Mechanisms of IFN-γ–induced apoptosis of human skin keratinocytes in patients with atopic dermatitis

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Background: Enhanced apoptosis of keratinocytes is the main cause of eczema and spongiosis in patients with the common inflammatory skin disease atopic dermatitis (AD).
Objective: The aim of the study was to investigate molecular mechanisms of AD-related apoptosis of keratinocytes.
Methods: Primary keratinocytes isolated from patients with AD and healthy donors were used to study apoptosis by using annexin V/7-aminoactinomycin D staining. Illumina mRNA Expression BeadChips, quantitative RT-PCR, and immunofluorescence were used to study gene expression. *In silico* analysis of candidate genes was performed on genome-wide single nucleotide polymorphism data.
Results: We demonstrate that keratinocytes of patients with AD exhibit increased IFN-γ–induced apoptosis compared with keratinocytes from healthy subjects. Further mRNA expression analyses revealed differential expression of apoptosis-related genes in AD keratinocytes and skin and the upregulation of immune system–related genes in skin biopsy specimens of chronic AD lesions. Three apoptosis-related genes (*NOD2*, *DUSP1*, and *ADM*) and 8 genes overexpressed in AD skin lesions (*CCDC109B*, *CCL5*, *CCL8*, *IFI35*, *LYN*, *RAB31*, *IFITM1*, and *IFITM2*) were induced by IFN-γ in primary keratinocytes. The protein expression of IFITM1, CCL5, and CCL8 was verified in AD skin. In line with the functional studies and AD-related mRNA expression changes, *in silico* analysis of genome-wide single nucleotide polymorphism data revealed evidence of an association between AD and genetic markers close to or within the *IFITM* cluster or *RAB31*, *DUSP1*, and *ADM* genes.
Conclusion: Our results demonstrate increased IFN-γ responses in skin of patients with AD and suggest involvement of multiple new apoptosis- and inflammation-related factors in the development of AD. (J Allergy Clin Immunol 2012;129:1297-306.)

Key words: *Cytokine, mRNA expression array, atopic eczema, inflammation, allergy*

Atopic dermatitis (AD) is a common chronically relapsing skin disease that is characterized by the disturbance of epidermal barrier function, recurrent skin inflammation, and accompanying apoptosis of keratinocytes.[1-3] Linkage and association studies have identified several candidate genes possibly linked to either epidermal barrier function or to immune processes.[2,4] For instance, variants of *IL4/IL13* receptor,[5,6] *IL13*,[7] and the gene encoding the α-chain of the high-affinity receptor for IgE *(FCER1A)* have been shown to be associated with AD.[8] Concordantly, a predominant T_H2 bias with increased IgE levels is a widely recognized hallmark of AD. Nonetheless, in the chronic phase of skin inflammation, IFN-γ, as the characteristic cytokine for T_H1 cells, is dominant in the skin of patients with AD.[2,9,10] The presence of IFN-γ, excess of other cytokines and often accompanying skin infection lead to enhanced and disease-related apoptosis of keratinocytes in the eczematous lesions of patients with AD.[1-3] In contrast, keratinocytes in patients with psoriasis, the most closely analogous skin disease, undergo hyperproliferation and altered differentiation.[2,11] Apoptosis is known to occur through death receptors that are activated by their ligands. Keratinocytes have been shown to express TNF-α receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), fibroblast growth factor–inducible 14 (FN14), and TNF receptor superfamily

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Abbreviations used
7-AAD: 7-Aminoactinomycin D
AD: Atopic dermatitis
FAS: TNF receptor superfamily member 6
FN14: Fibroblast growth factor–inducible 14
KORA: Cooperative Health Research in the Region of Augsburg
qRT-PCR: Quantitative RT-PCR
SNP: Single nucleotide polymorphism
TNF-R: TNF receptor
TRAIL: TNF-related apoptosis-inducing ligand
TWEAK: TNF-like weak inducer of apoptosis

member 6 (FAS).[12] Previously, it was shown that IFN-γ–induced apoptosis occurs through FAS both in keratinocytes and IFN-γ–producing T cells.[13-15] Although IFN-γ appears to be a key factor, other cytokines, such as TNF-α, TNF-like weak inducer of apoptosis (TWEAK), and IL-32, can contribute to keratinocyte apoptosis in patients with AD.[16,17]

In the present study molecular mechanisms of AD-related apoptosis of keratinocytes were investigated. Interestingly, we found enhanced IFN-γ–stimulated apoptosis of keratinocytes from patients with AD, whereas no difference between the studied groups was found when other death ligands were used. To search for genes responsible for the increased sensitivity of keratinocytes from patients with AD to IFN-γ–stimulated apoptosis, we performed mRNA array analyses of keratinocytes and skin of patients with AD. Our results show that several differentially regulated immune system– and apoptosis-related genes are stimulated by IFN-γ in keratinocytes, which might be associated with AD.

METHODS

Keratinocyte cultures and apoptosis detection

Generation and maintenance of primary keratinocytes from healthy subjects, patients with AD, and patients with psoriasis and apoptosis assays are described previously.[16,17] In brief, all included subjects were older than 18 years and did not receive systemic treatment and topical corticosteroids during 1 week before the study. Keratinocytes were collected from unlesional skin from the atopic subjects. Viability represents the percentage of annexin V– and 7-aminoactinomycin D (7-AAD)–negative cells (ie, cells that were early apoptotic [annexin V-positive] and late apoptotic and necrotic [annexin V and 7-AAD-positive] were excluded).

Skin biopsy specimens for mRNA expression analysis

This study was approved by the Ethical Review Committees on Human Research of the University of Tartu and the University of Szeged. All participants signed a written informed consent form. In total, 10 patients with chronic AD and 10 healthy subjects older than 18 years were included. Eight patients had 6- to 14-day-long severe exacerbation of the disease, and 2 patients had more than 4-week-old dermatitis. No patients had been treated with systemic antihistamines and topical corticosteroid for at least 1 week before inclusion into the study. All the patients had blood eosinophilia. Two skin biopsy specimens (diameter, 4 mm), one from lesional skin and the second from uninvolved skin, were obtained from each patient. One biopsy specimen was taken from each healthy control subject. All biopsy specimens were snap-frozen in liquid nitrogen and stored at −80°C.

mRNA array analysis

mRNA profiling was performed on Illumina HumanHT-12 Expression BeadChips (Illumina, San Diego, Calif). Keratinocyte and skin mRNA array data are available at ArrayExpress as E-TABM-728 and E-MTAB-729, respectively.

For more information on proliferation assay, flow cytometry, immunofluorescence, RNA isolation, and quantitative RT-PCR, see the Methods section in this article's Online Repository at www.jacionline.org.

