

# Mechanisms of IFN- $\gamma$ -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 spongiolysis 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-aminocoumarin 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- $\gamma$ -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- $\gamma$  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- $\gamma$  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;■■■:■■■-■■■.)

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

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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.<sup>1-3</sup> Linkage and association studies have identified several candidate genes possibly linked to either epidermal barrier function or to immune processes.<sup>2,4</sup> For instance, variants of *IL4/IL13* receptor,<sup>5,6</sup> *IL13*,<sup>7</sup> and the gene encoding the  $\alpha$ -chain of the high-affinity receptor for IgE (*FCER1A*) have been shown to be associated with AD.<sup>8</sup> Concordantly, a predominant T<sub>H</sub>2 bias with increased IgE levels is a widely recognized hallmark of AD. Nonetheless, in the chronic phase of skin inflammation, IFN- $\gamma$ , as the characteristic cytokine for T<sub>H</sub>1 cells, is dominant in the skin of patients with AD.<sup>2,9,10</sup> The presence of IFN- $\gamma$ , 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.<sup>1-3</sup> In contrast, keratinocytes in patients with psoriasis, the most closely analogous skin disease, undergo hyperproliferation and altered differentiation.<sup>2,11</sup> Apoptosis is known to occur through death receptors that are activated by their ligands. Keratinocytes have been shown to express TNF- $\alpha$  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

**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).<sup>12</sup> Previously, it was shown that IFN- $\gamma$ -induced apoptosis occurs through FAS both in keratinocytes and IFN- $\gamma$ -producing T cells.<sup>13–15</sup> Although IFN- $\gamma$  appears to be a key factor, other cytokines, such as TNF- $\alpha$ , TNF-like weak inducer of apoptosis (TWEAK), and IL-32, can contribute to keratinocyte apoptosis in patients with AD.<sup>16,17</sup>

In the present study molecular mechanisms of AD-related apoptosis of keratinocytes were investigated. Interestingly, we found enhanced IFN- $\gamma$ -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- $\gamma$ -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- $\gamma$  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.<sup>16,17</sup> 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  $-80^{\circ}\text{C}$ .

**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](http://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](http://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://bit.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](http://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.<sup>18</sup> As control subjects, we chose 1996 subjects from the population-based Cooperative Health Research in the Region of Augsburg (KORA) S4/F4 survey.<sup>19</sup> We extracted SNPs from identified genes plus a surrounding region of  $\pm 50$  kb by using the UCSC genome browser (assembly GRCh37/hg19, February 2009).<sup>20</sup> 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<sup>21</sup> by using a  $\chi^2$  test for the  $2 \times 2$  table for each SNP. Odds ratios were derived from a  $2 \times 2$  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  $\chi^2$  test. Haplotype analysis was performed with the R-package haplo.stats<sup>22</sup> within R 2.13.0 software (<http://www.R-project.org/>).

**RESULTS****Increased IFN- $\gamma$ -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- $\gamma$  on keratinocyte apoptosis that was further enhanced when keratinocytes were exposed to IFN- $\gamma$  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 ( $67.6 \pm 8.1$ ) were viable in comparison with keratinocytes from patients with AD ( $51.4\% \pm 5.4\%$ ). Of keratinocytes from patients with psoriasis,  $62.4\% \pm 7.2\%$  were viable. Less cells were viable when treated with TWEAK or TRAIL in combination with IFN- $\gamma$ , although no differences among the 3 keratinocyte groups were observed (Fig 1, B). According to annexin V-positive/7-AAD-negative staining,  $27.1\% \pm 13.8\%$  keratinocytes from patients with AD,

