Original article

Reversal of atopic dermatitis with narrow-band UVB phototherapy and biomarkers for therapeutic response

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Background: Atopic dermatitis (AD) is a common inflammatory skin disease exhibiting a predominantly T_H2/"T22" immune activation and a defective epidermal barrier. Narrow-band UVB (NB-UVB) is considered an efficient treatment for moderate-to-severe AD. In patients with psoriasis, NB-UVB has been found to suppress T_H1/T_H17 polarization, with subsequent reversal of epidermal hyperplasia. The immunomodulatory effects of this treatment are largely unknown in patients with AD.
Objective: We sought to evaluate the effects of NB-UVB on immune and barrier abnormalities in patients with AD, aiming to establish reversibility of disease and biomarkers of therapeutic response.
Methods: Twelve patients with moderate-to-severe chronic AD received NB-UVB phototherapy 3 times weekly for up to 12 weeks. Lesional and nonlesional skin biopsy specimens were obtained before and after treatment and evaluated by using gene expression and immunohistochemistry studies.
Results: All patients had at least a 50% reduction in SCORAD index scores with NB-UVB phototherapy. The T_H2, T22, and T_H1 immune pathways were suppressed, and measures of epidermal hyperplasia and differentiation normalized. The reversal of disease activity was associated with elimination of inflammatory leukocytes and T_H2/T22- associated cytokines and chemokines and normalized expression of barrier proteins.
Conclusions: Our study shows that resolution of clinical disease in patients with chronic AD is accompanied by reversal of both the epidermal defects and the underlying immune activation. We have defined a set of biomarkers of disease response that associate resolved T_H2 and T22 inflammation in patients with chronic AD with reversal of barrier pathology. By showing reversal of the AD epidermal phenotype with a broad immune-targeted therapy, our data argue against a fixed genetic phenotype. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: *Atopic dermatitis, phototherapy, narrow-band UVB, T_H2, T22, biomarker, skin*

Atopic dermatitis (AD) is a common inflammatory skin disease[1] characterized by (1) immune activation, (2) marked epidermal hyperplasia, and (3) defective barrier function, reflecting underlying alterations in keratinocyte differentiation.[2-4] The pathogenesis of AD is still debated. A primary barrier dysfunction that leads to immune polarization (the outside-in hypothesis[4]) is supported by recent reports of the presence of filaggrin gene *(FLG)* mutations in some patients with AD,[5] as well as wide downregulation of terminal differentiation genes on chromosome 1q21 (ie, loricrin *[LOR]*, late cornified envelope 2B, small proline-rich proteins, and the cornified envelope gene corneodesmosin *[CDSN]*).[6,7] Mutations in additional candidate genes, such as serine proteinase inhibitor Kazal-type 5 gene *(SPINK5)* and kallikrein-related peptidase 7 gene *(KLK7)*, were also suggested to predispose to the impaired epidermal barrier.[8-11] The inside-out hypothesis, favoring epidermal abnormalities caused by underlying immune activation,[12] is suggested by broad cornification defects in patients without *FLG* mutations[7] and the association of immune abnormalities in nonlesional AD (ANL) skin with disease severity.[13]

AD shares many features with psoriasis, including immune activation and epidermal hyperplasia. However, major differences in immune polarization exist between these diseases.[2,7,14] Although psoriasis is considered a T_H1/T_H17 disease, AD is predominantly a T_H2/T22-polarized disease with some component of T_H1 polarization in the chronic phase and a relative impairment of the T_H17 pathway.[15] The T22 cytokine IL-22 was found to induce epidermal hyperplasia and inhibit keratinocyte terminal differentiation.[15,16]

Whereas topical agents (ie, corticosteroids and calcineurin inhibitors) are effective for patients with mild AD, these options are insufficient to control more severe disease.[17] Limited therapeutic options exist for patients with moderate-to-severe AD: (1) oral steroids; (2) cyclosporine A; and (3) phototherapy (including UVB, UVA with psoralen, and UVA-1).[17] Both oral

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Abbreviations used

AD: Atopic dermatitis
AL: Lesional atopic dermatitis
AMP: Antimicrobial protein
ANL: Nonlesional atopic dermatitis
CDSN: Corneodesmosin
DC: Dendritic cell
DEG: Differentially expressed gene
FLG: Filaggrin
FOXP3: Forkhead box protein 3
IDEC: Inflammatory dendritic epidermal cell
IHC: Immunohistochemistry
IVL: Involucrin
K16: Keratin 16
LOR: Loricrin
NB-UVB: Narrow-band
RT-PCR: Real-time PCR

steroids and cyclosporin A have major adverse effects, prohibiting long-term use.[17] Because UVA-1 phototherapy is expensive and not widely available in the United States, narrow-band UVB (NB-UVB) has emerged as an effective alternative.[18,19]

Although prior studies have reported the clinical efficacy of NB-UVB in reducing SCORAD index scores,[18,20] its immunomodulatory effects are largely unknown in patients with AD. Given that NB-UVB might be the only practical long-term treatment for moderate-to-severe AD, it is crucial to better understand the mechanistic properties of this disease. In patients with psoriasis, NB-UVB treatment has been found to suppress the T_H1/T_H17 immune axes with subsequent reversal of epidermal hyperplasia.[21-24] These investigations defined parameters for psoriasis symptom reversal and established an understanding of the pathogenic relationship between expression of immune-regulatory genes and disease activity.[21-23,25]

The present study analyzes the effects of NB-UVB on barrier and immune abnormalities in patients with AD, aiming to establish the reversibility of the disease and, if reversed, to determine a specific set of genomic and histologic measures of disease reversal. We hypothesized that NB-UVB might reverse both epidermal growth and differentiation defects, as well as underlying immune activation, lending support to the inside-out hypothesis. Conversely, the persistence of epidermal hyperplasia and terminal differentiation defects despite immune suppression with NB-UVB treatment would be a rejection of the inside-out hypothesis. Our study is the first to show clear genomic and tissue reversal of AD disease pathology and to highlight biomarkers of therapeutic response that could be implemented in testing of targeted therapeutics for patients with AD.

METHODS

Patients' characteristics and skin samples

Pretreatment and posttreatment lesional AD (AL) and ANL (≥10 cm from any active lesion) skin biopsy specimens and blood samples were obtained from 12 patients with moderate-to-severe chronic AD (9 male and 3 female patients; age, 24-51 years; median age 43 years) and 10 healthy volunteers under an institutional review board–approved protocol (see Table E1 in this article's Online Repository at www.jacionline.org). Patients received full-body NB-UVB 3 times weekly until clearance or up to 12 weeks (mean, 23.5 sessions; range, 9-48 sessions). Patients were allowed to use emollients only with no additional pharmacologic treatment during the study period. Pretreatment and posttreatment AL and ANL biopsy specimens were obtained from the same skin area to evaluate therapeutic effect. Pretreatment serum IgE levels were increased in 9 of 12 patients (range, 1-6965 kU/L; mean, 1387 kU/L; reference range, 0-160 kU/L), and serum eosinophil counts were increased in 1 patient (reference range, 0% to 7%). The SCORAD index was used to evaluate disease severity at enrolment and after completion of treatment (see the Methods section in this article's Online Repository at www.jacionline.org).[26] Pretreatment SCORAD index scores ranged from 28 to 97.5 (mean, 60); posttreatment SCORAD index scores ranged from 0 to 21 (mean, 10). A single-copy R501X mutation in the *FLG* gene was found in 1 patient (see Table E1).

Immunohistochemistry

Biopsy specimens were frozen in O.C.T. medium, and immunohistochemistry (IHC) techniques were performed in a standard manner (see Table E5 in this article's Online Repository at www.jacionline.org). Epidermal thickness and positive cells per millimeter were quantified by using computer-assisted image-analysis software (ImageJ 1.42; National Institutes of Health, Bethesda, Md). Pathologic epidermal thickness was defined as pre-AL minus pre-ANL epidermal thickness.

Quantitative real-time PCR and gene microarray analysis

RNA was extracted for real-time PCR (RT-PCR), and Affymetrix human-U133APlus2.0 arrays (Affymetrix, Santa Clara, Calif) were used as previously described (see the Methods section in this article's Online Repository).[15] The data in this publication have been entered in the National Center for Biotechnology Information's Gene Expression Omnibus (series accession no. GSE27887, see Tables E2 and E4 in this article's Online Repository at www.jacionline.org).

