies of normal subjects ($n = 11$), AD patients with acute lesions ($n = 12$), AD patients with chronic lesions ($n = 9$), or psoriasis ($n = 12$). p21 gene expression was evaluated using real-time RT-PCR. (b) Keratinocytes were cultured in Ca²⁺-rich environments in the presence and absence of IL-4 (100 ng ml⁻¹) and IL-13 (100 ng ml⁻¹). Protein expression was evaluated by western blot. Results of a representative experiment ($n = 4$) are shown.

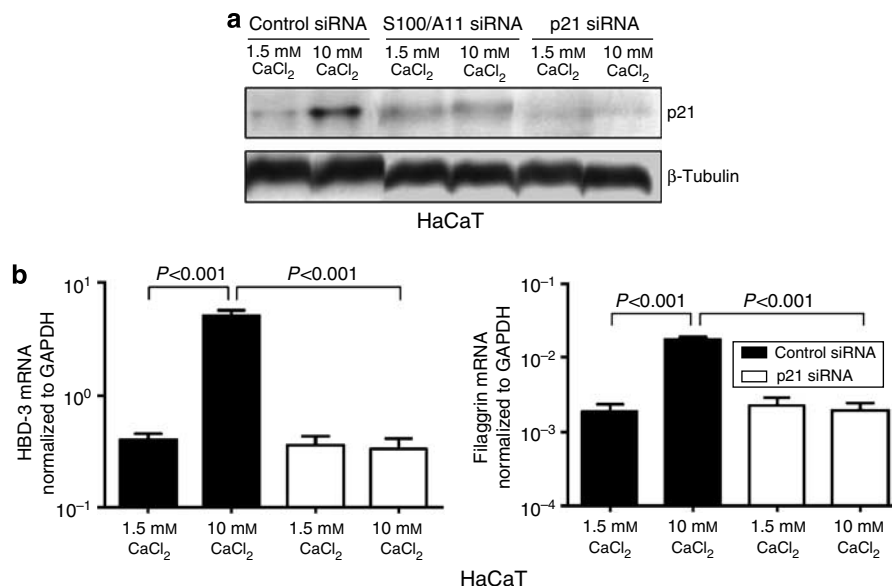


Figure 7. Requirement of p21 for calcium-induced HBD-3 and FLG expression. Keratinocytes were cultured in Ca²⁺-rich environments in the presence and absence of S100/A11 siRNA or p21 siRNA. (a) p21 protein expression was evaluated by western blot. Results of a representative experiment ($n = 4$) are shown. (b) HBD-3 and FLG gene expression were analyzed by real time RT-PCR. Columns and error bars represent the mean $\pm$ SEM.

DISCUSSION

The epidermal skin barrier serves as the first line of defense against invading pathogens and allergens. The skin of AD patients is characterized by significant barrier disruption, which increases susceptibility to allergic sensitization, as well as recurrent bacterial and viral skin infections (Leung *et al.*, 2004; Cork *et al.*, 2006). Recent studies have shown that the Th2 cytokine milieu in AD skin plays an important role in downregulating skin barrier function and host defense (Ong *et al.*, 2002; Howell *et al.*, 2006a,b, 2007a,b). The mechanisms, however, underlying these observations are unknown. Therefore, the current study used a hypothesis-free approach to investigate the global effects of IL-4 and IL-13 on protein expression in keratinocytes. Using proteomic technology, we focused our attention on proteins, which had a greater than two-fold absolute change in expression. As shown in Table 1, significant increases in protein levels were observed for squamous cell carcinoma antigen (SCCA), SCCA2 (leupin), and heat-shock protein 70 kDa, whereas decreased protein levels were found for PIN/LC8, and S100 calcium-binding protein A11 (S100/A11).

