

# High Expression Levels of Keratinocyte Antimicrobial Proteins in Psoriasis Compared with Atopic Dermatitis

Gys J. de Jongh,\* Patrick L. J. M. Zeeuwen,\* Martina Kucharekova,† Rolph Pfundt,‡ Pieter G. van der Valk,\* Willeke Blokx,§ Aynur Dogan,\* Pieter S. Hiemstra,¶ Peter C. van de Kerkhof,\* and Joost Schalkwijk\*#

\*Department of Dermatology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; †Department of Dermatology, University Medical Centre Maastricht, Maastricht, The Netherlands; ‡Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; §Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; ¶Department of Pulmonology, Leiden University Medical Centre, Leiden, The Netherlands; #Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands

**Recently, it was shown that lesional skin of atopic dermatitis patients expresses low levels of some antimicrobial peptides, compared with psoriasis patients. Here we performed microarray analysis on mRNA from purified lesional epidermal cells of patients with chronic plaque psoriasis and chronic atopic dermatitis, to investigate whether this is a general phenomenon for host defense proteins, and how specific it is for this class of molecules. Microarray data were confirmed on a selected set of genes by quantitative PCR and at the protein level by immunohistochemistry. We found overexpression of many antimicrobial proteins in keratinocytes from psoriatic skin compared with atopic dermatitis skin. Interestingly, we observed that markers of normal differentiation and the activated/hyperproliferative epidermal phenotype were expressed at equal levels. Chronic lesions of psoriasis and atopic dermatitis patients are remarkably similar with respect to cellular proliferation. We conclude that psoriatic epidermis expresses high levels of host defense proteins compared with atopic dermatitis epidermis, and this phenomenon appears to be specific for these proteins. It remains to be investigated whether this is caused by genetic polymorphisms in pathways leading to an epidermal antimicrobial response, or by differences in the cellular infiltrate in psoriasis compared with atopic dermatitis.**

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The stratum corneum of normal human epidermis is a cross-linked structure of insoluble proteins and lipids and as such represents a barrier against invasion by microbial agents. In addition to this physical barrier, human keratinocytes express a number of molecules that are part of the innate immune system and participate in recognition of “microbial non-self” and subsequent killing of invading microorganisms (Gallo and Huttner, 1998). Some of the toll-like receptors (TLR), which are involved in recognition of conserved microbial patterns, were recently demonstrated in human epidermis (Curry *et al*, 2003). The skin appears to be a particularly rich source of effector molecules as many proteins with bactericidal or bacteriostatic properties are constitutively or inducibly expressed in human epidermis. These include human  $\beta$ -defensin (hBD)-1, 2, 3, and 4, secretory leukocyte proteinase inhibitor (SLPI), LL-37, elafin/SKALP, dermcidin, calgranulins A, B and C, psoriasin, and cystatin A (Harder *et al*, 1997; Wingens *et al*, 1998; Simpson *et al*, 1999; Takahashi *et al*, 1999; Schitteck *et al*, 2001; Zaiou and Gallo, 2002; Harder *et al*, 2004; Glaser *et al*, 2005). Serial analysis of gene expression (SAGE) on cultured ker-

atinocytes or human skin biopsies has revealed that some of these molecules are found at high levels of expression (Jansen *et al*, 2001). There is evidence from genetic studies in mice that these molecules are vital for maintaining epidermal integrity (Ashcroft *et al*, 2000; Nizet *et al*, 2001). Elafin, psoriasin, LL-37, and hBD-2 are not normally present in human epidermis but are highly induced upon barrier disruption or chronic inflammation such as in the skin disease psoriasis. In psoriasis, the on-switch of these genes appears to be part of a stress response also referred to as regenerative maturation, which includes hyperproliferation and induction of a number of established marker genes such as cytokeratin (CK) 6, 16, and 17.

Psoriasis vulgaris and atopic dermatitis are two distinct clinical entities characterized by various different histological features depending on the stage of the lesion. These histological characteristics are most apparent in fully developed acute lesions. In the chronic phase of the disease, the histopathological differences are less pronounced and both diseases are characterized by acanthosis and a mononuclear infiltrate. A striking difference is the high frequency of bacterial colonization of non-lesional atopic dermatitis skin and recurrent skin infections by bacterial, fungal, and viral pathogens in lesional atopic dermatitis skin (Leung and Bieber, 2003). In contrast, a large epidemiological study on disease concomitance in psoriasis revealed that psoriasis

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Abbreviations: hARP, housekeeping gene human acidic ribosomal protein P0; hBD, human  $\beta$ -defensin; SLPI, secretory leukocyte proteinase inhibitor; TLR, toll-like receptor

patients have an increased resistance to bacterial and viral infections compared with controls and atopic dermatitis patients (Henseler and Christophers, 1995). Recently, a study was published showing that expression levels of two antimicrobial proteins hBD-2 and LL-37 are significantly decreased in lesional atopic dermatitis skin compared with lesional psoriatic skin (Ong *et al*, 2002). It was speculated that a relative deficiency in expression of innate immunity genes in atopic dermatitis patients could account for the susceptibility to skin infection with *Staphylococcus aureus*. In addition, these authors published a microarray analysis of full thickness biopsies from lesional skin of psoriasis and atopic dermatitis patients. Their study also revealed a specific difference in the profile of proinflammatory cytokines and chemokines. Here we sought to confirm and extend these previous findings. First, we wanted to limit our analysis to the epidermal compartment, and second, we wanted to investigate the specificity of the observed difference for keratinocyte gene expression. In other words: is expression of genes that are associated with keratinocyte activation/regeneration, but not involved in host defense, also different? Finally, we wanted to evaluate the expression at the protein level, which has not been extensively performed so far. Our data indicate that a large number of established and putative antimicrobial proteins are highly expressed in psoriasis compared with atopic dermatitis, both at the mRNA and protein level. We conclude that the epidermis of these two patient groups is remarkably similar in other respects, such as cellular proliferation and other established markers of the regenerative phenotype, emphasizing the specificity of the differential antimicrobial response.