Statistical analysis

Analysis of IHC and RT-PCR data was carried out by using a linear mixed model to account for the paired structure of AL and ANL skin and pre– to post–NB-UVB measures. Affymetrix CEL files were scrutinized for spatial artifacts by using the Harshlight package.[13] Expression values were obtained with the GCRMA algorithm. Probe sets with more than 5 samples with an expression of greater than 4 and an SD of greater than 0.2 were kept for analysis. *P* values were adjusted for multiple hypotheses by using the Benjamini-Hochberg procedure. Spearman rank correlations were used to correlate all variables measured by means of IHC and RT-PCR with clinical and histologic response to NB-UVB at a significance *P* value of less than .1.

RESULTS

After NB-UVB, all patients met the predefined criteria for response to therapy (a decrease of ≥50% in SCORAD index score), with marked clinical improvement. Overall, a mean 81.1% ± 8.7% reduction in SCORAD index scores was observed after an average of 23.5 NB-UVB sessions (see Table E1). Eleven of 12 patients were judged to be responders by using histologic (>40% reduction in pathologic epidermal thickness) criteria. Subsequent statistical analyses include the outcome of NB-UVB in the 11 responders.

To determine a specific set of cellular and molecular markers of disease reversal that correlate with a major reduction in SCORAD index scores after NB-UVB, we used the term biomarker and adopted its formal definition by the National Institutes of Health: "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response(s) to a therapeutic intervention."[27] We established biomarkers of disease activity in patients with

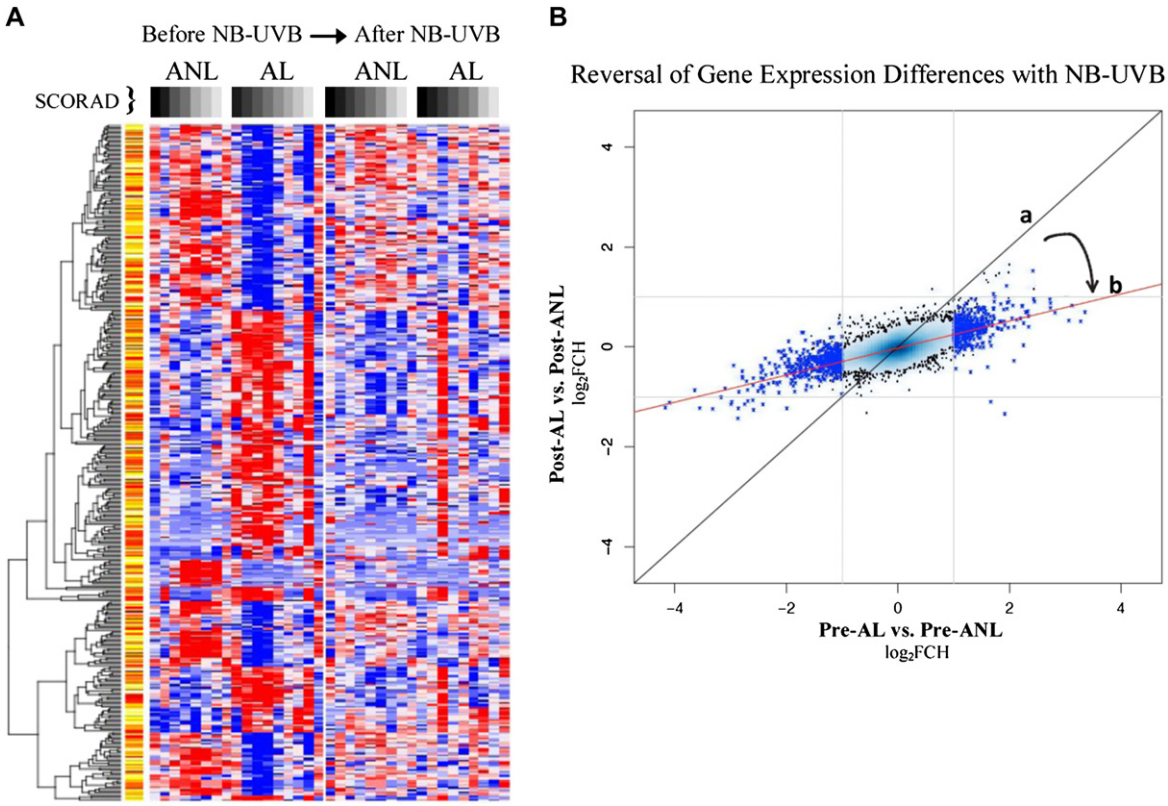

FIG 1. Genomic differences in patients with AD before and after NB-UVB phototherapy. **A**, DEGs in ANL and AL skin before and after NB-UVB (*red*, gene upregulation; *blue*, gene downregulation). **B**, Scatterplot of gene expression values in pre–NB-UVB *(x-axis)* and post–NB-UVB *(y-axis)* samples shows the substantial reduction (78%) in the AD disease phenotype with NB-UVB. *FCH*, Fold change.

baseline AD, as well as biomarkers of disease reversal with therapy (variables for which a significant change with NB-UVB treatment correlated with a reduction in SCORAD index score).

Genomic reversal of epidermal hyperplasia and inflammatory markers with NB-UVB therapy

Gene-array analysis was performed to compare genomic expression before and after phototherapy in both ANL and AL skin. We have defined the genomic differences in pre-ANL and pre-AL skin as the genomic phenotype of visible disease (see Table E2).[13] A heat map illustrating this distinction between pre-ANL and pre-AL skin displays disease reversal after NB-UVB, with post-ANL and post-AL skin showing similar expression levels (Fig 1, *A*). By organizing patients in order of SCORAD index scores, the partial retention of pre-AL expression levels can be appreciated in patients with higher scores (Fig 1, *A*; gray-spectrum boxes at the top of each column represent an increase in SCORAD index scores from white to black). When represented in a scatterplot, the substantial nullification of the genomic disease phenotype is also observed (Fig 1, *B*). Genomic expression differences in pre-AL versus pre-ANL expression (the AD disease phenotype) are graphed as an identity line (Fig 1, *B*, slope = 1, line a, black). In comparison, the linear regression line of genomic expression differences in post-AL versus post-ANL skin (Fig 1, *B*, line b, red) is reduced by 78%, representing a reduction in the AD disease phenotype.

The criteria of a fold change of greater than 2 and a false discovery rate of less than 0.1 were used to determine the differentially expressed genes (DEGs) between pre-ANL and post-ANL and pre-AL and post-AL skin. There was a downregulation of 372 genes and an upregulation of 192 genes in AL skin after phototherapy compared with pretreatment expression levels (see Fig E1 in this article's Online Repository at www.jacionline.org), with a downregulation of 35 and upregulation of 23 genes in ANL skin (see Fig E1). Markers of epidermal proliferation (Ki67, keratin 16 [K16], and keratin 6B), as well as many inflammatory mediators, were within this set of genes (see Tables E3 [the top 50 DEGs] and E4 [the complete list of DEGs] in this article's Online Repository at www.jacionline.org). Among the most significantly downregulated immune genes were markers of T cells (CD2), T-cell activation (CD69), T_H2-associated products (CCL13, CCL18, CCL26, and IL-10), T_H1-related products (TNF-α and IL-12), interferon-induced genes (MX-1 and OAS1), and dendritic cell (DC) antigens (ITGAM/CD11b, CD209, CD1c, FcεRI, and CD1a) and several keratinocyte-associated immune mediators (CCL2, TNF-α–induced proteins, and CCL5 and the IL-7 receptor; see Tables E3 and E4).

