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Scalp biomarkers during dupilumab treatment support Th2 pathway pathogenicity in alopecia areata

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Abstract

Background: The mechanisms driving alopecia areata (AA) are still unclear, hindering development of targeted therapeutics. Specific Th2 targeting with dupilumab in AA provides a unique opportunity to dissect its pathogenesis and explore the role of Th2 pathway.

Methods: We evaluated changes in scalp biomarkers in AA patients (with and without concomitant atopy) randomized to weekly dupilumab or placebo for 24 weeks, followed by

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AUTHOR CONTRIBUTIONS

Conceptualization: EGY, MGL, JGK. Methodology: EGY and JGK. Investigation: EGY, YRY, ABP, EDD, YDE, SB, PF, PJGA, MA. Statistical analysis: ABP, SG. Visualization: YRY, ABP, EDD, ADP. Validation: ADP. Data curation: JB, MC. Funding acquisition: EGY and JGK. Supervision: EGY and JGK. Writing—original draft: YRY, EGY, JGK. Writing—review and editing: YRY, ABP, EDD, EGY, JGK.

CONFLICT OF INTEREST

EGY has served as a consultant for AbbVie, Amgen, Allergan, Asana Bioscience, Celgene, Concert, Dermira, DS Biopharma, Escalier, Galderma, Glenmark, Kyowa Kirin, LEO Pharmaceuticals, Lilly, Mitsubishi Tanabe, Novartis, Pfizer, Regeneron, Sanofi, and Union Therapeutics; a member of advisory boards of Allergan, Asana Bioscience, Celgene, DBV, Dermavant, Dermira, Escalier, Galderma, Glenmark, Kyowa Kirin, LEO Pharma, Lilly, Novartis, Pfizer, Regeneron, and Sanofi; and a recipient of research grants from AbbVie, AnaptysBio, AntibioTx, Asana Bioscience, Boehringer-Ingelheim, Celgene, DBV, Dermavant, DS Biopharma, Galderma, Glenmark, Innovaderm, Janssen Biotech, Kiniska Pharma, LEO Pharmaceuticals, Lilly, Medimmune, Sienna Biopharmaceuticals, Novan, Novartis, Ralexar, Regeneron, Pfizer, UCB, and Union Therapeutics. MGL is an employee of Mount Sinai and receives research funds from: Abbvie, Amgen, Arcutis, Avotres, Boehringer Ingelheim, Cara therapeutics, Dermavant Sciences, Eli Lilly, Incyte, Janssen Research & Development, LLC, Ortho Dermatologics, Regeneron, and UCB, Inc., and is a consultant for Aditum Bio, Almirall, AltruBio Inc., AnaptysBio, Arcutis, Inc., Arena Pharmaceuticals, Aristea Therapeutics, Avotres Therapeutics, BiomX, Brickell Biotech, Boehringer-Ingelheim, Bristol-Myers Squibb, Cara Therapeutics, Castle Biosciences, Celltrion, Corevitas, Dermavant Sciences, Evommune, Inc., Facilitatation of International Dermatology Education, Forte Biosciences, Foundation for Research and Education in Dermatology, Hexima Ltd., LEO Pharma, Meiji Seika Pharma, Mindera, Pfizer, Seanergy, Trevi, and Verrica. JGK has received grants paid to The Rockefeller University from Amgen, Boehringer Ingelheim, Bristol Myers Squibb, Dermira, Innovaderm, Janssen, Kadmon, Kineta, Kyowa, LEO Pharma, Lilly, Novartis, Paraxel, Pfizer, Provectus, Regeneron, and Vitae and personal fees from AbbVie, Baxter, Biogen Idec, Boehringer Ingelheim, Bristol Myers Squibb, Delenex, Dermira, Janssen, Kadmon, Kineta, Lilly, Merck, Novartis, Pfizer, Sanofi, Serono, and XenoPort. All other authors declare no competing interests.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