## Results

**Experimental design of microarray analysis** We used a design in which we compared samples from lesional psoriasis epidermis with lesional atopic dermatitis epidermis on one microarray. Although the samples were chosen to be as homogeneous as possible with respect to stage of the disease, biopsy site, drug use, and age of the patient, we made a design of experiment in which these variables were confounded, under the assumption that the effect of disease would be much larger than these other factors. The experimental design chosen here is therefore quite different from previous large-scale expression studies (Oestreicher *et al*, 2001; Zhou *et al*, 2003). We deliberately did not compare normal skin with diseased skin because it generates an extremely large list of differential genes that is hard to interpret. By comparing two types of disease skin we eliminated a large number of changes in expression that are common to chronic inflammatory diseases, such as psoriasis and atopic dermatitis (e.g., increased metabolic state, hyperproliferation, altered differentiation). Using mRNA from purified epidermis rather than full thickness skin biopsies eliminated another important source of noise. An important point to consider is that we used microarray analysis only as a screening procedure to assess the global difference in gene expression. Because we used a 19,000 element microarray and a limited number of samples, the family-wise error will be huge. This is a general problem in microarray

analysis that cannot be solved by the usual procedures like Bonferroni correction (Draghici, 2002). Instead of correcting for multiple testing we just sorted the genes according to the chance that they were differentially expressed. Applying a criterion of at least 2-fold difference and a  $t > 5.5$  (see also Figs S1a and S1b) we found 183 genes to be differentially expressed. This procedure generates an estimated 10% false discovery rate. We used qPCR to verify the expression of 15 genes in this study by an independent quantitative method. For a limited number of genes we performed semi-quantitative histological analysis at the protein level. As normal epidermis was not included in the microarray analyses, the differences between psoriasis and atopic dermatitis are not informative for comparison with normal skin. In the qPCR and immunohistochemical validation, however, we have included normal skin. Here we focused our analysis on expression of genes involved in host defense; a comprehensive analysis of the complete data set will be published elsewhere. In Table I, we list a number of antimicrobial effector and membrane-bound signaling molecules that were present on the microarray and could be reliably analyzed. Antimicrobial effector molecules were defined as proteins with a proven bactericidal or bacteriostatic effect *in vitro*, based on Pubmed searches rather than relying on the annotation in Gene Ontology. Most of these genes were visible on all six microarrays. Genes whose expression level was below background in both channels (Cy3 and Cy5) were omitted from the analyses. Only genes that were visible on at least four microarrays were included in the analyses. Differences in expression were considered relevant when the fold-difference was  $> 2$  and the  $p$ -value  $< 0.05$  (uncorrected for multiple testing). For 10 out of 18 genes, we found a significantly higher expression of antimicrobial effector proteins in psoriasis compared with atopic dermatitis. The remaining genes were expressed at similar levels in both diseases. We could confirm the increase in expression of hBD-1 and hBD-2, elafin, calgranulin C, and iNOS previously found by others (see Table I last column). LL-37 and hBD-3, previously reported to be overexpressed in psoriasis, were also elevated in our microarray analysis but the difference was small and insignificant. Our analysis revealed an additional number of antimicrobial proteins to be significantly upregulated in psoriasis, such as psoriasin, calgranulin A and B, chitinase, and cystatin A. We found a significant upregulation in psoriatic epidermis for two of the  $\alpha$ -defensins but the fold-increase was only small. Upon examination of genes involved in receptor-mediated recognition of microbial structures, we found that none of the TLR family members present on the microarray (TLR 2, 3, 4, and 9) were differentially expressed (applying the criteria mentioned above), although they were clearly expressed in the epidermis. A significant difference was found for lipopolysaccharide-binding protein, which was overexpressed 2.7-fold in psoriatic epidermis. These findings raised the question if all genes normally associated with keratinocyte activation show elevated expression levels in psoriasis. To address this question we analyzed a number of epidermal proteins not known to be involved in host defense (see Table I). These include proteins that are normally present in skin but are strongly upregulated in psoriasis (involucrin,

**Table I. Antimicrobial effector and signaling proteins detected by microarray analysis**

Classification	Symbol	Mean of $^2\log[\text{ratio}]^a$	SD	$t$ test <sup>b</sup>	PS/AD <sup>c</sup>	Previous studies <sup>d</sup>
<i>Antimicrobial effector proteins</i>						
$\beta$ -defensin-1*	DEFB1	1.20	0.48	0.002	2.3	Nomura <i>et al</i> (2003b)
$\beta$ -defensin-2*	DEFB4	2.99	0.77	0.004	8.0	Ong <i>et al</i> (2002)
$\beta$ -defensin-3	DEFB103	0.60	2.13	0.522	1.5	Nomura <i>et al</i> (2003b)
$\alpha$ -defensin-4	DEFA4	0.42	0.68	0.244	1.3	
$\alpha$ -defensin-6	DEFA6	0.52	0.24	0.003	1.4	
Elafin*	PI3	1.97	1.13	0.008	3.9	Nomura <i>et al</i> (2003a)
Psoriasin*	S100A7	1.84	0.62	0.001	3.6	
Calgranulin A*	S100A8	2.88	1.52	0.006	7.4	
Calgranulin B*	S100A9	1.53	0.77	0.005	2.9	
Calgranulin C*	S100A12	2.97	0.86	0.001	6.9	Nomura <i>et al</i> (2003a)
Cystatin A*	CSTA	1.47	0.82	0.007	2.8	
LL-37	CAMP	0.30	0.99	0.498	1.2	Ong <i>et al</i> (2002)
iNOS*	NOS2A	2.19	0.50	0.001	4.6	Nomura <i>et al</i> (2003b)
Chitinase*	CHIT1	1.78	0.48	0.001	3.4	
Complement factor 1r	C1R	0.44	0.62	0.137	1.4	
Complement factor 1q	C1QA	-0.61	0.52	0.036	0.7	
Complement factor 5	C5	1.30	1.97	0.46	2.5	
Lactotransferrin	LTF	0.32	0.52	0.20	1.2	
<i>Antimicrobial signaling proteins</i>						
TLR2	TLR2	0.64	0.54	0.033	1.6	
TLR3	TLR3	0.81	0.68	0.034	1.7	
TLR4	TLR4	-0.53	0.85	0.303	0.7	
TLR9	TLR9	-0.63	0.50	0.087	0.6	
LPS-binding protein*	LBP	1.44	0.54	0.001	2.7	
<i>Non-antimicrobial epidermal proteins</i>						
Involucrin	IVL	-0.04	1.01	0.920	1.0	
Transglutaminase-1	TGM1	0.33	0.76	0.340	1.3	
CK10	KRT10	-0.65	0.49	0.022	0.6	
CK17	KRT17	-0.68	0.75	0.077	0.6	
PCNA	PCNA	0.14	0.61	0.607	1.1	

<sup>a</sup>This is the mean of the normalized  $^2\log$  of the Cy3/Cy5 ratio (see supplemental material).<sup>b</sup>p-value in two-tailed  $t$  test.<sup>c</sup>Fold difference of gene expression level in psoriasis compared with atopic dermatitis.<sup>d</sup>Refers to previous studies where differential expression between PS and AD was observed for a particular gene.\*Differences that were considered to be relevant (fold difference >2 and  $p < 0.05$ ).