Reduction of pathologic epidermal proliferation with NB-UVB

We evaluated tissue response by comparing epidermal thickness and keratinocyte proliferation (K16 staining) in pre-ANL, pre-AL, post-ANL, and post-AL samples (Fig 2, *A* and *B*). Pre-AL epidermal thickness was 141% greater (on average) than pre-ANL thickness and was significantly reduced after treatment

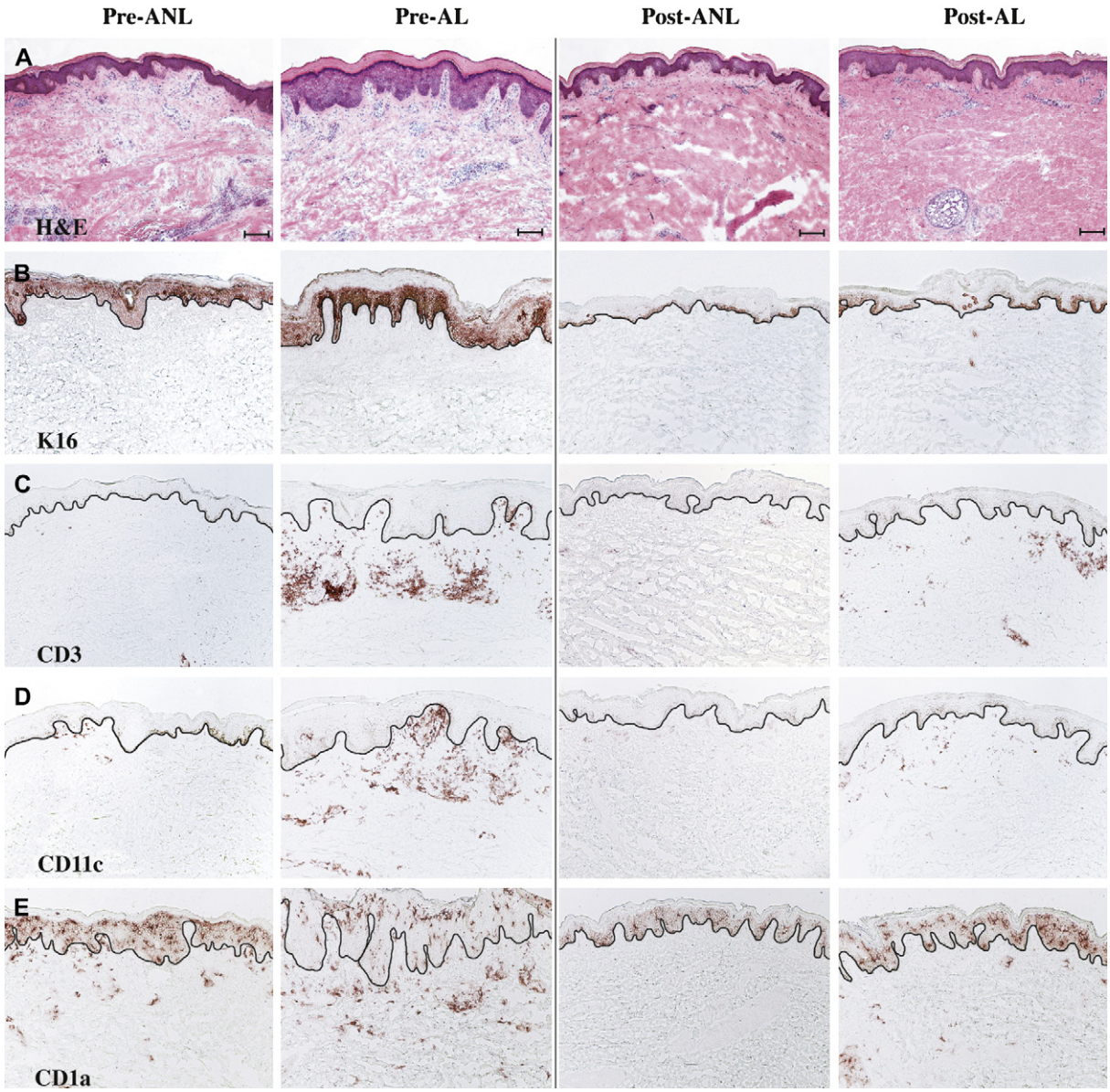

FIG 2. IHC of ANL and AL skin before and after NB-UVB. Staining of hematoxylin and eosin (*H&E*; **A**) and proliferation marker K16 **(B)** display reductions in epidermal hyperplasia and abnormal proliferation after NB-UVB. **C-E**, Reductions in dermal ($CD3^+$) T cells, myeloid ($CD11c^+$) DCs, and Langerhans ($CD1a^+$) cells. *Scale bar* = 100 μm.

($P < .005$), with a mean decrease of 115.6 ± 125.8 μm (a reduction of pathologic epidermal thickness of 86% ± 23.6%, Fig 3). A normalization of keratinocyte proliferation was seen after therapy: suprabasal K16 expression, which is not typically expressed in healthy skin, was present in only 1 post-AL sample (vs K16 positivity in all 12 pre-AL skin samples). These data were confirmed based on a significant reduction in K16 mRNA expression from pre-AL to post-AL skin ($P < .001$, Fig 4).

Suppression of inflammatory cell infiltrates after NB-UVB

The NB-UVB effect on immune cell infiltrates in AD skin was most evident in the papillary dermis, with some decrease in cell density in the reticular dermis (Fig 2, *C-E*). Comparison of cell counts in pre-AL and post-AL skin revealed significant decreases in $CD3^+$ and $CD8^+$ T cells ($P < .001$) and significant reductions in DC subsets, including myeloid ($CD11c^+$, $P < .05$), resident ($CD1c^+$, $P < .001$),[28] inflammatory (TNF-related apoptosis-inducing ligand positive, $P < .05$),[28] plasmacytoid (blood dendritic cell antigen 2 positive, $P < .01$), and mature ($CD83^+$, $P < .01$) DCs (Fig 3). The number of inflammatory dendritic epidermal cells (IDECs), quantified by $CD1b^+$, $Fc\epsilon R1^+$, and $CD206^+$ cells, also significantly decreased after NB-UVB therapy (Fig 3). Levels of CD1a, a marker of Langerhans cells and IDECs, were also significantly reduced ($P < .001$; Figs 2, *E*, and 3), as were levels of OX40 ligand, which is considered a marker of atopic DCs (Fig 3).[29] Low numbers of eosinophils (major basic