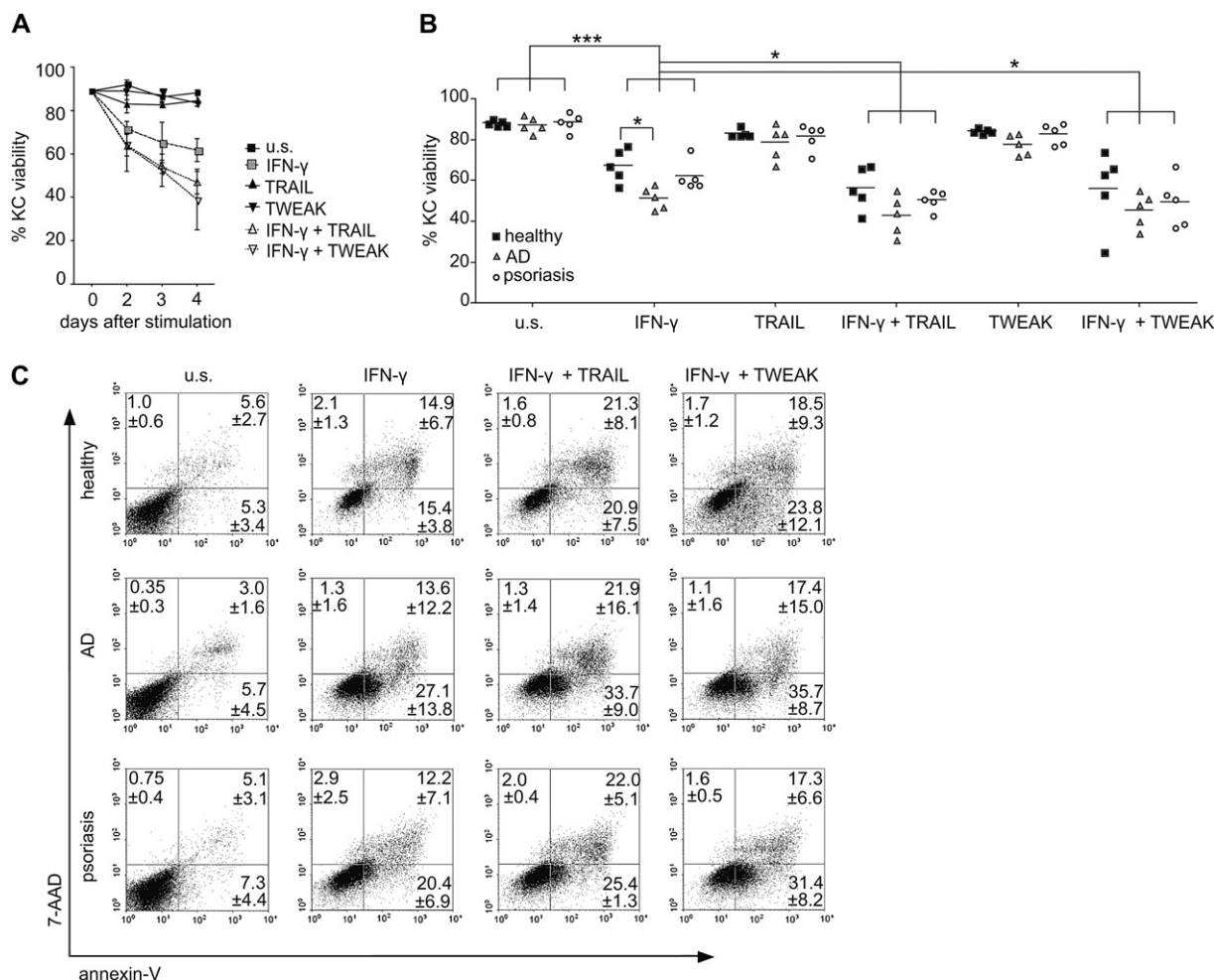


**FIG 1.** Increased IFN- $\gamma$ -stimulated apoptosis of keratinocytes (KC) from patients with AD. **A**, The viability (annexin V- and 7-AAD-negative cells) of unstimulated (*u.s.*) or stimulated primary keratinocytes is presented as means  $\pm$  SDs ( $n = 2$ ). **B**, The viability of primary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

15.4%  $\pm$  3.8% keratinocytes from healthy subjects, and 20.4%  $\pm$  6.9% keratinocytes from patients with psoriasis showed apoptosis after IFN- $\gamma$  stimulation, which indicates that mainly early apoptosis was enhanced in keratinocytes from patients with AD (Fig 1, C). Anti-FAS mAb, TNF- $\alpha$ , and their combinations with IFN- $\gamma$  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- $\gamma$ , TRAIL, TNF- $\alpha$ , and TWEAK among the 3 studied groups (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Together, these data demonstrate that keratinocytes from patients with AD are more susceptible to IFN- $\gamma$ -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- $\gamma$ -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- $\alpha$  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- $\gamma$  (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). There was no change in the expression of death receptors when either TNF- $\alpha$ , TWEAK, TRAIL, or anti-FAS mAbs were used (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://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).

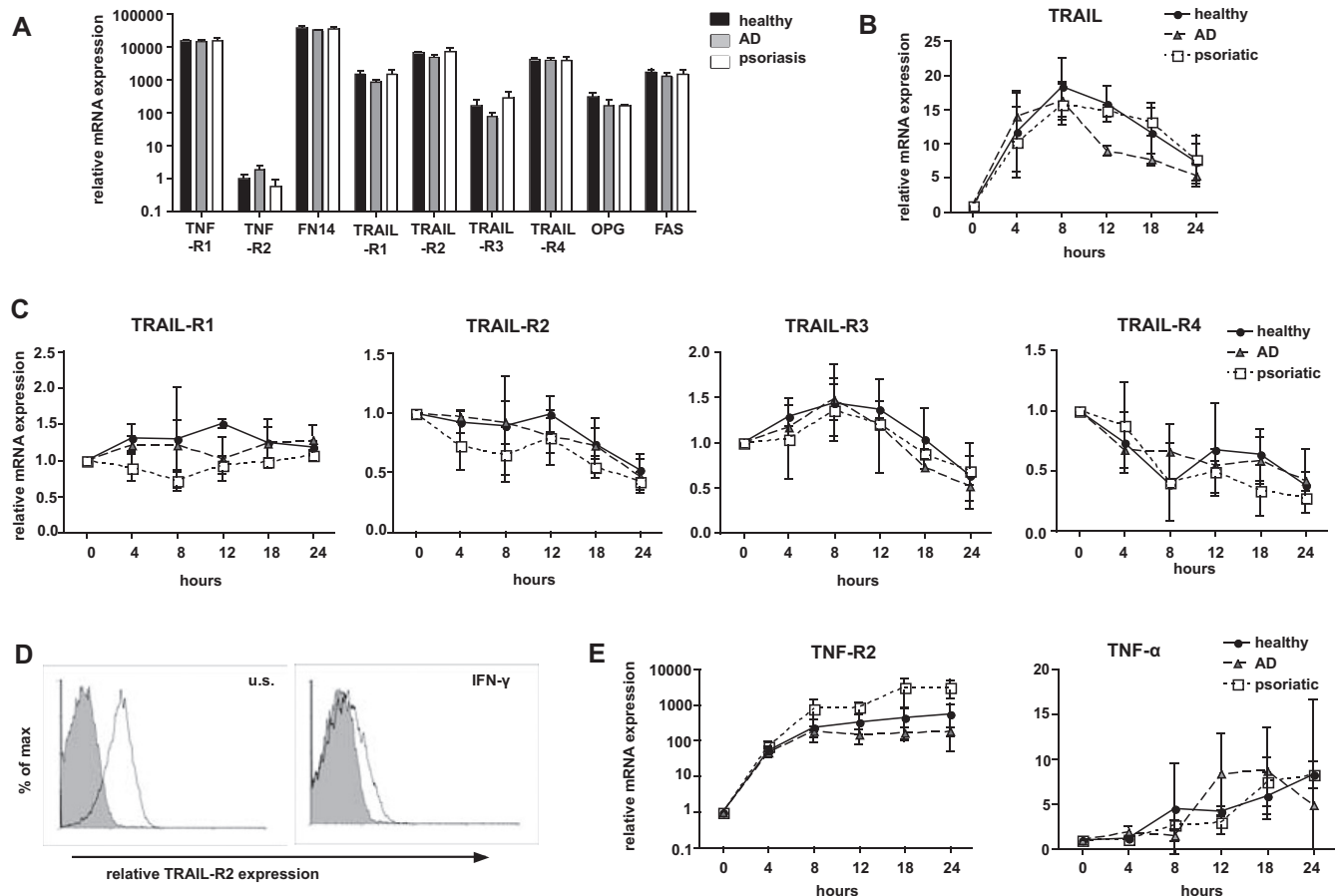


**FIG 2.** Influence of IFN- $\gamma$  on the expression of TRAIL, TNF- $\alpha$ , and their receptors in keratinocytes. **A**, Relative mRNA expression (mean  $\pm$  SD) compared with the expression level of TNF-R2 in keratinocytes ( $n = 3$  of each group). **B**, **C**, and **E**, Relative mRNA expression (mean  $\pm$  SD) after stimulation with IFN- $\gamma$  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- $\gamma$ -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](http://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](http://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).<sup>23</sup> In addition, ADM was assigned to be an apoptosis-related protein based on the literature.<sup>24</sup> qRT-PCR analysis confirmed that 5 of these genes, *ADM*, *NOD2*, *PCSK9*, *ANXA5*, and *INNP5*, 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- $\gamma$ -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](http://www.jacionline.org)). According to g:Profiler analysis, 21 of the differentially expressed genes had annotations suggesting a role in immune system