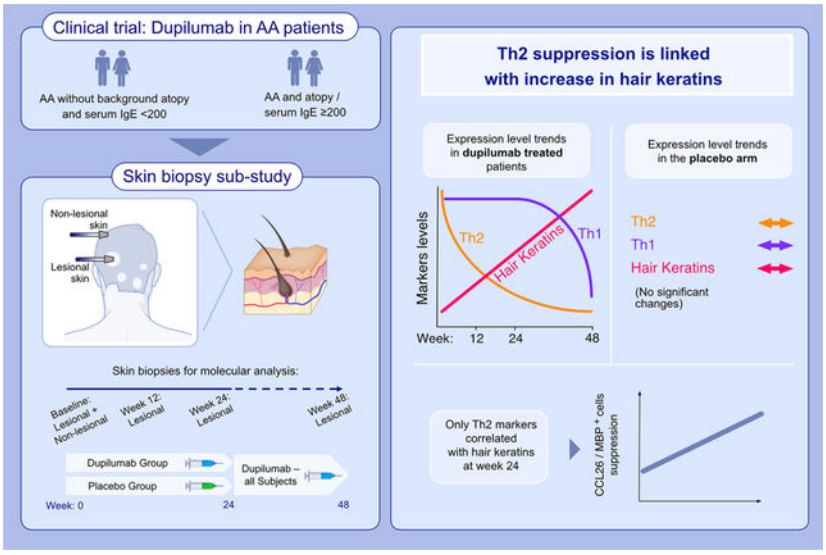
open-label dupilumab for 24 weeks. Changes in biomarker levels were measured at weeks 12, 24, and 48 and were also correlated with clinical hair regrowth.

Results: At week 24, preceding clinical hair regrowth outcomes, only dupilumab-treated patients presented significant suppression of cellular infiltrates, and multiple Th2-related, markers (CCL13/MCP-4, CCL18/PARC, CCL26/eotaxin-3, CCL24/Eotaxin-2), coupled with significant upregulation in the hair keratins. Th1-related suppression was evident later (week 48) when all patients received open-label dupilumab. Results were more pronounced in atopic AA patients, that showed 48% and 97% improvements in the lesional AA scalp profile at weeks 24 and 48, respectively, while 2% worsening was seen in the placebo arm at week 24. Moreover, placebo-treated patients presented 54% worsening in hair keratins when compared with baseline at week 24. At week 24, increases in hair keratins showed significant correlations only with decreases in Th2-related markers.

Conclusions: Scalp biomarkers provide evidence of dupilumab efficacy in AA, detected even prior to clinical response, with exclusive correlations between early suppression of Th2 markers and increased hair keratins. These findings strengthen previous reports suggesting a possible role for Th2 cytokines as AA drivers.

This biopsy sub-study provides a unique opportunity to explore the pathogenicity of Th2-related activation in alopecia areata. Only dupilumab-treated patients presented Th2 pathway suppression, coupled with significant upregulation in hair keratins. Th1-related suppression was evident later (week 48). At week 24, increases in hair keratins correlated only with decreases in Th2-related markers, underscoring the role of Th2 in AA.

Graphical Abstract



Keywords

atopic dermatitis; biologics; clinical immunology; dermatology; IgE

1 | INTRODUCTION

Alopecia areata (AA) is the most common inflammatory skin disease resulting in reversible hair loss, affecting up to 2% of the population worldwide.[1] The pathogenesis of AA is not fully understood,[2] hindering the development of specific therapeutics. Thus, despite the great burden of AA on patients' well-being[3] and the increased prevalence of associated anxiety, depression, and even suicidal ideation,[4–7] treatment options are limited and only baricitinib has been recently FDA-approved for moderate to severe AA.[8]

Alopecia areata is induced by a chain of events initiated by an unknown trigger, leading to compromised immune privilege of the hair follicle. The resultant immune attack and inflammatory milieu had been studied in AA animal models,[9] but the players of this complex process in human AA are still elusive.

