Redistribution of the nuclear protein IFI16 into the cytoplasm of ultraviolet B-exposed keratinocytes as a mechanism of autoantigen processing.

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Bulleted statements: In peripheral tissues, as in the skin of SLE patients, a potentially crucial role in the autoimmune reaction cascade has been attributed to UV-induced cell death. In this study, we demonstrated that the interferon-inducible protein IFI16, an autoantigen normally detected in the nucleus of human keratinocytes, can relocate to the cytoplasm under conditions of UV-induced cell injury. This translocation was also observed in the diseased skin sections of SLE patients.

Summary

Background The skin has long been recognized as a prominent target tissue in Systemic Lupus Erythematosus (SLE) which plays a crucial role in the initiation and perpetuation of the autoimmune reaction cascade as a consequence of UV-induced keratinocyte apoptosis. Antibodies against the interferon-inducible protein IFI16 have been detected in SLE patients sera.

Objectives To verify whether the induction of autoimmunity against IFI16 involves redistribution of this nuclear protein in keratinocytes during UVB-induced cell death.

Methods In this study, an in vitro epidermal model was developed to investigate the fate of the IFI16 protein in keratinocytes after irradiation with UVB; both keratinocyte monolayers and human skin explants were used. IFI16 expression and localization was also analyzed in diseased skin sections of SLE patients.

Results We demonstrated that IFI16, normally restricted in the nucleus, can be induced to appear in the cytoplasm under conditions of UVB-induced cell injury. This nucleus to cytoplasm translocation was also observed in skin explants exposed to UVB and in the diseased skin sections of SLE patients. In addition, IFI16 was found in the supernatants of UVB-exposed keratinocytes.

Conclusions The finding that IFI16 is present in the cytoplasm of diseased skin cells from SLE patients, together with the demonstration of IFI16 in the supernatants of UVB-exposed keratinocytes, suggest that UVB irradiation or other stimuli may favour an abnormal IFI16 presentation to the afferent limb of the immune system and potentially an autoimmune response against the protein itself.

Introduction

Systemic Lupus Erythematosus (SLE) is the prototype of a systemic autoimmune disease, characterized by the production of autoantibodies[1] mainly directed against ubiquitous nuclear targets. The skin has long been recognized as a prominent target tissue in SLE. Skin lesions are present in at least 80% of patients and constitute the primary sign in about 25% of these individuals[2,3]. Following the delivery of UVB irradiation to cultured keratinocytes, the early enrichment (8h) of lupus autoantigens, including SS-A/Ro, SS-B/La, snRNP and Sm, is observed in apoptotic surface blebs on the cell surface of the cultured keratinocytes. This finding has led to the notion that the surface blebs of apoptotic cells are important immunogenic particles in lupus[4,5]. To date, the mechanisms leading to the loss of self tolerance in SLE are still matter of research.

The IFI16 gene that encodes for a nuclear phosphoprotein was originally identified as a target of interferons[6,7]. IFI16 protein is specifically expressed in vascular endothelial cells, keratinocytes and hematopoietic cells[8]. We have previously demonstrated that oxidative stress and various proinflammatory cytokines (e.g. TNF-alpha and IL-1beta) can also trigger IFI16 expression[9,10]. In addition a role of IFI16 as inducer of proinflammatory molecules (e.g. ICAM1, RANTES and CCL20) in endothelial cells has also been observed, supporting its role in the initial steps of the inflammatory processes that precede the onset of autoimmune syndromes[11,12]. IFI16 protein is also a target for autoantibodies. We showed that anti-IFI16 autoantibodies are present in 26% of SLE, 50% of SjS, 21% of SSc patients and 5% of healthy controls[10,13].

Regarding the pathogenic mechanism of IFI16, we hypothesize that its overexpression and extranuclear appearance during cell death contributes to its release into the extracellular milieu and eventually to the induction of specific autoimmunity. To test this hypothesis, we have established an *in vitro* epidermal model to investigate the fate of the IFI16 protein in keratinocytes after UVB irradiation. In the current study, we find that the IFI16 protein, the expression of which is normally restricted to the

nucleus, can be stained in the cytoplasm of keratinocytes exposed to UVB irradiation. This nucleus to cytoplasm translocation is also observed in skin explants exposed to UVB irradiation and in the diseased skin sections of SLE patients. IFI16 protein was also found in the supernatants of UVB-exposed keratinocytes, providing evidence of the extracellular release of the protein under such conditions.

Materials and methods

Human skin explants

Human split skin samples (0.3–0.5 cm thick) were obtained from the chest of 8 healthy individuals undergoing surgery at Ospedale Maggiore della Carità (Novara). After excision, each split skin sample was divided into 4 equal pieces and kept in Petri dishes containing phosphate-buffered saline (PBS). Epidermal sides were set overnight at the air–liquid interface in DMEM Sigma (Immunochemical, Milan, Italy) at 37° C in a humidified 5% CO_2 atmosphere.

SLE biopsies

Skin biopsies, taken from clinically active lesions of 12 SLE patients (classified according to the classification system of Hoechberg et al.[14]), and 5 corresponding sera were obtained from Fondazione Policlinico, Mangiagalli e Regina Elena (Milano). In all cases, patients had given informed consent before skin biopsy was removed. The patients were all Caucasian females aged between 17 and 70 years old (mean age: 48 years). Biopsies were all taken for diagnostic purposes in stages of active skin disease and came from photoexposed areas, and patients were not taking immunosuppressive therapy. Five skin biopsies (females aged between 23 and 61 years old) were taken from cosmetic surgical interventions of normal skin. All skin samples (explants and biopsies) were formalin-fixed and paraffin-embedded. Forty-five sera from SLE patients were obtained

by the Division of Rheumathology, Isituto G. Pini, Milan. Controlos sera were obtained from 50 sex- and age-matched healthy individuals.

This study was conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from all the patients.

Cell Cultures and treatments

Primary human foreskin keratinocytes (HFKs) obtained from normal human foreskins pooled from three to five donors, were obtained from Lonza Verviers SPRL (Verviers, Belgium) and cultured as previously described. Cells used in irradiation experiments were subconfluent and in an exponential growth phase.

Doxorubicin and etoposide (VP-16) were purchased from Sigma (Immunochemical, Milan, Italy).

All UV irradiations were carried out in PBS. UV radiation was provided by a UVB lamp, HD 9021 (Delta OHM SRL, Padova, Italy), which emits most of energy within the UVB range (280-315nm), with an emission peak at 312 nm. Irradiation intensity was monitored by a UVB irradiance meter cosine corrector with spectral range 280-319 nm, LP 9021 RAD (Delta OHM SRL). Mock UVB-irradiated controls were treated in an identical manner except that the UVB lamp was turned off. Doses of UVB were chosen based on the World Health Organization guidelines for sun exposure[15]. Following irradiation with the required UVB dose, cells and skin explants were incubated in complete medium for the required time period at 37° C in a humidified 5% CO_2 atmosphere. Cells were fixed in PBS with 2% paraformaldehyde and skin explants in 10% neutral buffered formalin.