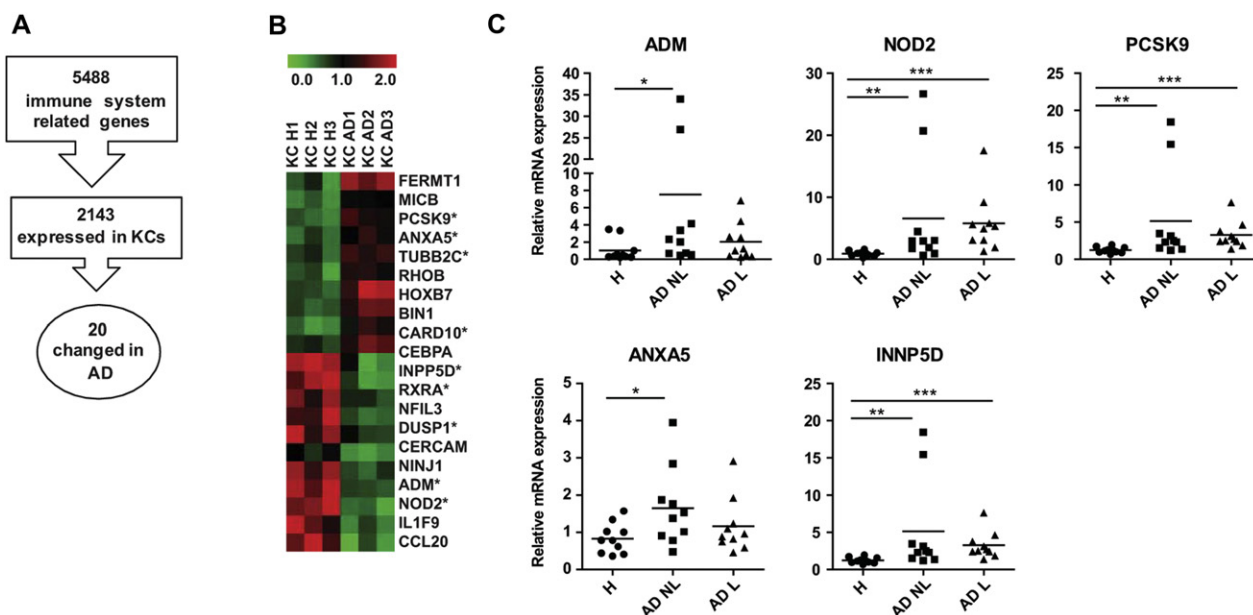


**FIG 3.** Apoptosis-related genes are differentially expressed in keratinocytes (KCs) and AD skin. **A** and **B**, The selection process (Fig 3, A) and heat map of log<sub>2</sub> values (Fig 3, B) of differentially expressed immune system-related genes. Keratinocytes from healthy subjects (H1-H3) 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 | <i>TUBB2C</i> , <i>RXRA</i> , <i>PCSK9</i> , <i>NOD2</i> , <i>INPP5D</i> , <i>DUSP1</i> , <i>CARD10</i> , <i>ANXA5</i>                                                                                                                                                                                             |
|                | 3.91e-06      | 1911                         | GO:0006950 | Response to stress      | <i>TUBB2C</i> , <i>RXRA</i> , <i>PLSCR1</i> , <i>PCSK9</i> , <i>NOD2</i> , <i>NINJ1</i> , <i>DUSP1</i> , <i>CCL20</i> , <i>ANXA5</i> , <i>ADM</i>                                                                                                                                                                  |
|                | 1.72e-05      | 595                          | GO:0009611 | Response to wounding    | <i>RXRA</i> , <i>PLSCR1</i> , <i>NINJ1</i> , <i>CCL20</i> , <i>ANXA5</i> , <i>ADM</i>                                                                                                                                                                                                                              |
| Skin           | 5.73e-06      | 203                          | GO:0008544 | Epidermal development   | <i>LCE3D</i> , <i>LCE3A</i> , <i>UGCG</i> , <i>CSTA</i> , <i>TGM1</i> , <i>TGM3</i> , <i>S100A7</i> , <i>KRT15</i>                                                                                                                                                                                                 |
|                | 4.27e-10      | 1248                         | GO:0002376 | Immune system process   | <i>CCL5</i> , <i>IFI35</i> , <i>IRF8</i> , <i>CCL8</i> , <i>DPP4</i> , <i>TIMP1</i> , <i>CST7</i> , <i>SH2B3</i> , <i>PLEK</i> , <i>IL7R</i> , <i>IFITM3</i> , <i>LYN</i> , <i>F12</i> , <i>IFITM2</i> , <i>MMP9</i> , <i>FCN1</i> , <i>ARHGDI1B</i> , <i>S100A7</i> , <i>DOCK2</i> , <i>CD3D</i> , <i>TNFRSF4</i> |

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- $\gamma$  in keratinocytes. To do so, we reanalyzed a published<sup>25</sup> and unprocessed dataset from Gene Expression Omnibus Database, which revealed upregulation of 439 genes in response to IFN- $\gamma$  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](http://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](http://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