SCCA1 (SERPINB3) and SCCA2 (SERPINB4) are members of the ovalbumin-serpin proteinase inhibitor family (B clade) that fold into unique structures to inhibit serine and cysteine proteinases (Silverman *et al.*, 2001). These proteins have previously been shown to be upregulated in the skin of AD patients (Mitsuishi *et al.*, 2005) and serum of acute asthmatics (Nishi *et al.*, 2005). Consistent with our current observations, IL-4 and IL-13 have been previously reported to induce SCCA expression in keratinocytes (Mitsuishi *et al.*, 2005). Heat-shock proteins are induced by environmental (that is, heat shock) and physiological (that is, inflammation) stress in order to protect cells. These proteins are constitutively expressed in normal skin and serve as chaperones by stabilizing unfolded proteins for transport across cellular membranes (Schlesinger,

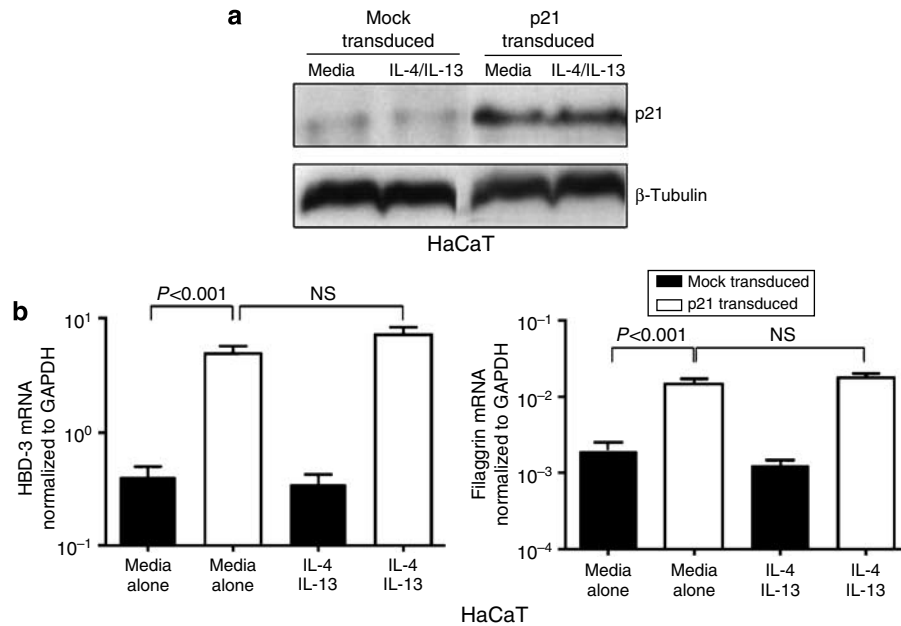


Figure 8. Recombinant p21 protein induces HBD-3 and FLG. Recombinant p21 protein was transduced into keratinocytes cultured in standard Ca^{2+} conditions. (a) p21 protein overexpression was confirmed by western blot. Results of a representative experiment ($n=4$) are shown. (b) HBD-3 and FLG gene expression were analyzed by real time RT-PCR. Columns and error bars represent mean $\pm$ SEM.

1990; Maytin, 1995). Heat-shock protein-70 proteins have also been previously reported to have increased expression in the skin of AD patients with highest expression observed in those individuals with severe AD (Ghoreishi *et al.*, 2000). PIN/LC8 is an 8-kDa dynein light chain that plays a role in intracellular protein trafficking (Liang *et al.*, 1999). McCauley *et al.* (2007) recently demonstrated that PIN/LC8 binds to neuronal nitric oxide synthase and inhibits nitric oxide production in mast cells. Additionally, it was suggested that this interference is responsible for blocking leukotriene production. On the basis of lack of novelty and their likelihood of a functional role in AD, we chose not to pursue the role of SCCA, SCCA2 (leupin), heat-shock protein-70, and PIN/LC8 as targets for Th2 cytokine inhibition of HBD-3 and FLG.

S100/A11 (calgizzarin) is a member of the EF-hand Ca^{2+} -binding family that is activated by increased Ca^{2+} concentrations and interferes with Ca^{2+} -signaling pathways by binding to and regulating target proteins (Unlu *et al.*, 1997). Calcium-sensor target proteins have been identified, which impact protein phosphorylation, cell growth, motility, and cell survival (Unlu *et al.*, 1997; Hrebicek *et al.*, 2007). Robinson *et al.* (1997) initially identified S100/A11 as an important component of the cornified envelope in the skin. The epidermal barrier, including the cornified envelope, serves as the first line of defense against invading pathogens and allergens. Recently there has been an interest in understanding the role of the epidermal differentiation complex in AD. S100/A11 is encoded within the same region of the epidermal differentiation complex on chromosome 1q21 (Eckert *et al.*, 2004). Combined with our proteomic data, which confidently (90% sequence matching) showed that IL-4 and IL-13 down regulates S100/A11

expression, we therefore investigated the role of S100/A11 on skin barrier integrity and innate host defense.

