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Non-lesional atopic dermatitis (AD) skin is characterized by broad terminal differentiation defects and variable immune abnormalities

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Abstract

Background—Atopic dermatitis (AD) is a common inflammatory skin disease with a Th2 and “T22” immune polarity. Despite recent data showing a genetic predisposition to epidermal barrier defects in some patients, a fundamental debate still exists regarding the role of barrier abnormalities versus immune responses in initiating the disease. In order to explore whether there is an intrinsic predisposition to barrier abnormalities and/or background immune activation in AD patients an extensive study of non-lesional AD (ANL) skin is necessary.

Objective—To characterize ANL skin by determining whether epidermal differentiation and immune abnormalities that characterize lesional AD (AL) are also reflected in ANL skin.

Methods—We performed genomic and histologic profiling of both ANL and AL skin lesions (n=12 each), compared to normal human skin (n=10).

Results—We found that ANL is clearly distinct from normal skin with respect to terminal differentiation and some immune abnormalities, and it has a cutaneous expansion of T-cells. We also showed that ANL skin has a variable immune phenotype, which is largely determined by disease extent and severity. Whereas broad terminal differentiation abnormalities were largely similar between involved and uninvolved AD skin, perhaps accounting for the “background skin phenotype,” increased expression of immune-related genes was among the most obvious differences between AL and ANL skin, potentially reflecting the “clinical disease phenotype.”

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Conclusion—Our study implies that systemic immune activation may play a role in alteration of the normal epidermal phenotype, as suggested by the high correlation in expression of immune genes in ANL skin with disease severity index.

Keywords

non-lesional; atopic dermatitis; altered terminal differentiation; immune activation

Introduction

Atopic dermatitis (AD) is the most common chronic inflammatory skin condition and has an increasing incidence worldwide.[1, 2] There are two competing hypotheses for primary pathogenesis of the disease. The "inside-out" (or immune-driven) hypothesis suggests that underlying immune activation triggers epithelial barrier dysfunction, whereas the "outside-in" (or barrier-driven) hypothesis favors an intrinsic epidermal abnormality that precedes immune activation, which is regarded as epiphenomenon.[1–4]

AD is a polarized T-cell disease, and activation of Th2 and "T22" T-cells contributes to the AD phenotype.[5, 6] More recently, discovery of mutations in the gene encoding the filament aggregating protein filaggrin (*FLG*) has suggested that an epidermal barrier abnormality may underlie the pathogenesis of AD.[7, 8] However, only a subset (up to 30%) of AD patients has a genetic predisposition to keratinocyte terminal differentiation abnormalities.[1] Prior work has also identified major deficiencies in epidermal lipid synthesis in AD skin, which raises the possibility that the cornification abnormalities are far more extensive than those resulting from the *FLG* mutation alone.[9, 10]

We have previously established that diseased skin from patients with chronic AD shows major genomic differences from normal skin, differences that relate to both a disrupted epidermal barrier and abnormal immune activation. Lesional skin from chronic AD patients has broad alterations in expression of terminal differentiation genes that form the cornified envelope (CE) and epidermal differentiation complex (EDC); we have also previously identified many differences in highly inflammatory genes in AD lesional (AL) skin.[11]

Previous investigations have identified that clinically unaffected or non-lesional AD (ANL) skin is different than normal skin.[12, 13] Decreased hydration and impaired synthesis of lipids have been found in ANL skin in comparison to normal skin.[10, 12, 14–16] A single report has shown abnormal epidermal proliferation in ANL with increased expression of the proliferation marker (keratin 16), in association with altered expression of terminal differentiation proteins, including loricrin (*LOR*), involucrin (*IVL*), and *FLG*.[10] Additionally, an increased infiltrate of inflammatory T-cells has been demonstrated in ANL skin compared to skin from healthy volunteers.[12]

We sought to characterize ANL skin and determine whether the terminal differentiation and immune abnormalities that characterize the active disease stage[11] are also broadly reflected in visibly normal AD skin, regardless of clinically apparent disease. We found that in chronic AD patients ANL skin is clearly distinct from normal skin with respect to keratinocyte terminal differentiation and some inflammatory pathways, and has expansion of cutaneous T-cells. However, altered expression of a key subset of genes in ANL skin is correlated with extent of clinical disease, as quantified by SCORing of AD (SCORAD) index,[47,48] potentially accounting for the somewhat variable ANL phenotype.

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Materials and Methods

Patients and Skin Samples

Skin biopsies were collected from 15 patients with moderate-to-severe AD (10 males, 5 females; ages 16–81 years, median 38 years) and 10 healthy volunteers under IRB-approved protocols and written consent was obtained. Patients with an acute exacerbation of chronic AD and without any therapy for >4 weeks were included (Table E1). Biopsies were obtained from both AL and ANL (at least 10 cm away from any active lesion) skin. Biopsies were frozen in OCT for immunohistochemistry (IHC) and liquid nitrogen for RNA extraction (see Online Repository (OR) S2).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on cryostat tissue sections using purified mouse anti-human monoclonal antibodies (see OR S2). Positive cells per millimeter were counted manually using computer-assisted image analysis software (ImageJ 1.42, National Institutes of Health, Bethesda, MD).

Sample preparation for real-time (RT)-PCR and gene-chip analysis

We used the Affymetrix human U133Aplus2 arrays (Affymetrix Inc, Santa Clara CA) as previously described.[18] Primers and probes for TaqMan RT-PCR assays were generated with Primer Express algorithm, version 1.5, using published genetic sequences (NCBI-PubMed). The RT-PCR reaction was performed using EZ-PCR Core Reagents (Applied Biosystems) and custom primers were generated as previously described[19] (see OR S4-S6).