Although AA is traditionally considered as a Th1-related activation compromising immunological tolerability in the hair bulb,[10–12] there is strong rationale to also test the role of Th2 antagonism in AA: AA patients have elevated Th2 markers in blood and skin[13–16]; GWAS studies suggest a role for Th2-related genes in AA pathogenesis[17–19]; large population studies show strong association between atopic diseases and AA[20,21]; some AA patients have high levels of serum IgE levels even without background atopy[22–24]; and anecdotal reports on hair regrowth in patients with concomitant AA and atopic dermatitis (AD) treated with dupilumab.[25–27] We thus recently reported the results of a clinical trial in which AA patients were treated with dupilumab, an IL-4R inhibitor blocking Th2 signaling.[28]

Sixty adult AA patients with and without history of AD were randomized 2:1 to dupilumab or placebo arms for 24 weeks, following another 24 weeks of open-label dupilumab extension period. At week 24, worsening of AA was reported in the placebo arm while AA severity was stabilized in the dupilumab arm. After week 24, gradual, consistent improvement in AA was found across both study arms treated with weekly dupilumab. A sub-analysis on patients with background atopy or elevated serum IgE levels (≥200 IU/ml) showed significantly improved hair regrowth in this sub-population when compared with patients with no atopy and low IgE levels at baseline.[28]

Herein, to further explore the role of Th2 in AA pathogenesis and the effects of Th2 inhibition on tissue immune- and hair-related biomarkers, we report a biopsy sub-study of this clinical trial. This sub-study includes scalp skin biopsies collected at baseline, at weeks 12 and 24 (during the randomized, placebo-controlled phase), and at week 48 (where all patients were treated with dupilumab), to establish the molecular circuits underlying hair regrowth in AA patients treated with IL-4R antagonism.

2 | METHODS

2.1 | Patient characteristics

Sixty participants were randomized in a 2:1 ratio to receive dupilumab or placebo in a randomized, double-blind, multicenter study, which was approved by the institutional review

board (IRB) at both study sites (NCT03359356). All patients signed IRB-approved informed consents. Scalp hair loss severity was assessed using SALT, ranging from 0 (no hair loss) to 100 (complete hair loss). Full trial protocol was previously published.[28] Scalp skin punch biopsies (4.5 mm) from non-lesional and lesional skin were collected at baseline, and lesional scalp biopsies were collected at weeks 12, 24, and 48.

2.2 | RNA-sequencing and quantitative real-time polymerase chain reaction

RNA was extracted from skin biopsy samples in Qiazol solution using tissue sonicator and processed downstream with QIAGEN miRNeasy Mini kit, as per company protocol (QIAGEN catalog#217004). Libraries were generated using TruSeq Stranded mRNA Library Prep kit (Illumina). mRNA was first extracted from 240 ng of total RNA using oligo-dT magnetic beads and fragmented at high temperature. A double-stranded cDNA library was then prepared by reverse transcription and second strand synthesis reagents. Indexing adaptors were ligated to the cDNA libraries and amplified on thermocycler. Next-generation sequencing was performed on the amplified libraries using Novaseq 6000 (Illumina Inc.) on 100 cycle single-ended read configuration. Our sequencing depth ranged between 35 and 45 M reads and the reference genome used was GRCh38/hg38.

For RT-PCR, reverse transcription to complementary DNA (cDNA) was carried out using the High-Capacity cDNA reverse transcription (Thermofisher). Pre-amplification was performed on all samples. TaqMan Low Density Array (TLDA) cards (Thermofisher) were used for quantitative RT-PCR (qRT-PCR). Primers are listed in Table S1. 100 ng total RNA was used for reverse transcription and resulting cDNA was used for PreAMP pool and TLDA. Eukaryotic 18 S recombinant RNA was used as an endogenous control. Expression values were normalized to *Rplp0*.

2.3 | Immunohistochemistry

Immunohistochemistry was performed on frozen skin sections as previously described[29,30] using antibodies as listed in Table S2. Counts were performed on full-depth biopsy sections including hair follicles (where applicable), and the average count per section across these X20 images was included for analysis. Cell counts were quantified using ImageJ V1.42 software (National Institutes of Health).