Statistics

For apoptosis, viability, and proliferation assays, statistical analysis between paired conditions (noninduced and induced keratinocytes from the same subject) was performed by using the Wilcoxon signed-rank test. The comparison between the groups was performed with nonparametric Mann-Whitney *U* tests (Fig 1, *B* and *C*, and see Fig E1, *B*, in this article's Online Repository at www.jacionline.org). Statistical analysis of quantitative RT-PCR (qRT-PCR) results was performed by using the nonparametric Mann-Whitney *U* test. The results were considered significant at a *P* value of less than .05 and highly significant at *P* values of less than .01 and .001.

mRNA array data were analyzed with Genomestudio software by using the custom rank invariant method (Illumina) for normalization. Genes with differential expression *P* values of less than .05 were considered differentially expressed. Pathway analysis was performed with g:Profiler (http://biit.cs.ut.ee/gprofiler/) by using default parameters. Detailed description of analysis and visualization procedures can be found in the Methods section in this article's Online Repository at www.jacionline.org.

For *in silico* candidate gene analysis, differentially regulated genes grouped by means of pathway analyses were investigated by using existing single nucleotide polymorphism (SNP) data from 533 patients with AD recruited in Munich and Kiel and were part of a recently published genome-wide association study.[18] As control subjects, we chose 1996 subjects from the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4/F4 survey.[19] We extracted SNPs from identified genes plus a surrounding region of ±50 kb by using the UCSC genome browser (assembly GRCh37/hg19, February 2009).[20] SNPs were filtered according to a call rate of greater than 0.97, a Hardy-Weinberg equilibrium deviation *P* value of less than .001, and a minor allele frequency in control subjects of greater than 0.05. The case-control analysis was carried out with PLINK[21] by using a χ^2 test for the 2×2 table for each SNP. Odds ratios were derived from a 2×2 contingency table. Because we investigated candidate genes with *a priori* evidence from mRNA expression analysis, we defined a significance threshold *P* value of less than .01 according to the χ^2 test. Haplotype analysis was performed with the R-package haplo.stats[22] within R 2.13.0 software (http://www.R-project.org/).

RESULTS

Increased IFN-γ–induced apoptosis of keratinocytes from patients with AD

To study AD-related keratinocyte apoptosis, we first evaluated the viability of healthy primary keratinocytes, which revealed a strong influence of IFN-γ on keratinocyte apoptosis that was further enhanced when keratinocytes were exposed to IFN-γ in combination with TRAIL or TWEAK. TRAIL and TWEAK alone did not influence the viability of primary keratinocytes (Fig 1, *A*), whereas the HaCat cells were sensitive to these cytokines (see Fig E1, *A*). We next investigated whether there is a difference in susceptibility to apoptosis between keratinocytes from healthy subjects, patients with AD, and patients with psoriasis. Keratinocytes from 5 different donors in each group were studied. Interestingly, significantly more healthy keratinocytes (67.6 ± 8.1) were viable in comparison with keratinocytes from patients with AD (51.4% ± 5.4%). Of keratinocytes from patients with psoriasis, 62.4% ± 7.2% were viable. Less cells were viable when treated with TWEAK or TRAIL in combination with IFN-γ, although no differences among the 3 keratinocyte groups were observed (Fig 1, *B*). According to annexin V–positive/7-AAD–negative staining, 27.1% ± 13.8% keratinocytes from patients with AD,

