

Atopic dermatitis displays stable and dynamic skin transcriptome signatures

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Background: Skin transcriptome studies in atopic dermatitis (AD) showed broad dysregulation as well as “improvement” under therapy. These observations were mainly made in trials and based on microarray data.

Objectives: Our aim was to explore the skin transcriptome and the impact of systemic treatment in patients of the TREATgermany registry.

Methods: Biopsy specimens from 59 patients with moderate-to-severe AD before and 30 patients 12 weeks after start of systemic treatment (dupilumab [n = 22] or cyclosporine [n = 8]) and from 31 healthy controls were subjected to mRNA sequencing. Differential expression, pathway enrichment, correlation, and coexpression network analysis were conducted.

Results: Both lesional and nonlesional skin showed a stable “core” signature characterized by disturbed epidermal differentiation and activation of IL-31/IL-1 signaling. A second dynamic signature showed progressive enrichment for type 2 inflammation, T_H17 signaling, and natural killer cell function. Markers correlated with disease activity have functions in epidermal barrier properties and immune modulation. *IL4RA* was among the top 3 central dysregulated genes. Cyclosporine led to a more pronounced global transcriptome reversion and normalized T_H17 cell/IL23

signaling, whereas dupilumab led to a stronger increase in level of epidermal differentiation markers. Both treatments strongly decreased levels of type 2 markers, but overall the residual profile was still profoundly different from that of healthy skin. Lower levels of *IL4RA* and *IL13* and high *IL36A* expression were related to a stronger clinical response to dupilumab.

Conclusion: The AD core signature is characterized by dysregulation of genes related to keratinocyte differentiation and itch signaling. A dynamic signature reflects progressive immune responses dominated by type 2 cytokines with an additional role of T_H17 and natural killer cell signaling. (J Allergy Clin Immunol 2020;■■■■:■■■-■■■.)

Key words: Atopic dermatitis, transcriptome, RNA-sequencing, gene expression, dupilumab, cyclosporine, type 2 inflammation, residual signatures

Atopic dermatitis (AD) is the most common chronic inflammatory skin disorder, affecting up to 20% of children and 10% of adults.¹ Its pathophysiology results from a complex interaction of genetic and environmental factors leading to epidermal dysfunction and cutaneous inflammation, which is driven by excessive

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Abbreviations used

AL:	Lesional AD skin
AN:	Nonlesional AD skin
NN:	Healthy skin
DEG:	Differentially expressed gene
EASI:	Eczema Area and Severity Index
FDR:	False discovery rate
LFC:	Log ₂ fold change
NK:	Natural killer
oSCORAD:	Objective Scoring of AD
POEM:	Patient-Oriented Eczema Measure
RNA-Seq:	RNA sequencing

T-cell activation and an increased production of inflammatory cytokines.²

Microarray and quantitative RT-PCR studies on skin specimens have revealed a large number of dysregulated genes and pathways, in particular in lesions such as type 2 cytokines and chemokines (such as IL-13, IL-31, CCL17, CCL18, CCL22, and CCL26), epidermal differentiation and proliferation markers (such as KRT16, FLG, LOR), and T_H17/T_H22 cell activity-related genes (such as IL-17A, IL-22, IL-23A/p19, IL-12B/p40, CCL20, DEFB4A, and STAT3).³⁻⁵ A recent meta-analysis also identified lipid abnormalities (FA2H, FAR2, and ELOVL3) and their correlation with T_H2 cell immune activation in lesional skin (AL) versus nonlesional skin (AN).⁶ Several clinical trials have indicated that systemic treatment with biologics and immunosuppressants modifies cutaneous gene expression and that clinical improvements are associated with a shift in expression levels of many of the genes dysregulated in marker lesions toward the levels observed in AN skin of patients with AD.⁷⁻¹¹ Most of these analyses were done in the context of clinical trials and their specific settings, were based on microarray profiling, and so far lack independent replication. Here, we have set out to study the cutaneous AD transcriptome and its changes under systemic therapy in a routine care patient population from the German TREATgermany registry by using RNA sequencing (RNA-Seq) as a powerful alternative to microarrays, thus providing higher precision and accuracy.¹²⁻¹⁷ To date, there are only few AD transcriptome studies based on RNA-Seq data.^{5,18,19} We have also included healthy controls to distinguish between inflammation-related signatures and basal AD signatures.

TREATgermany is a real-world academia-led disease registry that longitudinally collects clinical and lifestyle data from adult patients with moderate-to-severe AD at more than 30 study centers in Germany.²⁰ Biosamples were collected at baseline and after 24 months, as well as before and 3 months after initiation of a systemic treatment.

METHODS

Patient characteristics and skin samples

This study included adult patients with moderate-to-severe AD from the German TREATgermany registry²⁰; they were recruited between July 2017 and February 2019 at 6 of the 17 study centres that agreed to participate in the additional and optional bioanalytics module. Intrapersonal AL and AN biopsy specimens (4 mm) were collected from 59 patients before the initiation of systemic therapy under a protocol approved by the Medical Faculty of the Christian-Albrechts-University, Kiel, Germany (B 261/16), and the responsible local ethics committees at the other participating sites. Of those patients,

34 and 11 received anti-IL-4R α dupilumab and cyclosporine, respectively; follow-up biopsy specimens at month 3 were available from 22 and 8 of those patients at the time when molecular analysis was conducted. AN samples were taken at least 5 cm from the active lesion. Biopsy specimens were preferably collected from the upper extremities, otherwise from the trunk. In addition, RNA from biopsy specimens of 31 healthy volunteers with no history of atopic or chronic inflammatory disease who were included in a previous skin transcriptome study⁵ under an institutional review board-approved protocol (A 110/12) was available for this study. Biopsy specimens from healthy controls had been taken from the upper extremities. Disease activity was evaluated by using the following assessment instruments: Eczema Area and Severity Index (EASI), Investigator's Global Assessment (IGA), objective Scoring of AD (oSCORAD), and Patient-Oriented Eczema Measure (POEM). Response to therapy was defined as achieving at least a 50% improvement in EASI, and high response was defined as achieving at least a 75% improvement of EASI plus an EASI of 7 or lower.

RNA-Seq

RNA later-fixed skin tissue samples were disrupted by using innuSPEED Lysis Tubes W (1.4- to 1.6-mm steel beads and 3.5-mm ceramic beads) (Analytik Jena, Jena, Germany) in a SpeedMill Plus (3 times at 1-minute intervals) (Analytik Jena) in Lysis/Binding Buffer (Life Technologies, Carlsbad, Calif). Total RNA was isolated by using the mirVanaTM miRNA Isolation Kit (Life Technologies) following the manufacturers specifications with an additional on-column DNase treatment. Quality control of concentration and integrity of the isolated RNA was performed with the Qubit 2.0 Fluorometer (Qubit RNA HS Assay [Life Technologies]) and the 2200 Tape Station (R6K ScreenTape Assay [Agilent, Santa Clara, Calif]) following the manufacturer's instructions. RNA libraries were prepared by using the Illumina Truseq Stranded mRNA protocol including poly-A enrichment. Libraries were sequenced on the HiSeq4000 with 2 × 75 bp, producing paired-end reads according to the manufacturer's protocol (Illumina, San Diego, Calif). Raw mRNA sequencing data were processed by using Cutadapt (version 1.15),²¹ Tophat2 (version 2.1.1)²² together with Bowtie 2 (version 2.3.2),²³ Samtools (version 1.5),²⁴ and HTSeq (version 0.10.0),²⁵ and annotated according to the Gencode version 27 annotation gtf file.

Statistical analysis

Technical batch effects and outliers were assessed by using principal component analysis. Differential expression analysis between different conditions was conducted by using the DESeq2 Bioconductor package (version 1.24.0).²⁶ Analysis was performed by using the parametric Wald test and independent filtering of the results. A false discovery rate (FDR) less than 5% was used to declare significance, and genes with an absolute log₂ fold change (LFC) of 1 or more were defined as differentially expressed genes (DEGs). LFC estimates were corrected by using the DESeq2 inbuilt Log₂ Fold Change shrinkage function with the *apecglm*²⁷ method. For descriptive statistics and visualization, R software (version 3.6.1) with various R and Bioconductor packages and normalized or transformed gene counts were used. Pathway analysis was performed by using FunMappOne software²⁸ (for more details, see the Methods section in the [Online Repository at www.jacionline.org](http://www.jacionline.org)). Coexpression network analysis was performed using INFORM.²⁹

RESULTS

After quality control, full data sets were available for 57 AL and 54 AN pretreatment transcriptomes, as well as for 30 AL and 27 AN posttreatment transcriptomes. In all, 31 healthy skin (NN) (ie, control skin) transcriptomes were available for comparison. Demographics and clinical characteristics of the participants from the TREATgermany and the control cohort are shown in [Table E1](http://www.jacionline.org) (in this article's Online Repository at www.jacionline.org). An average of 25,139, 25,230, and 25,090 genes were expressed in