Statistical Analyses

CEL files quality control was assessed by Harshlight[20] and arrayQualityMetrics packages from R/Bioconductor. Expression measures were obtained using GCRMA. Gene-wise group differences were assessed by moderated *t*-test[21] and *P*-values were adjusted by Benjamini-Hochberg procedure. Multivariate correlations between gene-sets and SCORAD were obtained through *muStat* package as previously described.[22, 23] Raw data was deposited in Gene Expression Omnibus repository. See OR S6 for detailed statistics.

Results

Patient characteristics

We studied non-lesional and lesional skin biopsies from 15 adult patients with moderate-to-severe chronic AD with SCORAD range 28 to 97.5, (mean 60), and a body surface area (BSA) involvement of 11% to 63% (mean 36%) (Table E1). A single-copy R501X mutation in *FLG* gene was observed in one patient (Table E1) (see OR S3).

Non-lesional AD (ANL) shows abnormal proliferation and immune infiltration as compared with normal skin

To determine whether ANL maintains specific features of diseased skin, we compared epidermal thickness, protein expression of the proliferation markers K16 and Ki67, and T-cell and myeloid dendritic cell (mDC) markers (CD3 and CD11c, respectively) among AL, ANL and normal skin.

A significant increase (30%) in epidermal thickness and $Ki67^+$ proliferation index (218%) was observed from normal to ANL skin with an additional increase of $Ki67^+$ cells (145%) from ANL to AL skin (Fig 1A–B, E,G). Normal skin showed no expression of K16 in suprabasilar keratinocytes, while ANL skin samples demonstrated variable K16 staining

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with continuous suprabasilar staining in 5/12 cases (Fig 1A). The epidermal thickness of ANL skin samples was positively correlated with SCORAD (r=0.483, p<0.05) (Fig 1F).

ANL also showed an 86% (P<0.001) increase in the number of infiltrating dermal $CD3^+$ T-cells and a 56% (P<0.056) increase in $CD11c^+$ DC's as compared to normal skin (Fig 1C–D, H–I). Applying T-cell counts to BSA with adjustment for relative skin involvement, nearly 6 times the number of total cutaneous T-cells was observed in AD patients compared to normal volunteers (P<0.01) (Fig E1A; OR S1). When adjusting the cell counts in ANL for BSA of involved skin, we found a 17% expansion of T-cells/m^2 in ANL as compared to normal skin (Fig E1B).

General analysis strategy of non-lesional (ANL) *versus* lesional (AL) chronic atopic dermatitis (AD) and normal skin

Affymetrix HGU133Plus2 arrays were used to identify differentially expressed genes (DEG) in ANL in comparison with AL and normal skin. Our approach allows an understanding of whether ANL maintains the disease-related expression abnormalities that characterize AL skin with respect to normal biology.

Non-lesional AD (ANL) partially shares the lesional AD (AL) phenotype

Principal component analyses (PCAs) on two sets of gene expression data, including microarray (Fig 2A) and PCR (Fig 2B), represented ANL skin samples as an intermediary phenotype between AL and normal, in closer proximity to AL (see OR S6). Most ANL and AL skin samples clustered according to their SCORAD, as illustrated by size of the circles (larger circles indicate a higher SCORAD) (Fig 2A–B). The ANL skin samples of patients with higher SCORAD were closer to AL samples (Fig 2A–B). This is in contrast with psoriasis, in which non-lesional and normal skin overlapped and were clearly separated from lesional skin.[24]

We identified DEG between ANL or AL versus normal skin, using criteria of fold change (FCH) ≥2, and false discovery rate (FDR) <0.05 (Fig 2C–D). 809 probe-sets (575 unique genes by ENTREZ identifier) were upregulated and 1,294 (986 genes) were downregulated in ANL as compared to normal skin (Fig 2C–D). A higher number of DEG were identified in AL in comparison with normal skin. A relatively small set of DEG distinguished AL and ANL skin, with 328 probe-sets (240 genes) upregulated and 312 probe-sets (223 genes) downregulated (Fig 2C–2D, Tables E2–E3).

A large set of DEG (almost 70%) between ANL and normal skin were also significantly different when comparing AL with normal skin, with only 293 probe-sets (207 genes) uniquely upregulated and 335 probe-sets (249 genes) downregulated in ANL alone versus normal skin (Fig 2B–C). Overall, these data suggest that ANL partially displays the AL phenotype.

Abnormal keratinocyte terminal differentiation characterizes background and diseased skin

Figure 3A illustrates DEG in either AL or ANL versus normal skin, showing no apparent differences between AL and ANL (Fig 3A, highlighted box indicates similarly expressed genes; Table E3). Within this gene-set, which represents the genomic "background skin phenotype" of chronic AD patients, there are many EDC and terminal differentiation genes {e.g., loricrin (*LOR*), FLG, etc.} that are expressed at strikingly lower levels in both ANL and AL as compared to normal skin (Fig 3B, Tables E2–3). As seen in the highlighted box in Fig 3B, genes encoding for major terminal differentiation proteins, including *LOR*, *CDSN*, *FLG*, *IVL*, LCE, psoriasis 1 candidate 2 (PSORS1C2), and small proline rich protein

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4 (SPRR4), are commonly downregulated in AL and ANL as compared with normal skin. For example, *CDSN*, LCE2B, and *LOR* were decreased between 90–100-fold in both AL and ANL versus normal skin (Tables E2–3). Furthermore, PCA of probe-sets related to terminal differentiation revealed a clear overlap between ANL and AL skin samples, with a distinct separation from normal skin (Fig E2A). This terminal differentiation component of the background skin phenotype (highlighted box in Fig 3B) showed a weak multivariate correlation with the SCORAD in ANL skin samples (r=0.24, P<0.45) (see OR S6).