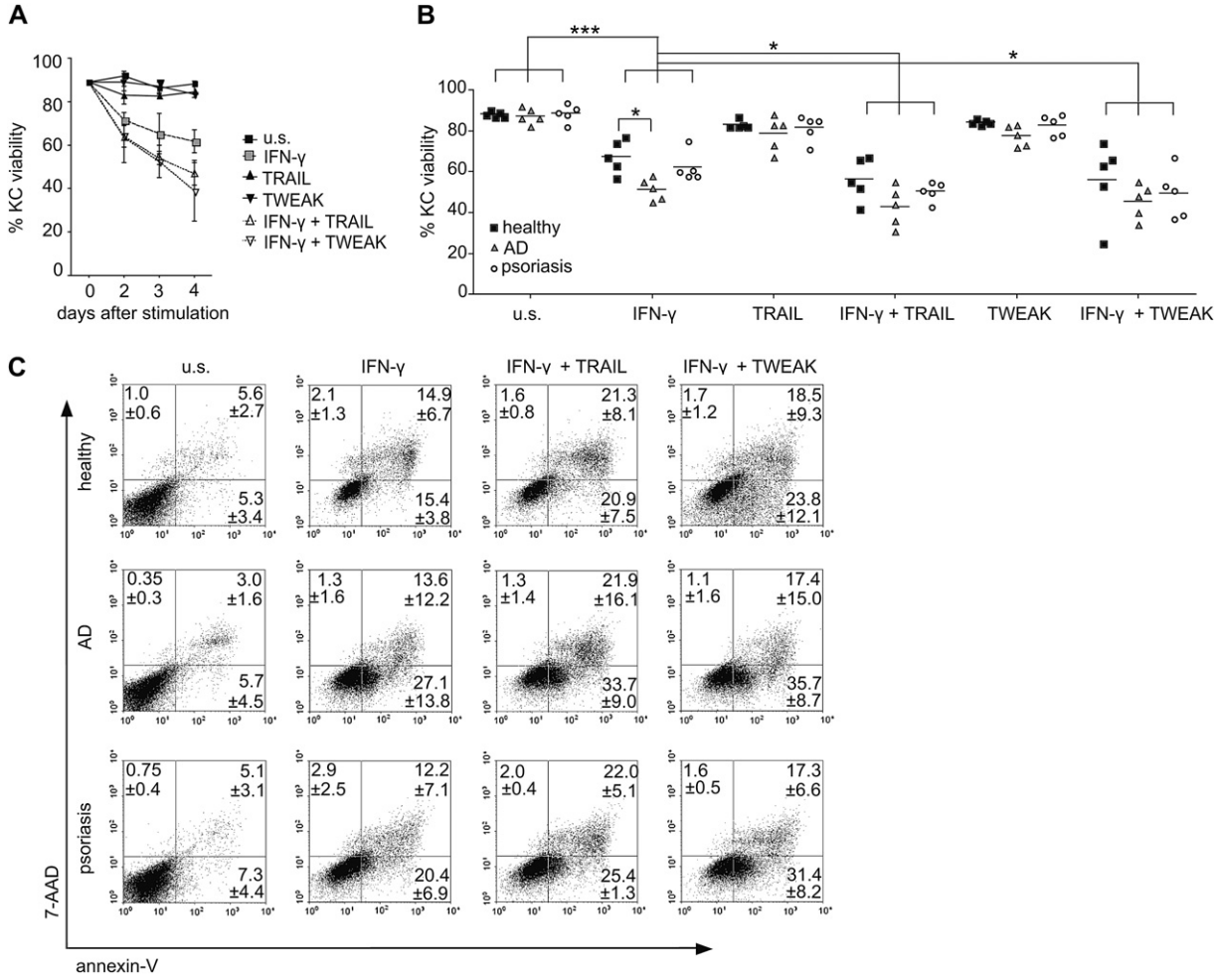

FIG 1. Increased IFN-γ–stimulated apoptosis of keratinocytes *(KC)* from patients with AD. **A,** The viability (annexin V– and 7-AAD–negative cells) of unstimulated *(u.s.)* or stimulated primary keratinocytes is presented as means ± SDs (n = 2). **B,** The viability of primary keratinocytes 4 days after stimulation (n = 5 for each group). **C,** One representative experiment is presented. Means ± SDs of all experiments are shown in the quadrants. **P* < .05 and ****P* < .001.

15.4% ± 3.8% keratinocytes from healthy subjects, and 20.4% ± 6.9% keratinocytes from patients with psoriasis showed apoptosis after IFN-γ stimulation, which indicates that mainly early apoptosis was enhanced in keratinocytes from patients with AD (Fig 1, *C*). Anti-FAS mAb, TNF-α, and their combinations with IFN-γ resulted in no difference on the level of apoptosis among keratinocytes from healthy subjects, patients with AD, and patients with psoriasis (see Fig E1, *B*). There was no significant difference in proliferation of keratinocytes treated with IFN-γ, TRAIL, TNF-α, and TWEAK among the 3 studied groups (see Fig E2 in this article's Online Repository at www.jacionline.org). Together, these data demonstrate that keratinocytes from patients with AD are more susceptible to IFN-γ–induced apoptosis.

The expression of death and decoy receptors and their ligands in keratinocytes is not different between healthy subjects, patients with AD, and patients with psoriasis

We next analyzed the mRNA expression of several known death and decoy receptors in keratinocytes from healthy subjects, patients with AD, and patients with psoriasis. TNF-R1 and FN14 exhibited the highest level of basal mRNA expression, whereas expression of TNF-R2 was the lowest. There was no difference in expression levels among the 3 groups (Fig 2, *A*). In the IFN-γ–treated cells TRAIL was upregulated 20-fold during the first 8 hours of stimulation. TRAIL-R2, TRAIL-R3, and TRAIL-R4 levels were downregulated up to 2-fold, and TRAIL-R1 levels remained unchanged (Fig 2, *B* and *C*). Downregulation of the TRAIL-R2 protein from the surface of keratinocytes was also observed (Fig 2, *D*). A greater than 100-fold and 8-fold increase in TNF-R2 and TNF-α mRNA expression, respectively, was detected (Fig 2, *E*). Other apoptosis-related proteins, such as FN14, TWEAK, TNF-R1, and FAS, did not respond to IFN-γ (see Fig E3 in this article's Online Repository at www.jacionline.org). There was no change in the expression of death receptors when either TNF-α, TWEAK, TRAIL, or anti-FAS mAbs were used (see Fig E4 in this article's Online Repository at www.jacionline.org). None of the studied death or decoy receptors or their ligands showed differences in their mRNA expression between keratinocytes from healthy subjects, patients with AD, and patients with psoriasis (Fig 2, *B-E*).