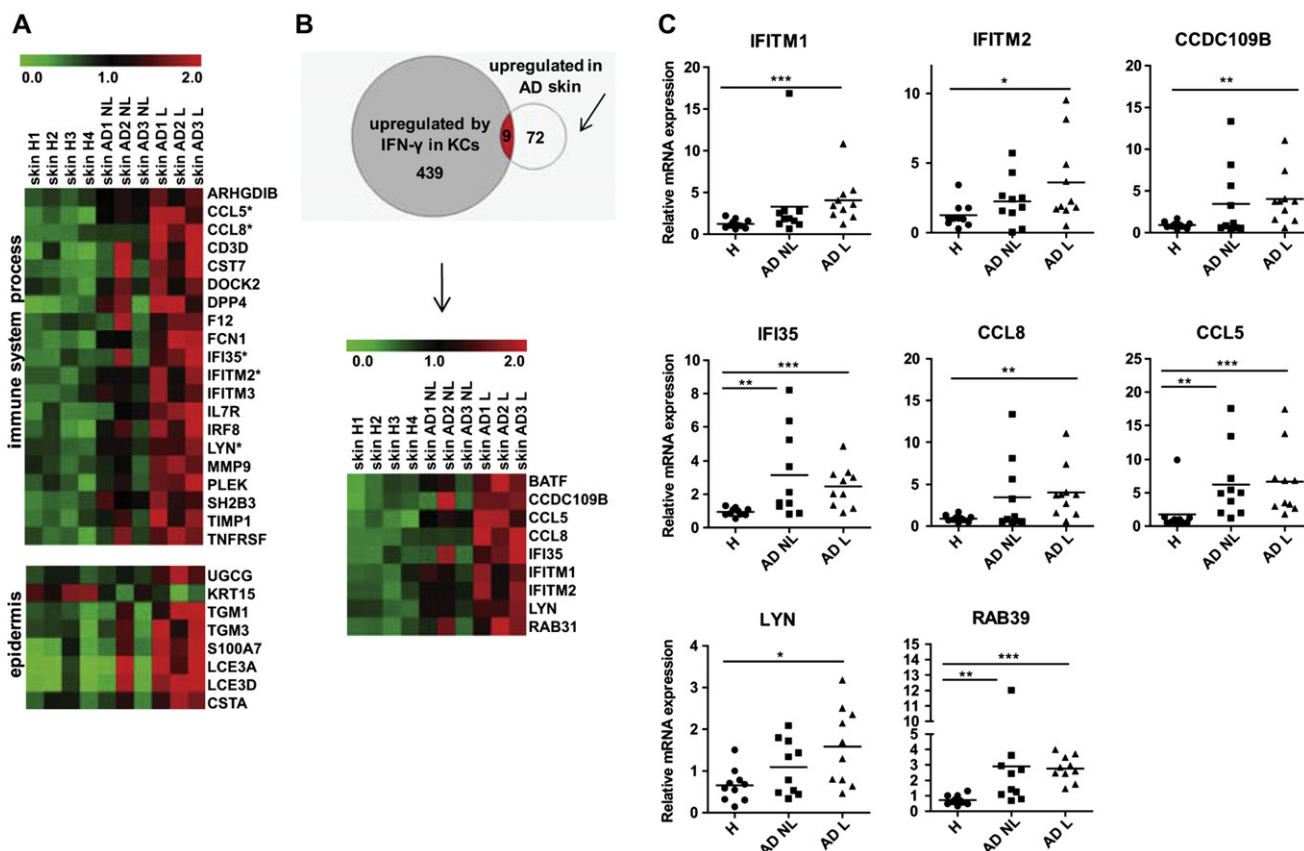


**FIG 4.** Increased expression of IFN- $\gamma$ -regulated genes in lesional biopsy specimens from patients with AD. **A** and **B**, Venn diagram of the selection process and heat maps of  $\log_2$  values of selected differentially expressed genes. mRNA from lesional (L) and nonlesional (NL) skin from patients with AD and healthy control subjects (H) was analyzed. The asterisks in Fig 4, A, designate IFN- $\gamma$ -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](http://www.jacionline.org)).

### IFN- $\gamma$ 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- $\gamma$  in keratinocytes. Three apoptosis-related genes, *ADM*, *NOD2*, and *DUSP1*, were upregulated in response to IFN- $\gamma$  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](http://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- $\gamma$  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- $\alpha$ , and *IFITM2* was upregulated by IL-4.

We next analyzed how these genes respond to IFN- $\gamma$  in keratinocytes from patients with AD compared with keratinocytes from healthy subjects and patients with psoriasis at different time points after IFN- $\gamma$  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- $\gamma$ , 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](http://www.jacionline.org)).

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

Of the detected IFN- $\gamma$ -regulated genes, the promoter polymorphism -401A of *CCL5* was previously shown to be associated with AD.<sup>26,27</sup> 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.<sup>18</sup> As control subjects, we used 1996 subjects from the population-based KORA S4/F4 survey with existing genome-wide SNP data.<sup>19</sup> We extracted SNPs within or close to the IFN- $\gamma$ -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](http://www.jacionline.org)). Eight were