To our knowledge, this is the first demonstration that S100/A11 is down-regulated by the Th2 cytokines, IL-4 and IL-13. Additionally, we demonstrate that S100/A11 is decreased in the skin of AD patients. Since S100/A11 is an important component of the epidermal differentiation complex, this may explain, in part, the significant acquired barrier dysfunction observed in AD skin. To further understand the role of S100/A11, we investigated the immunomodulatory effect of S100/A11 on FLG and HBD-3. These proteins have been shown to be integral in maintaining an intact skin barrier (Howell *et al.*, 2007a) and protecting against invading pathogens due to potent anti-viral and anti-bacterial activity (Harder *et al.*, 2001; Howell *et al.*, 2007b), respectively. Therefore, we first demonstrated that culturing NHK and the immortalized HaCaT keratinocyte cell line in the presence of increased calcium significantly induced S100/A11, FLG and HBD-3 expression. Inhibiting S100/A11 gene transcription using siRNA, however, prevented the Ca^{2+} -mediated induction of FLG and HBD-3. This observation is significant since FLG deficiency has been recently linked to severe AD (Palmer *et al.*, 2006; Weidinger *et al.*, 2006; Sandilands *et al.*, 2007) and progression of the atopic march leading to the development of asthma. Additionally, the down-regulation of HBD-3 increases the susceptibility of individuals to recurrent skin infections with bacterial and viral pathogens (Harder *et al.*, 2001; Howell *et al.*, 2007b).

S100 proteins bind to target proteins to regulate Ca^{2+} -signaling pathways. p21 (WAF1) is a cyclin-dependent kinase inhibitor downstream of S100/A11 that is involved in the cell growth and differentiation (Weinberg and Denning, 2002; Sakaguchi *et al.*, 2005). Therefore, we further investigated the

role of p21 in our experimental system. We first investigated the relationship between p21 and S100/A11. While increased Ca^{2+} concentrations induced p21 protein expression, this was inhibited by interfering with S100/A11 using siRNA. Similarly, the addition of IL-4 and IL-13 inhibited p21 expression in keratinocytes cultured in high Ca^{2+} concentrations. As a result, we investigated the effects of p21 on FLG and HBD-3. To do this, we inhibited p21 mRNA using siRNA and then cultured keratinocytes in the presence of increased Ca^{2+} to induce FLG and HBD-3. Ca^{2+} mediated induction of FLG and HBD-3 was inhibited by interfering with p21 expression, suggesting an important role for p21 in the regulation of the barrier integrity and host defense. This observation was further supported by transducing keratinocytes with recombinant p21 protein and demonstrating increased FLG and HBD-3 expression without the addition of high Ca^{2+} concentrations. Additionally, IL-4 and IL-13 failed to inhibit the induction of FLG and HBD-3 in p21 protein-transduced keratinocytes.

Our current study therefore demonstrates that S100/A11 and p21 are decreased in the skin of AD patients and that IL-4 and IL-13, previously reported to be increased in AD, can downregulate S100/A11 and p21. Furthermore, we demonstrate that S100/A11 and p21 play an important role in regulating the expression of FLG and HBD-3. This may contribute to the significant barrier disruption and AMP deficiency observed in the skin of AD patients. Importantly, it identifies novel targets to enhance keratinocyte differentiation and skin innate immune responses in this common skin disease.

MATERIALS AND METHODS

Subjects

The subjects included 12 healthy persons with no history of skin disease (mean age: 29.8 ± 3.8 years), 21 patients with mild-to-moderate AD (mean age: 34.7 ± 3.4 years), and 12 patients with psoriasis (mean age: 52.1 ± 4.8 years). None of the patients had received systemic corticosteroids or cyclosporine previously, and none had received topical corticosteroid or calcineurin inhibitors for a period of at least 1 week before enrollment. These studies were conducted according to the Declaration of the Helsinki Principles and were approved by the institutional review board at National Jewish Medical and Research Center in Denver and all subjects gave written informed consent prior to participation in these studies.