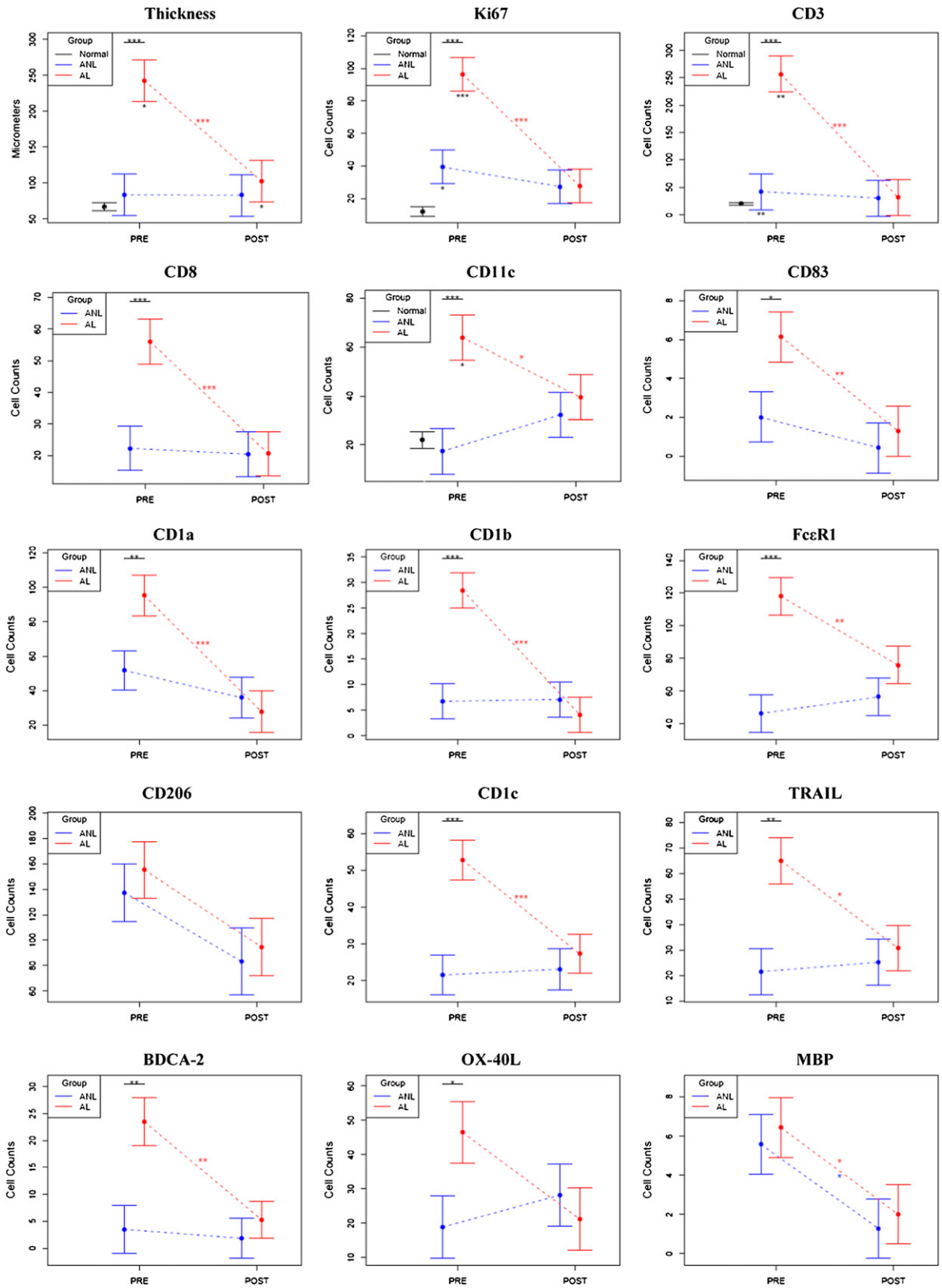

FIG 3. Reductions in epidermal thickness and immune cell infiltrates quantified by means of IHC in AL *(red lines)* and ANL *(blue lines)* skin after NB-UVB (*asterisks above lines* = significance of the change). *Black error bars* and *asterisks* represent the SEM in healthy skin samples and significance of difference in AL or ANL versus healthy skin (when available), respectively. $*P < .05$, $**P < .01$, and $***P < .001$. *BDCA-2*, Blood dendritic cell antigen 2; *MBP*, major basic protein; *OX-40L*, OX40 ligand; *TRAIL*, TNF-related apoptosis-inducing ligand.

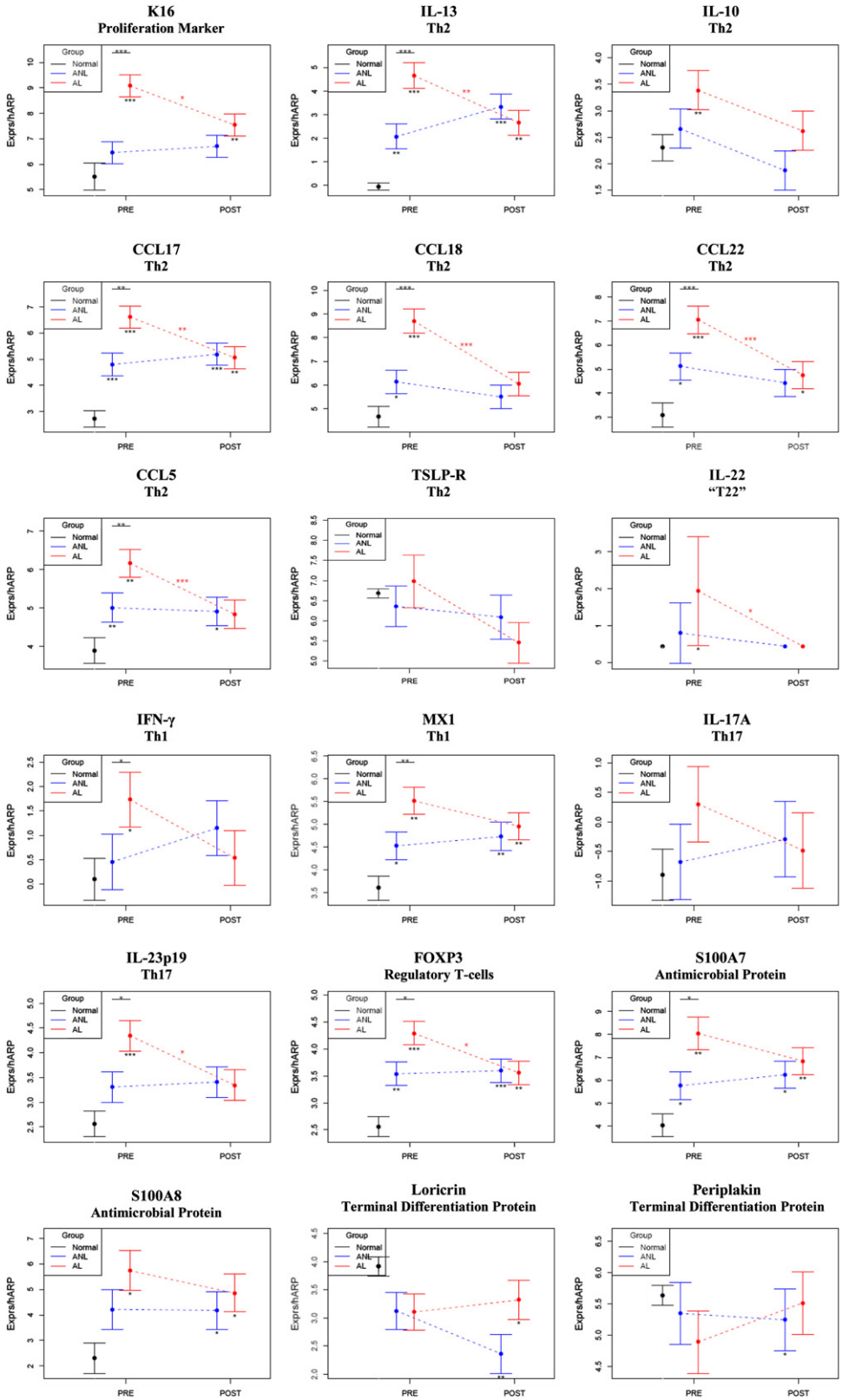

FIG 4. RT-PCR expression of selected genes in AL *(red lines)* and ANL *(blue lines)* skin after NB-UVB UVB (*asterisks above lines* = significance of the change). Associated inflammatory pathways (T_H1, T_H2, T_H17, or T22) are indicated for each cell type. *Black error bars* and *asterisks* represent the SEM in healthy skin samples and significance of difference in AL or ANL versus healthy skin (when available), respectively. $*P < .05$, $**P < .01$, and $***P < .001$. *FOXP3*, Forkhead box protein 3; *TSLP-R*, thymic stromal lymphopoietin receptor.