PS, psoriasis; AD, atopic dermatitis.

transglutaminase-1) or downregulated in psoriasis (CK10) when compared with normal skin or uninvolved psoriatic skin. In addition, genes are listed that are generally induced in hyperproliferative (psoriatic) epidermis such as CK17, or are associated with hyperproliferation such as proliferating

cell nuclear antigen (PCNA). Remarkably, none of these genes were differentially expressed in our microarray analysis. Other well-known hyperproliferation associated markers like cytokeratins CK6 and CK16 were not contained in our probe set. Our findings suggest that, although the

epidermis is activated in both diseases, as witnessed by the high expression levels of marker proteins (involucrin, CK17, and PCNA) a large number of host defense effector molecules are specifically overexpressed in psoriasis.

Our approach of using pure epidermis minimizes the contribution of infiltrating cells and cells normally present in full thickness skin biopsies, such as hair follicles, sweat glands, sebaceous glands, fibroblasts, blood vessels, and so on. The contribution of infiltrating inflammatory cells in the epidermis can obviously not be avoided. To address their presence, we analyzed two classes of signaling molecules that are also highly relevant for cutaneous host defense, i.e., chemokines and their cognate receptors. Some of these are known to be lymphocyte-specific, or not known to be expressed by keratinocytes, and can therefore be used to look at lymphocyte subsets in the lesional epidermis. The probe set of our microarrays contained 18 CCL chemokines, nine CXCL chemokines, nine CCR chemokine receptors, and two CXCR chemokine receptors. Most of them were either visible on all arrays or completely undetectable. Some chemokines such as CCL2 (MCP1), CCL5 (RANTES), CCL27 (CTACK), and CXCL14 are known to be produced by epidermal keratinocytes, and these were clearly present on our arrays although not differentially expressed between psoriasis and atopic dermatitis. Other chemokines, that are known to be expressed by lymphocytes or dendritic cells but not by keratinocytes were clearly found, such as CCL17 (TARC), CCL21, CCL22, CXCL9. Only CCL17/TARC was found to be differentially expressed between psoriasis and atopic dermatitis (5-fold upregulation in atopic dermatitis compared with psoriasis,  $p < 0.02$ ). The chemokine receptors CCR4 (Th1 lymphocytes), CCR5 (Th2 lymphocytes), CCR7 (memory T cells), and CCR8/MCP1 (T lymphocytes) were detected on nearly all arrays, but were not differential between psoriasis and atopic dermatitis.

Although purified epidermis contains >90% keratinocytes as the major cell population, Langerhans cells and melanocytes are resident epidermal cells that contribute to the mRNA profile generated by microarray analysis. Cell specific transcripts such as langerin and tyrosinase were clearly present in all microarrays examined, although never differential between the two patient categories (not shown).

#### Validation of microarray data by quantitative real-time

**PCR** As indicated above, microarray analysis is used as a screening procedure because it does not supply accurate quantitative data on expression levels and it generates a number of false positives. Therefore, validation by independent methods is required, such as qPCR analysis and (ideally) protein analysis, which was performed for a limited number of genes. Our microarray analysis was performed by pair-wise hybridization of purified lesional epidermis from psoriasis and atopic dermatitis patients. In order to address the question how the observed expression levels related to that of normal epidermis we included qPCR analysis of mRNA derived from purified epidermal sheets of skin biopsies from five healthy individuals for comparison with lesional skin of 10 psoriasis and six atopic dermatitis patients. We performed qPCR on 15 genes selected on the basis of

microarray analysis (see Table S1, Table S2 of supplemental online material for experimental details of the qPCR procedures). Table II lists the findings of the qPCR analyses. In general, there was a reasonable correlation between the fold difference in gene expression of microarray data and qPCR data, such as found for hBD-2, elafin, calgranulin A, calgranulin B, and psoriasin, which were all found to be upregulated in psoriasis. Particularly, the upregulation of hBD-2 is striking. hBD-2 is virtually absent from normal epidermis but was found to be expressed at more than 20-fold higher levels in psoriasis than in atopic dermatitis. The other genes showed modest upregulation (2.8–4.1-fold) and were significant (calgranulin B and psoriasin) or of borderline significance (elafin and calgranulin A). Interestingly, for elafin and calgranulin A, we could confirm the observed difference at the protein level, which was significant. We could not confirm the upregulation of hBD-1, by qPCR. Our qPCR data corroborated the microarray data on LL-37 and hBD-3, which did not indicate significant differential expression between psoriasis and atopic dermatitis. Expression of TLR3, which was considered non-significant by microarray (less than 2-fold up in psoriasis and  $p < 0.05$  in an uncorrected *t* test), was now again upregulated 1.8-fold, reaching borderline significance ( $p = 0.05$ ), suggesting that this difference, albeit small, is consistent.

We analyzed four genes involved in epidermal differentiation (involucrin and the cytokeratins 10, 16, and 17). None of these showed a significant upregulation in psoriasis compared with atopic dermatitis. In contrast to microarray analysis, which showed no differences, we now observed a significantly higher expression of involucrin and cytokeratin 17 in atopic dermatitis.

**Histopathology** Biopsies taken from chronic lesions of both patient groups were analyzed for parakeratosis, orthokeratosis, spongiosis, elongated rete ridges, acanthosis, and the presence of neutrophils. Histology confirmed that atopic dermatitis lesions were of the chronic/subacute type. Acanthosis and elongation of rete ridges was observed in nearly all biopsies. Regular elongation of rete ridges, however, was found in six of nine psoriasis patients and in none of the atopic dermatitis patients. Parakeratosis was found in all psoriasis patients, and in seven of 10 atopic dermatitis patients, mostly with a patchwise distribution. Spongiosis was clearly present in 10 of 10 atopic dermatitis patients, but could also be observed in seven of nine psoriasis patients, although far less prominent. Microabscesses with neutrophils were found in six of nine psoriatic patients, but in none of the atopic dermatitis patients. These observations indicate that chronic lesions of the two diseases share a number of histopathologic characteristics.