AL, AN, and NN (control) samples, respectively, whereas there were 985, 5787, and 3739 DEGs (FDR-adjusted $P < .05$ by pairwise Wald test and absolute LFC ≥ 1) between AL and AN, AL and NN, and AN and NN, respectively (see [Tables E2–E4](#) in this article's Online Repository at www.jacionline.org). On a transcriptome-wide level, AL, AN, and NN samples clustered distinctly ([Fig 1, A](#)), whereby the distribution of pairwise distances (each among the NN, AN, and AL samples) showed that the AL specimens in this study were more heterogeneous than were AN from the same patients ($P = 1.487e-10$), and both were significantly more heterogeneous than the NN ($P < 2.2e-16$; $P < 2.2e-16$) (see [Table E5](#) in this article's Online Repository at www.jacionline.org). The top 10 expressed genes across all samples (highest expression in AL, AN, and NN) are known skin/epithelial specific keratins, as well as mitochondrial and ribosomal “housekeeping” genes such as the *keratin* genes *1*, *2*, *5*, *10*, and *14*; the mitochondrial genes *MT-CO1*, *MT-CO3*, *MT-ND4*, and *MT-RNR2*; and the *EEF1A1* gene encoding a protein involved in protein synthesis. Two genes, *DEFB4B* and *CLEC3A*, were detected in AN but not in NN. Both were detected in AL as well, whereby *DEFB4B* showed a distinctly higher expression in AL than in AN. We detected a notable expression of *IL19* and *SLC18A3* in AL, but not in AN and NN (for *IL19*, FDR-adjusted $P = .02$ in AL vs in AN by the paired Wald test; $P = 1.8e-124$ in AL vs NN by the unpaired Wald test).

The AD core transcriptome signature is characterized by disturbed epidermal differentiation and activation of itch pathways

Several genes were dysregulated to a comparable extent in both AL and AN as compared with in NN (“core signature” $AL = AN \neq NN$, based on FDR-adjusted $P < .05$ by the pairwise Wald test). This core signature comprised a total of 7466 genes (see [Table E6](#) in this article's Online Repository at www.jacionline.org), of which 2162 revealed an absolute LFC of 1 or more in AL versus in NN. The core transcriptomic signature included genes involved in skin homeostasis, such as *KRT7* (upregulated), occurring in basal and suprabasal layers as well as in sebaceous glands; *KRT10*, a marker for early keratinocyte differentiation in suprabasal layers of the epidermis; genes involved in lipid metabolism such as *ELOVL1* (downregulated) and *FABP3*, *FABP4*, and *FABP9* (all upregulated); and the kallikrein genes *KLK5*, *KLK9*, *KLK11*, and *KLK14* (all downregulated), encoding proteases. Together, these observations indicate that a disturbance of epidermal homeostasis is a general feature of AD ([Fig 1, B](#)). Further, several interleukin receptor signaling complexes showed a dysregulation in both AL and AN (eg, upregulation of IL-1R1 along with a slight increase in IL-1B level and a decrease in level of the IL-1 antagonist IL-1RN). Likewise, the level of IL-31RA was upregulated in both AL and AN (along with a gradual [$AL > AN > NN$] increase of the receptor partner oncostatin M receptor [OSMR] and a slight increase of IL-31 level in AL, but not in AN). Further, both AL and AN showed an increased expression of JAK1 and STAT5B, which encode downstream molecules of the IL31RA/OSMR receptor complex. We identified *IL12A/p35* among the core gene set, indicating elevated T_H1 cell differentiation signaling in AD. In contrast, *IL12B/p40* and the receptor subunit genes *IL12RB1* and *IL12RB2* showed a stronger upregulation in AL skin than in AN skin, indicating progressive upregulation of the IL-23 receptor signaling complex, where *IL12B/p40*

(besides *IL23A/p19*) encodes a necessary ligand for T_H17 cell signal transduction. Further genes that showed dysregulation to a comparable extent in both AL and AN include *IL6ST/CD130*, *TSLP*, and *IL1RL2* (upregulated), and *NFKBIL1* (downregulated). Together, these observations indicate that the transcriptome in AD shows a generally disturbed expression of genes mainly related to keratinocyte differentiation and skin barrier function, as well as to itch signaling.

The AD dynamic transcriptome signature is characterized by progressive activation of type 2 and T_H17 cell signaling

A more progressive dysregulation was observed for 4713 genes (“dynamic signature”; $AL > AN > NN$ or $AL < AN < NN$, according to FDR-adjusted $P < .05$ by pairwise Wald test) (see [Table E7](#) in this article's Online Repository at www.jacionline.org), of which 2603 genes revealed an absolute LFC of 1 or more in AL versus NN.

A progressive upregulation ($AL > AN > NN$) was seen for classical type 2 genes such as *IL13*, *IL22*, *CCL13*, *CCL17*, *CCL18*, *CCL22*, and *CCL26*, but also T_H1 and T_H17 cell genes, and genes involved in natural killer (NK) cell function, such as granzyme B (*GZMB*), perforin (*PRF1*), *KLRD1/CD94*, *FCGR3A/CD16A*, *CD226/DNAM-1*, *CRTAM*, and *PRDM1* ([Fig 1, C](#)). Along with this, there was a gradual downregulation of several structural genes related to epidermal barrier function, such as *LOR* (*LOR* being the main protein component of the cornified envelope), *FLG* and *KRT1* (genes of the LCE group 1), and *ELOVL3* and *ELOVL6* (enzymes important for skin lipid homeostasis),³⁰ whereas the expression of *LCE3* genes (which have antimicrobial activity³¹) showed an upregulation ([Fig 1, C](#)).

On the pathway level, this was reflected in a gradual upregulation of immune system pathways such as activation of cytokine and chemokine signaling, cell adhesion, T_H1 and T_H2 cell differentiation pathways; TNF, NF- κ B, and PI3K-Akt signal transduction pathways; and signatures associated with other immune-mediated diseases such as asthma and rheumatoid arthritis, as well as infectious diseases ([Fig 1, D](#)). In contrast, the AD core transcriptome signature was not enriched for any pathway directly related to immune system but was instead characterized by an upregulation of several signal transduction pathways related to cell proliferation, remodeling, trafficking, and junction formation, pathways related to the nervous system such as glutamatergic, γ -aminobutyric acid-ergic, and cholinergic synapse, as well as neuroactive ligand-receptor interaction pathways ([Fig 1, D](#)).

Of note, the most discriminating feature (with regard to gene expression fold change) between AL and AN was the upregulation of IL17 signaling. Although some genes related to NK cell function were part of the core signature (*CD56/NCAM1* and *Nkp80/KLRF1*), a much more pronounced and progressive upregulation of markers related to NK cell function was seen in lesional skin, and a gene set enrichment analysis by using differences (LFC) in all genes covered by sequencing between AL and AN (see [Table E8](#) in this article's Online Repository at www.jacionline.org) revealed the natural killer NK cell-mediated cytotoxicity pathway as 1 of the top enriched upregulated pathways, along with the IL-17 signaling pathway and T_H17 cell differentiation ([Fig 2, A](#)), indicating that genes involved in those pathways separate AL from AN. Unsupervised hierarchic