2.4 | Statistical analysis

The statistical language R (www.r-project.org) was used to perform all analyses. Next-generation sequencing was performed on Illumina NovaSeq 6000 with single-ended 100 red cycles. RNA-seq sample quality was assessed using FastQC (Cambridge, United Kingdom) and MultiQC.[31] Samples were aligned to the human reference genome using the STAR RNA-seq aligner,[32] and sequencing reads were assigned to genomic features by featureCounts.[33] Data were subsequently $\log_2$ transformed with voom transform[34] and modeled using a mixed-effects model with time, treatment, and tissue as a fixed effect and random effect (using the R limma package) for each patient. The Benjamini-Hochberg procedure was used to adjust p values for multiple hypotheses by controlling the false discovery rate (FDR). Genes with fold change ($|FCH| > 1.5$ and $FDR < 0.05$ in any post-versus pretreatment comparison were considered differentially expressed. qPCR values

were normalized to Rplp0 by negatively transforming cycle threshold (Ct) values to dCt. Immune gene–specific subsets were quantified using gene-set variation analysis, a method of unsupervised sample-wise enrichment that results in an activity score for a subset of genes or pathway for each sample. Modeling was performed using the same approach described for single genes. The association of biomarkers and gene-sets with clinical responses was evaluated using Pearson correlation coefficients. Unsupervised clustering analysis using Pearson correlation and a McQuitty agglomeration scheme was performed.

3 | RESULTS

3.1 | Patient characteristics and clinical response

Of 60 patients included in the clinical trial (40 in the dupilumab arm and 20 in the matching placebo arm),[28] 56 were included in the biopsy sub-analysis at baseline: 38 and 18 in the dupilumab and placebo arms, respectively. Of these, 27 and 10 participants (in the corresponding arms) also donated baseline non-lesional scalp sample (not collected from patients with alopecia totalis or universalis). Participants' characteristics are presented in Table 1.

In this clinical trial, while the placebo arm presented AA worsening (a least-squares mean SALT change of −6.5) at week 24, the dupilumab arm presented disease stabilization (SALT change of 2.2) ($p < .05$).[28] At week 48, 32.5%, 22.5%, and 15% of patients achieved $SALT_{30}/SALT_{50}/SALT_{75}$ improvement, respectively. Baseline serum total IgE levels predicted treatment response with 83% accuracy; of patients with baseline IgE ≥ 200 IU/ml, 53.8%, 46.2%, and 38.5%, achieved $SALT_{30}/SALT_{50}/SALT_{75}$ improvement, respectively. As this biopsy sub-study includes most of the participants of the original clinical trial (95% and 90% of the dupilumab and placebo arms, respectively), it presents similar clinical results as the full study population.[28]

3.2 | Th2-related cells and eosinophil counts are suppressed with dupilumab treatment

Immunostaining was performed to demonstrate changes in inflammatory infiltrates of T-cell ($CD3^+$, $CD8^+$ cells), dendritic cell (DCs) ($CD11c^+$ cells), and Th2-related cell counts ($FcER1^+$ and $OX40^+$ cells, and eosinophils, marked by major basic protein/MBP^+) during placebo and dupilumab treatment (Figure 1).

Overall, cellular infiltrates were most commonly identified in proximity to hair follicles, extending into the deep dermis and intra-follicular regions in samples with extensive infiltrates. While $CD3^+$ and $CD8^+$ T-cells showed decreasing counts with dupilumab treatment, no significant changes in counts were recorded in the placebo arm. $CD11c^+$ DC and $FcER1^+$ cell counts decreased as early as week 12 of dupilumab treatment, with significant decreases noted in comparison with baseline counts as well as with counts in the placebo arm at weeks 12 and 24. Similar results were found for $OX40^+$ cells and eosinophils, where infiltrates were largely found in intra-follicular skin (Figure 1).

3.3 | Global transcriptomic improvement toward the non-lesional signature is only detected in dupilumab-treated AA

RNA-sequencing was performed to assess the global transcriptomic changes in lesional skin versus baseline non-lesional skin across study time-points, using criteria of fold change (FCH) > |1.5| and FDR < 0.05. A gradual improvement was observed in the dupilumab arm from week 12 onward, as indicated by the transition toward the baseline non-lesional signature. However, the placebo arm did not show molecular reversal, as seen in Figure 2A,B, where a minor profile worsening was detected in placebo patients at both weeks 12 and 24 (−3% and −6%, respectively), versus improvements of 17% and 30% in the dupilumab arm at the corresponding weeks, respectively. During the open label extension at week 48 (including those patients treated with placebo in the blinded period, until week 24), an improvement of 63% was detected. Furthermore, while similar dysregulation was seen at baseline in scalp profiles of both dupilumab- and placebo-treated patients, the up- and downregulated lesional profiles of the dupilumab arm has been largely reversed toward that of the non-lesional profile (marked by *dotted* line), in contrast to the placebo arm (Figure 2C).