**Immunohistochemistry** The ultimate test for relevance of differentially expressed genes is the presence and localization at the protein level. To investigate if overexpression of host defense molecules as found at the mRNA level is indeed found at the protein level we examined the immunolocalization of elafin, hBD-2, hBD-3, and calgranulin A in the skin of psoriasis and atopic dermatitis patients, and in healthy controls. Elafin was not detectable in the interfollicular epidermis of normal human epidermis

Table II. Validation of microarray data by qPCR

Classification	Delta PCR cycles			p-values				Ratio of PS/AD
	NS	PS	AD	Main <sup>a</sup>	NS versus PS <sup>b</sup>	NS versus AD <sup>b</sup>	PS versus AD <sup>b</sup>	
Antimicrobial effector proteins								
β-defensin-1	4.4	3.8	4.2	0.1062	–	–	–	1.4
β-defensin-2	15.6	0.9	5.4	0.0000	0.0001	0.0002	0.0057	22.1
β-defensin-3	11.8	5.3	4.7	0.0000	0.0002	0.0001	0.617	0.7
Elafin	10.0	0.8	2.8	0.0000	0.0001	0.0002	0.126	3.9
Calgranulin A	6.6	−1.4	0.1	0.0000	0.0001	0.0002	0.08	2.8
Calgranulin B	4.7	−3.4	−1.3	0.0000	0.0001	0.0002	0.0028	4.1
Psoriasin	5.9	−1.9	−0.2	0.0000	0.0001	0.0002	0.0438	3.2
LL-37	14.1	12.2	12.4	0.0758	–	–	–	1.2
Antimicrobial signaling proteins								
TLR2	10.9	8.9	9.2	0.0000	0.0001	0.0002	0.4	1.2
TLR3	11.1	10.3	11.2	0.0492	0.067	0.808	0.0500	1.8
TLR4	17.7	15.0	15.7	0.0228	0.0069	0.0285	0.421	1.6
Non-antimicrobial epidermal proteins								
Involucrin	7.0	7.2	5.4	0.0171	0.758	0.0200	0.0133	0.3
CK10	−0.9	2.1	2.1	0.0000	0.0002	0.0001	0.938	1.0
CK16	5.5	1.0	0.6	0.0000	0.0002	0.0001	0.5320	0.7
CK17	5.1	3.9	1.4	0.0002	0.085	0.0001	0.0019	0.2

<sup>a</sup>Main effect significance from univariate *F* test.

<sup>b</sup>Significance from a Duncan multiple range test for the indicated groups.

Quantitative PCR was performed on mRNA from purified epidermal cells of normal skin (*n* = 5 individuals), lesional psoriasis (*n* = 10 patients), and lesional atopic dermatitis (*n* = 6 patients). Data in the column "Delta PCR cycles" are expressed as the means of the difference between the threshold of PCR cycles of each gene minus the number of threshold cycles of the reference gene. An *F* test for analysis of variance was performed to test the main effect (p-value in the column "main"). For those genes where the main effect was significant at the 5% level a Duncan multiple range test was used to test the significance of the difference between each of the indicated groups. The ratio of expression in psoriasis and atopic dermatitis is given for comparison with Table I.

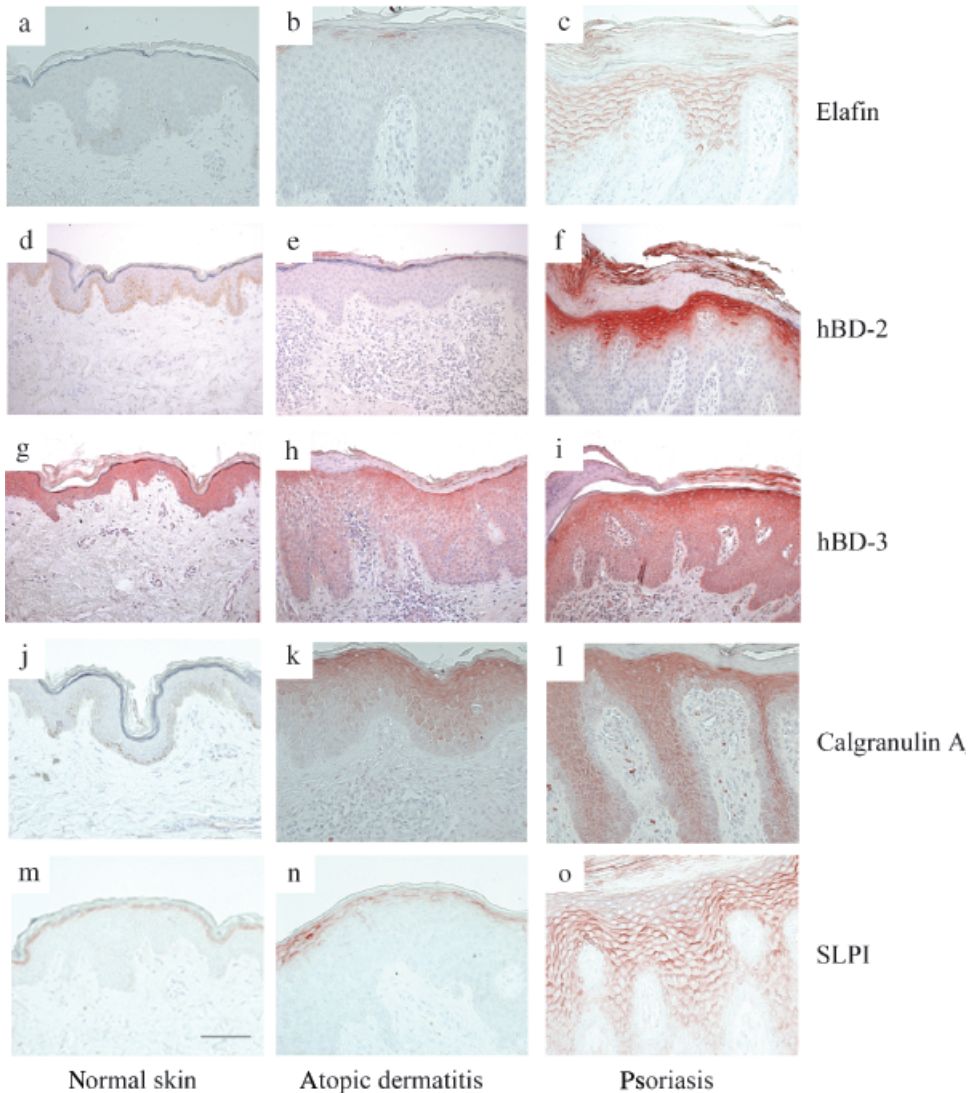
NS, normal skin; PS, psoriasis; AD, atopic dermatitis.