Immunohistochemistry

Sections (5 µm thick) were processed as previously described[8]. As negative controls, appropriate slides were incubated with PBS instead of primary antibodies and underwent the same staining procedure.

Immunofluorescence microscopy

Cells grown on glass slides were fixed in paraformaldehyde and then permeabilized Triton-X100 in PBS. After blocking, coverslips were incubated with rabbit polyclonal anti-human-C-terminal IFI16 antibodies[8], and then with a fluorescein-isothiocyanate (FITC)-labelled secondary antibody (Alexa Fluor 488, Molecular Probes, Oregon USA) in the dark at RT and then counterstained with propidium iodide (PI). Immunofluorescence was observed using a confocal laser scanning microscope (Leica Microsystems, Milan, Italy).

Skin explants were processed using a double immunostaining reaction by combining anti-IFI16 and anti-Pospho-Stat3 antibodies (Cell Signaling Technology Inc. Pero, Italy). After antigen unmasking by microwaving, tissues were incubated with a mixture of the two primary antibodies (anti-IFI16 and anti-Phospho-Stat3). Afterwards, slides were incubated with a mixture of the two secondary antibodies, respectively labelled with Alexa Fluor 488 and Tx Red (Molecular Probes, Oregon USA). Images were processed using a Leica fluorescence microscope (Leica Microsystems, Milan, Italy) equipped with a digital camera.

Western Blot Analysis

Cells were lysed as previously described[16]. Collected supernatants were concentrated with 25% TCA. The following antibodies were used for immunoblotting analysis: anti-IFI16 antibody (clone 1G7, Santa Cruz, CA, USA), anti-β-actin antibody (clone AC-15, Sigma, Milan, Italy), anti-PARP cleaved antibody (clone GTX24830, GeneTex, CA, USA).

Lactate dehydrogenase (LDH) leakage assay

The assay was performed using the Promega CytoTox-ONE^{TM} assay (Promega, Madison, WI, USA). HFK cells were seeded in 96-well plates in 100 µl complete medium (20,000 cells/well) and used for experiments performed in triplicate 24h after seeding. Fluorescence was measured at 560/590 nm.

Untreated cells were defined as 'no leakage' and total LDH release was defined as '100% leakage' by lysing cells with 0.1% Triton X-100.

Determination of antibody titers towards human recombinant IFI16 by ELISA

Anti-IFI16 autoantibodies in sera were detected by an indirect assay as previously described[13].

Results

The IFI16 nuclear protein relocalizes in response to UVB exposure

It is well known that the cellular response to UVB mainly induces apoptosis that may favour the exposure of autoantigens and their presentation to the afferent limb of the immune system[4,5]. To test whether this was also the case for the IFI16 autoantigen, keratinocyte monolayers were irradiated with graded doses of UVB and at different time points after treatment cells were harvested and analyzed by immunoblotting to measure the levels of cleaved PARP[17] and IFI16. As shown in Figure 1a, IFI16 expression levels were not affected by UVB irradiation, with the exception of cells tested 6h hours after 400 J/m^2 UVB irradiation, when a slight increase in IFI16 protein was observed. UVB-irradiated keratinocytes showed a low level of apoptosis, as measured by PARP cleavage, when treated with 200 J/m^2 at both 6h and 16h post irradiation, while their levels significantly increased at 16h after treatment with both 400 J/m^2 and 800 J/m^2. Next, cell morphology and immunofluorescence patterns were investigated by confocal and phase-contrast microscopy. At 6h post-irradiation with 400 J/m^2 the cells started to round-up. This phenomenon was more pronounced and accompanied by an increased cell size at 800 J/m^2 (Supplementary Fig. S1a). IFI16 immunofluorescence was restricted to the nucleus at 400 J/m^2, although its intensity was lower than that of untreated control, and it showed a tendency to accumulate at the edge of the nucleus. At 800 J/m^2, the fluorescence signal was very strong in the cytoplasm of these enlarged cells while the nuclei showed a complete lack of anti-IFI16 staining,

although they were still visible by PI staining. At 16h post-irradiation (Fig. 1b and Supplementary Fig. S1b), the anti-IFI16 immunostaining localized to cytoplasmic granules. In unirradiated cells, as well as in cells exposed to 200 J/m^2 (data not shown), IFI16 staining was exclusively nuclear at all time points tested.

The IFI16 nuclear protein does not relocalize in response to other cell death stimuli

To address whether the observed IFI16 relocalization was the specific result of UVB irradiation or a general phenomenon related to cell death stimuli, keratinocytes were exposed to two other death stimuli, etoposide (VP-16) and doxorubicin (Doxo) treatments. Western blotting showed that PARP cleavage was induced by both stimuli, although the kinetics of induction were faster following treatment with etoposide (16h) (Fig. 2). Based on these results, keratinocytes, incubated in medium containing one of the drugs for 48h, were stained with PI and underwent the anti-IFI16 immunostaining procedure to investigate IFI16 distributions during etoposide- and doxorubicin-induced cell death. After both treatments, IFI16 immunostaining remained exclusively nuclear (Supplementary Fig. S2), indicating that the nuclear-cytoplasmic shuttling observed after UVB exposure was specifically induced by this treatment and not simply related to cell death.

The IFI16 protein is specifically released into the culture supernatants of UVB-treated keratinocytes

To investigate the fate of IFI16 beyond the plasma membrane, cells were treated with different UVB doses or with a single high dose of either doxorubicin or etoposide, and the presence of the protein in the culture supernatants was tested by immunoblotting. As shown in Figure 3a, the signal for the IFI16 protein was very low in culture supernatants after treatment of cells with the lowest UVB dose, while with higher doses it was clearly visible, suggesting that UVB-induced cell death was

responsible for the extracellular release of the autoantigen. Analysis of lactate dehydrogenase (LDH) activity in the same supernatants confirmed that the extracellular presence of this soluble cytoplasmic protein as a consequence of cell lysis was very low at 16h after irradiation with 400 J/m^2, when the IFI16 protein was already detectable. At later time points after irradiation, LDH activity increased in the extracellular medium, very likely due to cellular membrane rupture and the non-selective leakage of cytoplasmic proteins. Consistent with the results obtained with fluorescence microscopy, neither etoposide nor doxorubicin treatments caused the release of IFI16 into the supernatant, even though LDH release was very high after doxorubicin treatment (Fig. 3b).