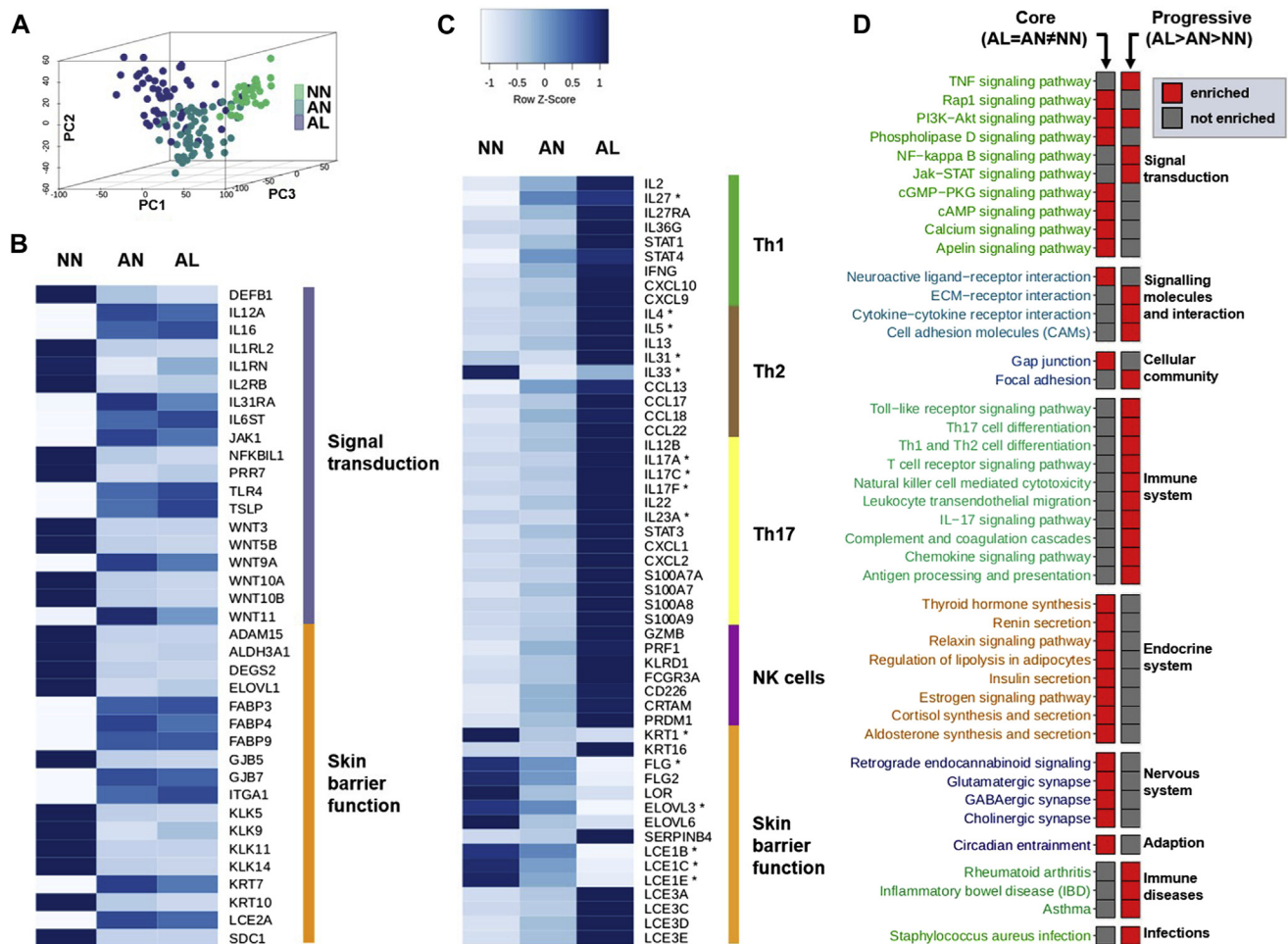


FIG 1. AD skin transcriptome signatures. **A**, First 3 principal components based on all measured genes (excluding genes on the X chromosome and Y chromosome). **B**, Heatmap of NN, AN, and AL mean expression values of selected genes of the AD core signature. Color code represents row z scores. **C**, Heatmap of NN, AN, and AL mean expression values of selected genes of the AD dynamic signature. Color code represents row z scores. *Genes show progressive dysregulation but do not fulfil predefined criteria of dynamic signature. **D**, Pathway enrichment analysis of genes of the AD core transcriptomic signature (AL = AN ≠ NN, based on FDR-adjusted $P < .05$ by the pairwise Wald test plus absolute LFC ≥ 1 in AL vs in NN) and progressively dysregulated genes (AL > AN > NN or AL < AN < NN, based on FDR-adjusted $P < .05$ by pairwise Wald test plus absolute LFC ≥ 1 in AL vs in NN). All significantly enriched pathways were upregulated.

clustering based on genes related to NK cell function indeed separated NN skin from AN and AL almost perfectly, and AN from AL to a reasonable extent (Fig 2, B).

Because AD is known to be dominated by type 2 mechanisms, we specifically examined the expression pattern of key type 2 markers such as IL-4, IL-5, IL-9, IL-13, TSLP, and CCL17/TARC and their correlation with each other and with markers of epidermal barrier function (see Table E9 in this article's Online Repository at www.jacionline.org). IL-13, TSLP, and CCL17/TARC were expressed at median levels of 10, 35, and 203 detected gene transcripts in AL and 3, 30, and 38 transcripts in AN, respectively, whereas IL-4, IL-5, and IL-9 were virtually absent in both AL and AN. Lesional expression levels of IL-13 correlated strongly and positively with other type 2 markers such as IL-22 (Spearman rho [p] = 0.79) CCL17/TARC (p = 0.77), and CCL22 (p = 0.77), and negatively with genes related to epidermal differentiation such as *FLG* (p = -0.53), *LOR*

(p = -0.43), and *KLK5* (p = -0.37), *KRT1* (p = -0.29), and *KRT10* (p = -0.35) (see Fig E1 in this article's Online Repository at www.jacionline.org). Additional genes showing strong positive correlation with *IL13* were *MMP12* (p = 0.85), an enzyme involved in tissue remodeling and skin fibrosis,³² *ALOX15* (p = 0.80), which contributes to corneocyte lipid envelope formation and affects the differentiation and function of dendritic cells,³³ and *IL4I1* (p = 0.81), which has antibacterial and immunomodulatory properties.³⁴ There was also a positive correlation of *IL13*/*IL4* receptor genes with type 2 markers, albeit of moderate strength, and a negative correlation with epidermal differentiation markers. Of note, *IL4RA* in particular showed a strong positive correlation with established AD marker genes expressed by keratinocytes such as the S100A genes *KRT16*, *KRT6A*, and *SERPINB4* (mean p = 0.76) and a strong negative correlation with *LOR* (p = -0.72), whereby type 2 markers IL-13, CCL17, and CCL22 showed a distinctly weaker correlation, indicating a

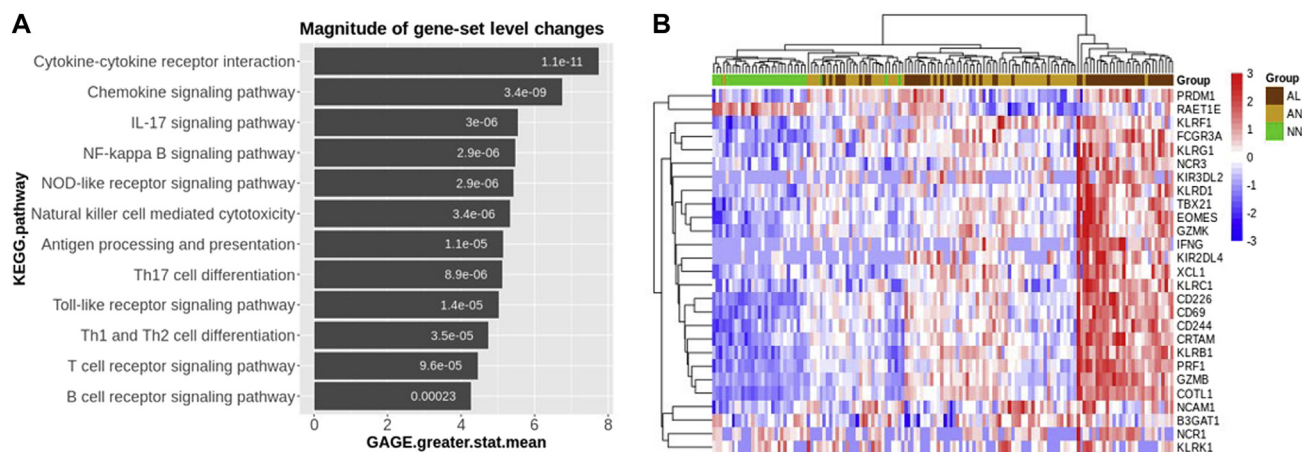


FIG 2. AD skin transcriptomic NK cell signature. **A**, Magnitude of gene set–level changes in AL vs in AN. The x-axis shows the greater statistical mean value, and white numbers show the *q* values from the gene set enrichment analysis (performed with Generally Applicable Gene Set Enrichment [GAGE]) for top enriched pathways. **B**, Unsupervised hierarchic clustering of AL, AN, and NN samples based on genes related to NK cell function. Color code represents row z score, which was restricted to values from -3 to 3.

strong link of *IL4RA* with markers of disturbed epidermal conditions.

To relate transcript levels to disease activity (as determined by EASI, oSCORAD, and POEM), we correlated AL and AN gene expression with AD severity measures. Generally, AL expression levels were more strongly correlated with severity than AN expression levels were; thus, only correlation in AL is reported (see Table E10 in this article's Online Repository at www.jacionline.org). We observed moderate correlations of EASI with genes related to cell junction and adhesion; water and urea transport across cell membranes; innate immune system; and signaling pathways involved in itch, such as aryl hydrogen receptor (AHR), CST6, aquaporin 3 (AQP3), IL36A, and OSMR ($\rho = 0.52, -0.5, 0.47, 0.46$, and 0.46 , respectively) (Fig 3, A). The POEM score yielded multiple additional correlations (eg, with tight junction protein 2 [TJP2] [$\rho = 0.58$], protocadherin 7 [PCDH7] [$\rho = 0.57$], NPEPPS [$\rho = 0.56$], apolipoprotein E [APOE] [$\rho = -0.53$], and MALT1 [$\rho = 0.52$]) that were significant after FDR correction (Fig 3, B). The genes most strongly correlated with disease activity (EASI) did not correlate strongly with type 2 marker genes (eg, although associated with AD *per se*, type 2 markers such as IL-13, IL-22, and CCL17/TARC overall showed only weak correlations with severity scores). In contrast, moderate-to-strong correlations of the genes most strongly correlated with EASI were seen with T_H17 cell/IL-23 markers. The strongest correlation was observed between PI3 and IL-36A ($\rho = 0.71$), and between IL-23A and IL-36A ($\rho = 0.64$). A correlation analysis of transcript levels in AL with itch intensity as determined by numerical rating scale (NRS) did not reveal any striking correlations (see Table E10). Of note, neither of the genes most strongly correlated with the EASI or POEM showed marked correlations with itch.