See online supplemental material for individual data of all biopsies for all genes.

(Fig 1a) but is highly expressed in psoriatic epidermis as shown in Fig 1c. In the epidermis of atopic dermatitis the distribution was often discontinuous, and the intensity as measured by the ratio of positive cell layers over the total number of cell layers was significantly different between the two diseases (Fig 1b and Table III). Staining for hBD-2 revealed a similar pattern as previously described by Ong *et al* (2002). hBD-2 was absent in normal epidermis (Fig 1d), but highly expressed in the suprabasal layers of psoriatic epidermis (Fig 1f). In atopic dermatitis epidermis, hBD-2 expression was far lower than in psoriasis (see Fig 1e and Table III). hBD-3 staining was strong in the stratum granulosum of normal skin and weak in the entire epidermis (Fig 1g). hBD-3 was clearly present in the stratum spinosum of the acanthotic lesional epidermis of psoriasis and atopic dermatitis patients. Although the expression in psoriasis tended to be somewhat stronger than in atopic dermatitis (Fig 1h and i) this was not significant, which was in accordance with microarray and qPCR data. Staining for calgranulin A was absent in normal skin (Fig 1j) but showed a continuous staining of the suprabasal compartment in both diseases (Fig 1k and l). Staining, however, was more intense

in psoriasis, and often extended to the first suprabasal layer (Fig 1l). The difference was of borderline significance (Table III). In addition to the aforementioned antimicrobial proteins that were all present on the microarray, we analyzed the presence of SLPI, a host defense protein that we have previously described in skin and lung (Tomee *et al*, 1997; Wingens *et al*, 1998), that was not present in the probe set used for the microarrays. SLPI staining in normal skin revealed a weak presence in the stratum granulosum (Fig 1m), whereas in psoriasis a strong staining throughout the stratum spinosum was found (Fig 1o) as described before. In epidermis of atopic dermatitis patients, SLPI staining was variable (Fig 1n). In some biopsies, it showed a discontinuous distribution and in others it resembled normal skin. In some cases, a few layers of the stratum spinosum were stained. Semi-quantitative scoring for distribution and intensity indicated that SLPI is significantly higher expressed in psoriasis than in atopic dermatitis (see Table III).

The structural protein involucrin is present in the stratum granulosum of normal epidermis (not shown), and is highly expressed throughout the stratum spinosum of psoriatic epidermis (Bernard *et al*, 1986). Figure 2a and b show that

**Figure 1**

**Host defense proteins in human skin.** Immunohistochemical staining of normal skin, atopic dermatitis skin, and psoriatic skin for elafin, human  $\beta$ -defensin (hBD)-2, hBD-3, calgranulin A, and secretory leukocyte proteinase inhibitor (SLPI). Note the strong difference in staining intensity for elafin, hBD-2, and SLPI between psoriasis and atopic dermatitis. Staining for hBD-3 was variable, and not significantly different between the two diseases. The brown staining of the basal layer in (d) and (j) is caused by melanin and does not represent positive staining for hBD-2 or calgranulin A. Scale bar: 50  $\mu$ m.

the staining pattern is similar between psoriasis and atopic dermatitis. No significant difference was found (Table III). The cytoskeletal protein CK16 is absent from normal epidermis (not shown) but is highly induced in psoriasis, as shown previously by others. As such it has been one of the earliest recognized markers for the regenerative differentiation pathway in epidermis. Figure 2 and Table III show that there is no significant difference in CK16 staining between the two diseases. These findings are in accordance with microarray and qPCR data.

It could be argued that the observed difference in expression level of host defense proteins is a consequence of gross differences in epidermal structure, the number of cell layers, or proliferative status of the tissues. Although histopathological differences exist, features such as parakeratosis, acanthosis, and spongiosis were observed in both conditions. To investigate cellular proliferation, we performed Ki-67 staining which is a marker for cycling cells. Figure 2e and f, and Table III show that the epidermis of atopic dermatitis patients is equally hyperproliferative as in psoriasis.

## Discussion

Several large-scale expression profiling studies have been published describing the differential gene expression profile between normal and psoriatic skin (Oestreicher *et al*, 2001; Zhou *et al*, 2003). These studies, using full thickness skin biopsies from lesional, uninvolved, and control skin have uncovered a wealth of data on genes associated with activated keratinocytes, and the role of T cells and lymphocytes in lesional psoriatic skin. The work of the group of Leung (Ong *et al*, 2002; Nomura *et al*, 2003a, b) has further extended these analyses by comparing lesional skin of psoriasis patients with lesional skin of atopic dermatitis patients. Their findings have raised the intriguing possibility that the increased susceptibility of atopic dermatitis patients for bacterial infections may be related to low expression levels of antimicrobial peptides (when compared with psoriasis patients). In this study we have extended these observations. We confirmed the differential expression of five antimicrobial proteins as reported by others at the protein level (hBD-1, hBD-2, elafin, calgranulin C, and iNOS)

**Table III. Semi-quantitative scoring of immunohistochemical staining**

Protein	Score	Psoriasis	Atopic dermatitis
Elafin	Distribution	2.0 ± 0.0*	0.8 ± 0.4
	Ratio	0.48 ± 0.14*	0.23 ± 0.11
SLPI	Distribution	2.0 ± 0.0\$	1.1 ± 0.6
	Ratio	0.49 ± 0.10#	0.28 ± 0.16
Calgranulin A	Distribution	2.0 ± 0.0	2.0 ± 0.0
	Ratio	0.78 ± 0.12¶	0.50 ± 0.30
β-defensin-2	Distribution	1.5 ± 0.5#	0.5 ± 0.5
	Ratio	0.35 ± 0.14\$	0.05 ± 0.06
β-defensin-3	Distribution	1.8 ± 0.5	1.4 ± 0.9
	Ratio	0.32 ± 0.13	0.19 ± 0.24
Involucrin	Distribution	2.0 ± 0.0	2.0 ± 0.0
	Ratio	0.59 ± 0.11	0.64 ± 0.05
CK16	Distribution	2.0 ± 0.0	2.0 ± 0.0
	Ratio	0.85 ± 0.14	0.84 ± 0.10
Ki-67	Positive cells per millimeter	89 ± 45	85 ± 45

Immunohistochemical staining was quantified as indicated in the Method section. Figures represent mean ± standard deviation of 10 atopic dermatitis patients and nine psoriasis patients. Values for elafin, SLPI, calgranulin A, and β-defensin-2 staining were significantly different between psoriasis and atopic dermatitis:

\* $p < 0.001$ ; \$ $p < 0.01$ ; # $p < 0.02$ ; ¶ $p < 0.05$ . No significant differences were found for β-defensin-3, involucrin, CK16 and Ki67. Mann-Whitney *U* test.