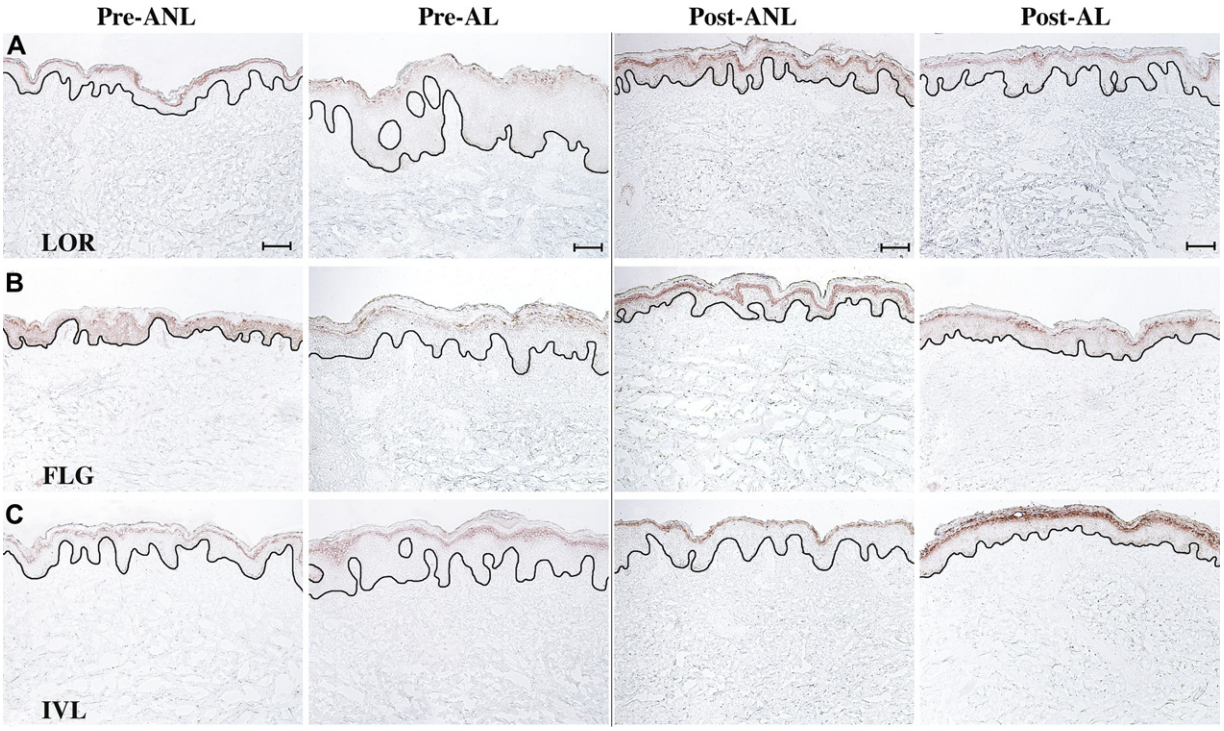

FIG 5. IHC of ANL and AL skin before and after NB-UVB. Staining of the terminal differentiation proteins LOR **(A)**, FLG **(B)**, and IVL **(C)** displays increased and more continuous granular layer staining after NB-UVB therapy. *Scale bar* = 100 μm.

protein positive, Fig 3) and mast cells (tryptase positive; see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) were observed in pre-AL skin; numbers of these cells were significantly reduced after NB-UVB ($P < .05$ and $P < .01$, respectively).

NB-UVB suppresses inflammation in patients with AD

We validated UVB's effects on inflammatory pathways with RT-PCR. T_H2-associated products (IL-13, CCL11, CCL17, CCL18, and CCL22), which perpetuate T_H2-associated inflammation, showed significantly reduced mRNA expression levels (Fig 4). Reductions of IL-10 and thymic stromal lymphopoietin receptor levels approached significance ($P = .346$ and $P = .097$, respectively; Fig 4). Levels of the T22 cytokine IL-22, which were significantly increased in pre-AL skin compared with healthy skin ($P < .05$), decreased significantly with NB-UVB ($P < .05$, Fig 4). A decrease was also observed in mRNA expression of the S100A7 and S100A8 antimicrobial proteins (AMPs), which are induced by IL-22 (Fig 4).[2,15]

The mRNA expression of the T_H1-associated cytokine IFN-γ and of the interferon-induced proteins MX-1 and CXCL-10 was increased in pre-AL skin compared with that seen in healthy skin ($P < .05$, $P < .01$, and $P = .097$, respectively) and was downregulated with treatment ($P = .119$, $P = .186$, and $P = .134$, respectively; Fig 4 and see Fig E2, *B*).

We detected decreases in the mRNA expression of IL-17A, IL-23p19 (Fig 4), and IL-23p40 (see Fig E2, *C*) with treatment ($P < .05$ for p19 alone). Finally, the mRNA expression of the regulatory T-cell marker forkhead box protein 3 *(FOXP3)* was significantly increased in pre-AL skin compared with that seen in healthy skin ($P < .001$) and decreased with NB-UVB ($P < .05$, Fig 4).

Normalization in barrier proteins with NB-UVB

Histologic evaluation of the terminal differentiation proteins LOR, FLG, and involucrin (IVL) revealed clear increases in granular layer expression (Fig 5). We observed a more continuous expression and a greater depth of expression (into the stratum spinosum layer) of LOR (10/10 patients; Fig 5, *A*), FLG (8/10 patients; Fig 5, *B*), and IVL (5/8 patients; Fig 5, *C*) in post-ANL and post-AL skin compared with that seen before NB-UVB, representing a relative normalization. The normalization in granular layer proteins correlated, in general, with reversal of hyperplasia markers (suprabasal K16 staining and epidermal thickness). Likewise, mRNA expression of terminal differentiation proteins (LOR and periplakin) was significantly inversely correlated with a reduction in the SCORAD index score, suggesting increased expression of these proteins with greater therapeutic response (Table I, *B*).

Suppression of inflammatory cells and their products correlates with clinical improvement

Several immune and terminal differentiation markers significantly correlate with disease severity (SCORAD index) in baseline pre-AL skin (before treatment). These include T-cell ($CD8^+$) and DC ($CD1a^+$, $CD206^+$, and OX40 ligand positive) markers, T_H2-associated products (IL-13 and CCL22), T22-associated IL-22, and the AMP S100A7, as well as the terminal

TABLE I. Correlation of epidermal and immune variables with AD severity at baseline and with disease improvement and reductions in inflammatory cytokine levels after NB-UVB phototherapy*

A. Variables significantly correlated with disease severity (SCORAD index) in baseline lesional AD skin

Disease variable	Spearman correlation	*P* value
CD206$^+$ cells (I)	0.87	.003
CD1a$^+$ cells (I)	0.57	.026
CCL22 (R)	0.62	.028
CD8$^+$ cells (I)	0.68	.032
OX40L$^+$ cells (I)	0.64	.044
TRAIL$^+$ cells (I)	0.51	.045
IL-13 (R)	0.43	.081
S100A7 (R)	0.43	.081
IL-22 (R)	0.42	.087
PPL (R)	−0.55	.032
LOR (R)	−0.51	.098

B. Variables significantly correlated with reduction of SCORAD index scores after NB-UVB

Disease variable	Spearman correlation	*P* value
CD1a$^+$ cells (I)	0.729	.004
CD1b$^+$ cells (I)	0.688	.007
FcεRI$^+$ cells (I)	0.665	.009
CD8$^+$ cells (I)	0.740	.018
CD83$^+$ cells (I)	0.663	.037
IL-22 (R)	0.532	.038
S100A8 (R)	0.583	.065
LOR (R)	−0.804	.008
PPL (R)	−0.755	.015

C. Variables significantly correlated with reduction of epidermal hyperplasia after NB-UVB

Epidermal thickness

Disease variable	Spearman correlation	*P* value
CD83$^+$ cells (I)	0.638	.044
TRAIL$^+$ cells (I)	0.467	.063
CCL17 (R)	0.487	.077
CD3$^+$ cells (I)	0.430	.081
CCL5 (R)	0.526	.090
BDCA-2$^+$ cells (I)	0.522	.092
CCL18 (R)	0.456	.093
CD1a$^+$ cells (I)	0.395	.102

K16 expression determined by means of RT-PCR

Disease variable	Spearman correlation	*P* value
BDCA-2$^+$ cells (I)	0.992	<.001
LCN2 (R)	0.93	<.001
Elafin (R)	0.913	<.001
CCL18 (R)	0.903	<.001
CCL5 (R)	0.883	<.001
CCL22 (R)	0.848	.001
S100A7 (R)	0.806	.002
S100A8 (R)	0.729	.008
IVL (R)	0.536	.055
CCL17 (R)	0.468	.086
Thickness (I)	0.428	.083

(*Continued*)

TABLE I. *(Continued)*

D. Variables significantly correlated with reduction in mRNA expression of the cytokine IL-22 after NB-UVB

Disease variable	Spearman correlation	*P* value
BDCA-2$^+$ cells (I)	0.752	.016
S100A8 (R)	0.678	.016
SCORAD	0.532	.038
OX40L$^+$ cells (I)	0.655	.039
CD1b$^+$ cells (I)	0.454	.069
S100A7 (R)	0.464	.088
CDSN (R)	−0.591	.036
LOR (R)	−0.590	.036

BDCA-2, Blood dendritic cell antigen 2; *I*, immunohistochemistry; *LCN2*, Lipocalin-2; *MBP*, major basic protein; *OX40L*, OX40 ligand; *PPL*, Periplakin; *R*, RT-PCR; *TRAIL*, TNF-related apoptosis-inducing ligand.