Two-millimeter punch biopsies were collected from the acute lesional skin of 12 AD subjects, chronic lesional skin of nine AD patients, lesional skin of 12 psoriasis subjects, and healthy skin from 12 normal subjects. Skin samples were submerged immediately in either Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) or 10% buffered formalin for real-time RT-PCR and immunohistochemical studies, respectively.

Keratinocyte cultures

The HaCaT human keratinocyte cell line was grown in Dulbecco's modified Eagle's Medium (Cellgro, Herndon, VA) as previously described (Howell *et al.*, 2006a). Cells were seeded at 2×10^5 cells ml^{-1} and allowed to adhere for 18–24 hours prior to stimulation. HaCaT cells were then stimulated with 100 ng ml^{-1} of

IL-4 (R&D Systems, Minneapolis, MN) and 100 ng ml^{-1} of IL-13 (R&D Systems) for 24 hours. Following the initial 24-h incubation, the concentration of calcium chloride (CaCl_2) was increased to 10 mM, cytokines were refreshed, and cells were cultured for an additional 24 hours.

Primary keratinocyte cell cultures (NHK) were obtained from Cascade Biologics (Portland, OR) and maintained in serum free EpiLife Medium (Cascade Biologics) containing 0.06 mM CaCl_2 as previously described (Kim *et al.*, 2007a). In some experiments, NHK cells were differentiated in 1.3 mM CaCl_2 and stimulated with 100 ng ml^{-1} of IL-4 (R&D Systems) and 100 ng ml^{-1} of IL-13 (R&D Systems) for 5 days.

Proteomics

The differential in gel electrophoresis was used to determine the protein composition of human keratinocytes. Proteins were precipitated from the cell culture lysate using a methanol/chloroform extraction method previously described (Wessel and Flugge, 1984). Precipitated protein pellets were then dissolved overnight in 7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate at room temperature. The final protein concentration was determined using Bradford method (Bradford, 1976).

Differential in gel electrophoresis covalent labeling with CyDyes

For each sample, 50 mg of total protein was covalently modified with one of the CyDyes (Amersham Biosciences, Piscataway, NJ). The pH was adjusted by addition of pH 8.5 Tris to a final concentration of 30 mM. The CyDye (1 μl , 200 pmol μl^{-1} in anhydrous dimethylformamide (Sigma-Aldrich, St Louis, MO)) was added to the protein and the reaction allowed to proceed (30 minutes, 4 °C, in the dark). Under these conditions approximately 1% of the lysine residues of the protein sample are covalently conjugated to the CyDye. The reaction was then quenched by the addition of 10 mM of lysine. After the dye reactions were complete, the labeled samples were combined to one tube and an additional 450 μg of unlabeled protein from each of the two samples (1,000 μg total) was added to facilitate protein identifications after electrophoresis. Pharmalytes (Amersham Biosciences) and saturated aqueous bromophenol blue (1 μl) were added. The samples were brought to a final volume of 450 μl with a composition of 7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 2% broad range Pharmalytes (3–10 NL) and 2% dithiothreitol.

Two-dimensional gel electrophoresis. After resuspension in the rehydration buffer, protein samples were passively rehydrated into 24-cm immobilized pH gradient strips (IPG 3–10 NL; Amersham Biosciences) for 15 hours and then focused (IPGPhor system; Amersham Biosciences) for at least 133,000 volt-hours. Two-dimensional gel electrophoresis was carried out as previously described (McDermott *et al.*, 2007).

Scanning of differential in gel electrophoresis-labeled images. Gels were scanned on a Typhoon 9400 Variable Mode Laser Imager (Amersham Biosciences) set to 100- μm resolution. Laser and filter settings were as follows: Cy3, excitation 532 nm,

emission 580 nm, bandpass 30 nm; Cy5, excitation 633 nm, emission 670 nm, bandpass 30 nm; and Cy2, excitation 488 nm, emission 582 nm, bandpass 40 nm. The preparative gel used for protein spot excision was stained with Deep Purple Dye (Amersham Biosciences) to assess any mass shift between CyDye-labeled and unlabeled protein that occurs at low molecular weight.

Image analysis. DeCyder software (v. 6.5; Amersham Biosciences) was used for spot detection and relative quantification of proteins based on the fluorescent images. Peak volumes were measured for each protein spot in the three fluorescent channels (Cy3, Cy5 and Cy2). Volume ratios were calculated to directly compare volumes of each protein spot between the two directly compared samples. Proteins to be excised and identified were selected using the DeCyder Differential In-gel Analysis Module if the volume ratio difference was >2.0 .