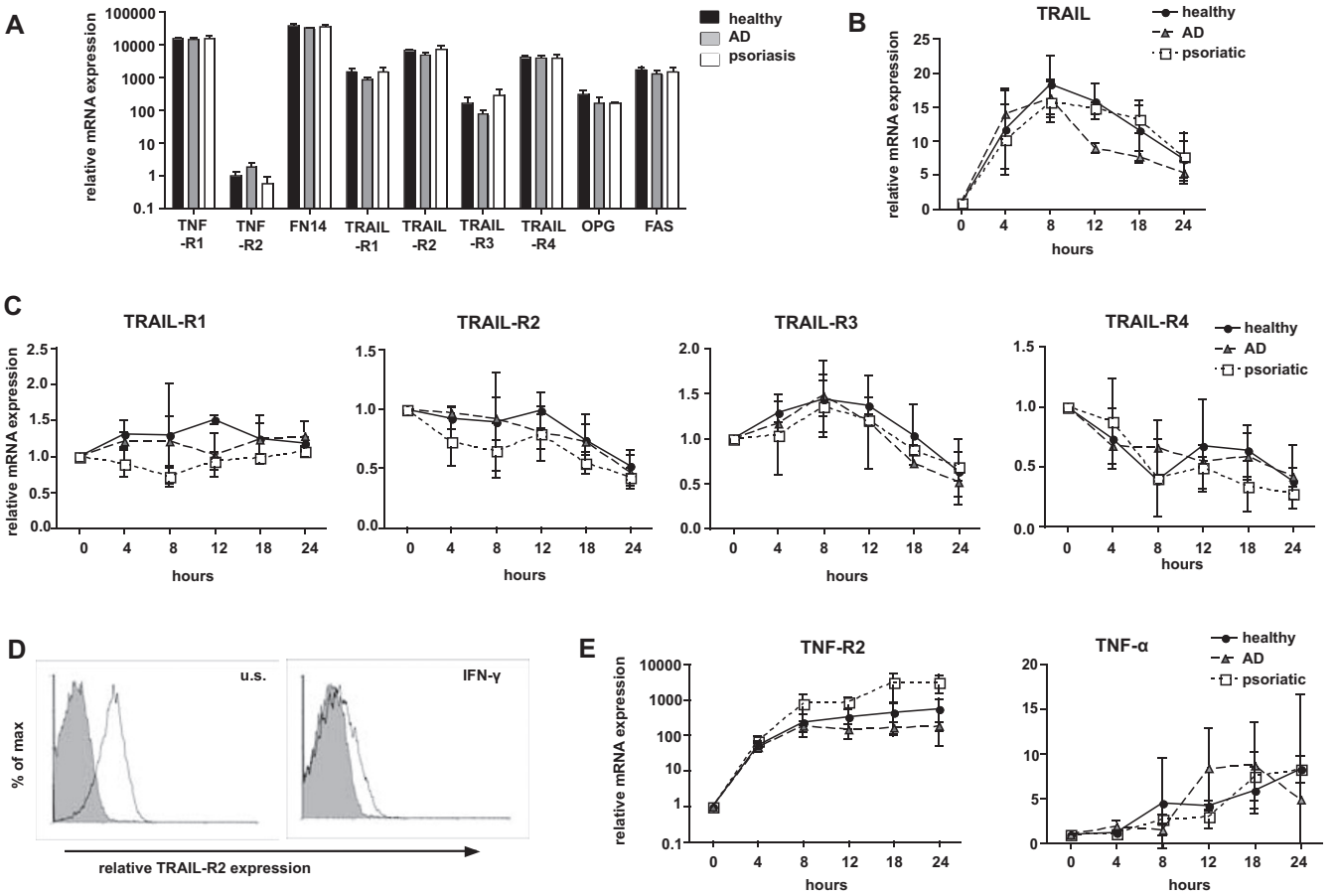

FIG 2. Influence of IFN-γ on the expression of TRAIL, TNF-α, and their receptors in keratinocytes. **A,** Relative mRNA expression (mean ± SD) compared with the expression level of TNF-R2 in keratinocytes (n = 3 of each group). **B, C,** and **E,** Relative mRNA expression (mean ± SD) after stimulation with IFN-γ compared with time point 0 (=1; n = 3 from each group). **D,** Surface expression of the TRAIL-R2 protein *(open curve)* and the isotype control *(solid curve). u.s.,* Unstimulated.

Several apoptosis-related genes are differentially regulated in keratinocytes from patients with AD

To search for cellular factors that can be responsible for the enhanced IFN-γ–induced apoptosis of keratinocytes from patients with AD, we next performed an mRNA array analysis of keratinocytes from patients with AD and healthy control subjects. A total of 85 genes were found to be differentially expressed in keratinocytes from patients with AD compared with those from healthy subjects (see Tables E1 and E2 in this article's Online Repository at www.jacionline.org). qRT-PCR analysis of 6 selected genes confirmed the array results (see Fig E5 in this article's Online Repository at www.jacionline.org). To determine whether and how many differentially expressed genes function in inflammatory responses, our results were compared with a list of 5488 immune system–related genes from https://www.immport.org, of which 2143 were expressed in keratinocytes of healthy subjects according to the selection criteria average signal of greater than 20.0. Among these, 20 genes were differentially expressed in keratinocytes from patients with AD (Fig 3, *A* and *B*). Interestingly, 8 of these genes are associated with apoptosis according to a search performed with g:Profiler (http://biit.cs.ut.ee/gprofiler/), which retrieves the most significant Gene Ontology groups: Kyoto Encyclopedia of Genes and Genomes and REACTOME pathways (Table I).[23] In addition, ADM was assigned to be an apoptosis-related protein based on the literature.[24] qRT-PCR analysis confirmed that 5 of these genes, *ADM*, *NOD2*, *PCSK9*, *ANXA5*, and *INNPD5*, were differentially expressed also in the chronic skin lesions of patients with AD (Fig 3, *C*), which further indicates a possible contribution of these genes to AD-related apoptosis of keratinocytes.

Increased expression of IFN-γ–regulated genes is observed in biopsy specimens from lesional AD skin

We next performed mRNA array analysis of skin biopsy specimens from chronically lesional and nonlesional skin of 3 patients with AD and 4 healthy subjects. Seventy-two genes were upregulated and 11 were downregulated in affected skin of patients with AD compared with skin from healthy subjects. Several of the upregulated genes also showed a trend toward increased expression in nonlesional skin of patients with AD (Fig 4; see Tables E3 and E4 in this article's Online Repository at www.jacionline.org). According to g:Profiler analysis, 21 of the differentially expressed genes had annotations suggesting a role in immune system

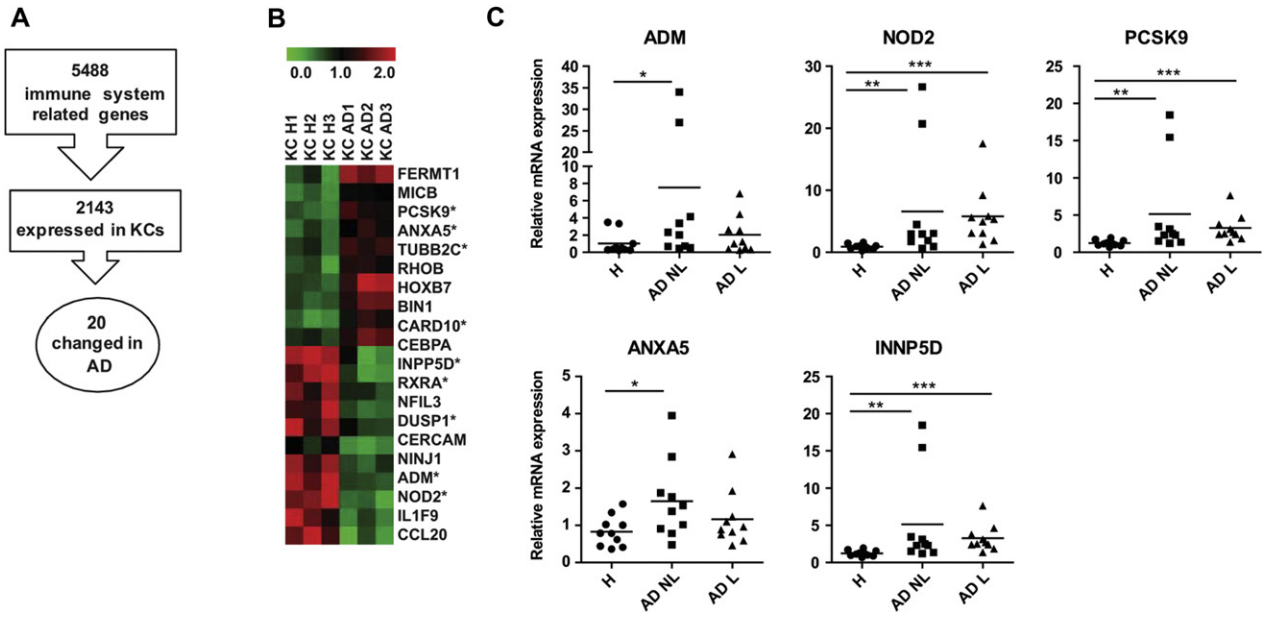

FIG 3. Apoptosis-related genes are differentially expressed in keratinocytes *(KCs)* and AD skin. **A** and **B,** The selection process (Fig 3, *A*) and heat map of $\log_2$ values (Fig 3, *B*) of differentially expressed immune system–related genes. Keratinocytes from healthy subjects *(H1-H3)* and patients with AD *(AD1-AD3)* were used. The *asterisks* in Fig 3, *B,* designate apoptosis-related genes. **C,** Relative mRNA expression (mean, n = 10) in lesional *(L)* and nonlesional *(NL)* AD skin is shown compared with the level seen in healthy control subjects (*H,* =1). **P* < .05, ***P* < .01, and ****P* < .001.

