

IL-32 is expressed by human primary keratinocytes and modulates keratinocyte apoptosis in atopic dermatitis

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Background: Keratinocyte (KC) apoptosis is an important mechanism of eczema and spongiosis in patients with atopic dermatitis (AD) and is mediated by IFN- γ , which is secreted by T_H1 cells. IL-32 is a proinflammatory cytokine that is involved in the inflammatory processes of rheumatoid arthritis, chronic obstructive pulmonary disease, and Crohn disease. Recently, it was shown that upregulation of IL-32 induces apoptosis.

Objective: The aim of the study was to investigate the expression and function of IL-32 in patients with AD.

Methods: The expression of IL-32 in KCs was analyzed by means of RT-PCR, ELISA, and flow cytometry. Transfections of small interfering RNA were performed in primary KCs, and apoptosis was analyzed by means of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, annexin-V, and 7-amino actinomycin D stainings. Immunofluorescence stainings were used to detect IL-32 in skin biopsy specimens, and serum levels of IL-32 were analyzed by means of ELISA.

Results: We report that IL-32 is expressed in human primary KCs on stimulation with IFN- γ , TNF- α , and T_H1 cells in contrast to T_H2, regulatory T (Treg), or T_H17 cells, which showed no effect. Transfection of primary KCs and artificial skin equivalents with small interfering RNA to IL-32, which

resulted in a clear decrease in IL-32 expression, significantly reduced KC apoptosis. Immunofluorescence staining demonstrated that IL-32 was expressed in AD lesional skin, whereas it was present in neither skin biopsy specimens from healthy donors nor in lesional skin from patients with psoriasis. Serum levels of IL-32 from patients with AD correlated with disease severity, but increased serum levels of IL-32 were also detected in asthmatic patients.

Conclusion: The present study demonstrates KCs as a source of IL-32, which modulates KC apoptosis and contributes to the pathophysiology of AD. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Human primary keratinocytes, atopic dermatitis, IL-32, apoptosis

T cells play an important role in the pathogenesis of atopic dermatitis (AD), with marked infiltration of activated CD4 and CD8 memory T cells in the skin.¹ At the initial stages of inflammation, T_H2 cells migrate into the dermis, where they acquire a T_H0/T_H1 phenotype under the influence of IL-12, which is produced by antigen-presenting cells or activated keratinocytes (KCs).² Whereas acute lesions display a T_H2 phenotype with increased levels of IL-4, IL-5, and IL-13, the chronic phase is dominated by the T_H1 cytokine IFN- γ .³ Several studies have demonstrated that KC apoptosis is an important component of eczema and spongiosis in patients with AD and epithelial shedding in patients with asthma and chronic sinusitis and is mediated through soluble Fas ligand, IFN- γ , or both.^{4,5} Epithelial cell interaction with T cells displays 2 stages characterized by initial cellular activation and proinflammatory cytokine/chemokine release, followed by apoptosis and shedding of epithelia.⁶

IL-32 was originally described as natural killer cell (NK) transcript 4 found in activated NK cells and T cells and later named IL-32.⁷ Human lung epithelial cells and intestinal epithelial cells express IL-32 on stimulation with TNF- α , IFN- γ , IL-1 β , and IL-18.^{8,9} The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into 8 exons. IL-32 lacks sequence homology to presently known cytokine families. There are 6 isoforms caused by alternative mRNA splicing, resulting in proteins with a molecular weight of between 14.9 and 26.7 kDa. The isoforms α , β , γ , and δ are expressed in several tissues and cell types, whereas the isoforms ϵ and ζ are only abundantly expressed in activated T cells.¹⁰ A receptor for IL-32 has not yet been described. IL-32 induces TNF- α , IL-8, and macrophage

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The author's laboratory is supported by the European Asthma and Allergy Center Davos (EACD), Swiss National Science Foundation grant no. 32-188226, and the Christine Kühne Center for Allergy Research and Education (CK-CARE).

Disclosure of potential conflict of interest: S. Woehrl receives research support from ALK-Abelló and the GA²LEN Network of Excellence EU 6th Framework Program and receives speaker's fees from Bencard Allergie. C. Rhyner is an employee of SIAF. P. Schmid-Grendelmeier receives research support from Novartis Pharma Switzerland. M. Akdis receives research support from the Swiss National Science Foundation and Invision and is a council member of Collegium Internationale Allergologicum. C. A. Akdis receives research support from Novartis, Stallergenes, the Swiss National Science Foundation, the Global Allergy and Asthma European Network, and the Christine Kühne Center for Allergy Research; is a fellow of the American Association of Allergy, Asthma & Immunology; is vice president of the European Academy of Allergy and Clinical Immunology; and is an ex-committee member WP leader for GA²LEN. The rest of the authors have declared that they have no conflict of interest.

Received for publication August 25, 2009; revised January 8, 2010; accepted for publication January 11, 2010.

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doi:10.1016/j.jaci.2010.01.016

Abbreviations used

AD: Atopic dermatitis
 DC: Dendritic cell
 KC: Keratinocyte
 NK: Natural killer
 siRNA: Small interfering RNA
 Treg: Regulatory T

inflammatory protein 2 in monocytes, macrophages, and PBMCs by activating nuclear factor κ B and p38 mitogen-activated protein kinase.⁹ In this context it has been recently demonstrated that silencing endogenous IL-32 by short hairpin RNA impairs the induction of the proinflammatory cytokines TNF- α and IL-1 β in monocytes.¹¹ IL-32 has been implicated in many inflammatory disorders, and its expression was correlated with disease severity in patients with Crohn disease, rheumatoid arthritis, and chronic obstructive pulmonary disease.^{8,12,13}

IL-32 also appears to play an important role in host defense against invading micro-organisms. Recent studies have shown that IL-32 synergizes with nuclear oligomerization domain ligands to stimulate IL-1 β and IL-6 release in a caspase-1-dependent manner.¹⁴ Moreover, it was described that IL-32 is induced in PBMCs and monocytes by *Mycobacterium tuberculosis*,¹⁵ and the depletion of endogenous IL-32 in HIV-infected PBMCs resulted in a marked decrease of T_H1 cytokines, suggesting that IL-32 could be a natural inhibitor of HIV-1.¹⁶ In addition to its proinflammatory characteristics, several studies have demonstrated that IL-32 plays a role in the apoptosis of Hela cells and leukemia cells.^{10,17}

In the present study we investigated the expression of IL-32 in patients with AD and psoriasis and healthy subjects. Silencing of IL-32 with specific small interfering RNA (siRNA) was used to deplete IL-32 production in KCs, and its role in KC apoptosis was demonstrated. IL-32 was highly expressed in biopsy specimens of lesional skin from patients with AD, and its serum levels correlated with disease severity.

METHODS**Culture of primary KCs and artificial skin equivalents and reagents**

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Real-time PCR mRNA expression analysis

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Differentiation of T_H1, T_H2, regulatory T, and T_H17 cells

T-cell subsets were generated as described previously.¹⁸ For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org. After culturing T cells for 12 days, the cells were restimulated with a combination of mAbs (0.5 μ g/mL anti-CD2, 1.0 μ g/mL anti-CD3, and 0.5 μ g/mL anti-CD28) for 6 hours and cocultured with KCs (3:1 ratio). After 72 hours of coculture, KCs were washed twice with PBS and analyzed by means of flow cytometry.

Small interfering RNA transfections

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Flow cytometric analysis, ELISA, and immunofluorescence staining

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org. Isotype controls for IL-32 flow cytometric staining and gating strategy are shown in [Figs E1 through E3](#) (available in this article's Online Repository at www.jacionline.org). Murine IgG2A isotype control for skin biopsy stainings is shown in [Fig E4](#) (available in this article's Online Repository at www.jacionline.org).