The *IL4RA* gene is a central gene in the lesional AD transcriptome network

Coexpression network analysis was performed to relate DEGs in AL versus in AN ($n = 985$ DEGs [see Table E2]) with each

other. After the network had been computed, genes were ranked by the combined influence of network centrality properties and differential expression scores (see Table E11 in this article's Online Repository at www.jacionline.org). According to our analysis, *SH2D2A* encoding a T-cell-specific adapter protein involved in T-cell activation,³⁵ *IL4RA* encoding the IL4R α -chain, and *TYMP* encoding an angiogenic factor attracting and promoting the growth of endothelial cells³⁶ were the top 3 central genes of transcriptome dysregulation in AL. Notably, the consensus network allocated a central role to *IL4RA*, but not to those genes encoding its receptor partners (*IL13RA1* and *IL2RG*) or to its ligand IL13. Other highly ranked genes included cathepsin C (*CTSC*), histamine receptor H2 *HRH2*), *FOXP3*, *KRT6A*, cadherin 3 (*CDH3*), and granzyme B (*GZMB*). We correlated the top 50 central genes with 8 specific target proteins (IL-4RA, IL-13, IL-22, IL-31, IL-33, TSLP, OX40, and OX40L) that are targets of mAbs under development as AD therapies (Fig 4). The transcription levels of *IL4RA* on average showed the strongest correlation with central genes (mean $\rho = 0.51$), which is explained by the fact that *IL4RA* itself was a top central gene, indicating that blocking *IL4RA* affects the expression of top central dysregulated genes in AD lesions (Fig 4). We observed weaker correlations for *IL13* (mean $\rho = 0.45$), *IL22* (mean $\rho = 0.44$), *OX40/TNFRSF4* (mean $\rho = 0.43$), *IL31* (mean $\rho = 0.32$), and *OX40L/TNFRSF4* (mean $\rho = 0.31$), and very weak correlations for *IL33* (mean $\rho = 0.15$) and *TSLP* (mean $\rho = 0.09$).

Dupilumab treatment leads to a stronger normalization of skin barrier-related genes, whereas cyclosporine treatment has more pronounced effects on T_H17 cell/IL-23 signaling in lesional skin

The mean reduction in EASI was 75% in patients treated with dupilumab and 48% in those treated with cyclosporine ($P = .02$ [see Tables E12 and E13 in this article's Online Repository at www.jacionline.org]). In all, 86.4% and 62.5% of the patients treated with dupilumab and cyclosporine, respectively, reached

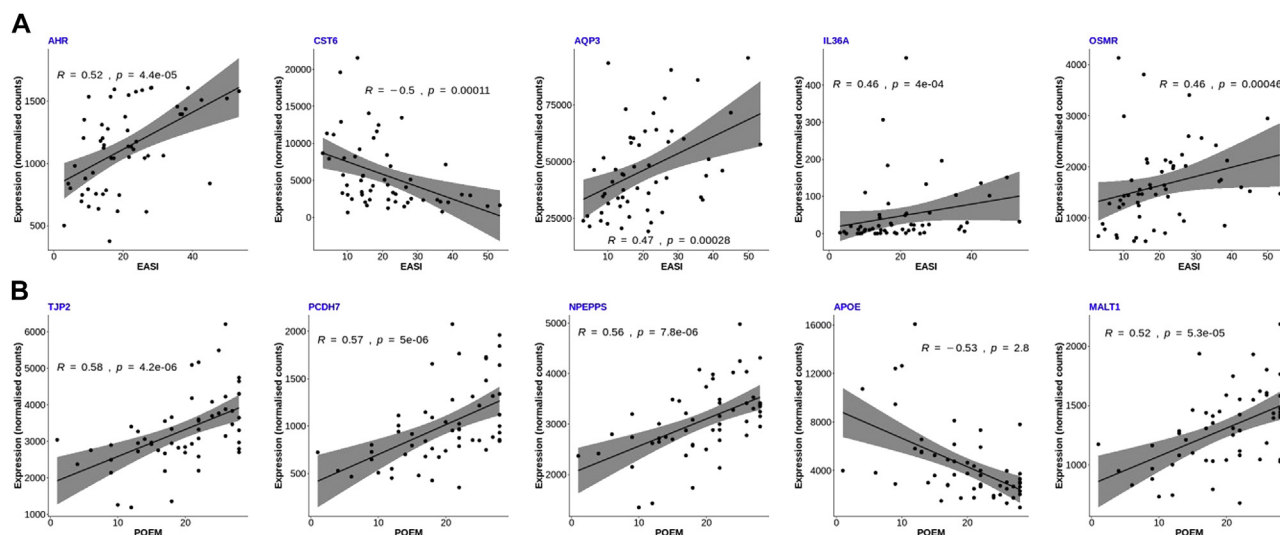


FIG 3. Gene expression associated with AD disease severity scores. **A**, Top Spearman correlations of pretreatment AL gene expression with the EASI. **B**, Top Spearman correlations of pretreatment AL gene expression with POEM.

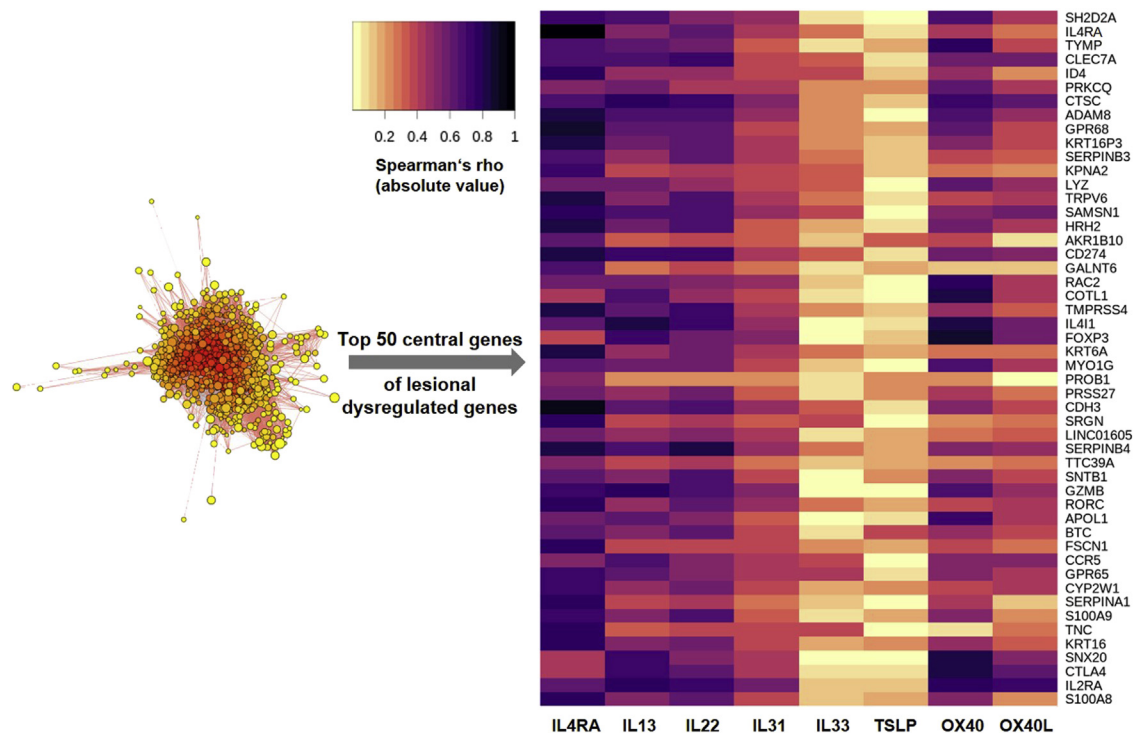


FIG 4. Coexpression network and its relation to (potential) therapeutic targets. *Left*, INfORM coexpression network with DEGs in AL and AN. Nodes are colored by rank from high rank (red) to low rank (yellow). The genes are ranked by the combined influence of network centrality properties and differential expression scores. *Right*, Top 50 central genes from the network analysis and their correlation with genes that can be blocked by targeted treatments and were tested in clinical trials of AD. Color code of the heatmap represents Spearman ρ (absolute value).

at least a 50% reduction in EASI at month 3. There were no marked differences in the pretreatment transcriptome profiles between groups (see Fig E2 in this article's Online Repository at www.jacionline.org). Despite the lower overall clinical efficacy, on a global level the shift of the AL transcriptome toward an

AN-like transcriptome was significantly more pronounced under cyclosporine treatment (a 65% mean reduction in expression fold change between AL and AN) than under dupilumab treatment (a 43% mean reduction) (for upregulated genes, $P < 2.2 \times 10^{-16}$; for downregulated genes, $P = 7.2 \times 10^{-5}$) (Fig 5, A). In the coexpression