Spot selection and enzymatic digestion. Protein spot excision and in-gel enzymatic digestion were performed automatically by the Ettan Spot Handling Workstation (Amersham Biosciences) as described previously (McDermott *et al.*, 2007).

Protein identification by mass spectrometry. All digests were analyzed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight MS (Voyager DE-STR; Applied Biosystems, Foster City, CA). After trypsin digestion, an aliquot (0.7 μ l) of each sample was deposited on a standard stainless steel 100-well Matrix-Assisted Laser Desorption target (Applied Biosystems). Agilent a-Cyano-4-hydroxycinnamic acid solution was used as the matrix (Agilent, Palo Alto, CA) and added to the sample on target (0.7 μ l). Spectra were collected over the range m/z 500–5,000 in positive ion mode using the instruments full reflector flight path. No cleanup of digests was performed. Peptide mass fingerprints were internally calibrated to monoisotopic trypsin autolysis peaks ($m/z=515.33$, 842.51, 1,045.56, 2,211.11). Spectra were processed using ProTS Data (Efecta Technologies, Steamboat Springs, CO) to generate centroided peak lists that were submitted to Mascot (Matrix Sciences, Boston, MA) for database searching. Spectral processing included defining baseline, noise, and signal-to-noise ratio as well as monoisotopic peak selection. A signal-to-noise ratio in ProTS data of >4 was required for inclusion in the peak list. Database searches were conducted using a human subset (153,190 sequences) of the non-redundant protein database (NCBI, database version 11/04/2006 with 4,099,826 sequences and 1,413,990,101 residues). Other settings in ProTS included peak amplitude set at 100, peak width 250, and chemical noise factor 1.5. Key Mascot parameters included the following: peptide error tolerance was set at ± 30 p.p.m., fixed modification of carbamidomethylation of cysteine side chains, and trypsin selected as the enzyme for peptide cleavage with one missed cleavage accepted. Searches were not constrained by pI or molecular weight. Tandem mass spectrometry was performed on a LC/MSD XCT ion-trap mass spectrometer (Agilent). A total of 5 μ l of digest solution was injected onto a c18 trapping column. The LC system (Agilent nanoflow 1100) was used to elute peptides off of the trap and through a heated 75- μ m c18 column with a gradient of increasing acetonitrile. MS/MS spectra were collected in a software-driven data-dependent analysis. Spectra were processed and analyzed using Spectrum Mill (Agilent).

RNA isolation and analysis

Total RNA was isolated from 2-mm skin biopsy samples using Tri-Reagent according to the manufacturer's guidelines (Molecular Research Center Inc.). RNeasy Mini Kits (Qiagen, Valencia, CA) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to further purify RNA from skin biopsies. RNA was reverse transcribed into cDNA and analyzed by real-time RT-PCR as previously described (Nomura *et al.*, 2003b). Primers and probes for GAPDH, S100(A11), p21, involucrin, loricrin, and FLG were purchased from Applied Biosystems. HBD-3 primers and probes were prepared as previously described (Nomura *et al.*, 2003b). Relative expression levels were determined as outlined in the manufacturer's technical bulletin (Applied Biosystems). To allow for comparisons between samples and groups, target gene expression was normalized to the corresponding GAPDH levels.

Transient and stable transfectants

HaCat cells were transiently transfected at 60% confluency with 100 nM of S100/A11 (Dharmacon, Lafayette, CO), p21 siRNA (AM51326; Ambion Inc., Austin, TX), or control siRNA (4611; Ambion, Inc.), using Lipofectamine 2000 according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA). The sequence targeting human S100/A11 was as follows: sense, 5'-CAACAGUG AUGGUCAGCU AUUtt-3'; antisense, 5'-UAGCUGACCAUCACUG UUGUtt-3'. The sequence targeting human p21 was as follows: sense, 5'-GGAGUCAGACAUUUUAAGAtt-3'; antisense, 5'-UCUUA AAAUGUCUGACUCtt-3'. In order to estimate the efficiency of transfection, siRNA was labeled with Cy3 on the 5' end of the antisense sequence.