*Spearman rank correlations were determined for all evaluated variables quantified by means of IHC and RT-PCR with baseline disease severity (**A**) and reductions in SCORAD index scores (**B**), epidermal hyperplasia (**C**), and IL-22 levels (**D**) after NB-UVB with results in order of significance. Variables with significant inverse correlations are listed after all positive correlations.

differentiation proteins LOR and periplakin (inversely correlated with disease severity; Table I, *A*).

We also evaluated which immune, terminal differentiation, and hyperplasia markers are associated with clinical and histologic disease reversal by determining the correlations of each variable measured by using IHC and RT-PCR with (1) reduction in SCORAD scores (clinical disease reversal; Table I, *B*) and (2) reduction in epidermal hyperplasia and K16 expression (histologic disease reversal; Table I, *C*).

Immune cell subsets were the most highly correlated variables with clinical improvement. These included CD8$^+$ T cells and several DC subsets (CD1a$^+$ IDECs, inflammatory [CD1b$^+$ and FcεRI$^+$] DCs, and mature [CD83$^+$] DCs; Table I, *B*). The T_H2-associated chemokines (CCL17, CCL18, CCL22, and CCL5), which are produced by myeloid and plasmacytoid DCs,[2] were highly correlated with histologic improvement (Table I, *C*).

IL-22 was the only cytokine for which its reduction in gene expression with NB-UVB was significantly correlated with the reduction in SCORAD index scores, suggesting the importance of this cytokine in disease activity (Table I, *B*). The reductions in expression of the IL-22–induced AMPs S100A7 and S100A8[30] were similarly highly correlated with clinical and histologic improvement (Table I, *B*). Moreover, considering all variables, the cytokine IL-22 appeared to be the most highly correlated across several characteristics of disease activity (T_H2-related products, SCORAD index scores, and inflammatory cell infiltrates; Table I, *D*). In comparison, the reduction in IL-13 mRNA expression was not strongly correlated with either clinical or histologic improvement, although it was highly correlated with expression of several terminal differentiation proteins, including LOR, transglutaminase-1 (TGM1), and CDSN (Spearman correlations of $r = 0.720$, $r = 0.621$, and $r = 0.569$, respectively). T_H1- and T_H17-related products were not correlated with disease improvement, but reduction in expression of the p40 cytokine subunit was correlated with the reduction in hyperplasia (Table I, *C*).

Taken together with the variables correlated with disease activity at baseline (Table I, *A*), our data show that the reversal of disease activity with NB-UVB is most strongly associated with elimination of CD8$^+$ T cells; several DC subsets; the cytokine IL-22, the T_H2 chemokines CCL17, CCL18, and CCL22;

the AMPs S100A7 and S100A8; and a normalized expression of barrier proteins (Table I, *B-D*).

DISCUSSION

Few studies have evaluated the mechanism of action of NB-UVB in patients with AD, and these have mostly concentrated on the clinical response.[31] There are also limited data on the molecular effects of UV radiation in patients with AD.[32-37] In a murine model UVB has been shown to improve barrier function through increased expression of terminal differentiation proteins (FLG and IVL) and AMPs, with altered expression of the AMPs human β-defensin 1 and 2 described in patients with AD treated with NB-UVB.[32] Other studies on UV in patients with AD have found reductions in cutaneous T cells,[34,36] with no significant changes in mast cells or FOXP3$^+$ cells.[18,34,37] The lack of mechanistic studies in therapeutic trials in patients with AD extends to other treatments, including cyclosporin A, corticosteroids, and calcineurin inhibitors,[38-48] and partially stems from an incomplete understanding of the pathogenic mechanisms underlying the disease.

However, recent reports have promoted the understanding of AD's pathogenesis,[7,14] establishing the following features: (1) regenerative hyperplasia,[7,13] (2) abnormal terminal differentiation in both AL and ANL skin,[13] and (3) activation of T_H2 and T22 subsets in AL skin.[14] Thus a complex immune pathogenesis characterizes chronic AD, with a relative deficiency of the T_H17 immune axis and an upregulation of the T22 axis in the context of ongoing T_H2 pathway dominance.[2,7,15] Although the T_H2 cytokines IL-4/IL-13 have been reported to inhibit T_H17 activation,[49] terminal differentiation proteins,[50] and AMPs,[51] T_H2 products have not previously been reported to promote epidermal hyperplasia.

There is increasing evidence linking IL-22 to many aspects of epidermal pathology in patients with AD, including inhibition of epidermal differentiation and induction of hyperplasia, and also associating it with disease activity in patients with AD.[15,16,52,53] Furthermore, the cytokine has been reported to strongly upregulate the AMPs S100A7 and S100A8,[14,16,49] although it is a much less potent inducer of AMPs than IL-17, which is relatively deficient in patients with AD.[15] Finally, staphylococcal exotoxins induce IL-22 secretion in keratinocytes, supporting an IL-22–dependent role of *Staphylococcus aureus* in AD severity.[54]

These advances allowed us to analyze the NB-UVB effects in patients with AD and to correlate the normalization of immunologic and histologic parameters with clinical improvement. We evaluate how T_H2/T22 immune suppression induced by NB-UVB influences reversal of the genomic and epidermal disease phenotype of AD and creates a set of measures for the assessment of disease reversal.

UV radiation has immunomodulatory effects that lead to improvement of inflammatory skin diseases.[36] In animal and human models UVB inhibits immune responses,[23,55,56] likely related to induction of T-cell apoptosis.[21,23,57] In patients with psoriasis, NB-UVB was initially shown to induce immune suppression followed by normalization of epidermal hyperplasia, supporting an immune regulation of the epidermal pathology in patients with this disease.[58] Subsequently, NB-UVB was found to suppress major T-cell pathways involved in disease pathogenesis, namely the T_H17/IL-23 and T_H1 pathways.[22,24,59,60] In these psoriasis studies there was a significant reduction in IFN-γ gene expression with NB-UVB, although the reductions in IL-17 and IL-22 expression were found to better correlate with the clinical response.[24] Similarly, our study shows strong suppression of the T_H2 and T22 axes in patients with AD after NB-UVB together with normalization of epidermal barrier function. We also found significant reductions in inflammatory cell subsets (including T cells, IDECs, and atopic DCs).

Our study extends the mechanism of action of NB-UVB to include suppression of T_H2 immune activation in patients with chronic AD. Thus NB-UVB can suppress all activated polar T-cell immune pathways within patients with inflammatory diseases. Overall, we show that in patients with chronic AD, NB-UVB induces significant immune suppression of the T_H2 and T22 axes and a less significant suppression of the T_H1 axis. Thus in both patients with AD and those with psoriasis,[24] the immune suppression changes seen with NB-UVB treatment reflect the major inflammatory pathways that characterize each disease and are correlated with the therapeutic response.

Interestingly, concordant with prior reports,[51,61] the immunoregulatory cytokine IL-10 showed upregulated mRNA expression in both AL and ANL skin compared with that seen in healthy skin, which decreased with NB-UVB. Increased levels of IL-10 have been postulated to indirectly contribute to the AMP deficiency in patients with AD, potentially accounting for an increased propensity for infections.[62] Higher IL-10 expression levels were reported in skin from patients with chronic AD compared with the acute stage, possibly because of an upregulation of the IL-10 receptor by IFN-γ.[63,64]

We present data for reversal of epidermal hyperplasia and aberrant terminal differentiation with NB-UVB and the correlation of these variables with T_H2 and T22 immune pathways. The suppression of T_H2 chemokines was highly correlated with the reversal of hyperplasia after NB-UVB therapy. We also found a high correlation between the reduction in IL-22 expression and the reduction in epidermal hyperplasia and reduction in expression of S100A7 and S100A8. The reduction in IL-22 expression after NB-UVB was also inversely correlated with the change in expression of terminal differentiation proteins. We hypothesize that IL-22 is critical in AD's pathogenesis because our observations link suppression of IL-22 to (1) a reduction in SCORAD index scores; (2) reversal of epidermal hyperplasia; (3) depletion of immune cell subsets, including Langerhans cells and plasmacytoid DCs (both recently reported to induce T22 cells)[65,66]; and (4) normalization of terminal differentiation proteins. Upregulation of the T22 axis might be the link between the immune and epidermal abnormalities seen in patients with AD.[14]

The novel approach we have taken to evaluate NB-UVB's effects in patients with AD allows us to define a set of biomarkers of disease response that associate resolved T_H2 and T22 inflammation with reversal of epidermal barrier pathology. We clearly show reversal of the AD epidermal phenotype with a broad-based immune-targeted therapy, which argues against a fixed genetic phenotype. Thus although not providing absolute proof for the inside-out hypothesis, we cannot refute this assumption. However, because NB-UVB might have direct effects on epidermal keratinocytes,[67] some therapeutic effects could also be modulated through keratinocytes. Future studies with specific immune antagonists coupled with the disease markers shown here are warranted to critically test the alternate hypotheses of disease pathogenesis: inside-out (immune-generated epidermal dysfunction) versus outside-in (defined by barrier-initiated immune activation).