Determination of apoptosis

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Statistical interpretations

Statistical analysis was performed with GraphPad Prism 4.0 software (GraphPad Software, Inc, San Diego, Calif). Results are shown as means $\pm$ SDs. Nonparametric statistical comparison between groups was performed by using the Mann-Whitney *U* test. Differences in *P* values of .05 or less were considered significant. For correlation analyses, the Spearman correlation test was used.

Subjects

Lesional skin biopsy specimens were obtained from 3 healthy control subjects, 5 patients with AD, and 4 patients with psoriasis diagnosed according to standard criteria at the Departments of Dermatology at the medical faculties of the University of Zurich and the University of Vienna. Blood samples were obtained from 17 healthy control subjects, 36 patients with AD, 24 patients with asthma, and 20 patients with psoriasis diagnosed according to standard criteria at the Department of Dermatology at the medical faculty of the University of Zurich and the Hochgebirgsklinik in Davos, Switzerland. All studies were approved by the responsible ethical commissions.

RESULTS**IL-32 is expressed in human primary KCs**

Human primary KCs were incubated with several cytokines that have been demonstrated to play a role in the pathogenesis of AD to investigate the regulation of IL-32 expression on KCs. Relative mRNA levels of IL-32 increased after stimulation with IFN- γ and TNF- α in a time-dependent manner. After 72 hours of incubation with IFN- γ or TNF- α , relative IL-32 mRNA expression increased 210-fold or 15-fold, respectively. The combination of both cytokines induced a 603-fold increase in IL-32 expression, demonstrating that TNF- α and IFN- γ synergize for the induction of IL-32 in human KCs ([Fig 1, A](#)). IL-4 and IL-13 had no effect on IL-32 mRNA levels in human KCs (see [Fig E5](#) in this article's Online Repository at www.jacionline.org). Next we examined which IL-32 isoform was predominantly expressed in KCs. IL-32 α , β , γ , and δ were detected in KCs, with IL-32 γ levels showing the highest increase and IL-32 β levels showing the lowest increase after stimulation ([Fig 1, B](#)). Overall, the relative mRNA expression of each isoform was altered after stimulation with IFN- γ and TNF- α as IL-32 γ levels increased and levels of the other isoforms decreased ([Fig 1, C](#)).

Next the induction of IL-32 by TNF- α or IFN- γ in primary KCs from healthy subjects and patients with AD was investigated.

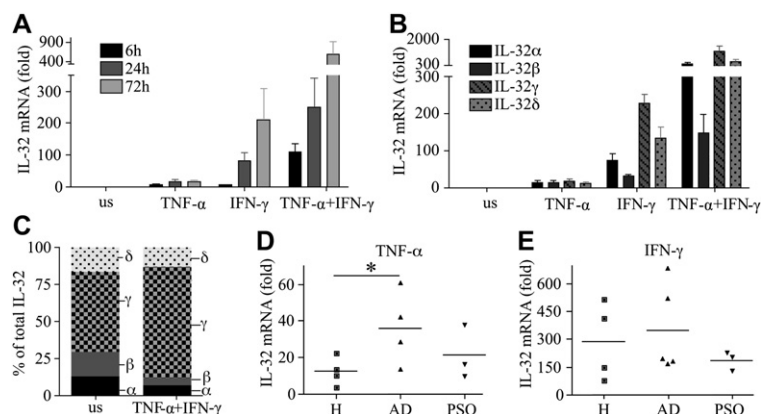


FIG 1. IL-32 mRNA is expressed in human primary epidermal KCs and regulated by TNF- α and IFN- γ . KCs were stimulated with TNF- α , IFN- γ , or the combination of TNF- α and IFN- γ for indicated time points, and relative mRNA expression of total IL-32 was analyzed (**A**). Its isoforms (**B**) and the ratio between the different IL-32 isoforms (**C**) were analyzed after 24 hours. Relative IL-32 mRNA expression in human primary KCs derived from patients with AD (AD), patients with psoriasis (PSO), or healthy volunteers (H) was analyzed after incubation with TNF- α or IFN- γ for 24 hours (**D** and **E**). Fig 1, A and B: Mean $\pm$ SD, $n = 3$. Fig 1, C: Percentage of total IL-32 for each sample was set 100%, $n = 3$. Fig 1, D and E: $n = 4$ for healthy control subjects and patients with AD and $n = 3$ for patients with psoriasis. * $P < .05$. us, Unstimulated.

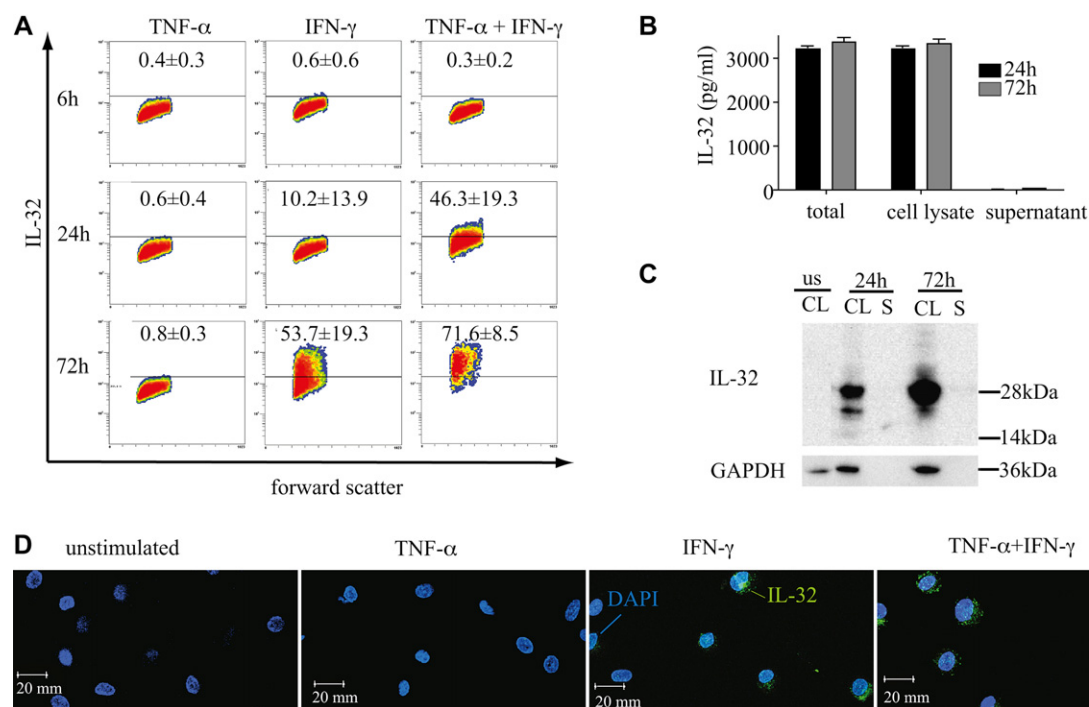


FIG 2. IL-32 production by human primary KCs. IL-32 protein expression in KCs was analyzed by means of flow cytometry after incubation with TNF- α , IFN- γ , or the combination of TNF- α and IFN- γ for the indicated time points (**A**). IL-32 concentrations of cell lysates or supernatants from KCs stimulated with the combination of TNF- α and IFN- γ for 24 or 72 hours were measured by means of ELISA (**B**) and Western blotting (**C**). KCs were incubated with the indicated stimuli for 72 hours; IL-32 was stained with Alexa Fluor 488 (green), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue; **D**). Fig 2, A and B: Mean $\pm$ SD, $n = 3$. Fig 2, C: One representative Western blot of $n = 2$. Fig 2, D: One representative staining, $n = 3$. CL, Cell lysate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; S, supernatant; us, unstimulated.