The recombinant p21 protein was introduced into cells using a BioTrek Protein Delivery kit (204140; Stratagene, La Jolla, CA). The p21 (908-281; Assay Designs, Ann Arbor, MI) protein and an FITC-antibody control protein were delivered into HaCaT cells (60% confluent) according to the manufacturer's guidelines (Stratagene). Four hours following protein delivery, the media was changed and the delivery efficiency was determined using fluorescent imaging. The protein delivery efficiency was approximately 80% for each experiment. The p21-transfected cells were then treated with low calcium plus or minus IL-4 and IL-13 and harvested after 24 hours for immunoblotting and real-time analysis.

Western blot analysis

Protein extracts were prepared by lysing the cells in radioimmuno-precipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) containing protease and phosphatase inhibitors. Proteins were electrophoresed using NuPage and transferred to a polyvinylidene fluoride membrane. Membranes were then blocked with 5% non-fat milk and probed with antibodies against anti-p21 (sc-817; Santa Cruz Biotechnology, Santa Cruz, CA), anti-S100A11 (BC001410; Proteintech Group Inc., Chicago, IL), and anti- β -tubulin (32-2600; Zymed, San Francisco, CA). Membranes were incubated with secondary Ig antibodies conjugated to horseradish peroxidase and developed using the enhanced chemiluminescence plus detection system (Amersham Biosciences, Little Chalfont Buckinghamshire, England, UK) followed by autoradiography.

To visualize proFLG and FLG, 20 μ g of total proteins were resolved under reducing conditions by 10% of SDS-polyacrylamide

gel (Bio-Rad, Hercules, CA) electrophoresis and transferred to reinforced nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline at 4 °C for overnight. After blocking the membrane was incubated for 2 hours at room temperature with anti-FLG mouse monoclonal antibody diluted 1:100 (Vector Laboratories Inc., Burlingame, CA) in 1% BSA/0.06% Tween 20 in Tris-buffered saline. The membrane was washed three times using Tris-buffered saline with 0.2% Tween 20 for 10 minutes each and then incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) at dilution of 1:10,000 in 1% bovine serum albumin/0.06% Tween 20 in Tris-buffered saline for 1 hour at room temperature. The membrane was washed three times for 10 minutes each using Tris-buffered saline with 0.2% Tween 20 and then developed with enhanced chemiluminescence Western Blotting Detection Reagents (Amersham Biosciences) according to the manufacturer's protocol.

Immunohistochemistry

Two-millimeter skin biopsies were paraffin embedded and cut into 5- μ m sections. Tissue samples were deparaffinized through a series of ethanol washes and then incubated with either a polyclonal rabbit anti-S100A11 (BC001410; Proteintech Group Inc.) antibody overnight. The cell and tissue staining kit for mouse and rabbit primary antibodies (R&D Systems) was used according to the manufacturer's protocol. Antibody specificity was confirmed using purified non-immune mouse or rabbit IgG (Southern Biotechnology, Birmingham, AL). All slides were coded prior to analysis and read blindly, to ensure patient anonymity. The intensity of the immunostaining was graded with the use of microscopy on a scale from 0 to 5, with 0 indicating no staining and 5 the most intense staining.

Statistical analysis

Statistical analysis was conducted using Graph Pad Prism, version 4.03 (San Diego, CA). Data were analyzed by a one-way analysis of variance and significant differences were determined by Tukey–Kramer test (Tukey, 1977). Differences were considered significant at $P \leq 0.05$. A minimum of three independent experiments were conducted to allow for statistical comparison.

For statistical analysis of the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass fingerprint results Mascot, version 2.1 (Matrix Sciences) was used to calculate a probability-based score. This software uses a probability-based implementation of the Mowse algorithm (Pappin *et al.*, 1993). The Mascot score is the absolute probability that the observed match is a random event and is reported as $-10\log_{10}(P)$, where P is absolute probability.

DeCyder, version 6.0 (GE Healthcare), differential analysis software, was used for automatic spot detection (co-detection algorithm), background subtraction, quantitation, normalization, and inter-gel matching of the fluorescent images. An internal standard, which consisted of pools of treated and untreated samples, was combined and labeled with Cy2. This internal standard was used to calculate standardized volume ratios using the biological variation analysis Module. Protein spot volume ratios were compared using a Student's *t*-test (two-tailed) in the biological variation analysis module.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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