TABLE I. Pathway analysis of the genes differentially regulated in keratinocytes and skin of patients with AD

Dataset	Significance*	No. of genes in target group	ID	Function	Genes
Keratinocytes†	1.04e-06	891	GO:0042981	Regulation of apoptosis	*TUBB2C*, *RXRA*, *PCSK9*, *NOD2*, *INPP5D*, *DUSP1*, *CARD10*, *ANXA5*
	3.91e-06	1911	GO:0006950	Response to stress	*TUBB2C*, *RXRA*, *PLSCR1*, *PCSK9*, *NOD2*, *NINJ1*, *DUSP1*, *CCL20*, *ANXA5*, *ADM*
	1.72e-05	595	GO:0009611	Response to wounding	*RXRA*, *PLSCR1*, *NINJ1*, *CCL20*, *ANXA5*, *ADM*
Skin	5.73e-06	203	GO:0008544	Epidermal development	*LCE3D*, *LCE3A*, *UGCG*, *CSTA*, *TGM1*, *TGM3*, *S100A7*, *KRT15*
	4.27e-10	1248	GO:0002376	Immune system process	*CCL5*, *IFI35*, *IRF8*, *CCL8*, *DPP4*, *TIMP1*, *CST7*, *SH2B3*, *PLEK*, *IL7R*, *IFITM3*, *LYN*, *F12*, *IFITM2*, *MMP9*, *FCN1*, *ARHGDIB*, *S100A7*, *DOCK2*, *CD3D*, *TNFRSF4*

GO, Gene Ontology.
*Significance designates the *P* value from the Fisher exact test showing the significance of the overlap between the target list and the indicated functional category.
†Analysis is carried out with 2143 immune system–related genes expressed in keratinocytes.

processes, whereas 8 genes were implicated in epidermal development (Table I and Fig 4).

We next examined whether the upregulated genes can be induced by IFN-γ in keratinocytes. To do so, we reanalyzed a published[25] and unprocessed dataset from Gene Expression Omnibus Database, which revealed upregulation of 439 genes in response to IFN-γ in keratinocytes. Comparing this list with the list of genes upregulated in AD skin, we identified 9 overlapping genes (Fig 4, *A* and *B*; see Table E5 in this article's Online Repository at www.jacionline.org). qRT-PCR analysis of biopsy specimens from 10 patients with AD with chronic lesions and 10 healthy subjects confirmed the array results (Fig 4, *C*). In addition, increased expression of *IL5*, *IL4R1*, *IL13RA*, and *IL22* was detected in AD skin (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org).

CCL5, CCL8, and IFITM1 proteins are highly expressed in AD skin

Protein expression of 3 genes, *CCL5*, *CCL8*, and *IFITM1*, which were highly and differentially expressed according to the AD skin array, was analyzed by means of immunofluorescence. Robust *CCL5* expression was visible in damaged areas of the lesional AD skin, whereas no expression of *CCL5* was detected in the skin of healthy subjects. A scattered staining pattern was observed for *CCL8* in both healthy control and AD skin in the epidermis. However, a greater number of cells expressed *CCL8* in AD skin. A strong cytoplasmic staining pattern in suprabasal keratinocytes of AD skin specimens was observed for *IFITM1*, whereas the *IFITM1* signal was weak and predominantly nuclear in healthy control subjects (Fig 5). The isotope controls did not