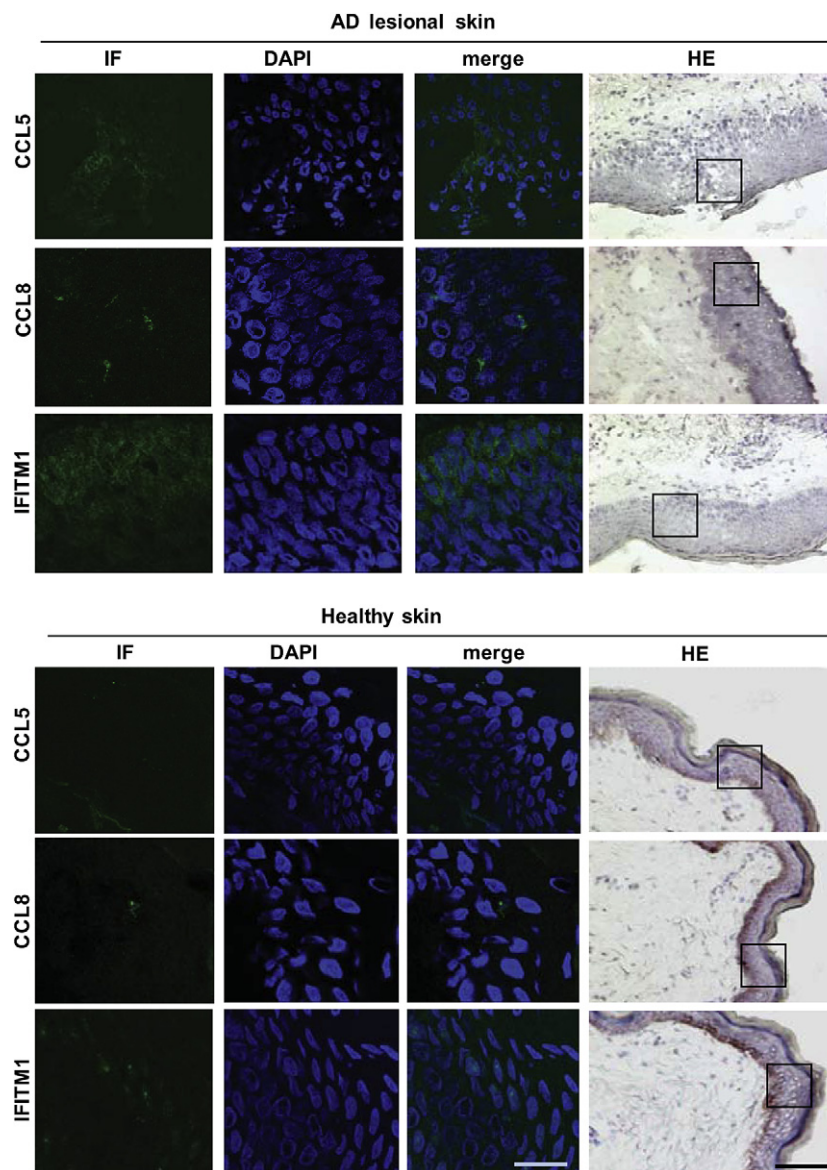


**FIG 5.** The *CCL5*, *CCL8*, and *IFITM1* genes are expressed in the epidermis of skin from patients with AD. Immunofluorescence (IF), 4',6-diamidino-2-phenylindole (DAPI), and hematoxylin (HE) staining of healthy and AD skin is shown. One representative example is shown ( $n = 3$  for each group). Bars correspond to 10  $\mu\text{m}$  (IF) and 60  $\mu\text{m}$  (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- $\gamma$ -regulated *RAB31* gene, and the *IFITM* locus (Table II). In line with previous studies, several SNPs within the epidermal differentiation complex<sup>18,28,29</sup> and the cystatin A (*CSTA*) gene<sup>30</sup> 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](http://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.<sup>31,32</sup> In the present study we demonstrate that IFN- $\gamma$  induces significantly more apoptosis in keratinocytes from patients with AD than in keratinocytes from healthy subjects. Accordingly, we show that several IFN- $\gamma$ -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

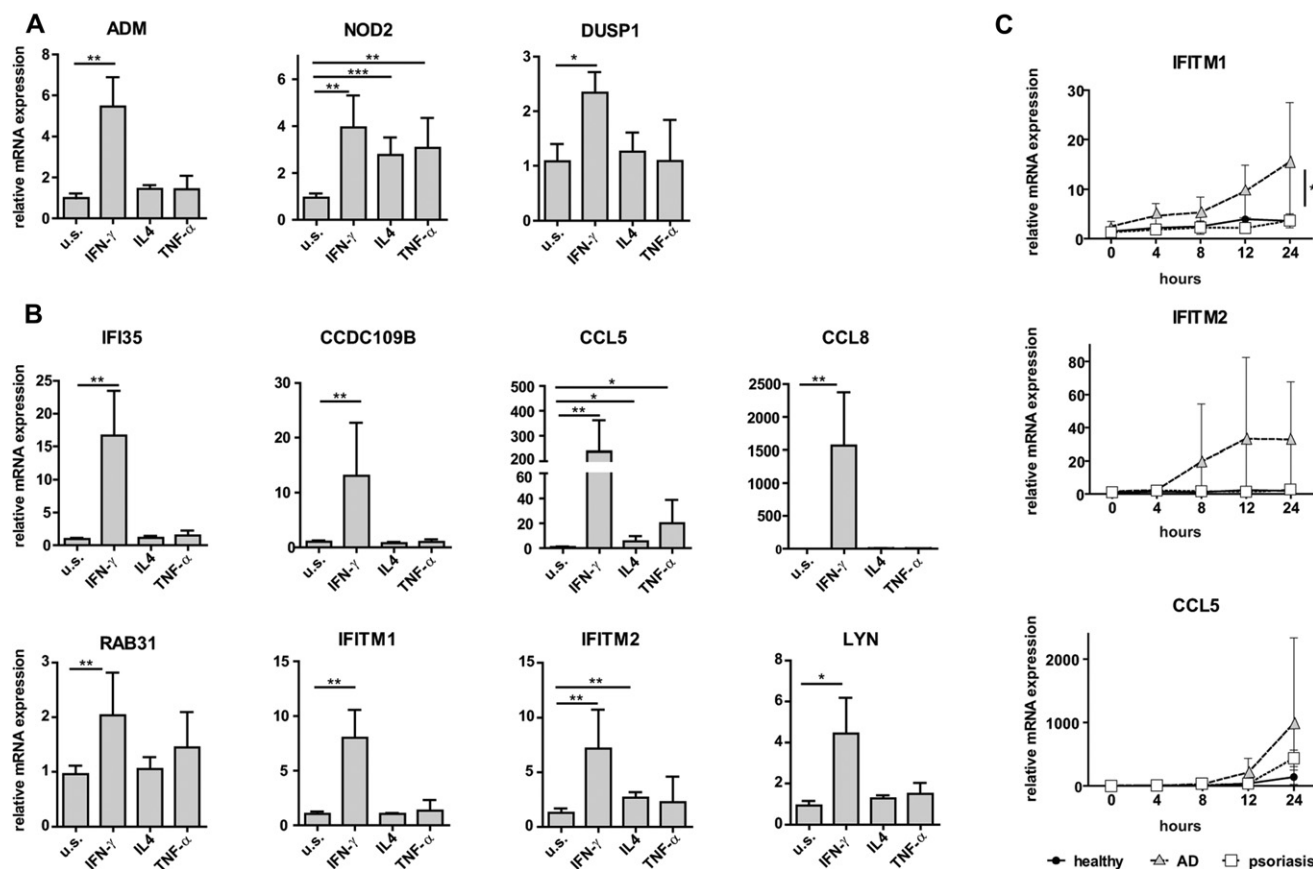


**FIG 6.** IFN- $\gamma$  response of differentially expressed genes in primary keratinocytes. **A** and **B**, Relative mRNA expression (mean  $\pm$  SD,  $n = 6$ ) of apoptosis-related genes (Fig 6, **A**) and genes differentially regulated in AD skin (Fig 6, **B**) in unstimulated (u.s., =1) or stimulated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