Increased immune-mediated inflammation is mostly a feature of lesional AD (AL)

We also determined DEG between AL and ANL skin, which define the "lesional" phenotype, containing 463 genes (Figs 2C–D, Fig 3C, highlighted box indicates similarly expressed genes; Tables E2–3). For this set, gene expression in ANL is more similar to normal skin. The set of up-regulated genes in AL contains many inflammatory gene products, including cytokines, chemokines, and cytokine-induced gene products in keratinocytes (Fig 3D, Tables E2–3). This immune gene-set comprises an increased Th2 genomic signal (i.e. CCL22, CCL17, CCL18, CCR1, IL-4R), as well as Th1/IFNγ signal, including STAT-1, OASL, MX-1, CXCL10, IL-12Rβ2, and strong inflammatory mediators (such as MMP12, IL-8, CXCL1). The T-cell trafficking chemokine, CCL19, and its receptor CCR7, were also among the genes uniquely increased in diseased skin (Tables E2–3). Expression levels of the highlighted inflammatory genes in ANL skin samples were highly correlated with SCORAD (r=0.66; P<0.02) (Fig 3D). This set of primarily immune genes (Fig 3D) that is largely up-regulated in AL skin alone may produce the visible skin lesion, constituting the "clinical disease phenotype" (Fig 3D, Tables E2–3).

Increased expression of Th2, Th22, and Th1 inflammatory products in non-lesional AD skin

Expression of representative Th1, Th2, Th22, Th17 and terminal differentiation proteins was performed by quantitative RT-PCR to confirm and extend microarray results (Fig 4). We found increased mRNA expression of Th1-regulated genes (including IFNγ, IL-8, and MX-1) in both AL and ANL in comparison with normal skin (Table E4). We also found an up-regulation of mRNA expression of Th2 pathway genes, in both involved and uninvolved AD, as compared with normal skin. The increase was significant for IL-13 cytokine, as well as for CCL17, CCL18, CCL22, CCL5, and CCL11. The mRNA expression of the T22 cytokine IL-22 was also elevated in both AL and ANL AD as compared with normal skin. Both subunits of IL-23 (IL-23p19 and IL-23p40) were significantly increased in AL, and ANL, in comparison with normal skin. CX3CL1/Fractalkine, postulated to have an important role in the trafficking of CX3CR1 leukocytes during the inflammation caused by AD,[25] showed a significant increase in both AL and even more in ANL as compared with normal skin. The mRNA expression levels of transglutaminase 1 (*TGM1*) terminal differentiation gene was downregulated in AL and ANL versus normal skin (significant for ANL alone). We also found increased mRNA expression of two antimicrobial genes, *S100A7* and *S100A8* in both AL and ANL as compared with normal skin (only *S100A7* was significant for ANL) (Fig 4). Since these strong inflammatory molecules are induced by IL-22[45], increased expression of IL-22 in ANL skin might drive the expression of these gene products, potentially contributing to ANL skin inflammation.

Delayed and abnormal expression of terminal differentiation proteins characterizes both involved and uninvolved atopic dermatitis skin

Expression of proteins associated with terminal differentiation was studied by IHC (Fig 5). In normal skin, as expected as part of homeostatic growth, there is a coordinated pattern of expression of terminal differentiation proteins, including *LOR*, *FLG*, *IVL* and *CDSN*, in a continuous manner confined to the granular layer (Fig B–E).[18,26] In contrast, both ANL and

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AL skin exhibit a largely delayed and discontinuous (to completely absent in certain areas) expression of terminal differentiation proteins in the granular layer (Fig 5B–E). Whereas normal skin retains expression of *CDSN* and *LOR* in the stratum corneum as part of normal CE formation (Fig 5B–E), in both AL and ANL skin, there is a relative absence of *CDSN* and *LOR* in corneocytes above the granular layer, suggesting abnormal formation or retention of these terminal differentiation structures in corneocytes. Thus, although ANL skin might have a normal appearance, it shows parallel abnormal epidermal differentiation as in lesional skin. Of note, the patient with the *FLG* mutation showed a granular layer as well as a discontinuous expression of *FLG*, suggesting residual *FLG* function (data not shown).

Background skin phenotype is influenced by the extent of disease

A PCA based on immune-mediated probe-sets shows that whereas AL is largely separated from normal skin, ANL skin samples cluster according to their SCORAD, with lower SCORAD samples clustering closer to normal skin, and higher SCORAD samples clustering in proximity to AL skin (Fig E2B).

Furthermore, clustering of immune and terminal differentiation mRNA gene expression in both ANL and AL skin shows a positive correlation of SCORAD with expression of inflammatory genes, and a weaker negative correlation with major terminal differentiation genes (Fig 6A–C). Among the immune genes that are correlated with disease activity in ANL skin, there are major Th2 (CCL22, CCL18, and IL-13) and T22 (IL-22) mediators, as well as MX-1, a specific sensor of IFN levels (Fig 6A, C). Genes of several strong inflammatory mediators (i.e IL-8, MMP12, S100A8) were correlated with SCORAD only for AL, but not ANL skin (Fig 6A–B). An impressive inverse correlation with SCORAD was found in ANL skin for EREG, an anti-microbial protein, reported to play a critical role in immune-related responses of keratinocytes.[27, 28] To clarify these observations, we used multivariate U-scores (mu-scores) to correlate multivariate measurements of epidermal and immune genes in ANL with the SCORAD, as previously described.[22, 23] Genes with absolute univariate correlation higher than 0.4 were considered in both immune and terminal differentiation sets. Mu-score correlations of 0.78 and 0.55 were found between SCORAD and the immune and terminal differentiation sets, respectively (Fig 6D). When considering both immune and epidermal sets together, a significant multivariate correlation of 0.82 was obtained for ANL skin samples. Thus, the SCORAD of ANL samples correlates with immune markers of Th2, Th1, and “T22” pathways, with a weaker inverse correlation with terminal differentiation variables.