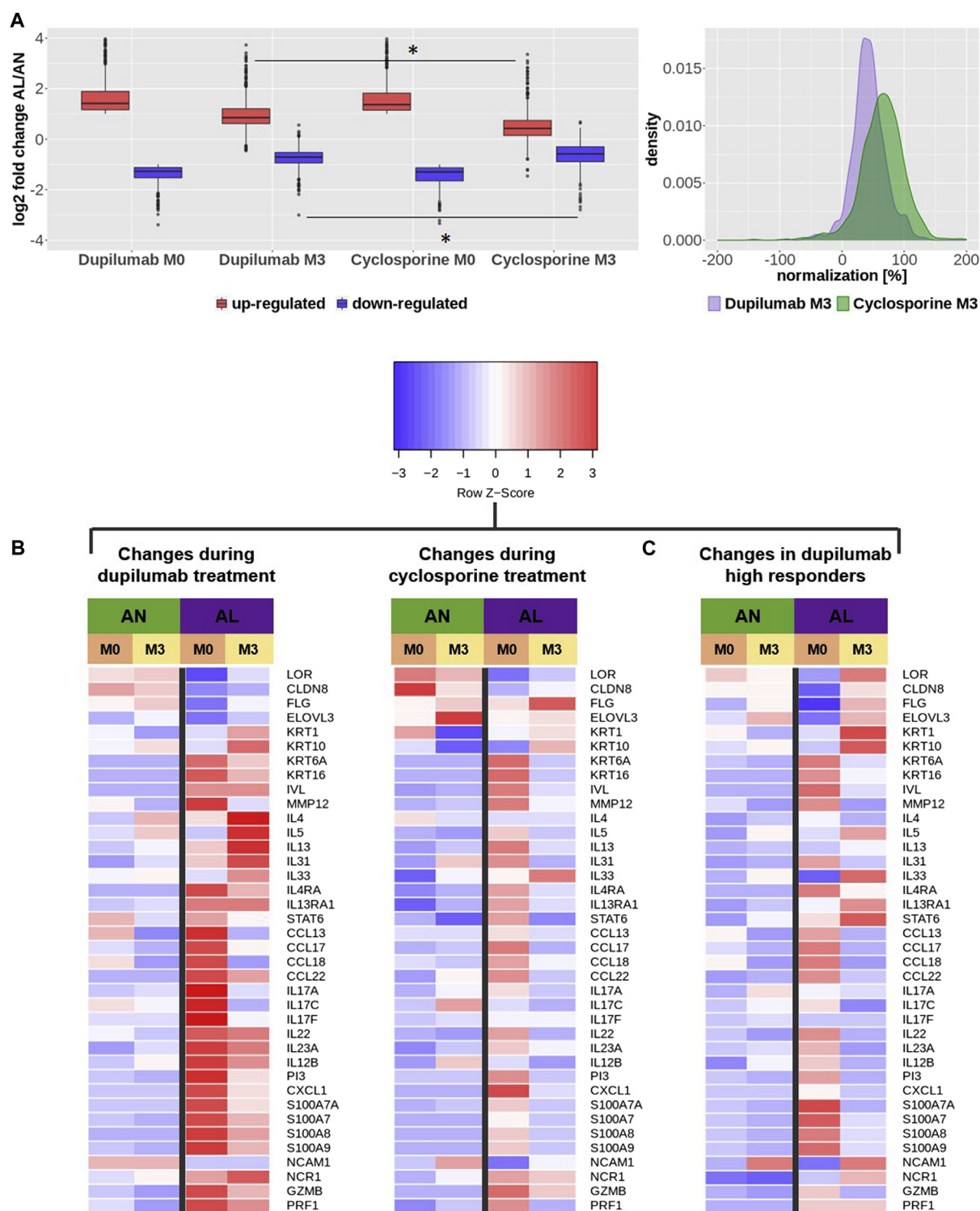


FIG 5. Transcriptomic changes under therapy with dupilumab and cyclosporine. **A, Left,** Shift of LFCs of DEGs in AL and AN (with absolute LFC ≥ 1) from baseline (month 0 [M0]) to month 3 (M3) in patients treated with dupilumab and cyclosporine, respectively. The upregulated and downregulated genes in AL compared with in AN are depicted in red and blue, respectively. * $P < .05$ by Wilcoxon test. **Right,** The distribution of normalization of AL gene expression toward AN expression levels. A 100% normalization means that the expression fold change between AL and AN after 3 months of therapy was 0. **B,** Heatmaps showing mean expression values (mean normalized counts) of selected AD-related genes in patients treated with dupilumab or cyclosporine, grouped according to time point and treatment. Rows are ordered by annotations of genes to functions/immune axes. **C,** Heatmap showing the same as in **(B)** for high responders to dupilumab only ($\geq 75\%$ improvement in EASI and EASI ≤ 7 ; $n = 7$ patients). Color code represents row z score above all 3 heatmaps together.

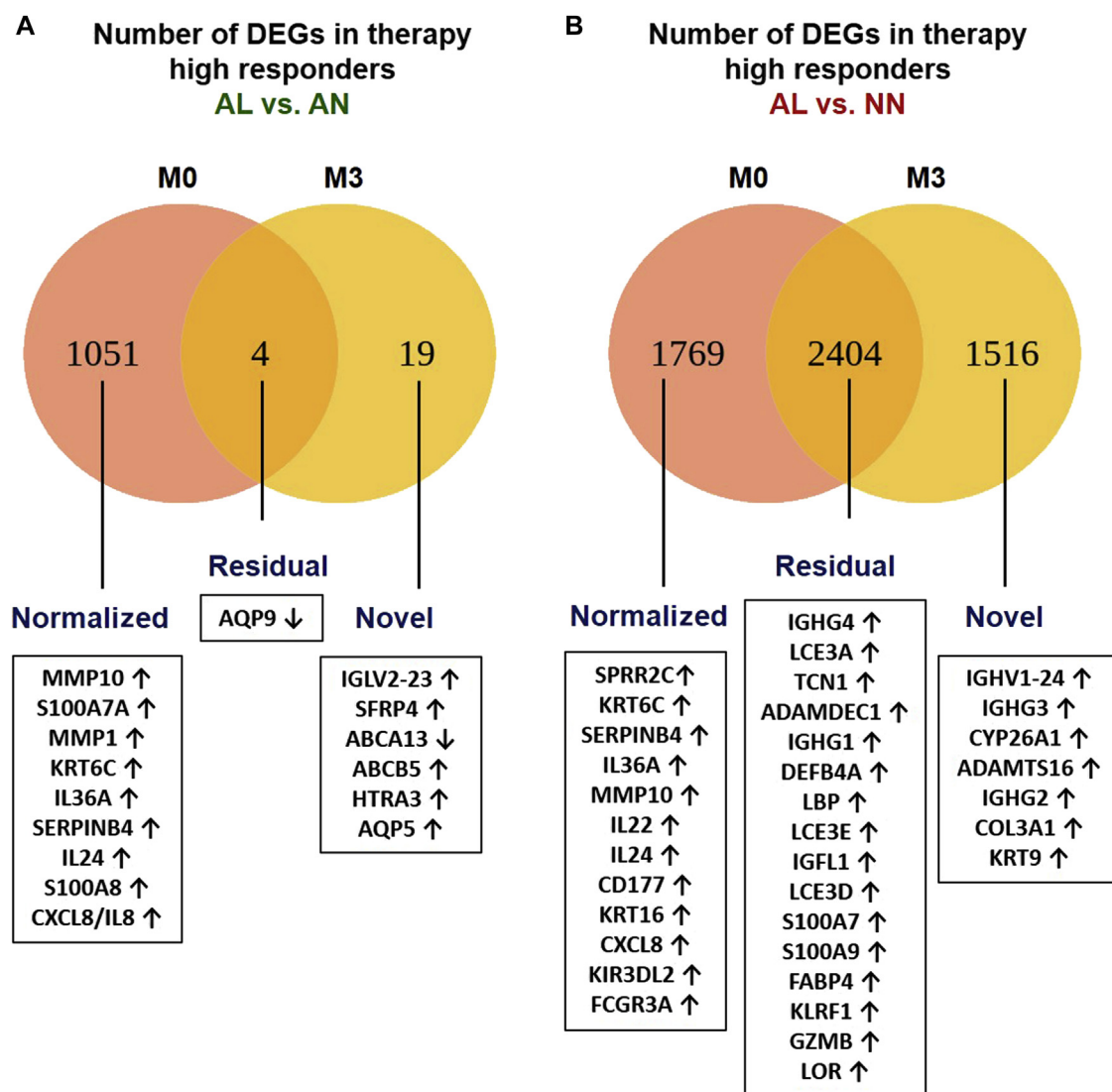


FIG 6. Reverted and residual genomic profile in responders to dupilumab. **A**, The number of DEGs in AL and AN before (month 0 [M0] [orange]) and after 3 months of treatment (M3) (yellow), and the overlap of both. All genes with an FDR-adjusted *P* value less than .05 were considered DEGs. **B**, The number of DEGs in AL and NN before (M0 [orange]) and after 3 months of treatment (M3 [yellow]), and the overlap of both. All genes with an FDR-adjusted *P* value less than .05 and an absolute LFC of 1 or more were considered DEGs.

network analysis, the levels of the top 50 central genes changed by 50% in patients treated with cyclosporine and by 34% in patients treated with dupilumab (see Fig E3 in this article's Online Repository at www.jacionline.org).