UVB irradiation induces cytoplasmic translocation of IFI16 in skin explants

To more accurately model the effect of UVB irradiation on IFI16 localization in epidermal keratinocytes *in situ*, skin explants were exposed to graded doses of UVB, and following different numbers of days post-UVB exposure, the explants underwent histological analysis[18]. As revealed by examination of H&E staining, at 24h post UVB exposure there was evidence of keratinocyte apoptosis (sun-burn cells), as demonstrated by pyknotic nuclei and hypereosinophilic cytoplasms, especially with 1000 and 2000 J/m^2 (Fig. 4a and Supplementary Fig. S3a). Corroborating the results from cultured keratinocytes, levels of cytoplasmic IFI16 staining progressively increased with number of days post-UVB exposure, paralleled with a decrease in nuclear staining. At 48h after UVB exposure, alteration of the epithelial morphology was very evident at the highest UVB doses (Fig 4b and Supplementary Fig. S3b). At this time point, anti-IFI16 immunostaining in the cytoplasm was more evident, and most of the nuclei lacked significant levels of staining, especially the explants that received the 2000 J/m^2 UVB dose. In unexposed epidermis, IFI16 staining remained restricted to the nuclei.

To confirm further that the observed UVB-induced IFI16 nuclear to cytoplasm translocation was a specific phenomenon and not a general consequence of nuclear protein exiting due to a loss of nuclear membrane integrity, tissue sections were co-stained with anti-IFI16 antibodies and antibodies

against the phosphorylated form of the nuclear protein Stat3[19] and processed for immunofluorescence analysis. As shown in Figure 5, in unexposed skin both Phospho-Stat3 and IFI16 staining was strictly nuclear, whereas 48h after exposure to a UVB dose of 400 J/m^2, IFI16 staining was also distributed within the cytoplasm.

Taken together, the results obtained with the skin explants, clearly indicate that the translocation of the IFI16 protein from the nucleus to the cytoplasm is a physiological response of keratinocytes to UVB exposure that occurs not only when keratinocytes are cultivated *in vitro* as monolayers, but also in the context of the natural stratified squamous epithelia.

Cytoplasmic IFI16 distribution in Systemic Lupus Erythematosus lesional skin

To explore whether the changes in IFI16 distribution observed in UVB-exposed keratinocytes or skin explants could also be present in diseased skin sections, skin samples obtained from patients suffering from Systemic Lupus Erythematosus (SLE) were analyzed by immunohistochemistry with anti-IFI16 antibodies and compared with skin sections from healthy donors. As shown in Figure 6, the distribution pattern of IFI16 staining in diseased skin differed substantially from normal skin. As expected, IFI16 expression in normal skin was restricted to the nuclei, with evident positive staining in the keratinocytes (Fig. 6a and Supplementary Fig. S4a and b). In SLE biopsies, IFI16 staining in keratinocytes was stronger and intense positive nuclei were also found in the upper epidermal layers, as shown in the representative images (Fig. 6b and c; Supplementary Fig. S4c to f). None of the patients had a history of excessive sun or UVB radiation exposure, and similarly photoexposed areas of healthy controls did not present a situation comparable to that of SLE patients (data not shown). As expected, IFI16 nuclear staining was also observed in the dermal inflammatory infiltrate and in endothelial cells, with no apparent cytoplasmic staining. These findings provided an internal negative control, indicating

that the cytoplasmic translocation of IFI16 was keratinocyte-specific (Fig. 6b and c; Supplementary Fig. S4c to f).

To gain further insight into the pathogenetic role of IFI16 as an autoantigen, 5 sera samples obtained from the same SLE patients from whom skin biopsies were taken, 45 from other SLE patients, and 50 from healthy donors were analyzed by indirect ELISA to verify the presence of anti-IFI16 autoantibodies. As shown in Figure 7, 4 out of the 5 sera from whom skin biopsies were analyzed, displayed high titres of anti-IFI16 autoantibodies. Consistent with previous findings[13,20], 46% of the SLE patients showed serum levels of anti-IFI16 antibodies above the cut-off value, compared to 6% of healthy controls.

Discussion

In peripheral tissues, as in the skin of patients with SLE, a potentially crucial role in the initiation and perpetuation of the autoimmune reaction cascade has been attributed to UV-induced keratinocyte apoptosis. These dying cells display clustering of autoantigens at the cell surface of apoptotic blebs which can be presented to the immune system and may trigger an autoimmune response in susceptible individuals[21,22].

In this study, we have demonstrated that the IFI16 protein, normally detected in the nucleus of human keratinocytes, can be induced to appear in the cytoplasm under conditions of UV light-induced cell injury. IFI16 staining was very strong in the cytoplasm of cells that increased in size after UVB exposure and in cytoplasmic granules. In unirradiated cells, as well as in cells exposed to a low UVB dose, IFI16 staining was exclusively nuclear. This phenomenon is not associated with cell damage in general since it was not observed in keratinocytes exposed to the pharmacological inducers of cell death, doxorubicin and etoposide. Importantly, IFI16 protein was also found in the supernatants of

UVB-exposed keratinocytes before changes of permeability occurred allowing the dispersion of molecules, such as LDH, into the extracellular milieu.

Overall, these data indicate that the redistribution of IFI16 into the cytoplasm and cytoplasmic granules of keratinocytes is an active process induced by UVB exposure and is not simply related to the passive leakage of this protein in dying cells.

The translocation of IFI16 into the cytoplasm was also observed when keratinocytes were exposed to UVB treatment in the context of their natural environment, the stratified squamous epithelium. Immunohistochemical analysis of skin explants exposed to graded doses of UVB revealed the presence of IFI16 cytoplasmic staining. This translocation was selective for IFI16 and did not occur for other nuclear proteins including the activated phosphorylated Stat3 protein. A similar situation was also found in tissue sections of skin biopsies from SLE patients. In these tissue sections, IFI16 expression was upregulated, with relocalization of the antigen to the cytoplasm.

Overall, the results presented in this study provide new insights into the dynamics of the release of IFI16 from the nuclei during cell death induced by UVB treatment of keratinocytes, favouring the exposure of IFI16 to an immune attack.

Golan *et al.*[23] reported that keratinocytes obtained from SLE patients show an enhanced and selective expression of soluble intracellular antigens on the cell membrane after 200 J/m^2 UVB irradiation *in vitro*. More than 10 years ago, Casciola-Rosen et al.[4] showed that keratinocytes undergoing apoptosis after exposure to UV light cluster a variety of macromolecules in blebs on the cell surface. Importantly, these small blebs contained the major autoantigens recognized by the sera from SLE patients. From these studies, it can be hypothesized that the persistence of these blebs might induce an immune response culminating in the production of autoantibodies. The finding from the present study that IFI16 translocates from the nucleus into the cytoplasm and cytoplasmic granules in UVB-exposed keratinocytes, and that IFI16 is localized in the cytoplasm in diseased skin of SLE

patients is consistent with this pathogenetic mechanism and the presence of anti-IFI16 autoantibodies in SLE and other autoimmune diseases.