Clinical implications: Resolution of T_H2- and T22-associated inflammation is strongly correlated with the therapeutic response to NB-UVB in patients with AD, and targeting these pathways might be beneficial in disease treatment.

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METHODS

Two 4- to 6-mm punch biopsy specimens from AL and ANL (≥10 cm from any active lesion) skin biopsy specimens and blood samples were obtained from 12 patients with moderate-to-severe chronic AD (9 male and 3 female patients; age, 24-51 years; median age, 43 years) before and after a course of NB-UVB phototherapy. Lesional biopsy specimens were taken from the "most inflammatory-appearing chronic AD skin lesions" (characterized by intense erythema and some lichenification). Pretreatment and posttreatment AL and ANL biopsy specimens were obtained from the same skin area to evaluate therapeutic effect. Four- to 6-mm punch biopsy specimens were also taken from 10 healthy volunteers from similar body locations as in study patients. This study was conducted from September 2008 through February 2010. All patients were enrolled in the fall season (in September or October 2008 or 2009). This study was conducted under an institutional review board–approved protocol.

Patients received full-body NB-UVB 3 times weekly until clearance or for up to 12 weeks (mean, 23.5 sessions; range, 9-48 sessions). Patients were allowed to use emollients only, with no additional pharmacologic treatment during the study period. Dosing of NB-UVB began at 50% of the minimal erythema dose and was increased 10% to 20% with each treatment session. The minimal erythema dose is defined as the dose of NB-UVB necessary to induce a just perceptible erythema with no marked borders.[E1]

Venous blood samples were taken from patients for measurement of serum IgE levels (reference range, 0-160 kU/L) and serum eosinophil counts (reference range, 0% to 7%) and for DNA sequencing (see below). Pretreatment serum IgE levels were increased in 9 of 12 patients (range, 1-6965 kU/L; mean, 1387 kU/L; reference range, 0-160 kU/L), and serum eosinophil counts were increased in 1 patient (reference range, 0% to 7%; see Table E1).

The SCORAD index was used to evaluate disease severity at enrolment and after completion of treatment. SCORAD index scores were used to classify AD in each patient as mild (<25), moderate (25-50), or severe (>50; maximum score, 103), as previously defined.[E2,E3] The SCORAD index formula is as follows:

$$A/5 + 7B/2 + C.$$

In this formula *A* is defined as the extent of disease (percentage of body surface area involvement arrived at by application of the rule of nines applied to a front/back drawing of the patient), *B* is the intensity (erythema, edema/population, excoriations, lichenification, oozing/crusts, and dryness), and *C* is the patient's subjective symptoms (daily pruritus and sleeplessness).[E3]

Because of the loss of tissue with laboratory processing and analysis, histologic analysis was performed on 12 patients for most antibodies and on 10 patients for 6 antibody stains (see Table E5). Gene microarray analysis was performed on 12 patients, and RT-PCR was performed on 10 patients.

IHC

Cryostat tissue sections of all patients were stained with hematoxylin (Fisher, Fair Lawn, NJ) and eosin (Shandon, Pittsburgh, PA). IHC was performed by using purified mouse anti-human monoclonal and rabbit anti-human polyclonal antibodies (Table E2).

Biotin-labeled horse anti-mouse antibody and goat anti-rabbit antibody (Vector Laboratories, Burlingame, Calif) were amplified with avidin-biotin complex (Vector Laboratories) and developed with chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich, St Louis, Mo). All immunostained sections were photographed and analyzed with ImageJ analysis to measure epidermal thickness and cell counts per millimeter (ImageJ 1.42).

FLG mutation typing

DNA samples from all patients included in the study population were sequenced for 2 *FLG* mutations: R501X and 2282del4. *FLG* R501X was typed after amplification of genomic DNA with FLG_3_cdn_N-F: 5′TGGTTCCTA CTTGTCCTGGG3′ and 5′ATGGGCACTCTGCAGACAG3′. Sanger resequencing was then performed with nested primers, 5′AGAGGAAGCTTCA TGATGACG3′ or 5′ATGGGCACTCTGCAGACAG3′, to detect the 2 alleles. 2282del4 was typed after amplification of genomic DNA with FLG_3_cdn_M-F: 5′GATTGCTCGTGGTGGGAC3′ and FLG_3_cdn_M-R: 5′ AACACGAAATGAGGAACAATCA3′. Sanger resequencing was then performed with the nested primers DEL4.F2: 5′TCC CGC CAC CAG CTC C3′ or FLG_3_cdn_M-F: 5′GATTGCTCGTGGTGGGAC3′. One patient had a single-copy *FLG* R501X mutation. None of the patients had a 2282del4 mutation.

Sample preparation for gene-chip analysis

We used human U133APlus2.0 GeneChip probe arrays (Affymetrix) containing approximately 54,000 probe sets to assess expression of more than 47,000 transcripts, including approximately 38,500 genes and unigenes. The labeled target was fragmented and hybridized to probe arrays, as previously described.[E4] Total RNA was extracted from tissues frozen in liquid nitrogen with the RNeasy Mini Kit (Qiagen, Valencia, Calif). DNA was removed with on-column DNAse digestion by using the Qiagen RNAse-free DNAse Set. Total RNA (2 μg) was reverse transcribed, amplified, and labeled as previously reported.[E4] mRNA was isolated and converted to double-strand cDNA and then to biotinylated cRNA (BioArray High Yield RNATranscription Labeling Kit; Enzo Biochem, Inc, Farmingdale, NY). After fragmentation and quality confirmation with Affymetrix Test-3 Array, 15 μg of the biotinylated cRNA was hybridized to human U133APlus2.0 GeneChips. The chips were washed, stained with streptavidin-phycoerythrin, and scanned (HP GeneArray Scanner; Hewlett-Packard, Palo Alto, Calif). On each chip, the human housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* served as controls. Suite 5.0 software normalized the expression values by using these controls.

RT-PCR analysis

The primers and probes for the TaqMan RT-PCR (Applied Biosystems, Foster City, Calif) assays were generated with the Primer Express algorithm (Applied Biosystems), version 1.0, by using published genetic sequences (National Center for Biotechnology Information-PubMed) for each gene. Primer sequences were as follows: K16-forward, GCGAGGATGCCCACCTTT; K16-reverse, GAAGACCTCGCGGGAAGAAT; K16-probe, CCCAGCAAGCATCT GGCCAATCC-TAMRA; LOR-forward, AAGGGCGTCCCGATCTG; LOR-reverse, CGGGTAGCATCATGAGAGCTCTA; and LOR-probe, 6FAM-CACC AGACCCAGCAGAAGCAGGC-TAMRA. The primers and probes for IL23(p19) (assay ID Hs00372324), IL23(p40) (assay ID Hs00233688), MX-1 (assay ID Hs00895608), IL22 (assay ID Hs01574154), IL13 (assay ID Hs99999038), IFN-γ (assay ID Hs00989291), CCL22 (assay ID Hs00171080), CCL17 (assay ID Hs0017107), CCL18 (assay ID Hs00268113), S100A7 (assay ID Hs00161488), S100A8 (assay ID Hs00374264), CCL5 (assay ID Hs00982282), CCL11 (assay ID Hs99999025), transglutaminase-1 (TGM1; assay ID Hs00165929), IL10 (assay ID HS00961622), IL17A (assay ID Hs00369400), CDSN (Hs00381831), elafin (Hs00160066), periplakin (Hs00160312), lipocalin-2 (Hs01008571), IVL (Hs00902520), and FOXP3 (Hs01085834) were designed by Applied Biosystems.