Discussion

In contrast to psoriasis, in which non-lesional skin is largely similar to normal skin[24] and disease tendency cannot be determined based on abnormalities in non-lesional skin,[29] AD non-lesional (ANL) skin is viewed as abnormal by most authors,[10, 12–16] although conflicting results have been observed.[30] Prior studies have analyzed alterations in only a few genes of interest[31] and/or in a small number of patients,[12, 32] so that a global characterization of gene and protein expression in ANL skin was not possible. Most of these reports focused on epidermal hydration and lipid content in the epidermis.[12, 14–16] A single report has demonstrated uniformly abnormal expression patterns of proliferation markers (Ki67 and K16) and terminal differentiation proteins in both AL and ANL skin by IHC.[10] In isolation, IHC results for EDC genes are difficult to interpret, since regenerative growth activation of the epidermis leads to altered and premature expression of EDC proteins in spinous keratinocytes. Although an abnormal ANL phenotype has been suggested by few studies, these have not determined whether the observed abnormalities are consistent or

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heterogeneous across different AD patients, according to the presence or absence of the *FLG* mutation, overall burden of disease, or IgE status.[10, 12, 14–16]

Our study represents the first comprehensive genomic and histological comparison of chronic ANL with both AL and normal skin. We have found that ANL is clearly distinct from normal skin with respect to terminal differentiation and some immune pathways. Our data show an impressive reduction in expression of EDC gene products (including *LOR*, *IVL*, *FLG*, *CDSN*) at the level of both mRNA and protein expression in ANL as compared with normal skin, while AL skin also shows reduced protein expression in relationship to other regenerative conditions, such as psoriasis.[18] Interestingly, although expression of a large set of immune genes in ANL is more similar to normal than to AL skin, the expression level of many immune genes in ANL skin samples is highly correlated with the SCORAD. Moreover, clustering of immune mRNA gene expression variables in ANL skin showed a positive correlation of the SCORAD with major Th2, Th1, and "T22" inflammatory products, as well as a weaker inverse correlation with terminal differentiation genes. Considering immune and epidermal measures together, a significant multivariate correlation with the SCORAD was obtained for ANL skin samples. Overall, we have shown that background AD skin has an abnormal phenotype, with variable elements that are correlated with disease extent and severity. Unlike the clear correlation with the SCORAD, no associations were found between the ANL phenotype and other variables (i.e. IgE/eosinophil levels, the *FLG* mutation, or familial atopy).

In contrast with background skin, active skin lesions have higher expression of immune cytokines. This perhaps explains the inflammatory, "erythematous" nature of the "clinically visible" disease phenotype. This set of inflammatory genes includes Th2 mediators, such as IL4R, as well as few Th1/IFNγ genes, such as MX-1, and strong inflammatory products such as IL-8. It also included CCL19 and its receptor CCR7, potentially accounting for the chronic accumulation of T-cells in AD skin.[31]

There are two possible pathogenic hypotheses for the abnormal ANL phenotype: 1) Inherited epidermal differentiation genes determine an abnormal, fixed phenotype, which presents from birth and precedes disease activity. If inherited abnormalities were the basis for the ANL phenotype, one might expect to find an *FLG* mutation in the majority of cases, whereas only one of our cases had a mutation, and even this case was not associated with a regenerative epidermal phenotype. However, there are additional mutations in *FLG* genes that were not genotyped in this study, and AD has complex genetics which may involve additional epidermal barrier genes, such as SPINK5, *LOR*, *IVL*, and K16, as suggested by linkage studies.[33] 2) Influence of systemic immune abnormalities as determined by extent of clinical disease. These potential abnormalities include T-cell and eosinophil expansion, increased circulating Th2 cytokines and chemokines (IL-4, IL-5, CCL17, CCL18, CCL22), or increased IgE levels.[34–38] The strong association in our study between burden of disease activity and expression of immune cytokines in ANL skin together with an overall expansion of T-cells may collectively support the second hypothesis. The extent and severity of AD potentially initiate the systemic immune activation and increase in circulating cytokines, which in turn alters terminal differentiation in background AD skin. This hypothesis is supported by prior data suggesting that Th2-derived cytokines sustain Th2 T-cell expansion,[39] induce altered epidermal responses, and inhibit terminal differentiation proteins, such as *LOR* and *FLG*.[40, 41] Systemic immune abnormalities might also result from genetic defects in immune function-regulating genes, i.e. IL-4, IL-4RA, and IL-13, which have been linked with AD pathogenesis.[33, 42] However, unlike epidermal gene defects, which are fixed, defects in immune genes may show functional changes over time according to the immune activation status.

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The extent to which immune activation and/or an increase in circulating cytokines affect keratinocyte differentiation in ANL skin cannot be fully determined from these data. Since adult patients with chronic AD were studied, ANL skin could have been previously diseased, thus, an interpretation bias favoring the systemic immune model cannot be excluded.