The skin involvement is one of the most frequent features of the SLE. Nuclear antigens, potentially targeted by SLE autoantibodies, can be abnormally expressed in the skin and the whole machinery required for autoantigens processing by antigen presenting cells (APCs)[24] is present in this tissue. The disease model we propose is as follows: i) IFI16 expression in lesional skin may be enhanced by local production of type I IFN or by other proinflammatory stimuli[13,25] and ii) IFI16 relocalization and release triggered by UVB exposure or other stimuli lead to the breakdown in tolerance to this self antigen, as confirmed by the generation of specific anti-IFI16 autoantibodies. This model is supported by the observation that up to 46% of SLE patients present anti-IFI16 autoantibodies[13,20] and could be also applied to other systemic autoimmune diseases developing anti-IFI16 autoantibodies, such as SSc where we have already found IFI16 overexpression in the skin[13].

Although future investigations are required to understand the pathogenetic role of extracellular IFI16 in different disease settings, the current study indicates that the release and recognition of this protein following UV light exposure of human skin required in depth examination. Further elucidation of new factors that contribute to the UV initiation and perpetuation of autoimmune responses may lead to the development of more specific pharmaceuticals beyond UV filters to prevent induction and exacerbation of LE and to counteract the detrimental effects of UV irradiation in this disease.

References

1. Shoenfeld Y, Szyper-Kravitz M, Witte T, et al. Autoantibodies against protective molecules--C1q, C-reactive protein, serum amyloid P, mannose-binding lectin, and apolipoprotein A1: prevalence in systemic lupus erythematosus. Ann N Y Acad Sci 2007; **1108**:227-39.
2. Rothfield N, Sontheimer RD, Bernstein M. Lupus erythematosus: systemic and cutaneous manifestations. Clin Dermatol 2006; **24**(5):348-62.
3. Crowson AN, Magro C. The cutaneous pathology of lupus erythematosus: a review. J Cutan Pathol 2001; **28**(1):1-23.
4. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med 1994; **179**(4):1317-30.
5. Caricchio R, McPhie L, Cohen PL. Ultraviolet B radiation-induced cell death: critical role of ultraviolet dose in inflammation and lupus autoantigen redistribution. J Immunol 2003; **171**(11):5778-86.
6. Ludlow LE, Johnstone RW, Clarke CJ. The HIN-200 family: more than interferon-inducible genes? Exp Cell Res 2005; 308(1):1-17.
7. Mondini M, Costa S, Sponza S et al. The interferon-inducible HIN-200 gene family in apoptosis and inflammation: implication for autoimmunity. Autoimmunity 2010; **43**(3):226-31.
8. Gariglio M, Azzimonti B, Pagano M et al. Immunohistochemical expression analysis of the human interferon-inducible gene IFI16, a member of the HIN200 family, not restricted to hematopoietic cells. J Interferon Cytokine Res 2002; **22**(7):815-21.
9. Gugliesi F, Mondini M, Ravera R et al. Up-regulation of the interferon-inducible IFI16 gene by oxidative stress triggers p53 transcriptional activity in endothelial cells. J Leukoc Biol 2005; **77**(5):820-9.

10. Mondini M, Vidali M, Airo P, De Andrea M, Riboldi P, Meroni PL, et al. Role of the interferon-inducible gene IFI16 in the etiopathogenesis of systemic autoimmune disorders. Ann N Y Acad Sci 2007; **1110**:47-56.
11. Caposio P, Gugliesi F, Zannetti C et al. A novel role of the interferon-inducible protein IFI16 as inducer of proinflammatory molecules in endothelial cells. J Biol Chem 2007; **282**(46):33515-29.
12. Baggetta R, De Andrea M, Gariano GR et al. The interferon-inducible gene IFI16 secretome of endothelial cells drives the early steps of the inflammatory response. Eur J Immunol. 2010; [Epub ahead of print].
13. Mondini M, Vidali M, De Andrea M et al. A novel autoantigen to differentiate limited cutaneous systemic sclerosis from diffuse cutaneous systemic sclerosis: the interferon-inducible gene IFI16. Arthritis Rheum 2006; **54**(12):3939-44.
14. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997; **40**(9):1725.
15. WHO. World Health Organization, Geneva Switzerland. Global solar UV index: a pratical guide. 2002.
16. De Andrea M, Gioia D, Mondini M et al. Effects of IFI16 overexpression on the growth and doxorubicin sensitivity of head and neck squamous cell carcinoma-derived cell lines. Head Neck 2007; **29**(9):835-44.
17. Cepeda V, Fuertes MA, Castilla J et al. Poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors in cancer chemotherapy. Recent Pat Anticancer Drug Discov 2006; **1**(1):39-53.
18. Lehmann B, Genehr T, Knuschke P et al. UVB-induced conversion of 7-dehydrocholesterol to 1alpha,25-dihydroxyvitamin D3 in an in vitro human skin equivalent model. J Invest Dermatol 2001; **117**(5):1179-85.

19. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002; **3**(9):651-62.

20. Seelig HP, Ehrfeld H, Renz M. Interferon-gamma-inducible protein p16. A new target of antinuclear antibodies in patients with systemic lupus erythematosus. Arthritis Rheum. 1994; **37**(11):1672-83.

21. Bijl M, Reefman E, Limburg PC, Kallenberg CG. Inflammatory clearance of apoptotic cells after UVB challenge. Autoimmunity 2007; **40**(4):244-8.

22. Janko C, Schorn C, Grossmayer GE et al. Inflammatory clearance of apoptotic remnants in systemic lupus erythematosus (SLE). Autoimmun Rev 2008; **8**(1):9-12.

23. Golan TD, Elkon KB, Gharavi AE, Krueger JG. Enhanced membrane binding of autoantibodies to cultured keratinocytes of systemic lupus erythematosus patients after ultraviolet B/ultraviolet A irradiation. J Clin Invest 1992; **90**(3):1067-76.

24. Bave U, Magnusson M, Eloranta ML et al. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. J Immunol 2003; **171**(6):3296-302.

25. Lovgren T, Eloranta ML, Bave U et al. Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. Arthritis Rheum 2004; **50**(6):1861-72.

Figure legends

Fig. 1. (a) Western blot analysis to examine the expression of IFI16 and PARP after UVB exposure. Keratinocyte monolayers were UVB-irradiated at different doses (200, 400 or 800 J/m^2) or left unirradiated. At different time points following irradiation, cells were lysed, 30 μg protein aliquots separated by SDS-PAGE, blotted onto nitrocellulose membranes, reacted with the corresponding antibodies and visualized by chemiluminescence. Actin was used as equal loading control. **(b) IFI16 redistribution in UVB-irradiated human keratinocytes**. Representative immunofluorescence images of keratinocytes that were UVB-irradiated with 800 J/m^2 or left unirradiated. Sixteen hours after irradiation, cells were fixed and double stained with anti-IFI16 (green) and PI (red). Antibody and PI staining images were captured separately using confocal fluorescence microscopy and then merged (overlapping pixels appear orange/yellow).