(Ong *et al*, 2002; Nomura *et al*, 2003a, b). We did, however, not find a significant overexpression of LL-37 as previously reported (Ong *et al*, 2002). This could be explained by the fact that our analysis was performed on purified epidermal cells rather than full thickness skin biopsies, as it is known that infiltrating cells express high levels of this gene, compared with the epithelial cells. In our previous transcriptome studies, we used SAGE as a quantitative measure for gene expression. These studies suggested low levels of most host defense genes in normal epidermis (Jansen *et al*, 2001). Our qPCR and immunohistochemical data indeed show that antimicrobial genes are expressed at low levels in normal skin compared with inflamed skin of psoriasis and atopic dermatitis patients.

An important conclusion that can be drawn from our work and previous work by others, is that hBD-2 appears to be the strongest and most psoriasis-specific protein known so far. hBD-2 is absent in normal epidermis, although in some regions it can be found (e.g., the face). We did not find epidermal hBD-2 expression at the protein level in normal skin biopsies (from the trunk) in nine different individuals. Also, by qPCR it is barely detectable. In psoriatic epidermis hBD-2 is highly induced at the mRNA level (more than 20,000-fold by qPCR) compared with normal skin. In atopic dermatitis epidermis, hBD-2 is also induced considerably at the mRNA level compared with normal skin (more than 1000-fold), but at the protein level it is only occasionally

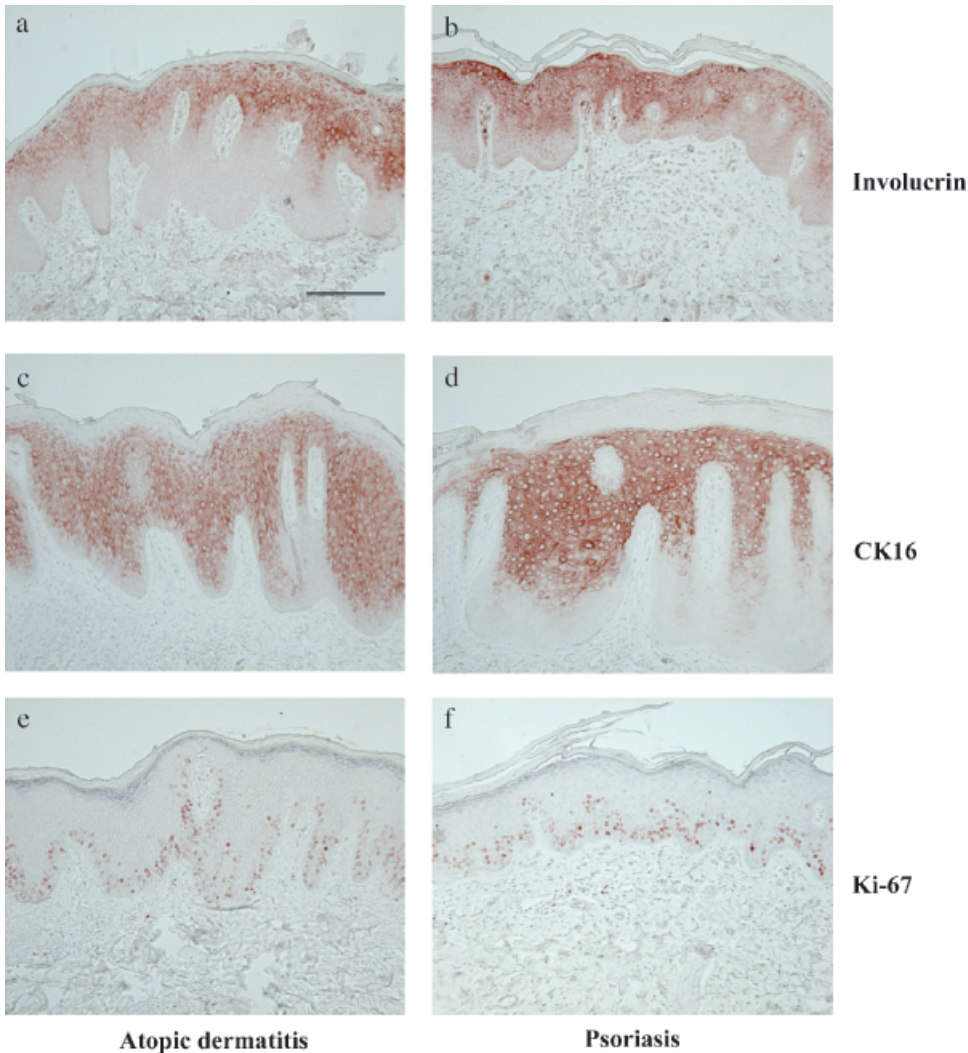
found in the stratum granulosum. Our analysis reveals a number of additional antimicrobial proteins to be overexpressed in psoriatic epidermis, such as psoriasin, SLPI, calgranulin A and B, cystatin A, and chitinase. Chitinase could, by virtue of its ability to degrade cell wall components, act as an antifungal enzyme, although its role as such has not yet been proven. Interestingly, chitinase has recently been implicated in the pathogenesis of asthma, which is also a multifactorial chronic inflammatory disease (Zhu *et al*, 2004). This raises the possibility that chitinase could be causally involved in disease, rather than being a protective enzyme. The other proteins have been implicated in the defense against a specific group of microorganisms (e.g., psoriasin against *Escherichia coli* (Glaser *et al*, 2005) calgranulin A/B against *Candida albicans* (Murthy *et al*, 1993), or against a broad range of microbes (like SLPI and elafin)).