IL-32 expression after TNF- α stimulation was significantly increased in primary KCs obtained from patients with AD compared with that seen in KCs from healthy control subjects, whereas there was no difference between levels seen in KCs obtained from patients with psoriasis and those seen in healthy

control subjects (Fig 1, D). Induction of IL-32 by IFN- γ was similar for KCs obtained from patients with AD, patients with psoriasis, or healthy subjects (Fig 1, E).

Although IL-32 mRNA was present in resting human KCs, we could not detect intracellular IL-32 protein in these cells by using

flow cytometry (see Fig E1, B, in this article's Online Repository at www.jacionline.org) and Western blotting (Fig 2, C). However, after incubation with IFN- γ for 24 and 72 hours, 10.2% and 53.7%, respectively of KCs expressed IL-32 protein (Fig 2, A). TNF- α alone did not induce IL-32 protein expression in KCs, but it synergistically enhanced the induction of IL-32 when it was used in addition to IFN- γ (Fig 2, A). After stimulation with IFN- γ and TNF- α for 24 hours, all IL-32 measured was found in the cell-associated fraction and not in the cell culture supernatant, suggesting that IL-32 is not secreted by KCs after stimulation (Fig 2, B and C). Similarly, after 72 hours, the vast majority of IL-32 remained cell associated. Moreover, immunofluorescence staining demonstrated that IL-32 was located in the cytoplasm of KCs after stimulation with IFN- γ and the combination of IFN- γ and TNF- α but not TNF- α alone (Fig 2, D). To determine the effects of immunosuppressive and anti-inflammatory drugs on the expression of IL-32, primary KCs were preincubated with dexamethasone, 13-cis-retinoic acid, rapamycin, and FK506 for 30 minutes, and subsequently, TNF- α and IFN- γ were added for 72 hours. Dexamethasone, 13-cis-retinoic acid, rapamycin, and FK506 did not influence the expression of IL-32 (see Fig E6 in this article's Online Repository at www.jacionline.org).

Induction of KC IL-32 expression by T cells

The influence of different T-cell phenotypes on the production of IL-32 by KCs was investigated. KCs cocultured with Th_H1 cells produced IL-32, whereas Th_H2, Th_H17, and Treg cells did not induce IL-32 in KCs. The percentage of IL-32-expressing KCs was 34.5% after coculture with Th_H1 cells for 72 hours. The Th_H1 cell-mediated induction of IL-32 was abrogated by neutralization of IFN- γ with anti-IFN- γ neutralizing mAb, demonstrating that activated Th_H1 cells induce IL-32 in KCs through secretion of IFN- γ (Fig 3).

Role of IL-32 in TNF- α - and IFN- γ -induced KC apoptosis

The expression of endogenous IL-32 was depleted in primary KCs to determine the role of endogenous IL-32 on KC death induced by IFN- γ and TNF- α . KCs were transfected with increasing concentrations of IL-32 siRNA, and matching concentrations of scrambled siRNA were used as negative controls. Under unstimulated conditions, IL-32 mRNA was reduced dose dependently in KCs (Fig 4, A). After stimulation with TNF- α and IFN- γ for 72 hours, the fraction of IL-32-expressing KCs was reduced from 63.9% to 24.1% after transfection with IL-32 siRNA (Fig 4, B). Cells transfected with IL-32 siRNA clearly showed less apoptosis compared with that seen in control cells transfected with scrambled siRNA. The total percentage of early and late apoptotic KCs decreased from 53.7% to 27.6% after silencing of endogenous IL-32 (Fig 4, C). Knockdown of IL-32 in artificial skin equivalents was performed to investigate whether IL-32 plays a role in KC apoptosis in 3-dimensional intact tissues (the structure of artificial skin equivalents is shown in Fig E7 in this article's Online Repository at www.jacionline.org). Transfection of IL-32 siRNA resulted in decreased IL-32 expression in artificial skin equivalents stimulated with IFN- γ and TNF- α (Fig 4, D). In addition, the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive cells decreased from

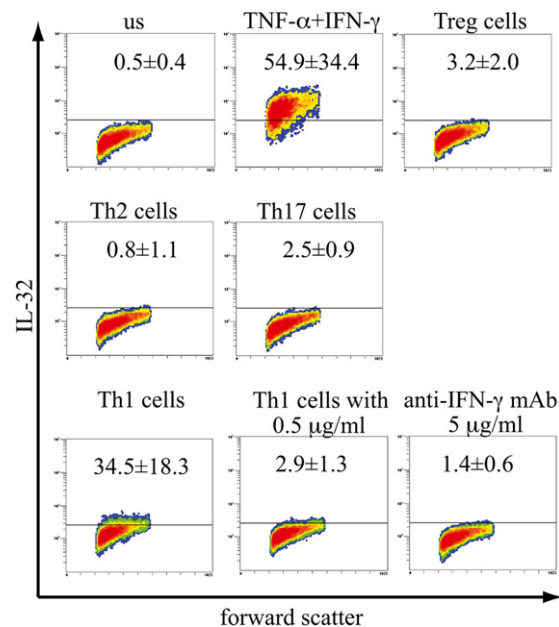


FIG 3. Th_H1 cells induce IL-32 through IFN- γ in KCs. KCs were cultured alone, with the combination of IFN- γ and TNF- α , or with Th_H1, Th_H2, Th_H17, and Treg cells. Th_H1 cells were added alone or in the presence of anti-IFN- γ -neutralizing mAbs (5 or 0.5 μ g/mL). After 72 hours of coculture, IL-32 expression in KCs was analyzed by means of flow cytometry. Mean $\pm$ SD, n = 3. us, Unstimulated.

34.1% to 16.7% after knockdown of IL-32 in artificial skin equivalents (Fig 4, E).

IL-32 is expressed in lesional skin of patients with AD

Skin biopsy specimens were stained with anti-IL-32 mAbs to evaluate the expression of IL-32 in the epidermis. IL-32 was detected in KCs of lesional skin biopsy specimens from patients with AD (Fig 5). A cytoplasmic scattered staining pattern was observed in suprabasal KCs. In contrast, IL-32 was not expressed by KCs in skin biopsy specimens derived from healthy donors or from lesional skin biopsy specimens derived from patients with psoriasis (Fig 5).

IL-32 levels are increased in sera of patients with AD

Although IL-32 was not released from intact human primary KCs, IL-32 is present in the serum. The mean serum level of patients with AD was 65.2 pg/mL compared with 32.7 pg/mL in healthy control subjects. Serum IL-32 levels were also determined in patients with psoriasis and asthma. Asthmatic patients also showed increased serum levels (104.7 pg/mL), whereas IL-32 serum levels of patients with psoriasis were not different from those of healthy control subjects (Fig 6, A). Subanalysis of patients with AD with and without concomitant asthma showed no difference in IL-32 serum levels between these 2 groups (Fig 6, A). In addition, the IL-32 serum levels of patients with AD correlated well with the disease severity score, as measured by using the SCORAD system (Spearman r = 0.54, P < .001; Fig 6, B). There was no correlation between IL-32 serum levels and IgE serum levels (Fig 6, C).