Fig. 2. Western blot analysis to examine the expression of IFI16 and PARP after doxorubicin and etoposide treatments. Keratinocyte monolayers were treated with 2μM doxorubicin (Doxo), 80μM etoposide (VP-16) or left untreated. At different time points, cells were lysed, 30 μg protein aliquots separated by SDS-PAGE, blotted onto nitrocellulose membranes, reacted with the corresponding antibodies and visualized by chemiluminescence. Actin was used as equal loading control.

Fig. 3. (a) Western blot analysis to examine the presence of IFI16 protein in culture supernatants. Keratinocyte monolayers were UVB-irradiated at different doses (200, 400 or 800 J/m^2), treated with either 2μM Doxo or 80μM VP-16, or left untreated. At 16h after treatment, culture supernatants of these cells were harvested, concentrated and analyzed on a SDS-PAGE gel. The proteins were blotted onto nitrocellulose membranes, incubated with anti-IFI16 antibodies and visualized by chemiluminescence. **(b) Percentage of lactate dehydrogenase (LDH) leakage into the cell culture medium following different cell death stimuli.** Keratinocyte monolayers were treated as in panel (a).

At 16h and 48h post treatments, culture supernatants of these cells were harvested and processed for LDH measurement. Results are expressed as mean ±SD of three independent experiments.

Fig. 4. IFI16 redistribution in UVB-irradiated human skin *ex vivo*. Human skin explants were prepared as described in the methods section. After 24h in culture media, explants were UVB-irradiated at 1000 J/m^2 then harvested at 24h **(a)** and 48h **(b)** post-UVB treatment and processed for hematoxylin-eosin staining and immunohistochemical staining of IFI16 (blue); no counterstaining method was used. Arrows indicate pyknotic nuclei and hypereosinophilic cytoplasms (sun burn cells). Representative images were taken using 40x magnification.

Fig. 5. Adjacent sections of the same explants shown in Fig. 4 were reacted with anti-Phospho-Stat3 and anti-IFI16 antibodies. Human skin explants were prepared as described in the methods section. After 24h in culture media, explants were UVB-irradiated at 400 J/m^2, then harvested at 48h post-UVB and processed for immunofluorescence staining. Sections were first reacted for IFI16 (green) and then for Pospho-Stat3 staining (red). Images were captured separately using confocal fluorescence microscopy and then merged (overlapping pixels appear orange/yellow).Representative images were taken using 40x magnification.

Fig. 6. Localization of the IFI16 protein in Systemic Lupus Erythematosus (SLE) skin lesions. Sections of two representative SLE skin lesions stained with anti-IFI16 antibodies and visualized with SG substrate (blue) are shown in panels (**b)** and (**c**). Normal skin processed in the same way is shown in panel (**a**). Representative images were taken using 40x magnification. Images taken using 10x and 20x magnification are shown in Fig. S4.

Fig. 7. Anti-IFI16 antibodies in SLE patients sera. IgG titers by ELISA assay against human recombinant IFI16 in patients with Systemic Lupus Erythematosus (SLE, N=50), and from healthy controls (CTRL, N=50). Each dot represents anti-IFI16 level of one sample (expressed in Arbitrary Units/ml on a Log2 scale). Larger dots represent the 5 sera from SLE patients whose biopsies were

analyzed by immunohistochemistry. The horizontal bars represent the median. Values over the dotted line represent the percentage of subjects with IgG titers above the cut off value (101 U/ml), calculated at the 95th percentile of the control population. Statistical significance: * $p<0.0001$ vs controls (Mann Whitney test).

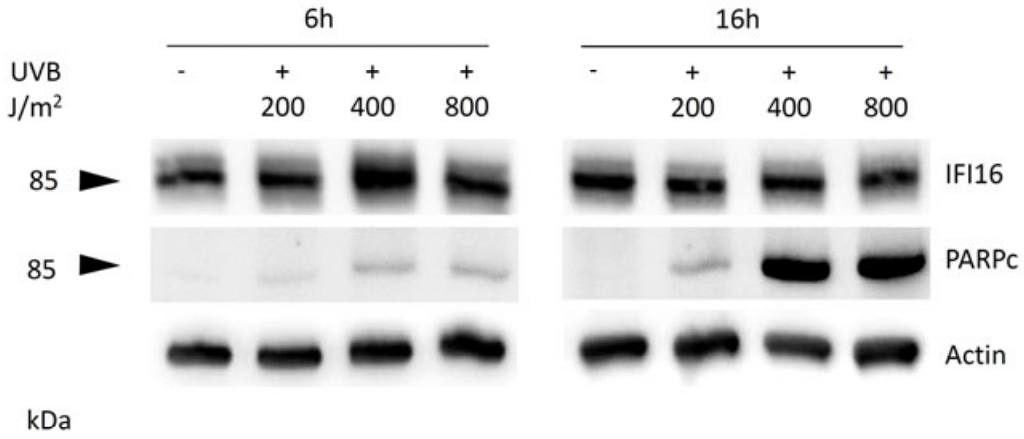
Supplementary Fig. S1 (a) and (b). IFI16 redistribution in UVB-irradiated human keratinocytes. Representative immunofluorescence images of keratinocytes that were UVB-irradiated with 800 J/m^2 (6h, panel a; 16h, panel b) or left unirradiated and double stained with anti-IFI16 (green) and PI (red). Antibody and PI staining images were captured separately using confocal fluorescence microscopy and then merged (overlapping pixels appear orange/yellow). (*) indicates panels that are also shown in Fig. 1b.

Supplementary Fig. S2. IFI16 redistribution after doxorubicin (Doxo) or etoposide (VP-16) treatments. Representative immunofluorescence images of keratinocytes treated with 2μM doxorubicin or 80μM etoposide and double stained with anti-IFI16 antibodies (green) and PI (red) 48h post-treatment. Antibody and PI staining images were captured separately by confocal fluorescence microscopy and then merged (overlapping pixels appear orange/yellow).

Supplementary Fig. S3. IFI16 redistribution in UVB-irradiated human skin *ex vivo*. Human skin explants were prepared as described in the methods section. After 24h in culture media, explants were UVB-irradiated at different doses (400 J/m^2 or 2000 J/m^2) then harvested at 24h **(a)** and 48h **(b)** post-UVB treatment and processed for hematoxylin-eosin staining and immunohistochemical staining of IFI16 (blue); no counterstaining method was used. Arrows indicate pyknotic nuclei and hypereosinophilic cytoplasms (sun burn cells). Representative images were taken using 40x magnification.