The RT-PCR reaction was performed with EZ-PCR Core Reagents (Applied Biosystems), according to the manufacturer's directions and as previously reported. The human acidic ribosomal protein gene *(HARP)*, a housekeeping gene, was used to normalize each sample and each gene. The primer sequences used were as follows: HARP-forward, CGCTGCTGAACATGCTCAA; HARP-reverse, TGTCGAACACCTGCTGGATG; and HARP-probe, 6-FAM-TCCCCC TTCTCCTTTGGGCTGG-TAMRA (Gene Bank Accession Number NM-001002). The data were analyzed and quantified by using the software provided with the Applied Biosystems PRISM 7700 (Sequence Detection Systems, version 1.7).

Statistical methods

All statistical analysis was carried out with open source R language (www.R-project.org) and packages from Bioconductor (www.bioconductor.org). Analysis of RT-PCR data ($\log_2$ scale of the normalized values), and cell counts were carried out by using a linear mixed-effects model to account for the paired structure of lesional and nonlesional measures and of pre– and post–NB-UVB measures.

Quality control, preprocessing, and filtering. Affymetrix CEL files for the human U133APlus2.0 chips were scrutinized for spatial

artifacts by using the Harshlight package.[E5] Classic microarray quality control was obtained by using Bioconductor's arrayQualityMetrics package. Expression values were obtained by using the GCRMA algorithm. Probe sets with more than 5 samples with expression of greater than 4 and an SD of greater than 0.2 were kept for further analysis; analysis included 29,960 probes representing 13,928 unique genes with a known ENTREZ identifier.

Model and hypothesis testing. Expression values in $\log_2$ scale were modeled by using a linear mixed-effect models with group as the fixed effect and a random intercept for each patient in the framework of Bioconductors' limma package. The comparisons of interest (pre-AL vs pre-ANL, pre-AL vs post-AL, pre-ANL vs post-ANL, and post-AL vs post-ANL, and the change from pre–NB-UVB to post–NB-UVB in ANL skin vs the change in AL skin) were tested by using the moderated *t* test,[E6] and resultant *P* values were adjusted for multiple hypotheses by using the Benjamini-Hochberg procedure. A gene was considered to be differentially expressed (DEG) if the false discovery rate was higher than 0.1 and the fold change was higher than 2.

Hierarchical clustering

Genes with similar patterns of expression were grouped together as hierarchical clusters and presented as color-coded heat maps. McQuitty linkage was used as an agglomeration method with Euclidean distance.

Correlations. Spearman rank correlations were determined for all variables evaluated by means of IHC and RT-PCR with clinical (reduction in SCORAD index scores) and histologic (markers of epidermal hyperplasia) response to NB-UVB. Variables with a significance *P* value of less than .1 are presented in Table I in order of correlation.

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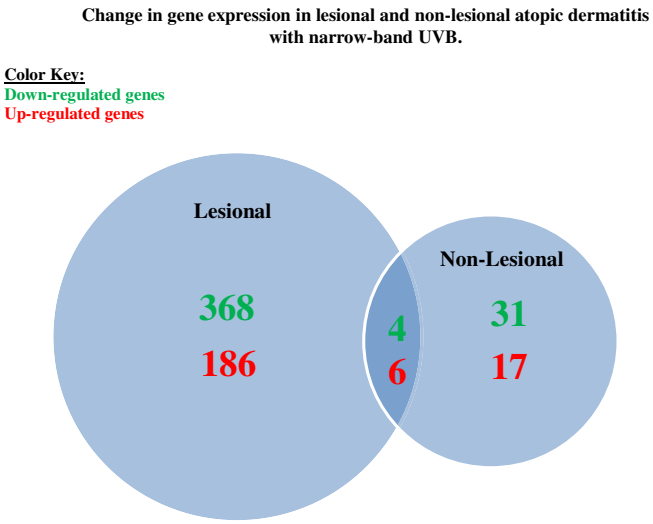

FIG E1. Number of DEGs between ANL and AL skin before and after NB-UVB phototherapy determined by using the criteria of fold change of greater than 2 and false discovery rate of less than 0.1. A complete list of DEGs with respective fold changes and false discovery rates are presented in Table E4.

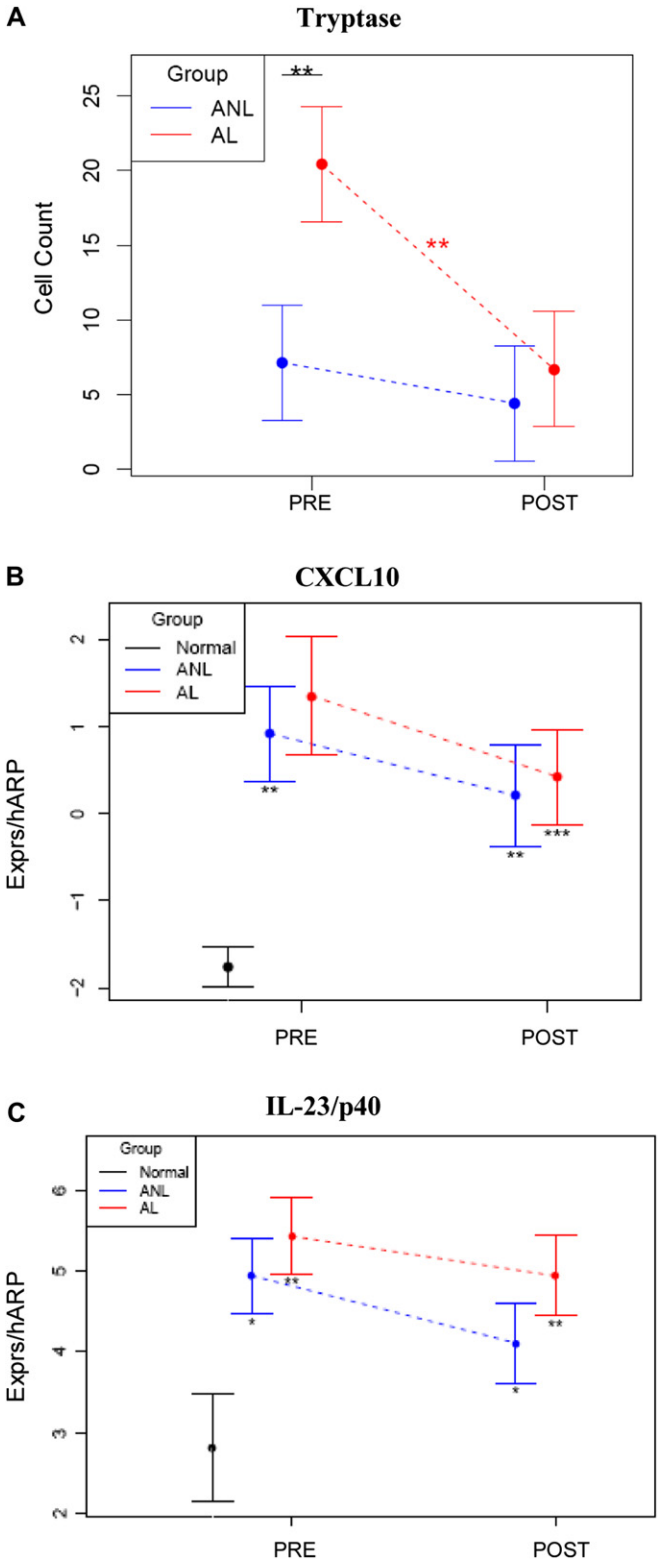

FIG E2. Quantification of tryptase-positive mast cells by means of IHC **(A)** and expression of the keratinocyte-produced chemokine CXCL10 **(B)** and the cytokine IL-23p40 **(C)** by means of RT-PCR in AL *(red lines)* and ANL *(blue lines)* skin before and after NB-UVB phototherapy (*asterisks above lines* = significance of the change). *Black asterisks* below error bars indicate the statistical significance of the difference in mean expression compared with healthy skin. $*P < .05$, $**P < .01$, and $***P < .001$.