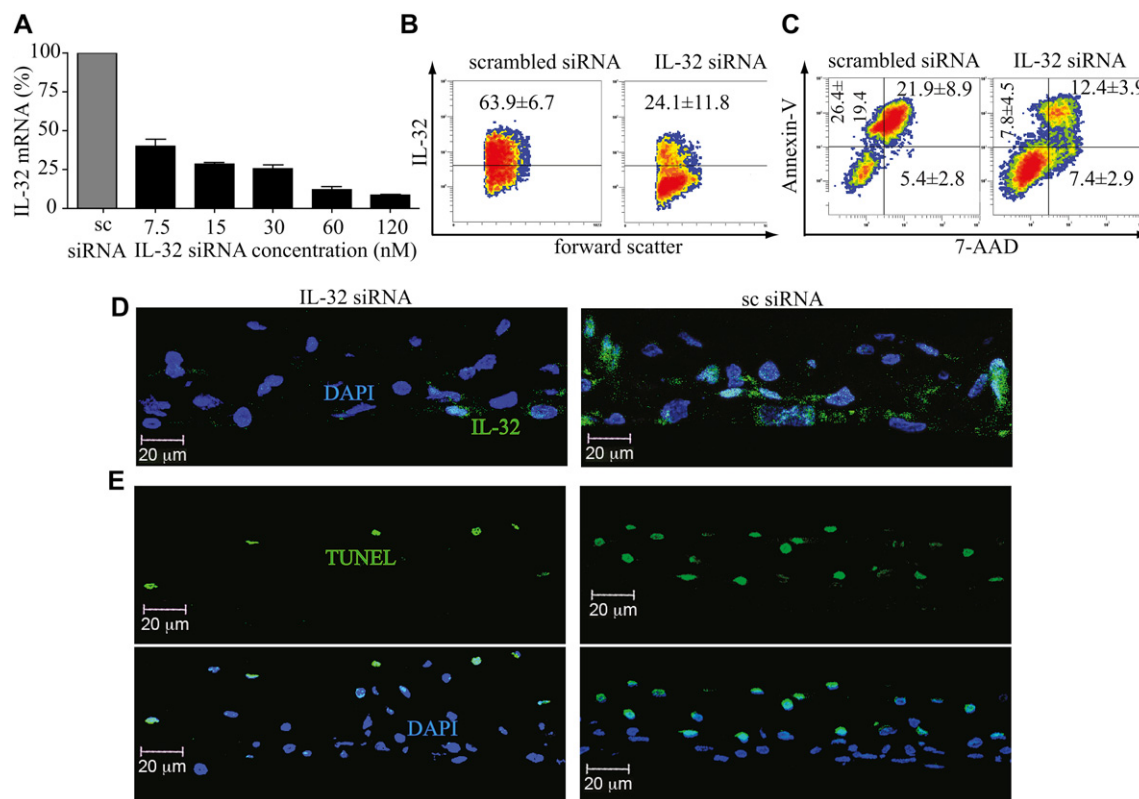


FIG 4. KC apoptosis induced by IFN- γ and TNF- α is IL-32 dependent. Relative IL-32 mRNA expression was analyzed in unstimulated KCs cultured for 24 hours after transfection with increasing IL-32 siRNA or scrambled (sc) siRNA concentrations. The changes in IL-32 mRNA levels were calculated by setting scrambled siRNA at 100% for each concentration (**A**). KCs transfected with IL-32 siRNA or scrambled siRNA were incubated with TNF- α and IFN- γ for 72 hours, and IL-32 expression was analyzed (**B**) and KC apoptosis was determined by means of 7-amino actinomycin D (7-ADD) and annexin-V staining (**C**). Artificial skin equivalents transfected with IL-32 siRNA or scrambled siRNA were stimulated with TNF- α and IFN- γ for 5 days. IL-32 was stained with Alexa Fluor 488 (green); nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue; **D**), and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining (Alexa Fluor 488, green) was performed (**E**, upper panels). Nuclei were stained with DAPI (blue), and merged pictures are presented in the lower panels of Fig 4, **E**. Fig 4, **A**: Mean $\pm$ SD, $n = 3$. Fig 4, **B** and **C**: Mean percentage $\pm$ SD, $n = 7$ ($P < .001$ for Fig 4, **B**, and $P = .017$ for Fig 4, **C**). Fig 4, **D** and **E**: One representative staining of 2 independent experiments. TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

DISCUSSION

In the present study we demonstrate that IL-32 is expressed at high levels in human epidermal KCs after stimulation with IFN- γ and TNF- α but is not secreted by KCs. T_H1 cells, but not T_H2, Treg, or T_H17 cells, induced expression of IL-32 in epidermal KCs. Neutralization experiments demonstrated that this effect was IFN- γ dependent. In addition, we investigated the functional effect of IL-32 on KCs. After siRNA knockdown of IL-32 in KCs and artificial skin equivalents, KC apoptosis was significantly decreased. Furthermore, we demonstrated that IL-32 is expressed in lesional skin of patients with AD and that IL-32 serum levels correlated with severity of AD disease.

IL-32 was initially detected in IL-2-activated T cells and NK cells.^{7,9} Subsequently, high levels of constitutive IL-32 have been demonstrated in endothelial cells of various origins.¹⁹ Our finding that IL-32 is expressed but not secreted by KCs is consistent with previous studies demonstrating that IL-32 was detected in the cytoplasm but was not secreted from PBMCs,

endothelial cells, or T cells.^{10,15,19} Overexpression of IL-32 in COS-7 cells resulted in secretion of IL-32,⁹ and the presence of IL-32 in the sera of patients with AD suggests that certain cell types can secrete this cytokine. However, the secretory mechanism for IL-32 still remains unclear. The late upregulation of IL-32 and the high increase in IL-32 mRNA after 72 hours compared with that at 24 hours suggest that intermediate cellular effectors or genes activated by IFN- γ are partially involved in the induction of IL-32.

AD is a chronic relapsing skin disorder involving multiple cell types, such as lymphocytes, fibrocytes, Langerhans cells, mast cells, and epidermal KCs.¹ Activated T cells infiltrating the skin play major roles in the pathogenesis of AD. Accordingly, we investigated the effects of different subsets of activated T cells on KCs and demonstrate that T_H1 cells induce KC IL-32 through IFN- γ . Moreover, several studies have identified additional IFN- γ -secreting cells, including NKT cells, macrophages, and dendritic cells (DCs), which are involved in the pathogenesis of AD.²⁰⁻²² After exposure to IFN- γ , KCs secrete a unique