Our data also does not settle the issue of primary pathogenesis (whether altered keratinocyte differentiation precedes the development of ANL phenotype), but demonstrates that broad differentiation abnormalities still manifest as normal appearing skin in chronic AD patients. Although altered expression of terminal differentiation genes alone does not determine the clinical-disease phenotype, inherited genes could predispose to barrier abnormalities (e.g., by altering antigen access to skin DCs or increased Langerhans-cell penetration), creating an initial Th2 and Th22 immune polarity that amplifies over time.[43, 44]

Perhaps, the arguments over an exclusive pathogenesis based only on keratinocyte or immune dysfunction are too simplistic, especially after disease presence for many years. An integrated model of pathogenesis that allows for contributions of both terminal differentiation defects and immune alterations might best fit our analysis of chronic AD, considering both ANL and AL components. Although our data advocates for a primary role of immune activation in producing the barrier defect that ultimately might create an antigenic insult sustaining local and systemic inflammation, future studies are required to determine the extent to which abnormal skin structure and function are influenced by genetic factors versus acquired immune alterations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

AD	Atopic Dermatitis
AL	Lesional
ANL	Non-lesional
BSA	Body surface area
CDSN	Corneodesmosin
CE	Cornified envelope
DEG	Differentially expressed genes
EDC	Epidermal differentiation complex
FC	Fold change
FDR	False discovery rate
FLG	Filaggrin
GSEA	Gene-set enrichment analysis
IHC	Immunohistochemistry
IVL	Involucrin
LOR	Loricrin
mDC	myeloid dendritic cell

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PCA	Principal component analysis
PSORS1C2	Psoriasis 1 candidate 2
SCORAD	Scoring Atopic Dermatitis Index
SPRR4	Small proline rich protein
TGM1	Transglutaminase 1

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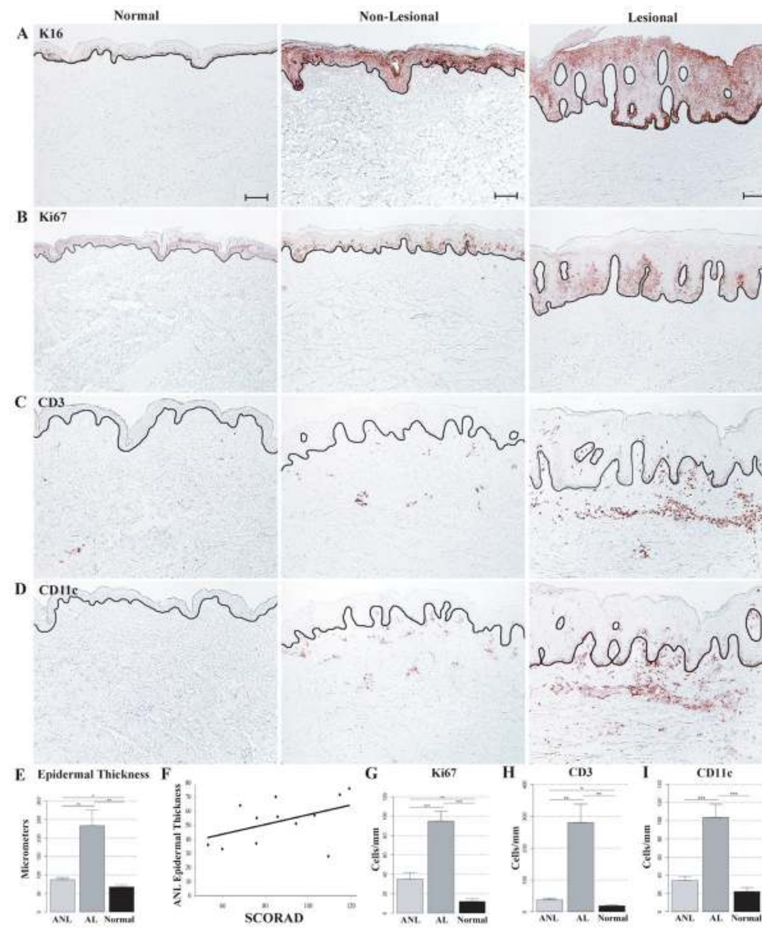

Figure 1.

Characterization of ANL and AL skin compared with normal. **A–D,** Representative immunohistochemistry staining of proliferation markers K16 (**A**) and Ki67 (**B**), and of T-cells ($CD3^+$) **(C)** and myeloid dendritic cells ($CD11c^+$) **(D)** in ANL, AL and normal skin. **E–H,** Quantification of epidermal thickness **(E),** $Ki67^+$ cells (**F**), $CD3^+$ **(G)** and $CD11^+$ **(H)** cells in ANL, AL, and normal; **P*<0.05;***P*<0.01;****P*<0.001. Scale bar 100 μm.