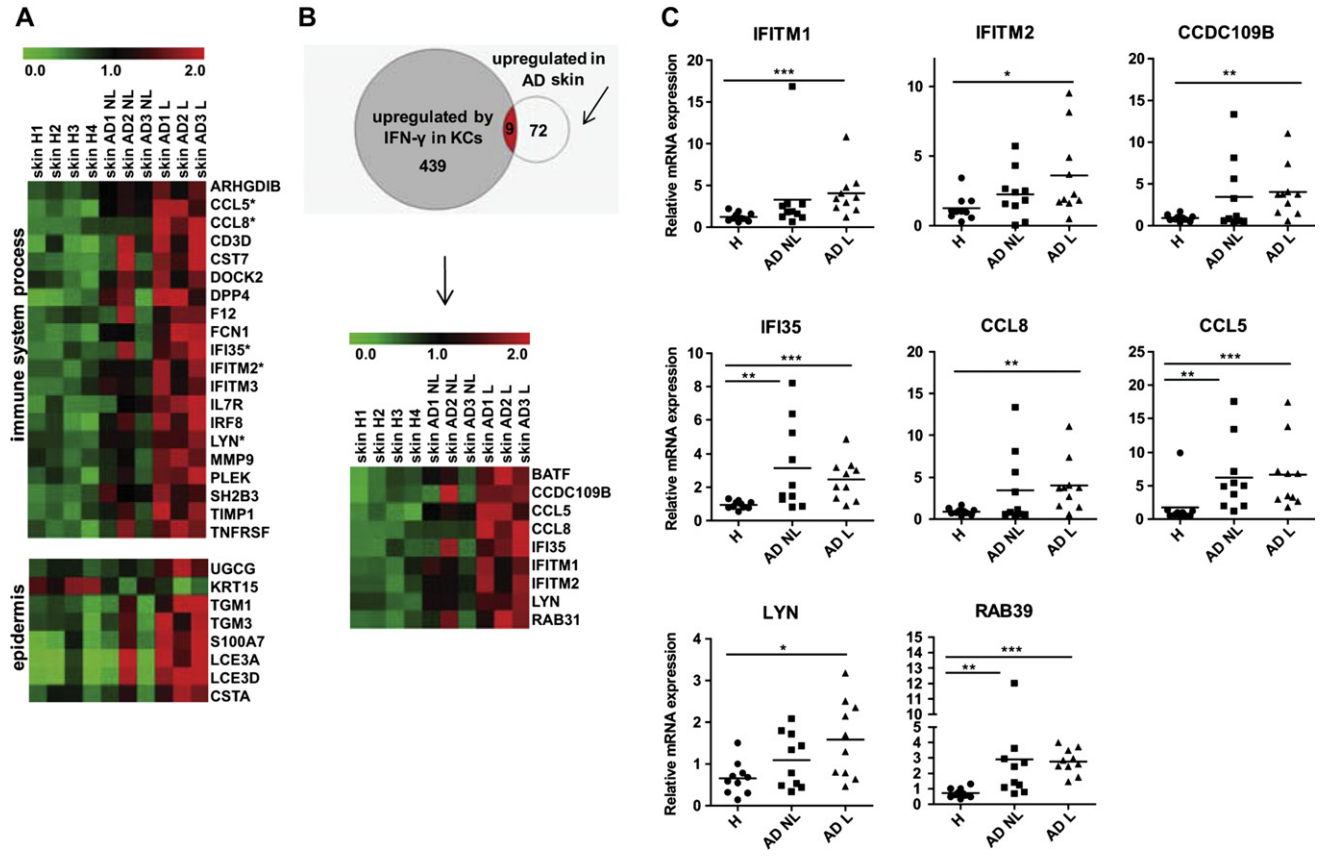

FIG 4. Increased expression of IFN-γ–regulated genes in lesional biopsy specimens from patients with AD. **A** and **B,** Venn diagram of the selection process and heat maps of $\log_2$ values of selected differentially expressed genes. mRNA from lesional *(L)* and nonlesional *(NL)* skin from patients with AD and healthy control subjects *(H)* was analyzed. The *asterisks* in Fig 4, *A,* designate IFN-γ–regulated genes. **C,** Same as Fig 2, *C.* **P* < .05, ***P* < .01, and ****P* < .001.

present any specific signal (see Fig E7 in this article's Online Repository at www.jacionline.org).

IFN-γ induces the expression of the potential target genes in keratinocytes

We next analyzed whether the selected differentially expressed genes can be indeed induced by IFN-γ in keratinocytes. Three apoptosis-related genes, *ADM*, *NOD2*, and *DUSP1*, were upregulated in response to IFN-γ in primary keratinocytes (Fig 6, *A*), whereas *RXRA*, *TUBB2C*, *PCSK9*, and *INPP5D* remained unchanged (see Fig E8 in this article's Online Repository at www.jacionline.org). In addition, 8 of the 9 overlapping genes (*IFI35*, *CCDC109B*, *CCL5*, *CCL8*, *RAB31*, *IFITM1*, *IFITM2*, and *LYN*) that were found to be upregulated in lesional skin biopsy specimens of patients with AD were induced by IFN-γ in keratinocytes (Fig 6, *B*). One gene, *BATF*, did not show a sufficiently strong signal in qRT-PCR. *CCL5* and *NOD2* were upregulated also by IL-4 and TNF-α, and *IFITM2* was upregulated by IL-4.

We next analyzed how these genes respond to IFN-γ in keratinocytes from patients with AD compared with keratinocytes from healthy subjects and patients with psoriasis at different time points after IFN-γ stimulation. Interestingly, *IFITM1*, *IFITM2*, and *CCL5* were more strongly upregulated in keratinocytes from patients with AD, and *IFITM1* expression was significantly increased at 12 and 24 hours after stimulation (Fig 6, *B*). Other genes responded to IFN-γ, but the differences among healthy subjects, patients with AD, and patients with psoriasis were not significant (see Fig E9 in this article's Online Repository at www.jacionline.org).

DUSP1, ADM, RAB31, and *IFTM* cluster gene variants are potentially associated with AD

Of the detected IFN-γ–regulated genes, the promoter polymorphism −401A of *CCL5* was previously shown to be associated with AD.[26,27] To investigate whether other genetic variants in the differentially expressed genes studied here show association with AD, we carried out an *in silico* candidate gene analysis using an existing genome-wide SNP dataset from 533 patients with AD from a recently published genome-wide association study.[18] As control subjects, we used 1996 subjects from the population-based KORA S4/F4 survey with existing genome-wide SNP data.[19] We extracted SNPs within or close to the IFN-γ–regulated genes (Figs 4, *B*, and 6), genes involved in apoptosis (Fig 3 and Table I), and other genes classified as immune system– or epidermal differentiation–related genes, for which we observed differential expression (Figs 3 and 4 and Table I). A total of 1518 SNPs located in 39 candidate loci were analyzed for association with AD. Fifty-two of these SNPs showed a suggestive association ($P < .01$, Table II and see Table E6 in this article's Online Repository at www.jacionline.org). Eight were