We show that increased expression of effector host defense genes in psoriasis is specific in a sense that many other genes associated with the activated phenotype are not differentially expressed or are even expressed at higher levels in atopic dermatitis (e.g., CK17 as assessed by qPCR). Our data suggest that expression of these genes is independent of proliferation or abnormal differentiation. In contrast, there seems to be little difference in the expression of host defence genes of the TLR family, possibly with the exception of TLR3.

It could be argued that a comparison between any two diseases would reveal differences in immunolocalization of host defense genes because of the differences in disease stage and severity, and the specific architecture of the affected epidermis in a particular disease. When, for instance, an acute lesion of atopic dermatitis is compared with a psoriatic lesion, the comparison would be less meaningful because of the reasons mentioned above. We took care, however, to study chronic lesions of both patient groups. Although the clinical diagnosis of the patients was clear-cut, the lesions showed a number of histopathological similarities, as indicated above. Some of the biopsies (five of nine) could not be unambiguously classified based on histopathology alone by a blinded observer who was not aware of the clinical diagnosis. Cellular proliferation and acanthosis measured as the number of epidermal cell layers were not different between the two diseases. A remarkable finding was that CK16, an established marker of keratinocyte activation in the context of hyperproliferation, was not differentially expressed.

How could we interpret the remarkable finding that there appears to be a difference in inducible cutaneous innate immunity between these two diseases? It could be caused by genetic factors that determine the host defense response of epidermal keratinocytes factors to environmental or endogenous stimuli, or it could be caused by polymorphisms in the regulatory sequences of the host defense genes themselves. To our knowledge, there are at present no data to support this possibility. Alternatively, one could argue that the difference in T cell infiltrate causes a distinct cytokine profile (Th1 vs Th2) that drives the keratinocyte response. Evidence for the latter possibility is provided by the observation that TNF-α stimulates expression of host defense genes whereas IL-4 is inhibitory (Nomura *et al*,





**Figure 2**  
**Differentiation and proliferation in psoriasis and atopic dermatitis.** Immunohistochemical staining of atopic dermatitis skin and psoriatic skin for involucrin, CK16, and Ki-67. Note the similar localization and staining intensities of these markers in the two diseases. Scale bar: 100  $\mu$ m.

2003b). It is conceivable to further address this issue experimentally, using the induction of antimicrobial proteins in response to standard injury of normal skin, or uninvolved skin of psoriasis and atopic dermatitis patients. The tape-stripping model would be an interesting option in this respect. This would, however, not address the question whether it is a cell-autonomous process or not, in other words: is the difference in epidermal innate immunity governed by the inflammatory infiltrate, or is it an intrinsic property of the keratinocyte? This could be investigated in keratinocyte cultures from both patient groups following stimulation with the appropriate factor to induce innate immunity genes *in vitro*. Previous studies have shown that serum, TNF- $\alpha$ , and detergents can induce elafin gene expression in cultured keratinocytes (Pfundt *et al*, 1996, 2000; van Ruissen *et al*, 1998). These studies and reports on elafin induction in other cellular systems have indicated that its expression is dependent on p38 MAPkinase and nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling (Bingle *et al*, 2001). hBD-2 and LL-37 were shown to be induced by various agents, including IL-1, TNF- $\alpha$ , IL-6, and bacterial products (Liu *et al*, 2002; Tsutsumi-Ishii and Nagaoka, 2002). Taken together, these data suggest that several of these antimicrobial pro-

teins may be expressed following TLR signaling or in the context of a Th1 immune response, where TNF- $\alpha$  is a dominant cytokine. Interestingly, an active TLR4 signaling complex has recently been described in keratinocytes (Song *et al*, 2002). It remains to be investigated whether the actual tissue levels of TNF- $\alpha$  or activity of TLR signaling pathways are higher in psoriasis than in atopic dermatitis lesions. Keratinocytes from psoriatic patients could be genetically programmed to respond earlier (at lower thresholds) or more vigorously to stimuli such as TNF- $\alpha$  or microbial products. In this case, one would expect that the psoriasis loci that have been identified in extensive linkage studies performed over the last years would yield candidate genes associated with innate immunity. The NOD2 gene would be a good candidate but this has been recently excluded for psoriasis (Nair *et al*, 2001). It is interesting to note that, in culture, differences in chemokine production have been found between psoriasis and atopic dermatitis keratinocytes (Giustizieri *et al*, 2001). This study suggests that psoriatic keratinocytes intrinsically produce more IL-8, MCP-1, and IP-10 than cells from atopic dermatitis patients. None of these chemokines were found to be differentially expressed in our study. We did observe, however,



that CCL27/TARC was overexpressed in atopic dermatitis epidermis, which probably reflects a difference in epidermal T cell subsets.

Another interesting issue is whether the *in vivo* constitutive expression of epidermal host defense genes is low in atopic dermatitis compared with psoriasis. This has not been studied so far for interfollicular epidermis. A recent paper, however, has reported that atopic dermatitis patients have less dermcidin, an antimicrobial peptide, in their sweat than healthy controls (Rieg *et al*, 2005). These data taken together with the data by the group of Leung and our own data suggest that both constitutive and inducible host defense is deficient in atopic dermatitis.

It is conceivable that psoriasis and atopic dermatitis patients represent the extremes of a population. Psoriasis patients may have a genetic background that predisposes towards a Th1-dominated T cell population in response to microbial stimuli and they might carry polymorphisms in TNF- $\alpha$  and NF- $\kappa$ B signaling that favor expression of epidermal host defense genes; on the other hand, atopic dermatitis patients favor a Th2 response (Hamid *et al*, 1994) and could possibly carry genetic polymorphisms conferring a poor epidermal innate immune response toward environmental microbial stimuli. The fact that psoriasis and atopic dermatitis are very common diseases strongly suggests that these predisposing genes confer a certain evolutionary advantage. A large population-based study by Henseler and Christophers (1995) has shown that psoriasis patients have an increased resistance to cutaneous bacterial infections. In this light, psoriasis could be the evolutionary cost of having too many otherwise favorable genetic polymorphisms.

To summarize, we think that the observations made by Ong *et al* (2002) and the data presented here, together with the recently proposed interaction between innate and adaptive immunity, can provide new insights in the immunobiology of common inflammatory skin diseases. The notion that polymorphisms in genes dealing with environmental (microbial) stimuli play a role in these conditions may enable us to gain greater insight in the pathogenesis of these diseases and even lead to more specific therapeutic avenues.