Treatment with dupilumab in particular decreased the expression of type 2-related chemokines such as CCL13, CCL17, CCL18, and CCL22 and had a strong effect on normalization of skin barrier function-related markers such as LOR, CLDN8, FLG, KRT1, KRT10, and in particular ELOVL3 (533%), whereas the expression levels of type 2 cytokines such as IL-4, IL-5, IL-13, and IL-31 showed an increase (Fig 5, B and see Table E14 in this article's Online Repository at www.jacionline.org). In general, expression changes correlated with the degree of clinical response and were most pronounced in "high responders" (Fig 5, C and see Table E14). Interestingly, patients with a particularly strong response to dupilumab (EASI75 plus EASI ≤ 7) had lower baseline

IL-4RA and IL-13 expression, a more pronounced decrease in IL-4RA expression, and less pronounced increases in IL-13 expression (Fig 5, B and C and see Table E14). Further, these patients showed a distinct increase (41%) in level of general NK cell markers such as CD56/NCAM1 and Nkp46, whereas levels of NK cell activity markers (granzymes, perforin/PRF1, TRAIL/TNFSF10, FAS, FASLG, and granzysin/GNLY) showed no marked changes (see Table E14).

Treatment with cyclosporine was also associated with a strong decrease in levels of key type 2 markers, including IL-13, IL-4RA, IL-13RA1, and IL-31, and a stronger reduction in levels of TH17 cell/IL23 signaling markers such as IL-22, IL-23A/p19, IL-12B/p40, and PI3/elafin, as well as in levels of S100A8, and S100A9 (Fig 5, B [see Table E14]). The level of expression of general NK cell markers did not show marked changes, but there was a decrease in level of NK cell activity markers by 24%. To

work out discriminating features in terms of expression changes induced by treatment, we examined genes that showed a difference of at least 30% between dupilumab and cyclosporine in terms of improvement as compared to healthy controls (see Table E15 in this article's Online Repository at www.jacionline.org). Overall, a total of 313 genes showed a stronger improvement in response to cyclosporine. These genes have annotations mainly related to immune responses, T-cell response, and cytokine and chemokine signaling (eg, *IL2RA*, *IL9R*, *IL12B*, *IL26*, *CXCL3*, *CXCL5*, *CXCL8*; *CXCR1* and *CD80*; and *CTLA4* and *OX40-R/OX40*), as well as to keratinocyte differentiation (eg, *KRT16*, *KRT6A*, *PI3*, and *LCE3A*). A total of 58 genes showed a considerably stronger improvement with dupilumab; these genes were mainly related to leukocyte recruitment (eg, *CCL3* and *CCL4*), linoleic acid metabolism (eg, *ALOX15* and *CYP1A2*), and regulation of T-cell responses (eg, *TNFSF18* and *TNFRSF13B*). To identify baseline signatures associated with the degree of clinical response, we compared AL of patients achieving the greatest reduction of EASI (upper quartile) with patients showing the least reduction of EASI (lower quartile). The top gene (according to fold change and *P* value) discriminating high from low responders was *IL36A*, which revealed a 5 times higher level of expression in high responders and decreased to levels seen in NN skin after 3 months of treatment (see Fig E4 and Table E16 in this article's Online Repository at www.jacionline.org). To determine the residual disease genomic profile ("molecular scar") in target lesions, we compared dysregulated genes before and 3 months after treatment, whereby we discriminated the residual profile as compared with those of AN and NN, respectively. In patients treated with cyclosporine, less than 1% and 53% of dysregulated genes remained significantly dysregulated whereas 38% and 73% of dysregulated genes remained dysregulated after 3 months of dupilumab treatment. When the analysis was restricted to high responders to dupilumab, of the 1055 genes differentially regulated between AL and AN at baseline, only 1 (skin-relevant) gene, namely AQP9 (aquaporin 9), was still characterized by significant (albeit improved) downregulation after 3 months of treatment (Fig 6, A). Of the 4173 genes differentially regulated at baseline between AL and NN, 2404 (58%) were still dysregulated after treatment (Fig 6, B). Thus, in patients achieving a high response to dupilumab, the transcriptome in lesions resembled the transcriptome in AN, but still showed large differences compared to NN. Notably, 41% of the AD core signature and 54% of the dynamic signature remained largely unchanged after 3 months of therapy. Interestingly, AN and AL may respond differently to treatment. Although T_H1-, T_H2-, and T_H17 cell-related genes mostly showed a decrease in AL during both dupilumab and cyclosporine treatment, some markers, such as *CCL22*, *IL17A*, *IL23A*, and *IL12B*, were slightly upregulated in AN (Fig 5, B and see Table E14). In contrast, in both AL and AN, *IL33* expression increased strongly under both treatments.

DISCUSSION

We provide evidence for a "core" AD gene expression profile found in both AL and AN, which is characterized by an activation of pathways related to epidermal barrier dysfunction and itch signaling. Of note, the AD core signature is not enriched for pathways directly related to immune response and inflammation. We further observed a progressive upregulation of immune responses, in particular, type 2, T_H17, and NK cell signaling

with inflammation ("dynamic" signature), along with a complex discoordinated expression of genes important for epidermal differentiation and skin lipid metabolism. In line with the findings of our previous RNA-Seq study on AD,⁵ the type 2 phenotype in AD skin is largely attributable to IL-13, whereas IL-4 and IL-5 are virtually absent. Interestingly, activation of IL-17 and NK cell signaling as part of the dynamic signature constitute a strong discriminating feature between AL and AN, which is in line with the findings of previous studies suggesting that IL-17 signaling is not a unique feature of psoriatic skin but is also dysregulated in AD,^{4,6,37,38} and with recent reports on an elevated NK cell transcriptional signature in AD skin lesions.³⁹ The NK cell signature observed appears to be derived from both NK1 and NK2 subsets previously defined on the basis of single-cell RNA-seq.⁴⁰ Key type 2 markers, in particular IL-13, were negatively correlated with structural genes related to barrier function and fatty acid metabolism, and positively correlated with each other, supporting previous observations of a close interaction between barrier abnormalities and cutaneous inflammation as well as progressively heightened and type 2-dominated immune signaling.^{4,5,18} Despite their strong correlation with disease status, type 2 markers such as IL-13, IL-22, and CCL17/TARC overall showed only a weak correlation with disease activity. In contrast, markers related to epidermal barrier properties and immune modulation, such as AQP3,⁴¹ TJP2,⁴² CST6,⁴³ AHR,⁴⁴ and IL-36A,⁴⁵ as well as OSMR encoding a subunit of the IL31RA/OSMR receptor complex involved in itch signaling,⁴⁶ showed a stronger correlation. The overall only moderate strength of correlation ($\rho = 0.4$ - 0.6) is in accordance with that in other studies involving correlation analysis of molecular markers with EASI and oSCORAD.^{10,37} Among the top 3 central genes in the lesional AD transcriptome network was *IL4RA*. Its transcription levels on average showed the strongest correlation with other central genes, implying that blocking *IL4RA* might positively influence the expression of top central dysregulated genes, as previous studies demonstrated that the activation level of a biologic signature can be characterized by its overall state of correlation, and that genes showing similar expression patterns may share biologic functions under common regulatory control.⁴⁷⁻⁵⁰ Indeed, in the coexpression network analysis, levels of the top 50 central AD network genes changed by 34% in all patients treated with dupilumab and by 63% in patients showing a high response (defined as $\geq 75\%$ improvement in EASI and absolute EASI ≤ 7 at month 3) to dupilumab. Interestingly, despite lower clinical efficacy, cyclosporine overall normalized the top 50 central genes to an even greater extent (50%). On the basis of our correlation analyses, blockade of IL-13, IL-22, and OX40/TNFRSF4 should also positively affect central dysregulated genes, whereas blockade of IL-31 and OX40L, and in particular IL-33 and TSLP, may have only little effect on the lesional AD transcriptome network.