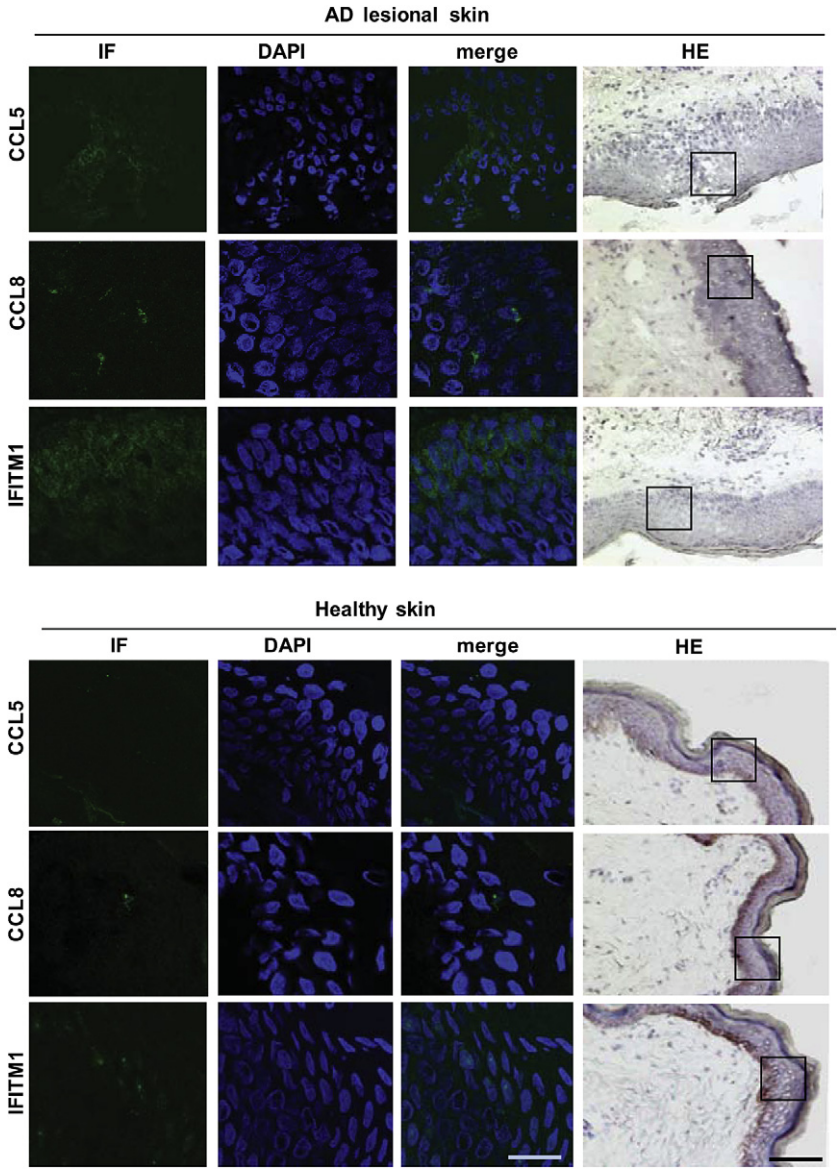

FIG 5. The *CCL5, CCL8,* and *IFITM1* genes are expressed in the epidermis of skin from patients with AD. Immunofluorescence *(IF), 4′*,6-diamidino-2-phenylindole *(DAPI),* and hematoxylin *(HE)* staining of healthy and AD skin is shown. One representative example is shown (n = 3 for each group). *Bars* correspond to 10 μm *(IF)* and 60 μm *(HE staining).* The IF-stained area is designated with a *square* on respective HE-stained sections.

located in the apoptosis-related genes (*DUSP1* and *ADM*), the IFN-γ–regulated *RAB31* gene, and the *IFITM* locus (Table II). In line with previous studies, several SNPs within the epidermal differentiation complex[18,28,29] and the cystatin A (*CSTA*) gene[30] were among the potentially associated variants (see Table E6). However, In an analysis adjusted for the most prevalent filaggrin *(FLG)* mutations in European populations (R501X, 2282del4), none of the epidermal differentiation complex SNPs remained significant (ie, the associated variants tag *FLG* haplotypes; data not shown). Haplotype analysis of the results presented in Table II showed that the *RAB31* haplotype A-C-A and the *IFITM* haplotype C-G-C are significantly associated with AD (odds ratios, 1.29 and 0.69; $P = .00188$ and .00343) compared with the reference haplotypes G-A-T and T-G-G, respectively. Haplotype frequencies are given in Table E7 in this article's Online Repository at www.jacionline.org.

DISCUSSION

Apoptosis is part of the normal process of epithelial cell renewal, but its excess in epithelium plays an important role in the pathogenesis of AD and asthma.[31,32] In the present study we demonstrate that IFN-γ induces significantly more apoptosis in keratinocytes from patients with AD than in keratinocytes from healthy subjects. Accordingly, we show that several IFN-γ–inducible genes are upregulated in chronic AD lesional skin and that several apoptosis-related genes are differentially expressed in primary keratinocytes from the skin of patients with

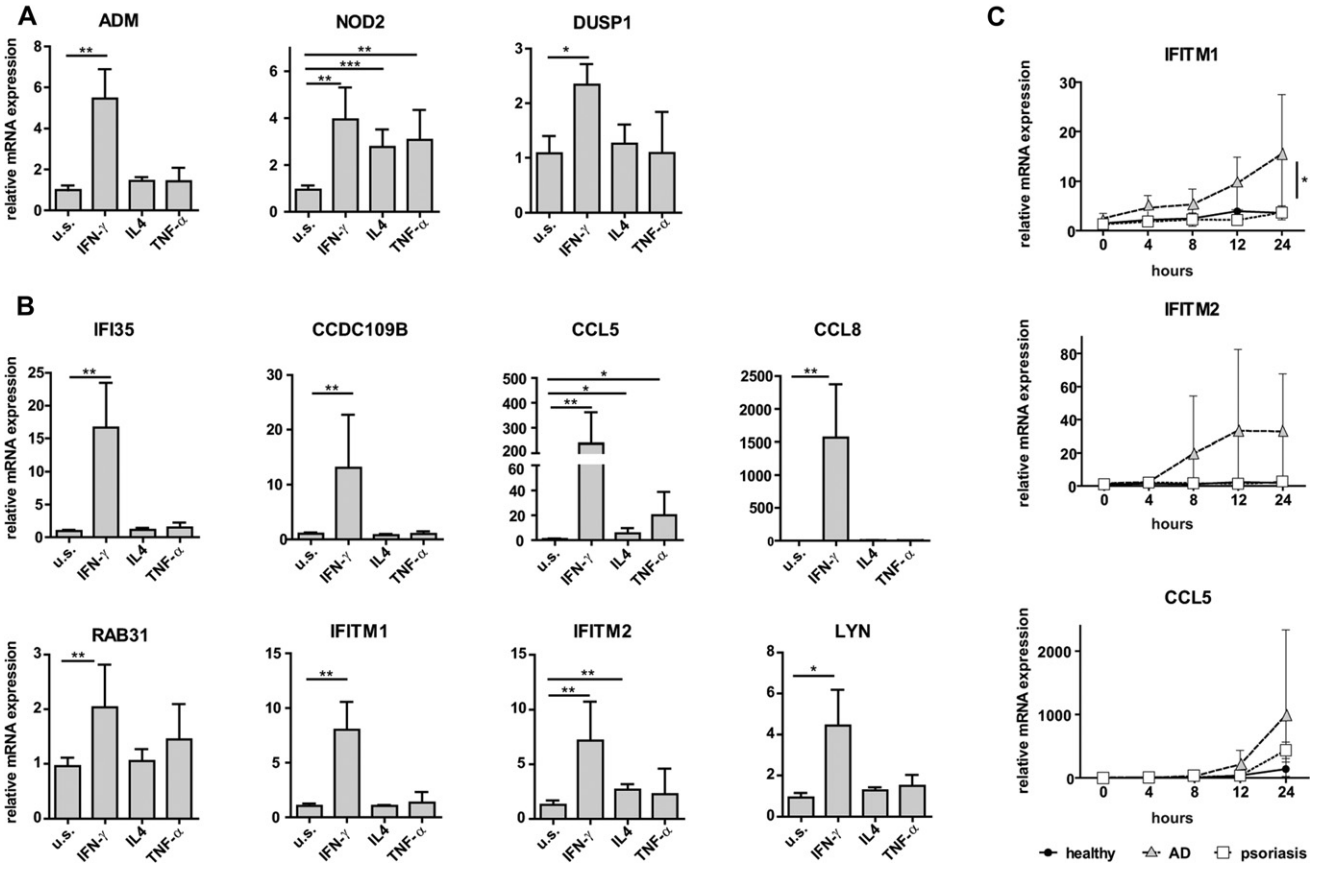

FIG 6. IFN-γ response of differentially expressed genes in primary keratinocytes. **A** and **B,** Relative mRNA expression (mean ± SD, n = 6) of apoptosis-related genes (Fig 6, *A*) and genes differentially regulated in AD skin (Fig 6, *B*) in unstimulated (*u.s.*, =1) or stimulated (24 hours) keratinocytes. **C,** Relative mRNA expression (mean ± SD, n = 4 for AD and healthy skin and n = 3 for psoriatic skin) after stimulation with IFN-γ compared with time point 0 (=1). **P* < .05, ***P* < .01, and ****P* < .001.