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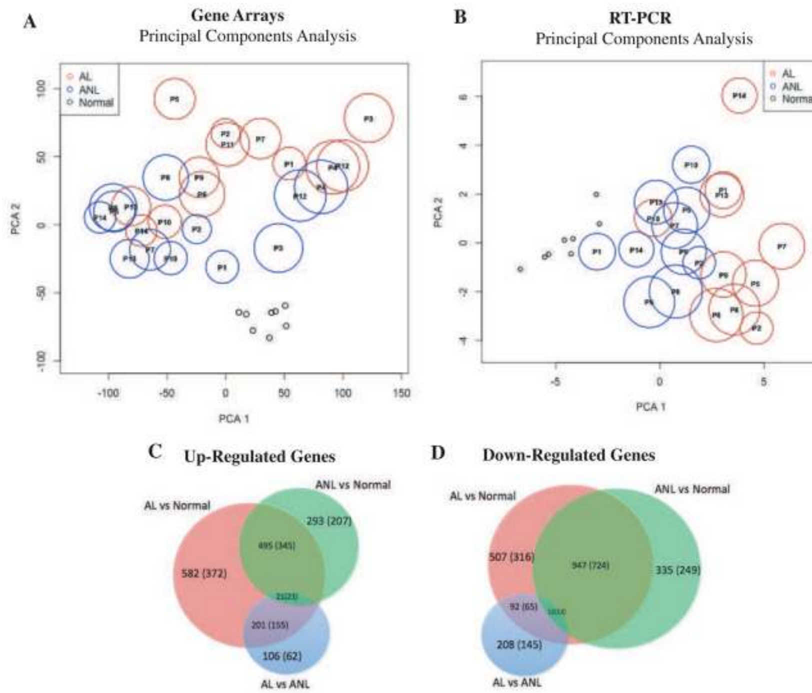

Figure 2.

A–B, Principal component analysis of gene arrays **(A)** and RT-PCR **(B)** in ANL, AL, and normal skin. Circle size is proportional to patient SCORAD index. **C–D,** Venn diagrams of up-regulated **(C)** and down-regulated **(D)** probe-sets (and genes) with very little overlap of AL *versus* ANL with ANL *versus* Normal, as illustrated by the lack of intersection of the blue and green circles alone.

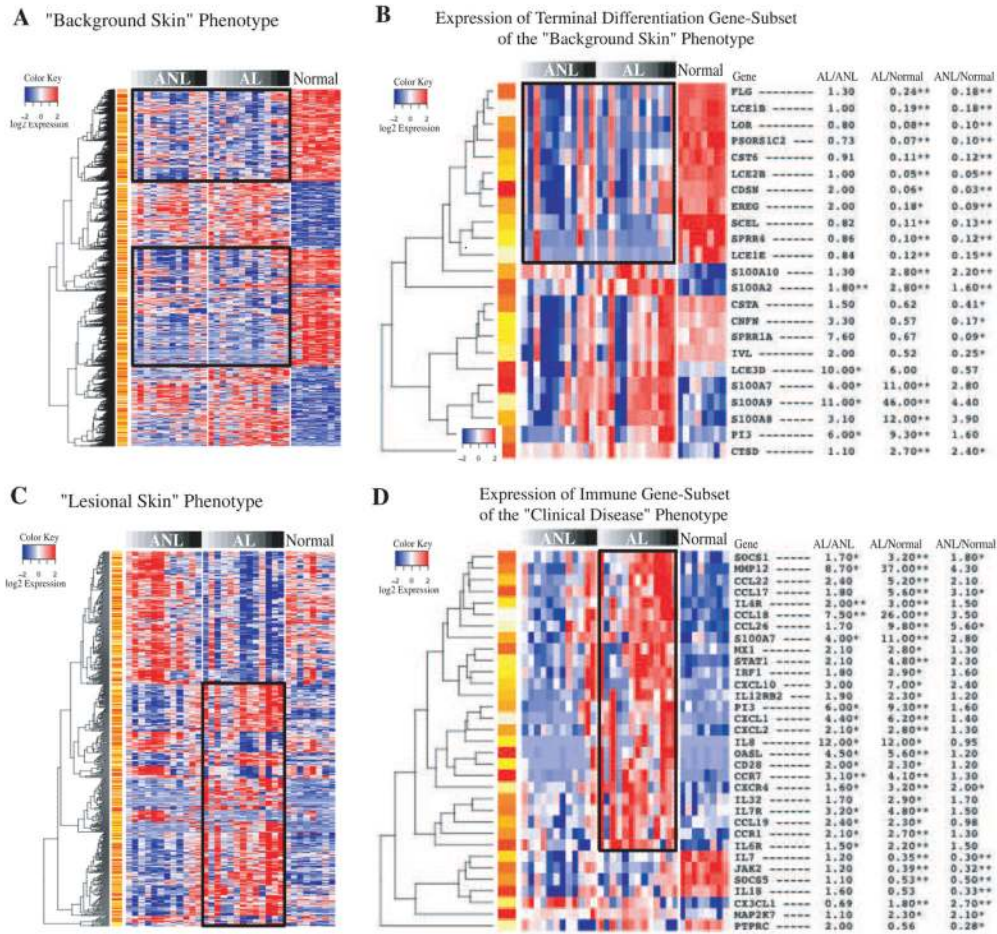

Figure 3.

Unsupervised hierarchical clustering of genomic expression (red: upregulated; blue: downregulated); black highlighting indicates areas of similarity; gray-spectrum boxes represent increasing SCORAD index from white to black. **A**, Differentially expressed genes (DEG) in either ANL or AL *versus* normal; **B,** As A for terminal differentiation subset; **C,** DEG in AL *versus* ANL; **D,** As C for immune subset, black highlighting indicates gene cluster correlating with SCORAD. *FDR<0.05, **FDR<0.01, ***FDR<0.001.