Both treatment with the IL4RA-blocking antibody dupilumab and the immunosuppressant cyclosporine resulted in a shift of the global AL transcriptome toward a profile seen in AN, an effect that was slightly more pronounced under cyclosporine treatment. Both treatments caused a strong decrease in cutaneous type 2 chemokine expression, as well as an increase in skin barrier-related genes, the latter of which was more pronounced for dupilumab. In addition, cyclosporine caused a decrease in the type 2 signature cytokines IL-4, IL-5, and IL-13, whereas they increased under treatment with dupilumab. This is to be expected,

given that cyclosporine blocks T_H2 cell activation (and therefore cytokine expression), whereas dupilumab blocks IL-4 and IL-13 action. For cyclosporine, stronger effects were observed on DEGs related to the T_H17 cell/IL23 axis, particularly IL-22, IL-12B/p40, and PI3/elafin. Dupilumab showed more specific effects and, for example, caused stronger improvement of ALOX15, which is involved in the cellular response to IL-13 and related to linoleic acid metabolism.^{51,52} In line with that, we observed a high correlation of IL-13 and ALOX15 expression levels in lesions at baseline. Lower baseline IL-4RA and IL-13 levels and high IL-36A expression levels were related to a more pronounced clinical response to dupilumab. Interestingly, in patients with a high response to dupilumab, we observed an increase in NK cell marker expression, whereas the expression of NK cell activity markers remained largely stable, implying effects on a potential counterregulatory mechanism to aberrant type 2 inflammation, which warrants further investigation. Likewise, the increase in IL-33 expression levels under both treatments and in both lesional and nonlesional AD skin deserves further study. Overall, dupilumab and cyclosporine showed largely comparable effects in reverting the dysregulation of the transcriptome in lesions toward the profile seen in nonlesional AD skin. Although not all individual molecular changes were correlated with clinical improvements, the overall degree of changes was correlated with the degree of clinical improvement, indicating that these changes relate to healing of lesions rather than to a specific treatment. However, compared with the residual transcriptomic profile in healthy control skin, the residual transcriptomic profile in target lesions of high responders to dupilumab still showed profound dysregulation of 41% of the DEGs of the AD core signature and 54% of the DEGs of the dynamic signature, indicating that a fraction of the AD-associated transcriptome remains unchanged despite clinical improvement. It should also be noted that the interpretation of molecular changes is not straightforward, and that molecular changes do not necessarily have immediate clinical implications.

Our study has several strengths, but also some limitations. We utilized samples collected in a highly standardized fashion with accompanying deep clinical information from the academia-led prospective TREATgermany registry as well as carefully matched controls, and we applied highly sensitive sequencing and novel analytic approaches with higher sensitivity and versatility compared with those of array-based approaches. However, the sample size in the current analysis is still rather low, which precluded robust responder analyses, and whole biopsy specimens composed of a mixture of cellular components were used rather than single-cell transcriptomics.

In summary, our study provides novel insights into core and dynamic transcriptome signatures in AD, as well as insights into the specific changes associated with targeted treatment with dupilumab compared with the broader immunosuppression with cyclosporine. With growing numbers of patients recruited to TREATgermany and novel drugs becoming available and used, future analyses should allow verification and extension of the preliminary findings and assumptions reported here.

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Key messages

- The skin in AD displays a core transcriptomic signature characterized by a dysregulation of genes driving skin barrier dysfunction and itch signaling, whereas a second, more dynamic signature reflects progressive inflammatory mechanisms such as activation of type 2, T_H17 , and NK cell function.
- Both dupilumab and cyclosporine led to a strong downregulation of type 2 markers and genes involved in T_H17 cell/IL23 signaling.
- After 3 months of therapy, the transcriptome in target lesions resembled the nonlesional transcriptome; however, despite clinical improvement, a profound fraction of the AD-associated transcriptome remained dysregulated as compared with that of healthy skin.

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Supplementary figures

FIG E1. Correlation of type 2 cytokines with other AD-related genes. Heatmap of Spearman correlation of type 2 cytokines IL13, TSLP and CCL22/TARC with other AD-related genes. Colour code denotes Spearman's rho. Rows and columns were ordered by unbiased hierarchical clustering. Dendrogram is shown for columns.

FIG E2. AL-AN global distances for dupilumab and cyclosporine treatment group. Distribution of pre-treatment lesional (AL) - non-lesional (AN) Euclidean distances (in log2 scale) for patients treated with dupilumab and cyclosporine, respectively (only patients for who post-treatment biopsies were available). The distance was computed based on the top three principal components and all measured genes (excluding genes on chrX and chrY). ns p-value >0.05 by Wilcoxon test.

FIG E3. Transcriptomic changes of top central genes under therapy with dupilumab and cyclosporine. Heatmaps showing mean expression values (mean normalised counts) of the top 50 central genes dysregulated in lesional skin grouped according to time point and treatment (dupilumab, cyclosporine, and dupilumab high responders). Rows are ordered by rank of the genes in the co-expression network. Colour code represents row Z-Scores.

FIG E4. Baseline dysregulation related to dupilumab response. Differentially expressed genes in baseline (M0) lesional (AL) skin of dupilumab high responders (patients achieving a reduction of EASI in the upper quartile) and dupilumab low responders (patients achieving a reduction of EASI in the lower quartile). Expression levels of healthy skin, baseline (M0) and dupilumab month 3 (M3) lesional skin are depicted. Error bars show standard error of the mean.

Supplementary tables

TABLE E1. Patients and healthy subject basis characteristics

Characteristic	Patients (n=57)	Controls (n=31)
Female sex, no. (%)	16 (28.1)	17 (54.8)
Age, mean $\pm$ SD	43.6 $\pm$ 15.3	32 $\pm$ 10.6
BMI, mean $\pm$ SD	26.1 $\pm$ 4.3	23.9 $\pm$ 3.8
EASI, mean $\pm$ SD	20.2 $\pm$ 11.7	-
IGA, mean $\pm$ SD	3.7 $\pm$ 0.8	-
POEM, mean $\pm$ SD	19.4 $\pm$ 6.9	-
Age of onset, mean $\pm$ SD (min, max)	8.1 $\pm$ 15.2 (0,77)	-
Duration of AD, mean $\pm$ SD (min, max)	35.2 $\pm$ 16.5 (1,78)	-

NA, Not available. SD, Standard deviation.

TABLE E2. Baseline differentially expressed genes in AL vs. AN

see separate excel file

TABLE E3. Baseline differentially expressed genes in AL vs. NN

see separate excel file

TABLE E4. Baseline differentially expressed genes in AN vs. NN

see separate excel file

Table E5. Euclidean distance between all pairs of samples within AL, AN, and NN skin

see separate excel file

Table E6. Genes of the AD core transcriptomic signature

see separate excel file

Table E7. Genes of the AD dynamic transcriptomic signature

see separate excel file

Table E8. Complete results of the baseline differential expression analysis in AL vs. AN

see separate excel file

TABLE E9. Spearman correlation matrix of type 2 cytokines and AD-related genes

see separate excel file

TABLE E10. Spearman correlation of disease activity scores and baseline AL gene expression

see separate excel file

TABLE E11. Rank table of DEGs from the co-expression network analysis

see separate excel file

TABLE E12. Characteristics of patients with dupilumab treatment (n=22)

Characteristic	Baseline	Month 3
EASI, mean $\pm$ SD	21 $\pm$ 11.5	4.9 $\pm$ 4.3
oSCORAD, mean $\pm$ SD	43.8 $\pm$ 13	20.3 $\pm$ 8.2
IGA, mean $\pm$ SD	3.6 $\pm$ 0.7	1.9 $\pm$ 0.7
% EASI50	-	86.4
% EASI75	-	63.6
% EASI $\leq$ 7	0	81.8

TABLE E13. Characteristics of patients with cyclosporine treatment (n=8)

Characteristic	Baseline	Month 3
EASI, mean $\pm$ SD	21.3 $\pm$ 9.6	9.5 $\pm$ 5.1
oSCORAD, mean $\pm$ SD	47 $\pm$ 7.3	34.1 $\pm$ 7.1
IGA, mean $\pm$ SD	3.9 $\pm$ 0.6	2.5 $\pm$ 0.5
% EASI50	-	62.5
% EASI75	-	25
% EASI $\leq$ 7	0	37.5