TABLE II. *In silico* candidate gene analysis of IFN-γ–induced and apoptosis-related genes

SNP	Chromosome	Allele	MAF	*P* value*	OR	Gene†	Functional group
rs591067	18	A<T	0.2856	.0054	1.228	Near *RAB31*	IFN-γ regulated
rs587351	18	C<A	0.4832	.0020	1.238	Near *RAB31*	IFN-γ regulated
rs684949	18	G<A	0.489	.0007	0.790	Near *RAB31*	IFN-γ regulated
rs3809112	11	C<T	0.3572	3.0E-05	0.731	Near *IFITM2*	IFN-γ regulated/immune system process
rs741738	11	A<G	0.2033	.0034	0.765	Near *IFITM2*	IFN-γ regulated/immune system process
rs7395116	11	C<G	0.255	6.6E-05	0.712	*BC040735*/near *IFITM3*	IFN-γ regulated/immune system process
rs17075181	5	G<A	0.1904	.0076	0.779	Near *DUSP1*	Regulation of apoptosis/IFN-γ regulated
rs4399321	11	G<A	0.3594	.0035	0.806	Near *ADM*	Regulation of apoptosis/IFN-γ regulated

MAF, Minor allele frequency in KORA control subjects; *OR,* odds ratio.
**P* value is calculated by means of comparison of allele frequencies with the Pearson χ^2 test.
†Gene annotation based on UCSC genome build 37, hg19.

AD. In addition, we found evidence of association of AD with genetic markers close to or within the IFN-γ–inducible genes (the *IFITM* cluster and *RAB31*) and the apoptosis-related genes *DUSP1* and *ADM*. These results suggest that altered expression of apoptosis-related and IFN-γ–inducible genes is responsible for increased IFN-γ–induced apoptosis of keratinocytes in patients with AD.

Previously, we have shown that activated T cells that infiltrate the skin during AD induce keratinocyte apoptosis through the production of IFN-γ.[3] Interestingly, the high IFN-γ–secreting T_H1 cells in peripheral blood selectively undergo activation-induced cell death and skew the immune response toward T_H2 cells in patients with AD.[33] Two very recent studies on PBMCs from patients with AD, one involving patients with a history of eczema herpeticum, reveal lower *IFNG* and *IFNGR* gene expression, whereas *IFNG* and *IFNGR1* SNPs were found to be significantly associated.[34,35] These studies highlight that IFN-γ signaling and IFN-γ–induced apoptosis is dysregulated in different cell types in patients with AD and indicate that either genetic variations or altered epigenetic modification patterns (or both)

can be associated with the development and severity of the disease.

We initially studied how IFN-γ influences the expression of death receptors and ligands. Although IFN-γ induced strongly TNF-R2 and TNF-α and influenced slightly TRAIL, TRAIL-R2, TRAIL-R3, TRAIL-R4, and FAS expression in keratinocytes, none of these genes showed expression differences when keratinocytes from patients with AD and healthy subjects were compared. Thus we concluded that other factors play a role and performed mRNA expression analyses of keratinocytes and skin from patients with AD and healthy subjects. We found several apoptosis-related genes to be differentially expressed in keratinocytes and skin from patients with chronic AD. In skin biopsy specimens from patients with AD, immune system–related genes and genes involved in epidermal development were differentially expressed. In agreement with previous mRNA profiling studies,[36-39] we observed expression changes also in nonlesional AD skin. As reported before,[37] increased expression levels of T_H2-related genes, such as *IL5*, *IL4R1*, and *IL13RA*, and *IL22* as the phenotype cytokine for T_H22 cells, were also detected. Comparing the skin array results with a previously published dataset of IFN-γ–inducible genes from keratinocytes,[25] we found 9 overlapping genes. We confirmed that 8 of these genes (*CCDC109B*, *CCL5*, *CCL8*, *IFI35*, *LYN*, *RAB31*, *IFITM1*, and *IFITM2*) are induced by IFN-γ. In addition, 3 apoptosis-related genes, *NOD2*, *DUSP1*, and *ADM*, were upregulated by IFN-γ in keratinocytes. Moreover, *IFITM1* expression was significantly increased in keratinocytes from patients with AD in response to IFN-γ when compared with that seen in healthy subjects. Enhanced expression of the *CCL5*, *CCL8*, and *IFITM1* genes was also determined at the protein level in AD skin. We were not able to detect IFN-γ, IL-4, and IL-13 themselves from the array and qRT-PCR analyses of AD skin. However, our results show that many IFN-γ–inducible gene products, which are most probably produced by keratinocytes, are present in chronic AD skin and might therefore contribute to the development of long-lasting lesions in patients with AD.

Consistent with expression and functional studies, an *in silico* genetic analysis showed that SNPs within the *IFITM* cluster or *RAB31*, *DUSP1*, and *ADM* genes are potentially associated with AD. In addition, the promoter polymorphism −401A of *CCL5*[26,27] was previously shown to be associated with AD. It is possible that altered expression levels of these genes lead to changes in the general protein expression profile and thereby influence the development of the disease. Two of the differentially expressed and IFN-γ–inducible genes, *CCL5* in human subjects[27,40] and *CCL8*[41] in mice, were previously shown to be functionally linked to AD. It would be interesting to study more closely how other apoptosis-regulated genes, IFN-γ–regulated genes, or both described here are involved in the development of AD. In addition, because long-lasting lesions in patients with AD are often accompanied by infection of the skin with *Staphylococcus aureus*, *Malassezia sympodialis*, and/or eczema herpeticum, studies in relation to these infections should be performed in the future.

In conclusion, we have shown that IFN-γ–induced apoptosis is enhanced in keratinocytes from patients with AD and thus might contribute to the development of eczematous lesions in AD skin. In addition, we propose that the differentially expressed apoptosis-related genes, IFN-γ–inducible genes, or both in keratinocytes identified herein might be involved in the pathogenesis of AD, especially in long-lasting and refractory cases of the disease. Although this process seems to be multifactorial, further studies would help to elucidate the effect of each novel candidate gene and would possibly help in the development of better therapeutics for AD.

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Clinical implications: The current study shows enhanced IFN-γ–induced apoptosis of keratinocytes from patients with AD and proposes several potential novel target molecules for the therapy of AD.

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