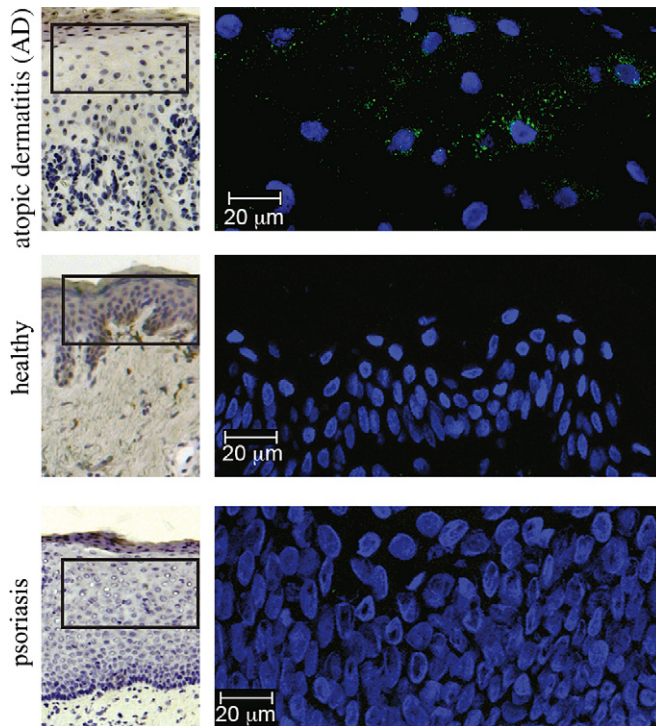


FIG 5. IL-32 is expressed in lesional skin of patients with AD. Hematoxylin-stained skin biopsy specimens from patients with AD, healthy subjects, and patients with psoriasis are presented (left panels). Solid rectangles in hematoxylin-stained skin biopsy specimens indicate the area of skin biopsy specimens from which immunostainings are shown. IL-32 was stained with Alexa Fluor 488 (green) in skin biopsy specimens from patients with AD, healthy subjects, and patients with psoriasis (right panels). IL-32 was detected in 5 lesional biopsy specimens from patients with AD and was not expressed in lesional biopsy specimens from patients with psoriasis ($n = 4$) or healthy donors ($n = 3$). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue).

profile of chemokines and cytokines, which attract immune cells, potentiating the inflammatory process.^{5,23} The aim of the treatment of AD is to break this inflammatory cycle with immunosuppressive drugs, such as FK506, glucocorticoids, and retinoic acid, which have been used for AD treatment with remarkable results.^{24–26} Interestingly, these anti-inflammatory drugs did not inhibit the induction of IL-32 in KCs by TNF- α and IFN- γ .

Activation of the immune system, particularly skin-homing T cells, transmigration of T cells to the skin, and effector functions, such as induction of KC apoptosis, IgE, and eosinophilia, represent cardinal features of allergic inflammation in patients with AD. The T_H0/T_H1 cells secrete significant amounts of the effector cytokines TNF- α , IFN- γ , and Fas ligand, which induce KC apoptosis, leading eventually to the eczematous lesions characteristic of AD.^{4,5,27} Organotypic skin models enabled the reproduction of several clinical hallmarks of eczematous dermatitis and demonstrated that T cells induce KC apoptosis, which leads to a reduced expression of E-cadherin and disruption of the epidermal barrier.²⁸ T cell-mediated KC apoptosis is located predominantly in suprabasal epidermal layers, suggesting that antiapoptotic mechanisms protect basal KCs.²⁹ Our observation that IL-32 increases KC apoptosis is consistent with recent studies showing that transfection of bone marrow stromal cells with IL-32 siRNA decreased IL-32 expression and reduced apoptosis

in leukemia cells.¹⁷ In addition, overexpression of IL-32 β in Hela cells was associated with increased apoptosis.¹⁰

Although AD is generally regarded as an immune cell-mediated disease, recent genetic studies have indicated the importance of abnormalities in the gene encoding filaggrin.^{30,31} In particular, patients with filaggrin mutations are predisposed to early-onset and extrinsic AD.³² Cell-autonomous differences have been described in bronchial epithelial cells derived from patients with atopic asthma and healthy nonatopic control subjects, showing a deficient innate immune response to rhinovirus in cells from asthmatic patients.³³ The increased IL-32 induction by TNF- α in KCs from patients with AD compared with KCs from healthy donors or patients with psoriasis suggests that certain patients with AD might have a specific genotype that contributes to increased levels of IL-32. Consequently, the increased IL-32 response to TNF- α in KCs from patients with AD could make them more susceptible to apoptosis.

Another source of IL-32 are DCs, which are in close contact to NK cells in AD lesions and can activate them through secretion of IL-12.³⁴ In addition to its proapoptotic role in KCs, intracellular IL-32 in DCs can influence IL-12 secretion and therefore probably decrease NK cell activation, resulting in a predisposition to viral infections in the skin.³⁵

Previous studies have established that the expression of IL-32 in tissues was associated with disease activity in patients with rheumatoid arthritis¹² and inflammatory bowel disease.⁸ We could detect IL-32 only in lesional skin from patients with AD and not in lesional skin of patients with psoriasis or healthy skin. In addition, serum IL-32 levels in patients with AD were significantly higher compared with levels in patients with psoriasis or healthy control subjects, and IL-32 serum levels correlated with AD disease severity. The cellular source of serum IL-32 in these patients is unclear, but it can be released from apoptotic KCs, or IL-32 could be secreted from other immune cells, such as macrophages, DCs, or T cells, which are involved in the inflammatory process of AD.^{9,13,36} Increased IL-32 serum levels in patients with asthma compared with those seen in healthy control subjects or patients with psoriasis support our hypothesis that IL-32 is released from apoptotic cells *in vivo*. The asthmatic epithelial response shows similarity to AD with increased apoptosis induced by T cells and eosinophils, with IFN- γ and TNF- α as key cytokines leading to epithelial cell shedding.³⁷ In contrast, the psoriatic epidermis shows a different KC response than AD. It is characterized by hyperproliferation and hyperkeratosis, whereas KC apoptosis is not a hallmark of this disease.³⁸ There is now evidence that T_H17 cells might be crucial in the pathogenesis of psoriasis,³⁹ whereas the IL-17/IL-23 axis is relatively reduced in patients with AD.⁴⁰ The difference in increased IL-32 levels in patients with AD compared with those seen in patients with psoriasis in the present study suggests that the inflammatory milieu in psoriasis does not support IL-32 expression in KCs. These results indicate that the evaluation of serum IL-32 levels could be a useful diagnostic tool for monitoring patients with AD or asthma, in which epithelial cell apoptosis is an important feature of the disease pathogenesis.

In conclusion, this study highlights IL-32 as a new cytokine increasing KC apoptosis, and IL-32 might play a role in the formation and maintenance of AD lesions. Disruption of IL-32 or its signaling mechanism might provide a novel target for the treatment of AD.

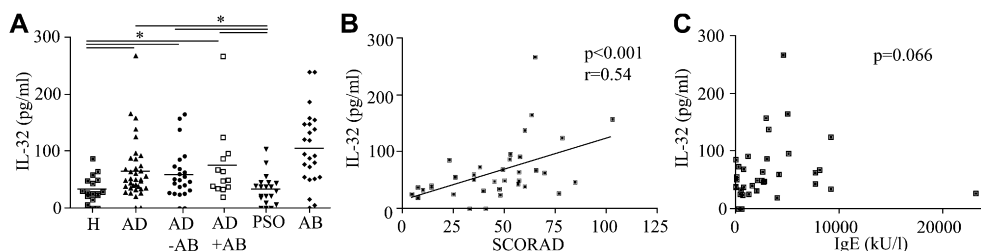


FIG 6. Serum IL-32 levels correlate with AD severity. IL-32 serum levels from patients with AD (AD, $n = 36$), healthy subjects (H, $n = 17$), patients with psoriasis (PSO, $n = 20$), and asthmatic patients (AB, $n = 24$) were analyzed with ELISA. Patients with AD were subdivided into patients with AD without (AD-AB, $n = 23$) or with (AD+AB, $n = 13$) concomitant asthma (A). IL-32 serum levels of patients with AD were correlated with the SCORAD values (B) and IgE levels (C). Fig 6, A: * $P < .05$; Fig 6, B: Spearman $r = 0.54$, $P < .001$; Fig 6, C: Spearman $r = 0.31$, $P = .066$.

Clinical implications: The current study shows a novel role for IL-32 in KC apoptosis and might provide a new target for the therapy of AD.