## Materials and Methods

**Patients** The study participants included 20 patients with a clinical diagnosis of moderate to severe chronic plaque psoriasis and 16 patients with moderate to severe chronic atopic dermatitis. None of the patients received systemic therapy or UVB-therapy at the time of biopsy taking, and patients were asked to discontinue the use of topical steroids at the biopsy site 2 wk before taking biopsy. Patients were allowed to use emollients. Punch biopsies were taken from chronic psoriasis plaques and from chronic atopic dermatitis lesions. Care was taken to exclude acute lesions or lesions with crusts or vesicles. Biopsies from normal skin were obtained from 11 healthy volunteers: six for immunohistochemistry and five for quantitative PCR. The medical ethical committee approved the study and all volunteers gave written informed consent. The study was conducted according to the Declaration of Helsinki principles.

**RNA isolation and microarray analysis** Detailed procedures for preparation of purified epidermis, RNA purification, linear RNA amplification, probe labeling, array printing, array hybridization, and microarray analysis are given as supplemental material. Briefly,

purified RNA was amplified (Van Gelder *et al*, 1990) with the aid of the Message Amp kit (Ambion, Huntingdon, Cambridgeshire, UK). Amplified antisense RNA was labeled by *in vitro* transcription ('t Hoen *et al*, 2003).

**Quantitative real-time PCR** Generation of first-strand cDNA was performed as described previously (Zeeuwen *et al*, 2001). The reverse transcriptase reaction products were used for quantitative real-time PCR amplification, which was performed with the MyiQ Single-Colour Real-Time Detection System for quantification with Sybr Green and melting curve analysis (BioRad, Hercules, California), as described previously (Franssen *et al*, 2005). Primer sequences and efficiency parameters for elafin, hBD-1, hBD-2, hBD-3, involucrin, CK10, CK16, CK17, LL-37, calgranulin A, calgranulin B, psoriasin, TLR2, TLR3, TLR4, and the housekeeping gene human acidic ribosomal protein P0 (hARP) are given as supplemental online material (Table SI). DNA was PCR amplified under the following conditions: 4.5 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 s. For all PCR, iQ Sybr Green Supermix (BioRad) was used in the reaction. All primer concentrations were 300 nM in a total reaction volume of 25  $\mu$ L. PCR reactions were performed in duplicate. The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of the hARP reference gene in the same sample. We used hARP that we have previously found to be very useful for normalization procedures when using RNA from biopsy material (Wingens *et al*, 1998). The hARP gene is, at least for skin biopsies, more suitable than commonly used genes such as actin or GAPDH, as hARP correlates far better with 28S ribosomal RNA (not shown). When fixed amounts of cDNA were used as template for qPCR, we found virtually identical numbers of threshold cycles for all biopsies, irrespective of the diagnosis. Relative mRNA expression levels of all examined genes were measured using the comparative  $\Delta$ - $\Delta C_t$  method (Livak and Schmittgen, 2001). The analyses were performed on epidermis of five healthy volunteers, 10 psoriasis patients and six atopic dermatitis patients. Primers for qPCR were only accepted if their efficiency was  $100 \pm 10\%$ . Thus, the PCR product concentration was doubled at each cycle and the threshold cycle will be linear with the log of the initial concentration. The log transform of gene expression is approximately normally distributed (Huber *et al*, 2002; Rocke and Durbin, 2003) and we used an *F* test to test the main effect. For those genes where the main effect was found to be significant at the 5% level a Duncan test was used to test the significance of the difference between each of the indicated groups. The calculated ratio of the expression level in psoriasis and atopic dermatitis (PS/AD in Table II) is not normally distributed and was not used for significance testing but is listed for reference only.

**Histological methods** Full thickness skin biopsies were fixed in buffered 4% formalin for 4 h and processed for routine histology. Tissues were embedded in paraffin and 5  $\mu$ m sections were cut. Sections were stained with hematoxylin-eosin (H&E) or processed for immunohistochemical staining using an indirect immunoperoxidase technique with avidin biotin complex enhancement. H&E-stained sections were blindly evaluated by an experienced pathologist (one of the co-authors: W. B.) to assess the diagnosis, and features such as parakeratosis, orthokeratosis, spongiosis, elongation of rete ridges, acanthosis, and the presence of neutrophils. For the immunohistochemical stainings, a panel of monoclonal and polyclonal antibodies was used: an antibody directed against the Ki-67 antigen was used to investigate epidermal proliferation (MIB-1, Immunotech, SA, Marseilles, France). To assess epidermal differentiation we used monoclonal antibodies against involucrin (MON-150) and CK16 (LL025, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). The dual function proteins SLPI and elafin (proteinase inhibitor and antimicrobial protein) were stained using polyclonal antibodies that we have previously described (Wingens *et al*, 1998). Calgranulin A (also known as MRP8) was stained with a polyclonal rabbit antiserum, kindly provided by Dr P. Madsen,

Aarhus, Denmark. Goat anti-hBD-2 polyclonal serum was from Peptotech (London, UK). Rabbit anti-hBD-3 was obtained from Biotrend (Cologne, Germany). The number of biopsies analyzed is indicated for each staining.

**Grading of immunohistochemical staining** Epidermal proliferation was measured by counting the number of Ki-67 positive nuclei per mm, by an observer who was unaware of the patient history (average of three measurements per section). Expression of elafin, SLPI, involucrin, CK16, calgranulin A, hBD-2, and hBD-3 was assessed in two ways. First the distribution was scored on a 3-point scale: 0, absent; 1, patchy; 2, continuous. See Fig 1b as an example of patchy staining, and Fig 1f for continuous staining. An additional parameter was the positively stained area of the epidermis, which was expressed as the mean ratio of positive cell layers divided by the total number of epidermal cell layers in a rete ridge (mean of three measurements). This could be easily determined for elafin, involucrin, and hBD-2, but was somewhat more difficult for calgranulin A and hBD-3 because staining tended to diminish gradually from the granular layer towards the basal layer.

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### Supplementary Material

The following material is available online for this article.

**Figure S1. (a) Epidermis after separation. (b) Dermis after separation**

**Table S1.** Primer pairs for qPCR

**Table S2.** qPCR data for all samples

**Supplemental text** Detailed description of tissue preparation, mRNA isolation and labeling, and microarray procedures.

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Address correspondence to: Joost Schalkwijk, Nijmegen Centre for Molecular Life Sciences, Radboud University Medical Centre Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Email: j.schalkwijk@derma.umcn.nl

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