**TABLE II.** *In silico* candidate gene analysis of IFN- $\gamma$ -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 <i>RAB31</i>                   | IFN- $\gamma$ regulated                         |
| rs587351   | 18         | C<A    | 0.4832 | .0020    | 1.238 | Near <i>RAB31</i>                   | IFN- $\gamma$ regulated                         |
| rs684949   | 18         | G<A    | 0.489  | .0007    | 0.790 | Near <i>RAB31</i>                   | IFN- $\gamma$ regulated                         |
| rs3809112  | 11         | C<T    | 0.3572 | 3.0E-05  | 0.731 | Near <i>IFITM2</i>                  | IFN- $\gamma$ regulated/immune system process   |
| rs741738   | 11         | A<G    | 0.2033 | .0034    | 0.765 | Near <i>IFITM2</i>                  | IFN- $\gamma$ regulated/immune system process   |
| rs7395116  | 11         | C<G    | 0.255  | 6.6E-05  | 0.712 | <i>BC040735</i> /near <i>IFITM3</i> | IFN- $\gamma$ regulated/immune system process   |
| rs17075181 | 5          | G<A    | 0.1904 | .0076    | 0.779 | Near <i>DUSP1</i>                   | Regulation of apoptosis/IFN- $\gamma$ regulated |
| rs4399321  | 11         | G<A    | 0.3594 | .0035    | 0.806 | Near <i>ADM</i>                     | Regulation of apoptosis/IFN- $\gamma$ 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  $\chi^2$  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- $\gamma$ -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- $\gamma$ -inducible genes is responsible for increased IFN- $\gamma$ -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- $\gamma$ .<sup>3</sup> Interestingly, the high IFN- $\gamma$ -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.<sup>33</sup> 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.<sup>34,35</sup> These studies highlight that IFN- $\gamma$  signaling and IFN- $\gamma$ -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- $\gamma$  influences the expression of death receptors and ligands. Although IFN- $\gamma$  induced strongly TNF-R2 and TNF- $\alpha$  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,<sup>36-39</sup> we observed expression changes also in nonlesional AD skin. As reported before,<sup>37</sup> increased expression levels of T<sub>H</sub>2-related genes, such as *IL5*, *IL4R1*, and *IL13RA*, and *IL22* as the phenotype cytokine for T<sub>H</sub>2 cells, were also detected. Comparing the skin array results with a previously published dataset of IFN- $\gamma$ -inducible genes from keratinocytes,<sup>25</sup> 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- $\gamma$ . In addition, 3 apoptosis-related genes, *NOD2*, *DUSP1*, and *ADM*, were upregulated by IFN- $\gamma$  in keratinocytes. Moreover, *IFITM1* expression was significantly increased in keratinocytes from patients with AD in response to IFN- $\gamma$  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- $\gamma$ , IL-4, and IL-13 themselves from the array and qRT-PCR analyses of AD skin. However, our results show that many IFN- $\gamma$ -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*<sup>26,27</sup> 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- $\gamma$ -inducible genes, *CCL5* in human subjects<sup>27,40</sup> and *CCL8*<sup>41</sup> 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- $\gamma$ -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- $\gamma$ -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- $\gamma$ -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- $\gamma$ -induced apoptosis of keratinocytes from patients with AD and proposes several potential novel target molecules for the therapy of AD.**

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# Online Repository

## Mechanisms of IFN- $\gamma$ -induced apoptosis of human skin keratinocytes in atopic dermatitis

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## **Online repository**

## **METHODS**

### **KC cultures**

Maintenance of primary KCs from healthy individuals, AD and psoriasis patients and apoptosis assays are described previously <sup>16, 17</sup>. The following cytokine concentrations were used: 100 ng/mL TWEAK, 25 ng/mL TNF- $\alpha$ , 100 ng/mL TRAIL, 500 ng/mL mAb anti-Fas (all from Beckman Coulter), 25 ng/mL IL-4 and 10 ng/ml IFN- $\gamma$  (both from R&D).

### **Proliferation assay**

KCs were incubated for two days with cytokines before <sup>3</sup>H thymidine (Hartmann Analytic) was added to an end-concentration of 1  $\mu$ Ci/ml. Actinomycin D (Sigma-Aldrich), used as positive control, and IFN- $\gamma$  completely stopped KC proliferation at a concentration of 10 ng/ml; TWEAK, TRAIL and TNF- $\alpha$  reduced it at a concentration of 100 ng/ml 60 - 90%. During cell-division, <sup>3</sup>H thymidine was incorporated into the replicating DNA. After 14 hours, cells were harvested onto a glass-fiber filter, mounted with a cocktail containing scintillation liquid and the counts were monitored.  $\beta$ -counts of the control cells were in the range of  $2.7 \times 10^4$  and  $1.2 \times 10^6$  counts per minute (cpm).

### **Flow cytometry**

Cell surface staining of TRAIL-R2 and FN14 was performed after the culturing of the cells with the cytokines for two days. Harvested cells were incubated for 30 minutes with labeled antibodies, TRAIL-R2 (R&D Systems), FN14 (eBioscience) and their appropriate isotope control (19).

### **Isolation of RNA, cDNA synthesis and qRT-PCR**

Total RNA from KCs or skin biopsies were extracted by using miRNAeasy Mini Kit or RNeasy Fibrous Tissue Mini Kit (Qiagen) according to manufacturer's instructions. Homogenization of the skin samples was done either by Ultra-Turrax T8 (IKA Labortechnik) or Precellys 24 (Precellys) homogenizer. All RNAs were treated with DNase I. cDNA was synthesized from 200 ng of total RNA using oligo-dT and SuperScript III Reverse Transcriptase (Invitrogen) or reverse transcription reagents with random hexamers (Fermentas) according to manufacturers protocols. Primers were designed with assistance of Primer 3 or 5. The sequences of qPCR primers and Taqman probes are provided in Supplemental methods. RNA concentration and quality was assessed with NanoDrop ND-1000 spectrophotometer and Agilent 2100 Bioanalyzer. For amplification of the PCR product either TAQMAN (Applied Biosystems) or SYBR/Rox-PCR (Fermentas) master mixes were used. qRT-PCR analysis was carried out on ABI Prism 7900, the relative gene expression levels were calculated using the comparative Ct ( $\Delta\Delta C_t$ ) method (Applied Biosystems).

### **mRNA array and pathway analysis**

mRNA profiling was performed on Illumina HumanHT-12 Expression BeadChips. The data were analyzed with Genomestudio (Illumina) using custom rank invariant method for normalization. Genes with differential expression  $p < 0.05$  were considered differentially expressed. Further analyses and visualizations were

performed using Microsoft Excel and Multi Experiment Viewer 4.6.1. Unsupervised hierarchical clustering was done using Euclidean distance and average linkage analysis. Heatmaps presented on Fig 3 and 4 were generated using Multi Experiment Viewer 4.6.1. Log2 values of expression levels that were mean-centered across all of the samples for each gene are presented. Color scale from green (lower expression) to red (higher expression) represents deviation from the mean (black).