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METHODS

Culture of primary KCs and artificial skin equivalents

Skin-shave biopsy specimens (3 × 3 cm) were obtained after achievement of local anesthesia from 3 subjects with psoriasis vulgaris and 4 subjects with AD to isolate human primary KCs. In subjects with psoriasis and AD, the biopsy specimens were collected from the buttock area and represented nonlesional skin. Human primary KCs were also purchased from Promocell (Heidelberg, Germany). Cells were cultured in serum free KC medium (Keratinocyte-SFM; Invitrogen, San Diego, Calif) supplemented with 5 ng/mL human recombinant epidermal growth factor, 50 µg/mL bovine pituitary extract, and 2 µmol/L L-glutamine (all from Gibco, Basel, Switzerland) in a humidified atmosphere containing 5% CO₂ at 37°C. Medium was changed every second day, and cells between passages 2 and 5 were used in all experiments. Artificial skin equivalents were purchased from CellSystems (St Katharine, Germany) and cultured according to the manufacturer's instructions. After 5 days, the artificial skin equivalents were washed and fixed according to the manufacturer's instructions (CellSystems) and placed in Tissue-Tek OCT compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and stored at -80°C. Seven-millimeter cryostat sections were prepared on slides (Menzel GmbH, Braunschweig, Germany). After air-drying the slides, immunofluorescence staining was performed.

Reagents

Primary KCs were incubated with IL-4 (R&D, Abingdon, United Kingdom), IL-13 (R&D), TNF-α (R&D), and IFN-γ (R&D). Dose titrations were performed. The optimal concentration for inducing IL-32 was 12.5 ng/mL for IFN-γ and 25 ng/mL for TNF-α. Dexamethasone, 13-cis retinoic acid, rapamycin, and FK506 were purchased from Sigma-Aldrich (St Louis, Mo).

siRNA transfections

Transfection reagents and siRNA were purchased from Applied Biosystems (Foster City, Calif). For transfection, a proprietary blend of polyamines that delivers siRNA into mammalian cells was used (siPORTTM Amine Transfection Agent; Ambion, Austin, Tex). The antisense sequence of IL-32 siRNA was 5'-UCUUUUUCAAGUAGAGGAGtg-3', and as a negative control, scrambled siRNA was used (Applied Biosystems). KCs were counted with trypan blue, centrifuged at 270g, and resuspended in prewarmed KC medium to 1 × 10⁵ cells/mL. The siRNA/transfection agent complexes were prepared according to the manufacturer's instructions and subsequently distributed into the culture well plates. KCs were transferred to the culture plate, and after a recovery period of 6 hours, KCs were either stimulated or left untreated. The corresponding amount of distilled water in which siRNA was solved was used for transfection to test whether IL-32 or scrambled siRNA is toxic to KCs. Twenty-four hours after siRNA transfection and distilled water coinoculation, the cell viability was analyzed (Cell-Titer Blue Reagent; Promega, Madison, Wis). There was no difference between viabilities of scrambled siRNA-transfected, IL-32 siRNA-transfected KCs, and distilled water-coincubated KCs. For transfection of artificial skin equivalents, siRNA/transfection agent complexes were prepared as described above and distributed into the culture well plates every 12 or 24 hours for the first 3 days of culturing. Because several reports have indicated that high siRNA concentrations could lead to nonspecific changes in gene expression,^{E1,E2} siRNA concentrations for cell culture experiments were between 7.5 and 120 nmol/L.

Real-time PCR mRNA expression analysis

Total RNA from KCs was extracted with the RNeasy kit (Qiagen, Basel, Switzerland), according to the manufacturer's instructions. Reverse transcription was performed by using reverse transcriptions reagents with random hexamers (Fermentas GMBH c/o Labforce AG, Nunningen, Switzerland). Relative quantification was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and the 2^{-ΔΔCt} formula.^{E3} The relative mRNA levels were compared with unstimulated cells based on the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. For analysis, the relative mRNA expression of the unstimulated control was

set at 1. Primers were designed to flank or cross an exon-intron border, and primer sequences are listed in Table E1 (available in this article's Online Repository at www.jacionline.org). In siRNA experiments relative IL-32 mRNA expression in IL-32 siRNA conditions was compared with relative IL-32 mRNA expression in scrambled siRNA conditions. For this, relative IL-32 mRNA expression of scrambled siRNA-transfected cells was set to 100%.

Differentiation of T_H1, T_H2, Treg, and T_H17 cells

PBMCs were isolated by means of Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation of peripheral venous blood. CD45RA⁺ T cells were isolated with the MACSTM system (Miltenyi Biotec, Bergisch Gladbach, Germany). Treg cells were generated in the presence of anti-IL-4 mAbs (5 µg/mL; R&D), anti-IL-12 mAbs (25 ng/mL, R&D), anti-IFN-γ mAbs (1 µg/mL, R&D), and TGF-β (5 ng/mL, R&D). T_H1 cells were differentiated in the presence of IL-12 (5 ng/mL, R&D) and anti-IL-4 mAbs (5 µg/mL, R&D). T_H2 cells were generated in the presence of IL-4 (25 ng/mL, R&D), anti-IFN-γ mAbs (1 µg/mL, R&D), and anti-IL-12 mAbs (25 ng/mL, R&D). T_H17 cells were differentiated in the presence of TGF-β (5 ng/mL, R&D), IL-6 (20 ng/mL; PeproTech EC, London, United Kingdom), IL-1β (10 ng/mL, PeproTech EC), IL-23 (10 ng/mL; eBioscience, San Diego, Calif), anti-IL-12 mAbs, and anti-IFN-γ mAbs (1 µg/mL, R&D). T-cell phenotypes were confirmed by measuring cytokine levels in culture supernatants by means of ELISA (Luminex; BioRad, Hercules, Calif), and transcription factor expression was quantified by means of real-time PCR. T_H1 cells secreted 29.2 ng/mL IFN-γ, T_H2 cells secreted 51.4 ng/mL IL-4 and 4.9 ng/mL IL-13, and T_H17 cells secreted 667.6 pg/mL IL-17. Relative forkhead box protein 3 mRNA expression in Treg cells was at least 47.5 times higher compared with that of other cell lineages.

ELISA

A sandwich ELISA for the detection of IL-32 was developed. Plates were incubated with capture mAb (KU32-56, final concentration of 0.25 µg/mL; BioLegend, San Diego, Calif) diluted in coating buffer for 1 hour at room temperature. Afterward, plates were blocked with blocking buffer (assay diluent, BioLegend) for 1 hour at room temperature. Samples and serially diluted recombinant IL-32α (BioLegend) were added to wells for 2 hours at room temperature. After washing the plates 4 times with PBS containing 0.05% Tween-20 (Fluka, Buchs, Switzerland), the detection mAb (biotinylated KU32-52, final concentration of 0.1 µg/mL, BioLegend) was added for 1 hour. Plates were washed 3 times with wash buffer, followed by horseradish peroxidase conjugate (BioLegend) incubation for 15 minutes, and were finally washed 6 times with wash buffer. ELISAs were developed with TMB substrate (BioLegend), and the absorbance at 450 nm was measured. The detection range was between 5 and 4000 pg/mL, and IL-32α and IL-32β isoforms were detected, but not γ and δ isoforms. The antibodies had no cross-reaction with other cytokines, such as IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-18, and TNF-α.^{E4}

The cell lysates for ELISA were prepared according to the following protocol. KCs were centrifuged for 5 minutes at 14,000g, and the pellet was resuspended in 30 µL of buffer C containing protease inhibitor (420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 20 mmol/L HEPES, 0.2 mmol/L EDTA, and 1 µmol/L NP-40) for 20 minutes at 4°C. After spinning at 14,000g for 1 minute, supernatants were collected, and 60 µL of buffer D (1.5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 20 mmol/L HEPES, 0.2 mmol/L EDTA, and 1 µmol/L NP-40) was added. Samples were stored at -80°C and analyzed in batches.