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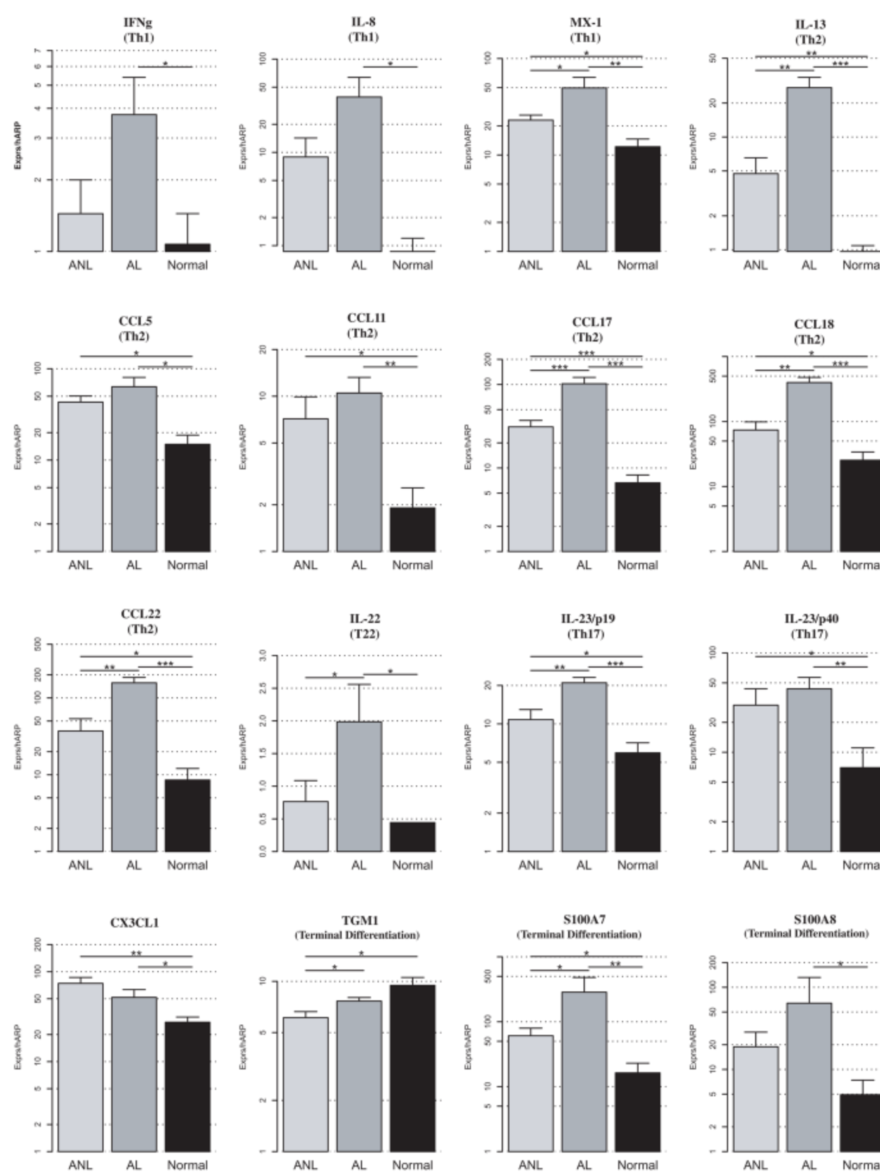

Figure 4.

RT-PCR of selected differentially expressed genes. Mean expression estimates normalized to hARP are represented. Increased expression of immune genes in ANL and AL *versus* normal and decreased expression of terminal differentiation proteins in both ANL and AL. *Error bars* indicate standard errors of the mean; *P<0.05, **P<0.01,***P<0.001.

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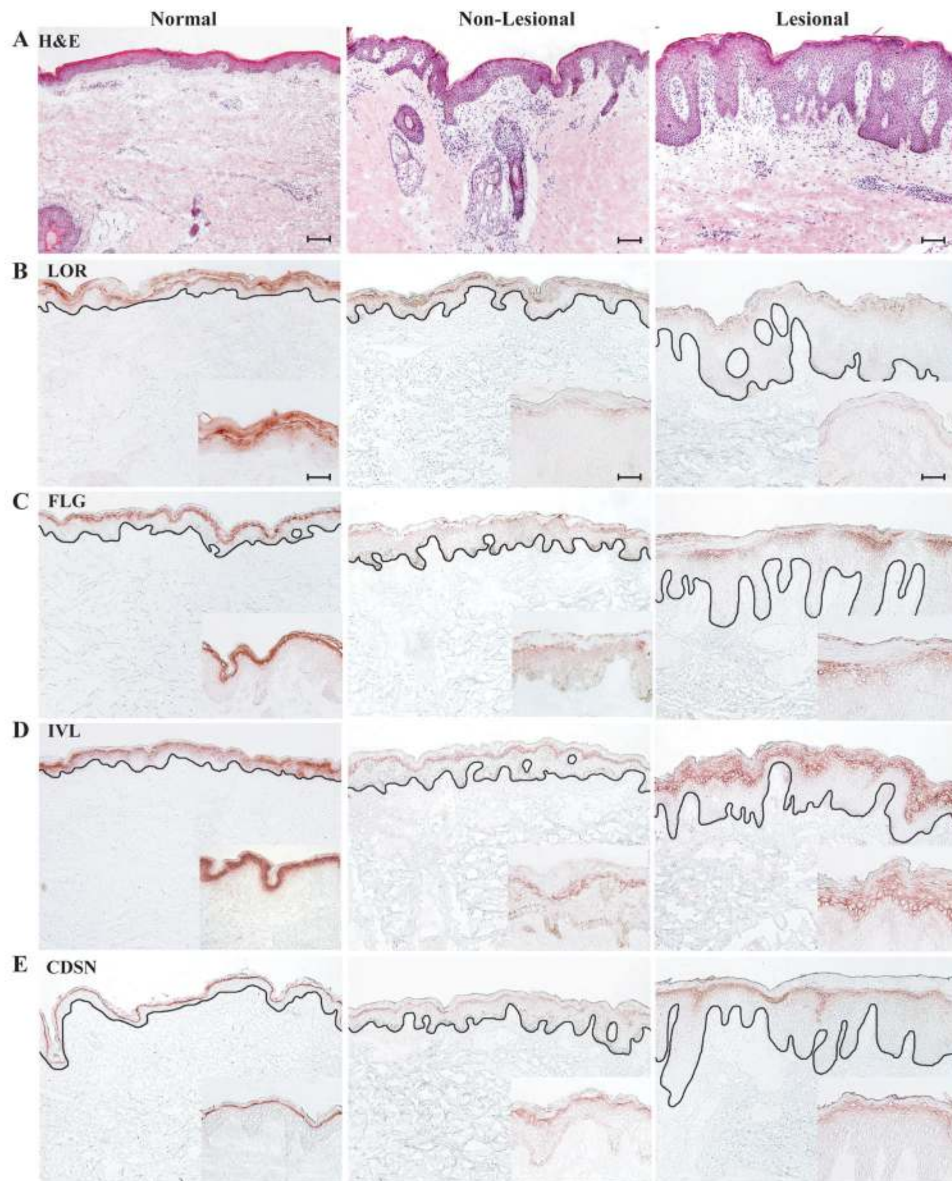