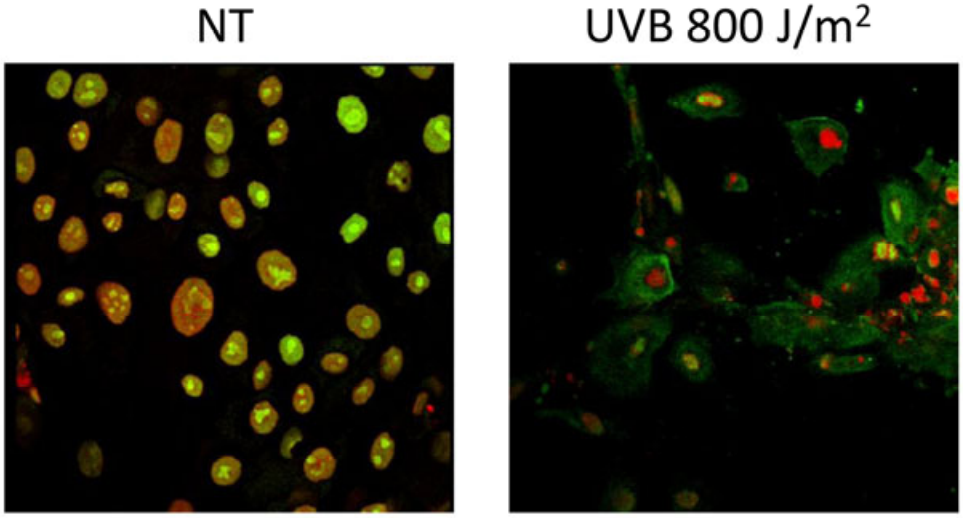
Supplementary Fig. S4. Localization of the IFI16 protein in Systemic Lupus Erythematosus (SLE) skin lesions. Sections of two representative SLE skin lesions stained with anti-IFI16 antibodies and visualized with SG substrate (blue) are shown in panels (**c)** to (**f)** at 10x or 20x magnification (40x magnification is shown in Fig. 6). Normal skin processed in the same way is shown in panels (**a)** and (**b**).

(a)

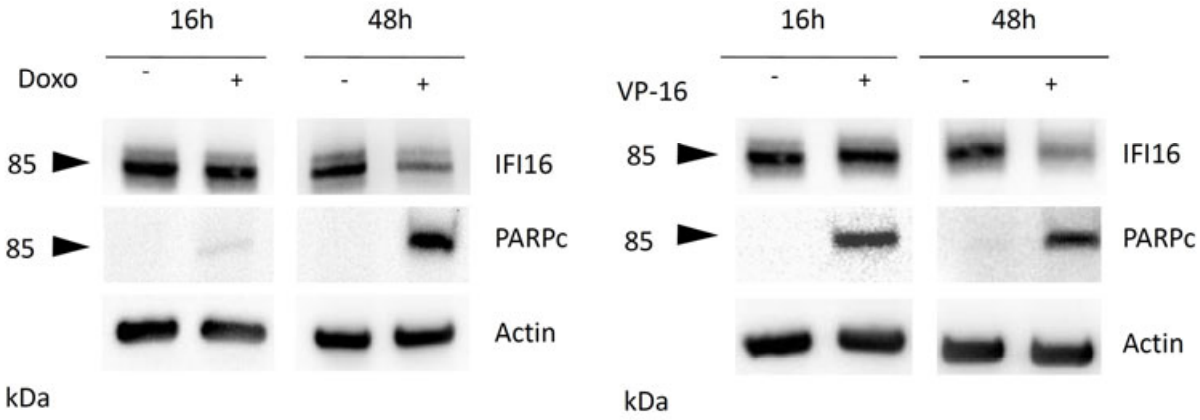


Costa et al., Fig. 1

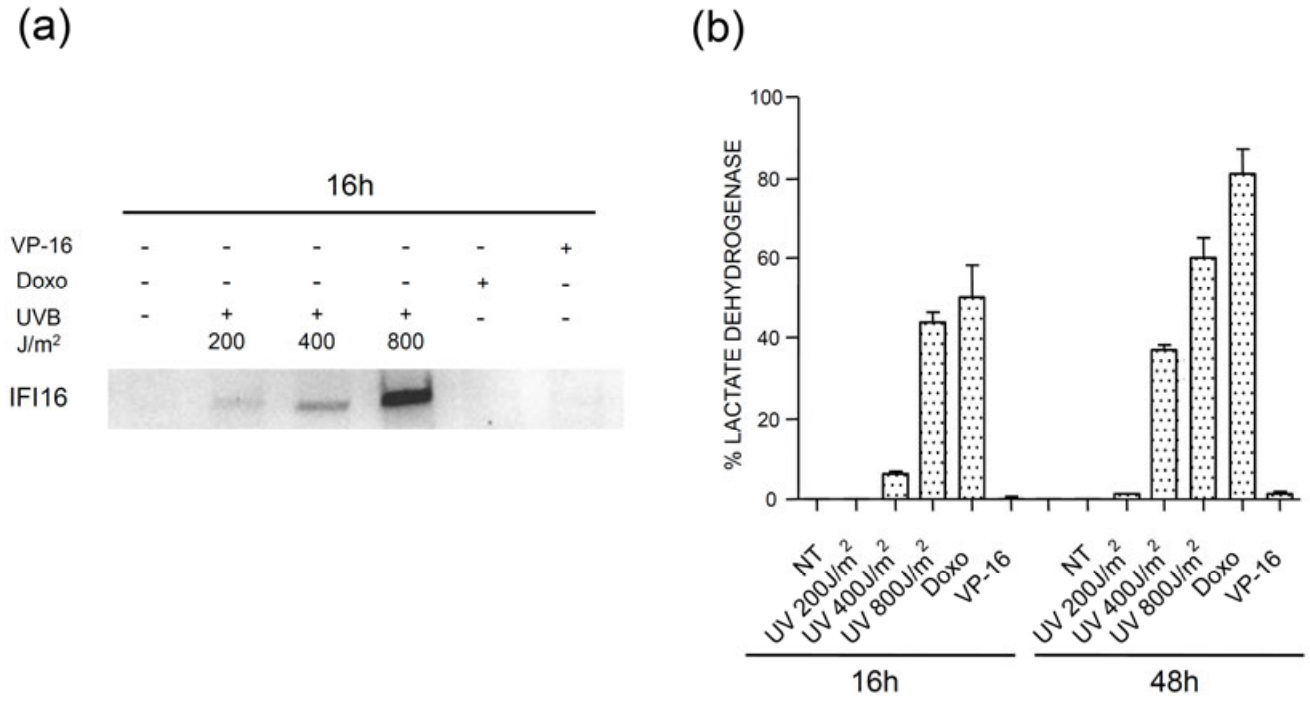
(b)