Western blot analysis

Protein extraction was performed as described in the ELISA section. Samples were loaded next to a prestained protein-mass ladder (Invitrogen) on a NuPAGE 12% bis-Tris gel (Invitrogen). The proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences, Glattbrugg, Switzerland). Unspecific binding was blocked with TBS-Tween 5% milk powder. After blocking, the membrane was incubated with an anti-IL-32 mAb (KU32-52, final concentration of 0.1 µg/mL, BioLegend) in blocking buffer overnight at 4°C. Next the membrane was washed and incubated with an

anti-mouse horseradish peroxidase-labeled antibody (GE Healthcare, Glattbrugg, Zurich, Switzerland; 1:10,000) at room temperature for 1 hour. The blot was developed with an ECL plus Western Blot Detection System (GE Healthcare) and visualized with an LAS 1000 camera (Fuji, Düsseldorf, Germany). Membranes were stripped, reblocked, and reprobed with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody To confirm equal sample loading and transfer (6C5, Ambion, 1:4,000).

Flow cytometric analysis

Anti-IL-32 mAb and murine IgG1A isotype control were purchased from R&D and labeled with Alexa Fluor 488 (Alexa Fluor 488 Monoclonal Antibody Labeling Kit; Invitrogen, Paisley, United Kingdom), according to the manufacturer's instructions. For fixation and permeabilization of KCs, the BD Cytofix/Cytoperm kit was used, according to the manufacturer's instructions (BD biosciences, San Diego, Calif), and subsequently, KCs were incubated in blocking solution (PBS, 0.5% BSA, and 10% rat serum) for 20 minutes at room temperature. Anti-IL-32 mAb or IgG1A isotype control was added for 45 minutes at room temperature, and after washing twice with PBS containing 0.5% BSA, cells were analyzed by means of flow cytometry, which was performed on an EPICS XL-MCL using the Expo32 software (Beckman Coulter, Fullerton, Calif).

Immunofluorescence staining

KCs grown on cover slips were fixed for 10 minutes with 1% formaldehyde (Fluka). At -25°C , 5- to 10-mm slides of artificial skin equivalents or skin biopsy specimens were cut with a cryomicrotome. Skin biopsy specimens were fixed for 8 minutes with 4% formaldehyde (Fluka), followed by permeabilization with detergent (0.1% Triton [Fluka] and 0.02% SDS [Roth, Karlsruhe, Germany] in PBS [Invitrogen, Basel, Switzerland]) for 5 minutes. Nonspecific staining was minimized by means of incubation with 2%

BSA (Fluka) for 30 minutes. Biopsy specimens were incubated for 1 hour with anti-IL-32 mAb (KU32-52, BioLegend) and fixed in 1% formaldehyde. After being washed in 2% BSA with PBS, cells were incubated with the secondary goat anti-mouse polyclonal antibody linked to Alexa Fluor 488. Slides were mounted with VectaShield mounting medium containing 4',6-diamidino-2-phenylindol (Vector Laboratories, Burlingame, Calif) and analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems AG, Glattbrugg, Switzerland).

Determination of apoptosis

Membrane phosphatidylserine redistribution and cell viability were determined by using phycoerythrin-conjugated annexin-V and 7-amino-actinomycin D (7AAD) staining and flow cytometry, according to the manufacturer's manual (Beckmann Coulter, Nyon, Switzerland). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling was performed on frozen sections of artificial skin equivalents, according to the manufacturer's manual (MEBSTATIN apoptosis kit II; MBL, Nagoya, Japan).

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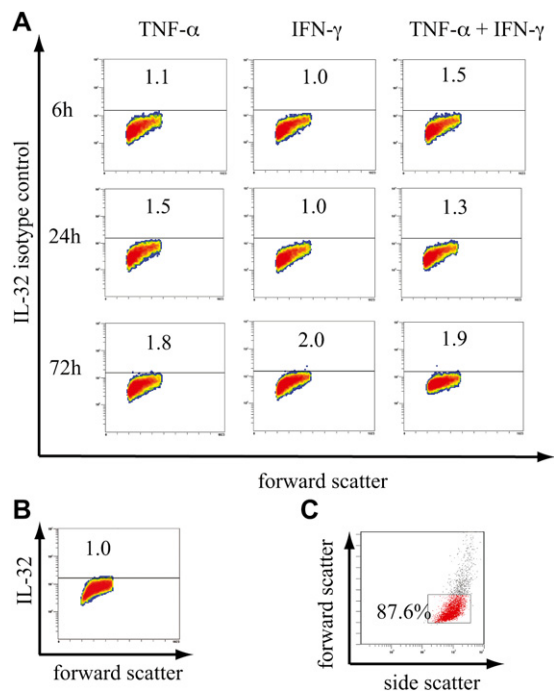


FIG E1. IL-32 isotype controls, unstimulated control, and gating strategy for Fig 2. IgG2A isotype controls for IL-32 staining for each condition (A), unstimulated control stained with anti-IL-32 mAb (B), and gating strategy of KCs (C) are presented.

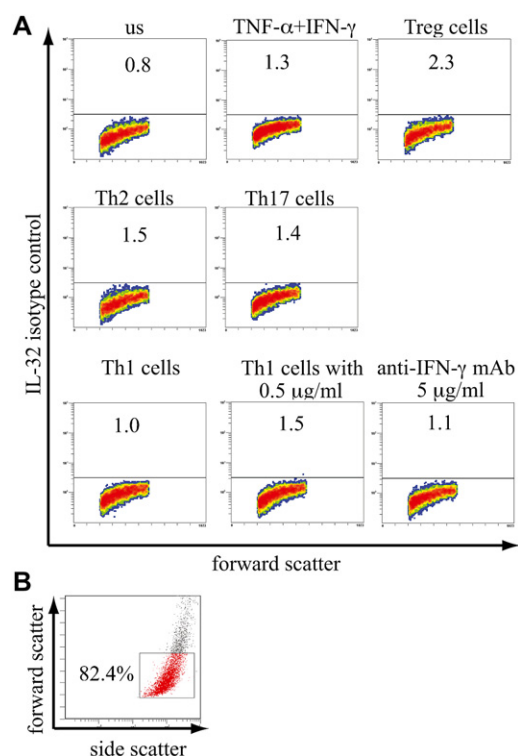


FIG E2. IL-32 isotype controls and gating strategy for Fig 3. IgG2A isotype controls for IL-32 staining for each condition (**A**) and gating strategy of KCs (**B**) are presented. *us*, Unstimulated.

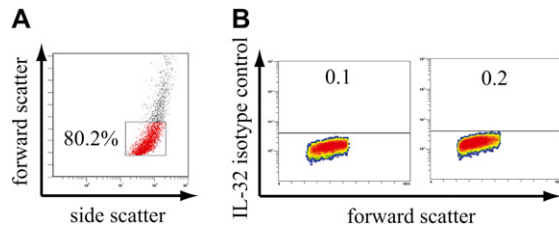


FIG E3. IL-32 isotype controls and gating strategy for Fig 4. Gating strategy of KCs for IL-32 staining (A) and IgG2A isotype controls for each condition (B) are presented.