Figure 5.

Representative H&E **(A)** and immunohistochemistry staining of terminal differentiation proteins **(B–E)** in ANL, AL and normal skin. Both ANL and AL skin show delayed and intermittent expression of *LOR* **(B)**, *FLG* **(C)**, *IVL* **(D),** and *CDSN* **(E)** with absent staining in stratum corneum of *LOR* **(B)** and *CDSN* **(E)** compared to retained expression in normal skin. Scale bar 100μm; inset (40x magnification) scale bar: 25μm.

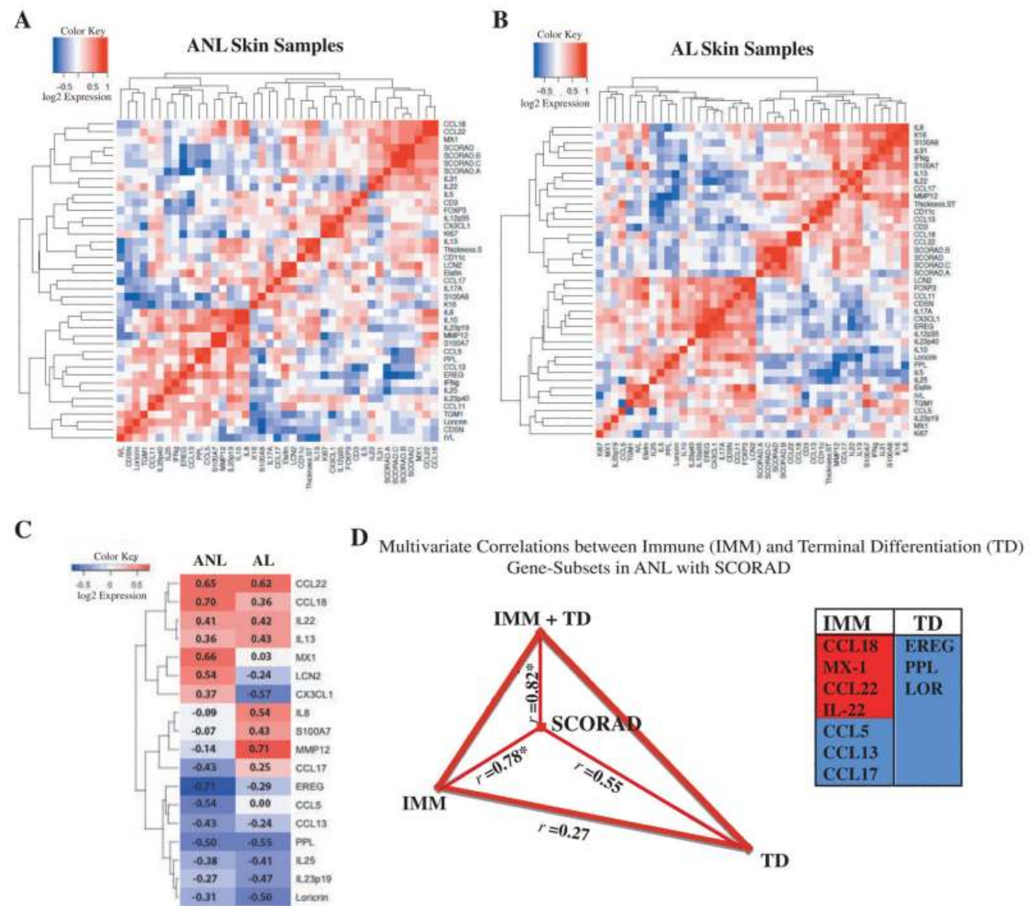

Figure 6.

Clustering of immune and terminal differentiation mRNA gene expression in ANL **(A)** and AL **(B)** skin by Spearman correlation with SCORAD index. **C,** Correlation of the SCORAD index with expression of selected genes (with absolute correlation above 0.4) in ANL and AL (red-positive; blue-negative Spearman correlation coefficient). **D,** Multivariate correlation of immune (IMM) and terminal differentiation (TD) genes in ANL with SCORAD. Line distance length indicative of the *r*-coefficient for the correlation of each gene-set to SCORAD, with shortest line (r=0.82) indicative of the strongest positive correlation to SCORAD (IMM and TD gene-sets combined). *P<0.05.

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