Costa et al., Fig. 1

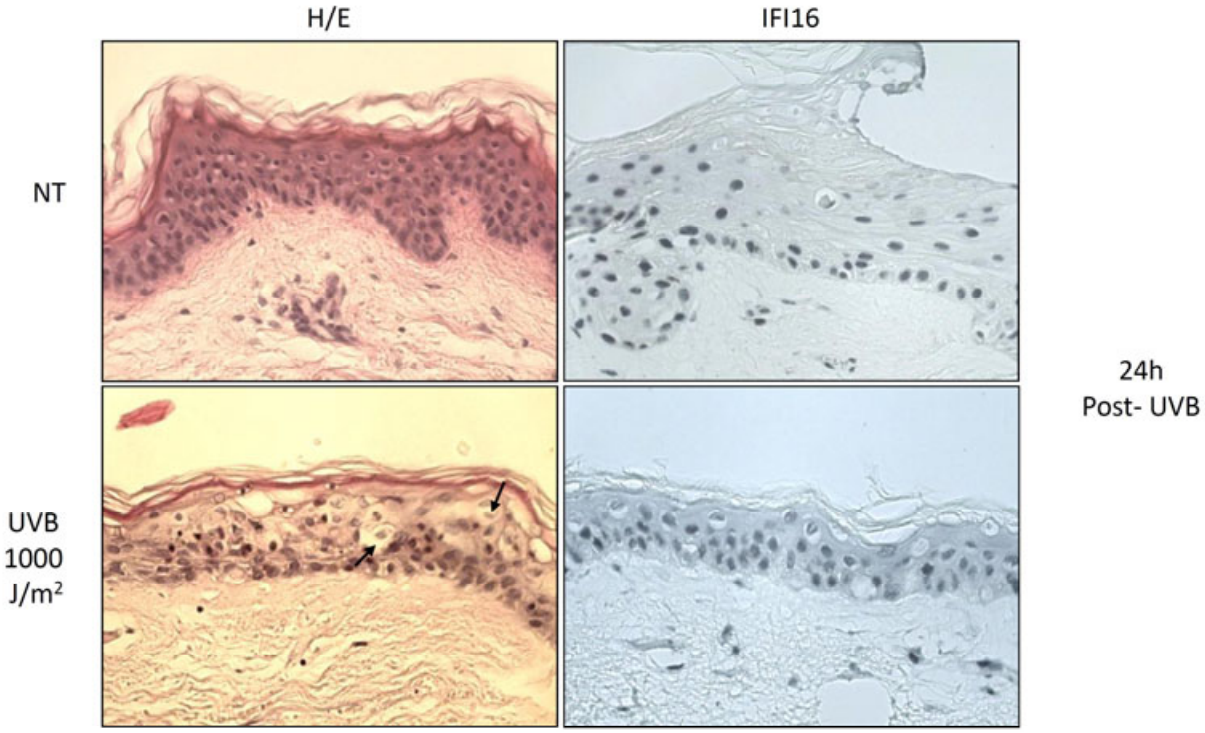


Costa et al., Fig. 2



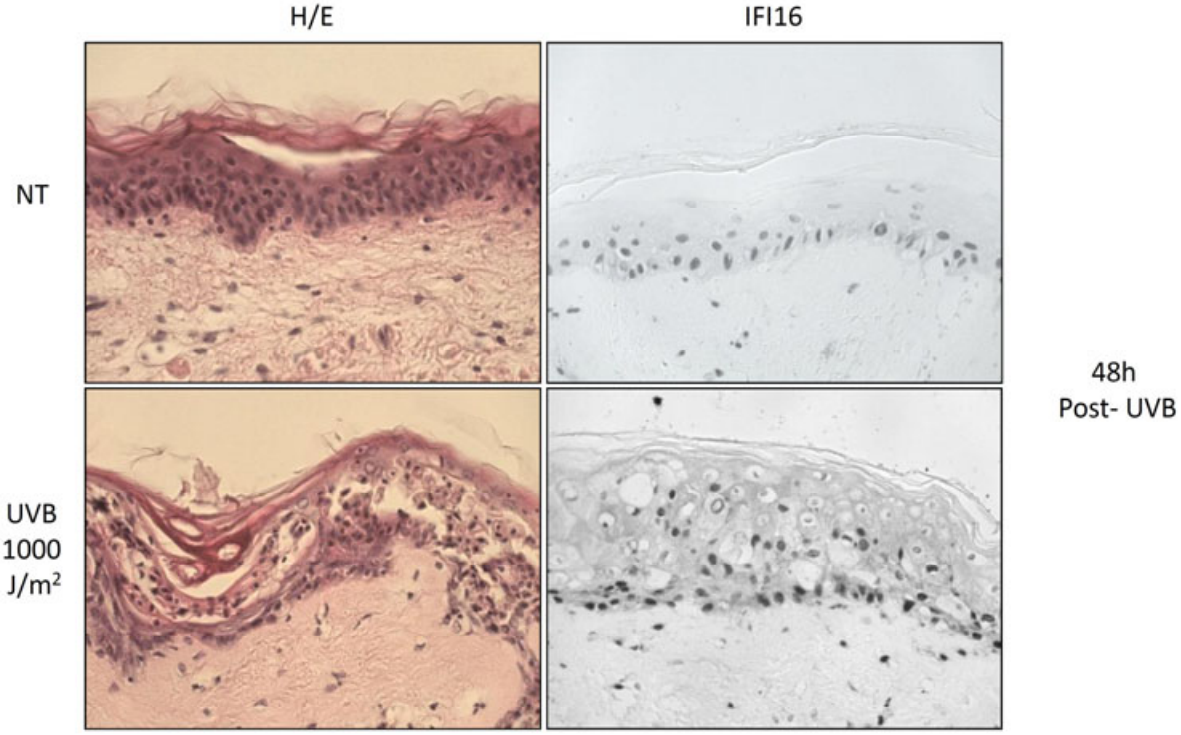
Costa et al., Fig. 3

(a)