We next evaluated changes in lesional scalp with dupilumab and placebo using a previously published immune gene-set[35–37] as shown in a heatmap comparing FCHs versus baseline (Figure 2D). General inflammation (MMP12) and cellular activation (CD1B, ITGAX/CD11c, CXCR1/IL-8RA) markers, multiple Th2-related chemokines (CCL13/MCP-4, CCL18/PARC, CCL26/eotaxin-3, CCL24/Eotaxin-2), and the Th2/regulatory marker IL-10 were all significantly downregulated starting from week 12 in the dupilumab arm (highlighted with a *red* box, FCH < −1.5, $p < .05$), with no changes recorded with placebo in these genes. Other markers, representing both innate immunity (e.g., IL-6, IL-8/CXCL8, IL-1B) and cellular activation (e.g., CD69, LAT) showed later response to dupiluamb, starting at week 24 or 48 (Figure 2D and Table S3). Th1-related responses varied as well; few Th1-related markers (e.g., CXCL10/11) showed minimal initial increase with dupilumab treatment, diminished at week 48, while multiple other Th1 markers, including CXCL9, IFNγ, IFNγR1, and CCL2/3/4, were not modulated by dupilumab or placebo at any time point (Table S3). Th17-related markers showed late (e.g., IL-17RA/RB) or no response (e.g., IL-17A, LCN2, PI3/Elafin) to dupilumab treatment. Similarly, most Janus kinase (JAK)/STAT markers (JAK1/2/3, TYK2, STAT3/4/6) were not significantly modulated by the drug (Table S3).

Using Gene-Set Variation Analysis (GSVA) of previously reported gene-sets,[35–38] we assessed changes in Th1 and Th2 pathways as well as in hair keratin gene-sets across study arms (Figure 2E–G). While the Th2 axis was significantly downregulated at all timepoints in dupilumab-treated participants (when compared with both placebo and baseline, $p < .001$), significant suppression of Th1 axis was only detected at week 48 ($p < .05$). Hair keratins showed upregulation only in the dupilumab arm already at week 24, with further increase at week 48 ($p < .05$; Figure 2G). More specifically, a 98% improvement in hair keratins was detected on week 48 when compared with baseline, versus a worsening of 54% in the placebo arm at week 24 (Figure 2H).

3.4 | RT-PCR further delineates Th2 suppression with hair keratins upregulations

To validate RNA-seq data and evaluate mRNA expressions of key inflammatory and hair keratin markers, some of which are not well detected by RNA-seq due to relatively low levels, we performed RT-PCR on a large panel of inflammatory and hair keratin genes (Figure 3 and S1).

Overall, PCR data were in line with RNA-seq results, showing early (already at week 12 for most markers) and significant suppression of Th2 cytokines only in dupilumab-treated scalp (CCL13/MCP-4, CCL17/TARC, CCL18/PARC, CCL26/eotaxin-3, IL-13; $p < .05$). However, Th1 markers, such as IFNγ and IL-12/IL-23p40, showed mild, non-significant and late (at week 48) decreases, except for STAT1, that was significantly downregulated at that timepoint. The Treg marker FOXP3 was minimally modulated across treatment arms, with mild increase in dupilumab-treated participants versus mild decrease in the placebo arm.

Hair keratins showed gradual and significant increases in the dupilumab arm while decreases were seen in the placebo arm (i.e., KRT85, KRT75, and KRT84; $p < .05$).

3.5 | Correlation analysis suggests early Th2 response versus late multi-faceted immune modulation in association with SALT change

To associate changes across immune and hair keratin markers with changes in disease severity (measured by change in SALT) and baseline serum IgE levels (a marker predictive of dupilumab response),[28] we conducted a Pearson correlation analysis.

At week 24, despite the minimal change in hair regrowth,[28] we already detected significant correlations between decreases in Th2 biomarkers (CCL26/eotaxin-3 and eosinophil/MBP$^+$ cell counts) and increases in early, middle and late hair keratins (e.g., KRT85/86/75; $r < -.35$; $p < .05$; Figure S2).