Pathway analysis was performed with g:Profiler using the g:GOST tool and default parameters. The version used at the time of analysis is currently available in the g:Profiler archive ([http://biit.cs.ut.ee/gprofiler\\_archive/r0818\\_e57\\_eg4/web/](http://biit.cs.ut.ee/gprofiler_archive/r0818_e57_eg4/web/)). g:Profiler calculates enrichment p-value using Fisher's one-tailed test.

A dataset GSE12109 of cytokine induced primary KCs was downloaded from Gene Expression Omnibus Database (<http://www.ncbi.nlm.nih.gov>) and analyzed using the R software package. For each probeset, a t-test between IFN- $\gamma$  and control group was performed to find differentially expressed probesets. P-values of the t-tests for multiple testing were corrected using False Discovery Rate (FDR). Probesets with corrected FDR<0.05 were considered as differentially expressed and mapped to gene names.

## **Immunofluorescence**

Skin biopsy specimens were from AD patients and healthy individuals that are described in (20). 7  $\mu$ m slices of skin biopsy specimens were fixed for 10 minutes with 4% formaldehyde in PBS followed by permeabilization/blocking step with 0.5% Triton-X-100, 1% BSA (Sigma) and either with 5% goat or rabbit serums for 15 min. Specimens were further incubated for 1 hour either with rabbit polyclonal anti-IFITM1 (ab106265), rabbit polyclonal anti-MCP2 (CCL8, ab9671) or goat polyclonal to anti-RANTES (CCL5, ab10590) (all from Abcam) or rabbit or goat IgG as isotype controls

in 1:100 dilutions. Alexa-488 labeled anti-goat-IgG and anti-rabbit-IgG (both from Invitrogen) in dilutions of 1:4000 were used as secondary antibodies. Before and after antibody incubations, specimens were washed 3x5 min with 0.01% Tween in PBS. Slides were mounted with VectaShield mounting medium containing 4',6-diamidino-2-phenylindol (DAPI) (Vector Laboratories) and analyzed with a Leica TCS SPE confocal microscope (Leica Microsystems).

### qRT-PCR primers

| Gene      | Forward                              | Reverse                                | Probe <sup>A</sup>                          |
|-----------|--------------------------------------|----------------------------------------|---------------------------------------------|
| TRAIL-R1  | TGT ACG CCC TGG AGT<br>GAC AT        | CAC CAA CAG CAA CGG<br>AAC AA          | TGT CCA CAA AGA ATC AGG<br>CAA TGG ACATAA T |
| TRAIL-R2  | CAC TCA CTG GAA TGA<br>CCT CCT TT    | GTG CAG GGA CTT AGC<br>TCC ACT T       | TCA CAC CTG GTG CAG<br>CGC AAG CAG          |
| TRAIL-R3  | CCC TAA AGT TCG TCG<br>TCG TCA T     | GGG CAG TGG TGG TGG<br>CAG AGT A       | CGC GGT CCT GCT GCC<br>AGT CCT AG           |
| TRAIL-R4  | ACA GAG GCG CAG CCT<br>CAA           | ACG GGT TAC AGG CTC<br>CAG TAT ATT     |                                             |
| OPG       | AAA GCA CCC TGT AGA<br>AAA CAC A     | GTT GCC GTT TTA TCC<br>TCT CTA C       |                                             |
| TNF-R1    | CGC TAC CAA CGG TGG<br>AAG TC        | TCC TTC AAG CTC CCC<br>CTC TT          | CTA CTC CAT TGT TTG TGG<br>GAA ATC GAC ACC  |
| TNF-R2    | GAG TGG TGA ACT GTG<br>TCA TGA       | GAG CTC GGC GCT GTG<br>ATC             | AAG GTG CCT CAC TTG CCT<br>GCC GAT A        |
| Fn14      | GCT CTG AGC CTG ACC<br>TTC GT        | CTC TCC GCC GGT CTC<br>CTC TA          | CCG CAG GAG AGA GAA<br>GTT CAC CAC CC       |
| Fas       | CTT TTC GTG AGC TCG<br>TCT CTG A     | CCC CAG AAG CGT CTT<br>TGA AC          |                                             |
| TRAIL     | CCC AAT GAC GAA GAG<br>AGT ATG AAC A | CTC AAA ATC ATC TTT<br>CTA ACG AGC TGA | CTG CTG GCA AGT CAA<br>GTG GCA ACT CC       |
| TNF-alpha | CCA GGC AGT CAG ATC<br>ATC TTC T     | GGA GCT GCC CCT CAG<br>CTT             | AGC CTG TAG CCC ATG<br>TTG TAG CAA ACC C    |
| TWEAK     | CGC CTT TCC TGA ACC<br>GAC TA        | CCG TGT TTT CCG GCC<br>TT              | TCG GCC TCG CAG AAG<br>TGC ACC T            |
| FERMT1    | TGA TGC AGC CAC CGG<br>GAT TCC A     | CGA TGA CCA CCT GCC<br>GGG TTT C       |                                             |
| CARD10    | AGT GTG CCC AGC GGA<br>AAG CC        | GAT GGC CCG GAT CCT<br>GCT GC          |                                             |
| ANXA5     | AGG CTT GGG CAC AGA<br>TGA GGA GA    | GCT GCA GAG ATT TCC<br>TGG CGC T       |                                             |
| IL1F9     | GCC CAC ATT GCA GCT<br>AAA AGA GCA   | AGG TCC TAC CAG TCT<br>TGG CAC GG      |                                             |
| ADM       | CTC TTA GCA GGG TCT<br>GCG CTT CG    | CCG ACG CGA CAT CCA<br>ACC GA          |                                             |
| BATF      | CCT GCA CCT GGA GAG<br>CGA AGA C     | GGC TGT TCA GCA CCG<br>ACG TGA A       |                                             |
| CCDC109B  | CGC CCC AGG TTT<br>TGCGTGTGA         | TTT CAT CAG GTG GCA<br>CCA CGG TAC T   |                                             |
| CCL8      | AGG GAC TTG CTC AGC<br>CAG ATT CAG T | TCT TGT GTA GCT CTC<br>CAG CCT CTG G   |                                             |