TABLE E14. Mean expression values of all genes separated for time point and treatment

see separate excel file

TABLE E15. Gene expression normalisation in AL vs. NN skin after treatment with dupilumab and cyclosporine

see separate excel file

TABLE E16. Baseline differentially expressed genes in AL skin of dupilumab upper vs. lower quartile of EASI reduction

see separate excel file

TABLE E17. Baseline AL vs. AN DEGs in patients treated with dupilumab

see separate excel file

TABLE E18. M3 AL vs. AN DEGs in patients treated with dupilumab

see separate excel file

TABLE E19. Baseline AL vs. NN DEGs in patients treated with dupilumab

see separate excel file

TABLE E20. M3 AL vs. NN DEGs in patients treated with dupilumab

see separate excel file

TABLE E21. Baseline AL vs. AN DEGs in patients treated with cyclosporine

see separate excel file

TABLE E22. M3 AL vs. AN DEGs in patients treated with cyclosporine

see separate excel file

TABLE E23. Baseline AL vs. NN DEGs in patients treated with cyclosporine

see separate excel file

TABLE E24. M3 AL vs. NN DEGs in patients treated with cyclosporine

see separate excel file

TABLE E25. Baseline AL vs. AN DEGs in dupilumab high responders

see separate excel file

TABLE E26. M3 AL vs. AN DEGs in dupilumab high responders

see separate excel file

TABLE E27. Baseline AL vs. NN DEGs in dupilumab high responders

see separate excel file

TABLE E28. M3 AL vs. NN DEGs in dupilumab high responders

see separate excel file

Supplementary methods

RNA sequencing

Isolation of mRNA was performed in batches of 10-12 samples after block randomisation based on the following variables in order to avoid batch effects: lesional skin, non-lesional skin, time point of sampling, individual patient. RNAlater fixed skin tissue samples were disrupted using innuSPEED Lysis Tubes W (1.4 – 1.6 mm steel beads & 3.5 mm ceramic beads) (Analytik Jena, Jena, Germany) in a SpeedMill Plus (3x 1min intervals) (Analytik Jena) in Lysis/Binding Buffer (Lifetechnologies). Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Lifetechnologies, Carlsbad, CA) following the manufacturers specifications with an additional on-column DNase treatment. Quality control on concentration and integrity of the isolated RNA was performed with the Qubit 2.0 Fluorometer (Qubit® RNA HS Assay) (LifeTechnologies, Carlsbad, CA) and the 2200 Tape Station (R6K ScreenTape Assay) (Agilent, Santa Clara, CA) following the manufacturer's instructions. RNA libraries were prepared using the Illumina Truseq® Stranded mRNA protocol including poly-A enrichment. Libraries were sequenced on the HiSeq4000 with 2x75bp, producing paired-end reads according to the manufacturer's protocol (Illumina, San Diego, CA). RNA samples were sequenced in pools of 12 samples per lane, with 8 lanes per flow cell and 1-2 flow cell per sequencing run on the HiSeq4000 machine.

RNA-seq data processing

Low quality reads were filtered from the data using the Illumina CASAVA FASTQ filter. Data quality was assessed using Picard (<http://broadinstitute.github.io/picard>), FastQC (version 0.11.5) (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC¹. Illumina standard adapters were trimmed using Cutadapt (version 1.15)². Paired reads were mapped to the human reference genome (GRCh38, Ensembl release 91) using Tophat2 (version 2.1.1)³ and Bowtie 2 (version 2.3.2)⁴, only uniquely mapped read pairs were retained. Mapped reads were cleaned and sorted using Samtools (version 1.5)⁵. Number of reads for each gene was counted using HTSeq (version 0.10.0)⁶ and annotated according to the Gencode version 27 annotation gtf file. The RNA processing was conducted according to the following parameter settings:

```
#!/bin/bash
```

```
cutadapt \
```

```
-m 35 -q 20 -O 3 \
```

```
-a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC \
```

```
-A AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT \
```

```
-o AD1_R1.cutadapt.fastq -p AD1_R2.cutadapt.fastq \
AD1_R1.filtered.fastq AD1_R2.filtered.fastq
```

tophat2 \

```
--transcriptome-index=tophat_transcriptome_index_Ensembl_release_91 \
--library-type fr-firststrand \
--b2-very-sensitive \
Ensembl_release_91/Homo_sapiens.GRCh38.dna.primary_assembly \
AD1_R1.cutadapt.fastq AD1_R2.cutadapt.fastq
```

```
samtools view -h -F 0x0008 accepted_hits.bam | samtools view -Shb -F 0x0004 - > AD1.cleaned.bam
```

```
samtools sort -n -o AD1.cleaned_sorted.bam AD1.cleaned.bam
```

htseq-count_0.10.0 \

```
-f bam -s reverse -m union -a 20 \
AD1.cleaned_sorted.bam \
gencode.v27.annotation.gtf \
> AD1.htseq \
```

Statistical analysis

Principle component analysis (PCA) was performed based on all measured genes excluding genes from the sex chromosomes to check for technical batch effects due to sequencing run (n=2), technician who isolated the RNA (n=3), and season in which the RNA was isolated (n=4). In order to identify samples that exceptionally vary from the other samples the following approach was used. PCA was performed for all subsets of samples (lesional (AL) and non-lesional (AN), each pre- and post-treatment, and healthy (NN)) based on all genes excluding genes from the sex chromosomes. Samples were defined as outliers if they deviated from the mean by more than three times the standard deviation for each principle component (PC) of those PCs that summed up to 60% of total variance (the first 5-9 PCs). Outliers were excluded from all analyses.

The pairwise Euclidean distances each among NN, AN, and AL samples were computed using the top three principal components. Reported p-values were computed by Wilcoxon test. Differential expression analysis between different conditions was conducted using DESeq2⁷ Bioconductor package (version 1.24.0). Analysis was performed using the

parametric Wald test and independent filtering of the results. A false discovery rate (FDR) <5% was used to declare significance. If not stated otherwise, significant genes with an absolute log2 fold change (LFC) ≥ 1 were defined as differentially expressed genes (DEGs). Log fold change estimates were corrected using DESeq2 inbuilt `lfcshrinkage()` function with the *apecglm*⁸ method in order to improve on the previous effect size estimator. The following R code described the general DESeq2 steps performed (biopsy as exemplary conditional variable):

In case of unpaired tests:

```
> ddsHTSeq = DESeqDataSetFromHTSeqCount(..., design = ~ biopsy)
```

In case of paired tests:

```
> ddsHTSeq = DESeqDataSetFromHTSeqCount(..., design = ~ patient + biopsy)
```

```
> dds = DESeq(ddsHTSeq, betaPrior = FALSE)
```

```
> res = results(dds, independentFiltering = TRUE, alpha = 0.05)
```

```
> resLFC <- lfcShrink(dds, coef="biopsy_AL_vs_AN", type="apecglm", res = res)
```

For descriptive statistics and visualisation, R (version 3.6.1) with various R and Bioconductor packages and normalised (`DESeq2::estimateSizefactors()`) or transformed (`DESeq2::vst()`, also includes normalisation) gene counts were used (see details below).

Differential gene expression was performed for lesional (AL) vs. non-lesional (AN), AL vs. healthy skin (NN), and AN vs. NN samples, whereby for AL vs. AN comparison, a paired test according to patient was performed (DESeq2 design formula: `design = ~ patient + biopsy`). The core signature was defined as genes differentially expressed in the same direction (FDR adjusted p-value <0.05) for AL vs. NN and AN vs. NN without being different (FDR adjusted p-value >0.05) between AL and AN in the same direction. The progressive signature was defined as genes being differentially expressed in the same direction for AL vs. NN, AN vs. NN, and AL vs. AN (FDR adjusted p-value >0.05). For gene enrichment analysis, we considered genes from the core and dynamic signature with an absolute LFC ≥ 1 in AL and NN. Gene enrichment analysis was performed using FunMappOne⁹ software with default settings. Enriched KEGG pathways were displayed on the third level of BRITE functional hierarchy. The input for FunMappOne was provided as Excel file containing two lists of DEGs (the core and the progressive DEG set) with gene modifications (shrunk LFCs according to `DESeq2 lfcshrinkage()` see above). Gene set enrichment analysis was performed based on the LFCs between AL and AN samples of all genes using GAGE (version 2.34.0)¹⁰ Bioconductor package with default settings and the set of KEGG signalling and metabolic pathways (`kg.hsa$sigmet.idx`). Heatmaps of core and progressive genes were created

plotting row Z-scores of mean NN, AN, and AL expression values. Disease activity scores were correlated with gene counts using the following R function and parameters:

```
> cor.test(x, y, method = "spearman", alternative = "two.sided")
```

whereby x was provided as numeric vectors of the activity scores (oSCORAD, EASI, and POEM) and itch score (NRS) and y was provided as vectors of normalised counts of the genes with the highest variance (upper quartile). Correlation analysis was performed separately for pre-treatment AL and AN samples.

Co-expression network was computed using the INfORM¹¹ software which combines multiple network inferences from an ensemble of methods to derive a gene co-expression network. Further, INfORM determines gene modules and ranks genes by the combined influence of network centrality properties and differential expression scores. The input for INfORM was provided as a table of DEGs (FDR adjusted p-values <0.05 and abs(LFC) >=1) (we included IL13RA1 despite an abs(LFC) <1) between pre-treatment AL and AN samples, as well as a table of normalised counts of the respective samples. INfORM was run with default settings, but without the use of the MRNETb inference algorithm to reduce computation time.

In order to identify baseline signatures that are associated with the strength of response to dupilumab, we compared AL skin of patients achieving the highest reduction of EASI (upper quartile, n=6 patients) with patients showing the lowest reduction of EASI (lower quartile, n=6 patients). Genes with a p-value <0.05 and an absolute LFC >=0.5 were examined.

In order to determine the residual disease genomic profile of patients who achieved a high response to dupilumab, we compared dysregulated genes before and 3 months after treatment, whereby we discriminated the residual profile as compared to AN and NN, respectively. For the AL vs. AN comparison, we accepted all differentially expressed genes with an FDR adjusted p-value <0.05 as DEGs. Due to the plenty of significant DEGs between AL and NN, we additionally filtered genes with an absolute LFC >= 1.

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