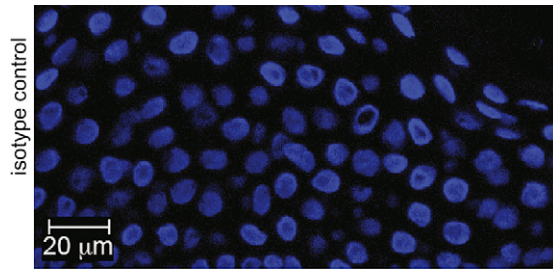


FIG E4. IL-32 isotype control. IgG2A isotype control stained with Alexa Fluor 488 (*green*) for Fig 5 (skin biopsy of a patients with AD) is shown. Nuclei were stained with 4',6-diamidino-2-phenylindole (*blue*).

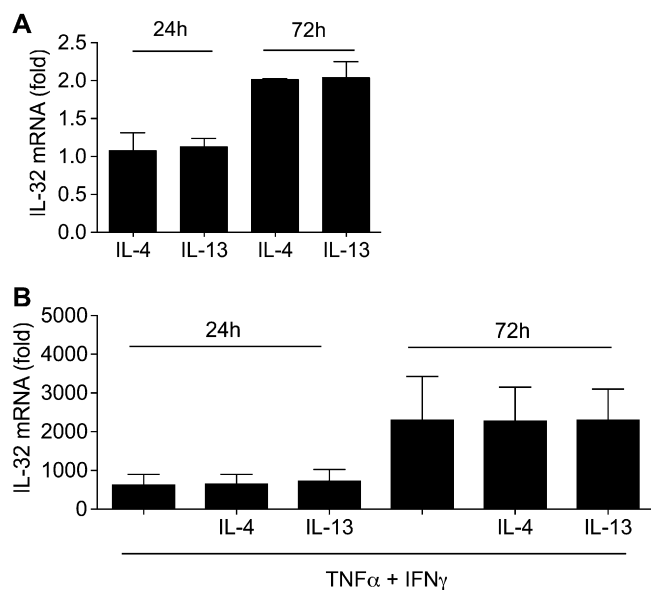


FIG E5. IL-4 and IL-13 have no effect on IL-32 mRNA expression in KCs. KCs were incubated with IL-4 (4 ng/mL) and IL-13 (10 ng/mL) for 24 or 72 hours, and relative IL-32 mRNA expression was analyzed by means of real-time PCR (**A**). Relative IL-32 mRNA expression was analyzed after incubation with TNF- α (25 ng/mL) and IFN- γ (12.5 ng/mL) with or without IL-4 (4 ng/mL) or IL-13 (10 ng/mL) after 24 or 72 hours (**B**). Real-time PCR quantification was based on comparative $\Delta\Delta CT$ method by using glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene and was normalized to the unstimulated control. Mean $\pm$ SD, $n = 3$.

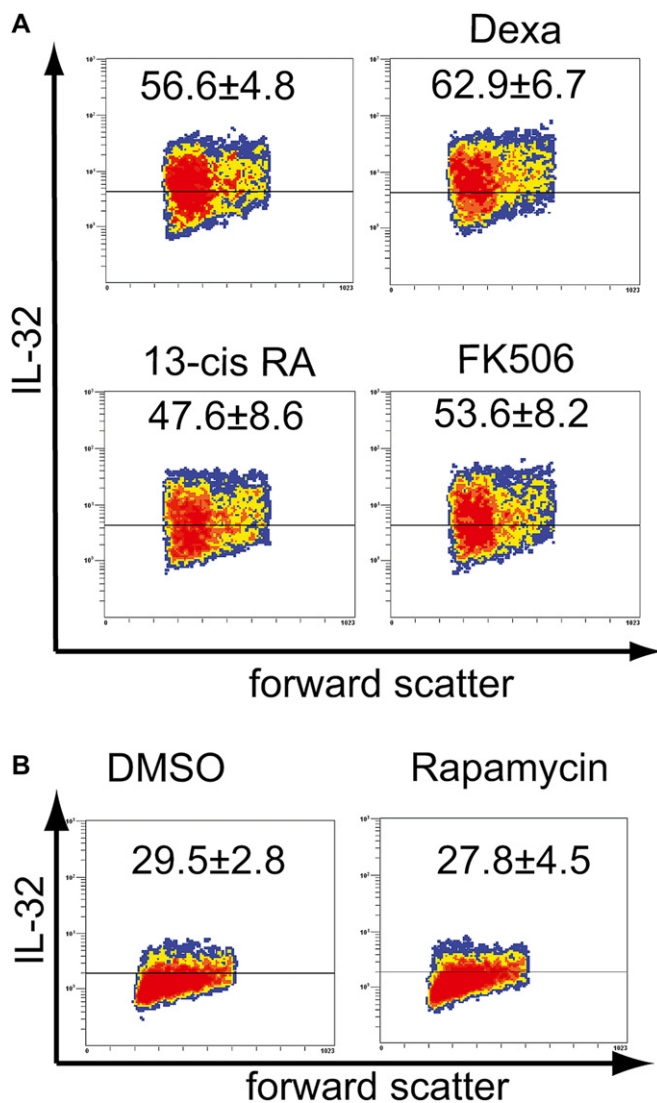


FIG E6. Induction of IL-32 by TNF- α and IFN- γ is not influenced by immunosuppressive drugs. KCs were preincubated with 1 μ mol/L dexamethasone (*Dexa*), 1 μ mol/L 13-cis retinoic acid (*13-cis RA*), or 1 μ mol/L FK506 for 30 minutes, and subsequently, TNF- α (25 ng/mL) and IFN- γ (12.5 ng/mL) were added for 72 hours (**A**). To exclude the involvement of the mammalian target of rapamycin (mTOR) pathway, 10 nmol/L rapamycin and the according amount of dimethyl sulfoxide (*DMSO*; for the control) were added, and KCs were stimulated with TNF- α (25 ng/mL) and IFN- γ (12.5 ng/mL) for 72 hours (**B**). IL-32 expression was analyzed by means of flow cytometry. Mean $\pm$ SD, n = 3.

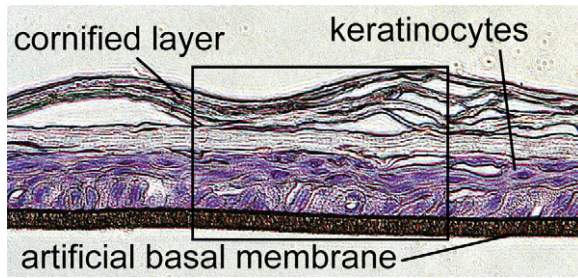


FIG E7. Structure of artificial skin equivalents. One representative hematoxylin staining of artificial skin equivalents is shown.

TABLE E1. Primer sequences

IL-32		
Forward		GAC AGT GGC GGC TTA TTA TGA G
Reverse		CCT CGG CAC CGT AAT CCA T
IL-32 α		
Forward		GAC AGT GGC GGC TTA TTA TGA G
Reverse		GCT CCG TAG GAC TTG TCA CAA A
IL-32 β		
Forward		CTGTCTCTCTCGGCTGAGTATTTGT
Reverse		TCT TCA TGT CAT CAG AGA GGA CCT T
IL-32 γ		
Forward		GTA ATG CTC CTC CCT ACT TC
Reverse		GCA AAG GTG GTG TCA GTA TC
IL-32 δ		
Forward		GAC GTG GAC AGG ACG ACT TCA
Reverse		CCT CGG CAC CGT AAT CCA T
GAPDH		
Forward		TGC ACC ACC AAC TGC TTA
Reverse		GGC ATG GAC TGT GGT CAT

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.