|           |                                   |                                    |  |
|-----------|-----------------------------------|------------------------------------|--|
| IFI35     | GGA ACG GAG GTG GCG<br>ATG TGG    | CAG ACG CTG AGC CAC<br>TCC ATC C   |  |
| IFITM1    | CAA CAC CCT CTT CTT GAA<br>CTG G  | GCC GAA TAC CAG TAA<br>CAG GAT G   |  |
| IFITM2    | GTT CAA CAC CCT CTT CAT<br>GAA CA | GAC GAC CAA CAC TGG<br>GAT GAT     |  |
| LYN       | AGC TCG TGA GGC TCT<br>ACG CTG T  | CAG CAA ACT GCC CTT<br>GGC CAT G   |  |
| RAB31     | GGG AGG TTC CCC TGA<br>AGG ATG CT | GGG TGG GAT CTG GCG<br>GCT GAT     |  |
| TUBB2C    | CGCCCGCTCTTCTGCTGCT<br>GTTT       | CCT CCC AAA ACT TGG<br>CGC CGA TT  |  |
| RXRA      | GCG CCA TCT TTG ACA<br>GGG TGC T  | GGG TTC GAG AGC CCC<br>TTG GAG T   |  |
| PCSK9     | ACG TCC TCA CAG GCT<br>GCA GC     | TTG GGC TGA CCT CGT<br>GGC CT      |  |
| NOD2      | TGA GGT GGC TCA GCC<br>TGG TGG    | TGC CAG CAT CAG TGC<br>CAA GGC     |  |
| INPP5D    | CTC CCG CAT CAC GGC<br>AAG CA     | GGG CTT CAC TGC ATG<br>GCA GTC C   |  |
| DUSP1     | GCG CGG AAT CTG GGT<br>GCA GT     | TTT CCA CCG GGC CAC<br>CCT GA      |  |
| CCL5      | AGT CGT CTT TGT CAC<br>CCG AAA    | TCT CCA TCC TAG CTC<br>ATC TCC AA  |  |
| CCL20     | CGG CAG CTG GCC AAT G             | TAT TTC ACC CAA GTC<br>TGT TTT GGA |  |
| IL1F9     | GCCACATTGCAGCTAAAA<br>GAGCA       | AGGTCCTACCAGTCTTG<br>GCACGG        |  |
| IL5       | GAC CTT GGC ACT GCT<br>TTC TAC TC | TGT ACA GGA ACA GGA<br>ATC CTC AGA |  |
| IL4R1     | GCACCAAGTGGCACAACCTC<br>CTACA     | AACAGGCAGACGGCCA<br>GGATGA         |  |
| IL13RA    | TGGGGTCCCAGTGTAGCAC<br>CAA        | CAGGATCACCTTCTGGG<br>GGTGAGA       |  |
| IL22      | CCT GCA TAT CCA GAG<br>GAA TGT G  | CCA GTT CTC CAA TTG<br>CTT TGA TCT |  |
| KRT10     | TCCCAACTGGCCTTGAAAC<br>AATCCC     | AGAGCTGCACACAGTAG<br>CGACCT        |  |
| HPRT      | GACTTTGCTTTCCTTGGTCA<br>GG        | AGTCTGGCTTATATCCA<br>ACA CTTCG     |  |
| EF1Aex7-8 | CCACCTTTGGGTCGCTTTG<br>CTGT       | TGCCAGCTCCAGCAGCC<br>TTCTT         |  |

<sup>A</sup> Taqman probes (Applied Biosystems) were used when probe sequence is shown. Other primers were ordered from Microsynth

## Online repository

## FIGURE LEGENDS

### **Figure E1. Cytokine induced apoptosis in HaCat cell-line and in primary KCs.**

HaCat cells (A) or primary KCs (B) were stimulated with indicated cytokines followed by Annexin V and 7AAD staining. (A) The viability (Annexin V and 7AAD negative cells) of HaCat cells either unstimulated (u.s.) or stimulated with indicated cytokines is presented. Data shown represent mean  $\pm$ SD of two independent experiments. (B) KCs from healthy individuals, AD and psoriasis patients were stimulated with indicated cytokines. Data are presented as percentage of viable KCs 4 days after stimulation with the cytokines, n=5 for each group.

**Figure E2. IFN- $\gamma$ , TRAIL, TNF- $\alpha$  and TWEAK suppress proliferation of KCs from healthy individuals, AD and psoriasis patients.** Proliferation was measured two days after stimulation with the cytokines at the indicated concentrations. Data are shown as percentage of suppression compared to Actinomycin D treatment (positive control, =100%). Data represent mean  $\pm$ SD, n=3 of each group.

**Figure E3. IFN- $\gamma$  has no influence on expression of FN14, TWEAK, FAS and TNF-R1 in KCs.** (A, C, D) Relative mRNA expression of indicated genes is shown as mean  $\pm$ SD of the cells harvested after stimulation with IFN- $\gamma$  for the indicated time-points and is shown compared to the time-point 0 (=1). n=3 for each group. (B) The histograms represent FN14 surface expression (unfilled curve) and the isotype

control (filled curve) measured by flow cytometry of unstimulated (u.s.) and IFN- $\gamma$ -stimulated KCs.

**Figure E4. The influence of TNF- $\alpha$ , TWEAK, TRAIL and anti-Fas mAb on the expression of TRAIL receptors TRAIL-R2 (A), TRAIL-R3 (B) and TRAIL-R4 (C).** Relative mRNA expression of indicated genes is shown as mean  $\pm$ SD of the cells harvested after at indicated time-points and is shown compared to the time-point 0 (=1). The analysis was performed on KCs from healthy individuals, AD and the psoriasis patients, n=3 for each group.

**Figure E5. Verification of the KC mRNA array results by qRT-PCR.** Relative mRNA expression of indicated genes is shown as mean  $\pm$ SD of the results from three different individuals for each group and is normalized to the value of one of the healthy individuals (=1).

**Figure E6. IL5, IL4R1, IL13RA and IL22 are differentially expressed in the skin biopsies from AD patients.** (A) Relative mRNA expression of indicated genes is shown as mean of the results from 10 different individuals for each group (NL: non-lesional; L: lesional; H: healthy) and is normalized to the value of one of the healthy individuals (=1). (B) HPRT and KRT10 were used as control genes.

**Figure E7. Isotype controls with rabbit and goat anti-IgG antibodies.** Immunofluorescence (IF), 4',6-Diamidino-2-phenylindole (DAPI) and hematoxylin

(HE) stainings of AD skin are shown. Goat and rabbit IgG were used as the isotype controls (IC). Original magnification of 60x and 10x for IF and HE staining were used, respectively. IF stained areas are designated with squares on HE stained panels.

**Figure E8. RXRA, TUBB2C, PCSK9 and INPP6 genes do not respond to IFN- $\gamma$  and TNF- $\alpha$  in healthy KCs.** Relative mRNA expression (mean  $\pm$ SD, n=6) of apoptosis-related genes unstimulated (u.s.,=1) or stimulated (24 hours) KCs.