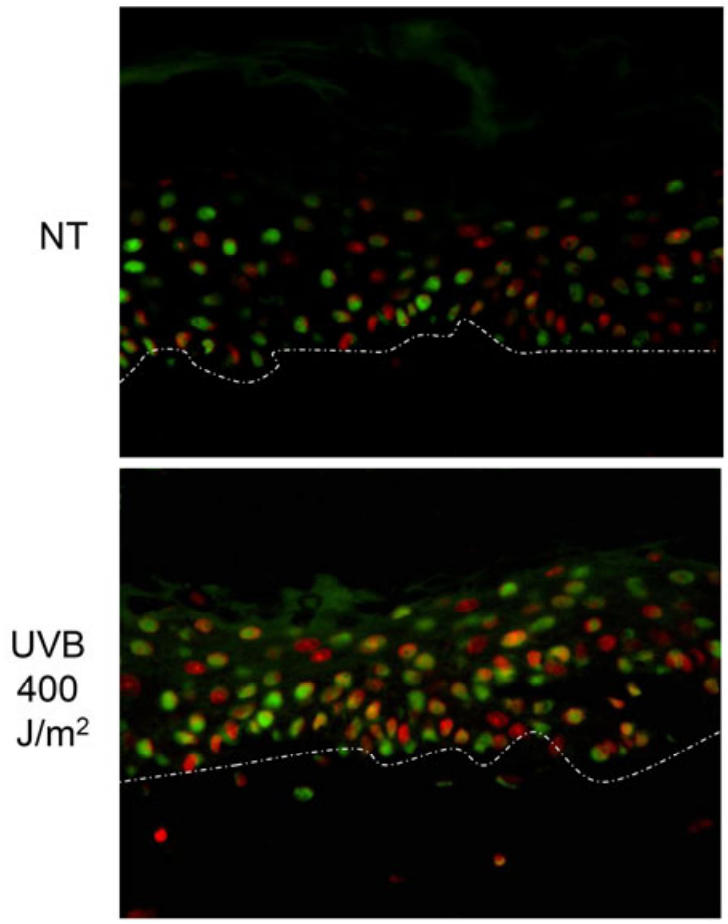


Costa et al., Fig. 4

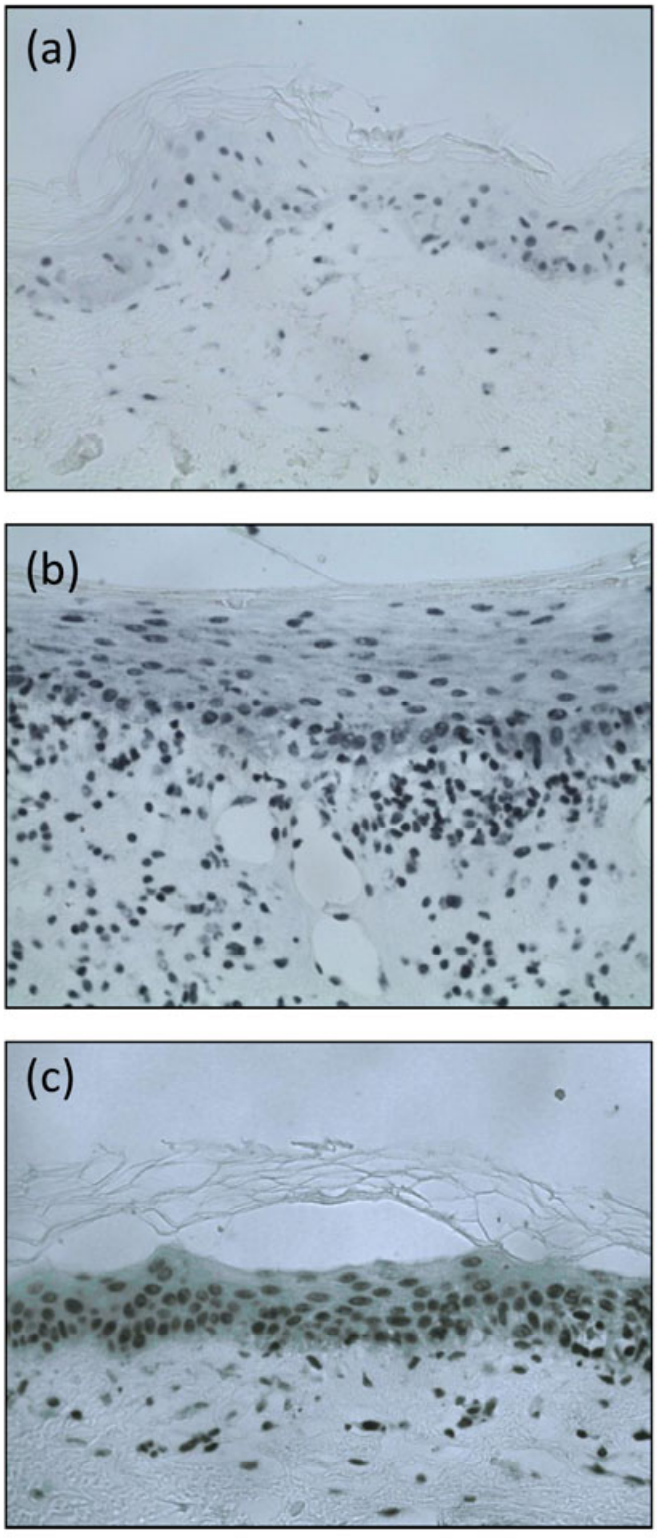
(b)



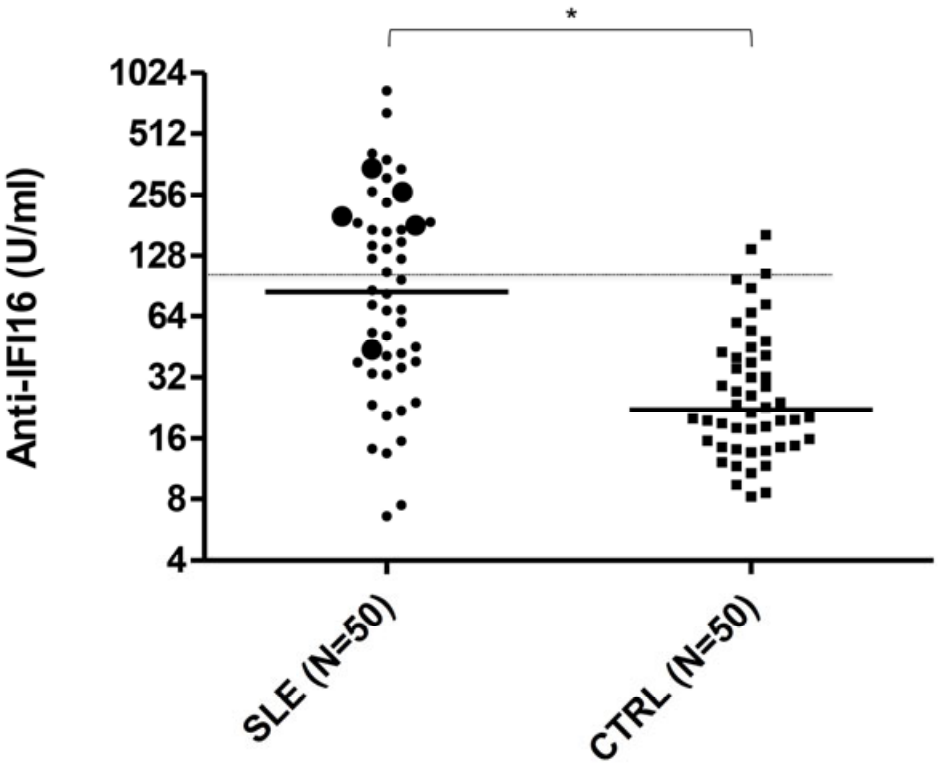
Costa et al., Fig. 4



Costa et al., Fig. 5



Costa et al., Fig.6



Costa et al., Fig. 7