Figure 4 presents a heatmap and table of selected robust correlations at week 48 ($r > |.8|$), showing significant, strong correlations between SALT change and decrease in markers related to general inflammation (MMP12), cellular activation (IL-2RA, IL-15), Th2 (IL-10, CCL18/PARC, CCL13/MCP-4), Th1 (IFNγ, CXCL10), and Tregs (FOXP3) ($r > .8$, $p < .1$ for all, highlighted in a *green* box). Hair keratins were negatively correlated with SALT changes, including KRTAP1, KRT86, depicting early and middle hair keratins, respectively ($r \leq -.8$, $p \leq .1$). Baseline serum IgE levels were strongly correlated with increase in hair keratins ($r \geq .92$, $p < .05$) and decrease in Th1 (CXCL10, IL-12/IL-23p40), cellular activation (IL-2RA), and Th2 (eosinophils/MBP$^+$ cell counts, CCL18/PARC, CCL13/MCP-4) markers ($r \leq -.84$, $p < .1$).

3.6 | Atopic AA patients present greater tissue improvements with dupilumab

To establish the role of background atopy on dupilumab response in AA, we also stratified patients based on existence of atopy, defined as personal or family atopy and/or baseline serum IgE levels ≥200 IU/ml.

As could be appreciated by the *blue* to *red* transition, atopic patients presented a 97% transcriptomic improvement toward non-lesional baseline levels (Figure 5A,B). Moreover,

modulation of downregulated genes exceeding baseline non-lesional levels (*dashed line*, Figure 5C) only in dupilumab-treated participants. In contrast, placebo-treated atopic participants presented an overall worsening, resembling the placebo response found in the entire cohort (Figure 5B).

We further evaluated changes in our previously published immune gene-set[35–37] in the group of dupilumab-treated patients, stratified by background atopy (Figure 5D). While both atopic and non-atopic participants displayed downregulations of Th2-related markers (CCL13/MCP-4, CCL18/PARC, CCL26/eotaxin-3, CCL24/eotaxin-2) starting from week 12 (highlighted in a *red* box), other gene modulations differed between the sub-groups. For example, atopic participants presented broader suppression of Th2-related markers (including CCL17/TARC, IL-13, IL-4, and IL-5), along with greater downregulations of innate immunity (IL-6, IL-8/CXCL8) and cellular maturation/activation (LCK, LAT, CD40LG, CD69, ICOS) markers. Moreover, while atopic participants showed late Th1 response (at week 48), including IFNγ, IL-12/IL-23p40, and IFNL1/IL-29, non-atopic participants showed early upregulations of some Th1-related markers (e.g., CXCL9/10/11) with no significant Th1-related downregulations throughout the treatment period. Using GSVA, Th2 suppression was demonstrated across both study arms at weeks 12 and 24 ($p < .05$), but not at week 48, where only atopic participants showed Th2 attenuation. We found decreased Th1 modulation (approaching significance for the atopic group) only at week 48 ($p < .1$; Figure 6A).

A heatmap and GSVA depicting hair keratins by atopic background (Figure 6B,C) show significant upregulation of hair keratins with dupilumab treatment occurring primarily in atopic individuals (e.g., KRT85/86/84), already starting week 24. These trends were not seen in atopic patients treated with placebo. While improvements of 55% and 155% were recorded in dupilumab-treated atopic participants at weeks 24 and 48, respectively, worsening of 50% was recorded in the placebo arm at week 24 (Figure 6D).

4 | DISCUSSION

While clinical studies with broader-acting agents, such as corticosteroids and even JAK inhibitors, can only inform on the effects of non-specific immune suppression on AA-affected hair follicles, our clinical trial with dupilumab, specifically inhibiting the Th2-pathway, provides a unique opportunity to further dissect the pathogenesis of AA and the role of Th2 skewing in patients with moderate-to-severe AA.

We found that Th2 targeting with dupilumab was able to largely reverse the AA phenotype, while minimal changes and even worsening was noted in the placebo arm at both 24 and 48 weeks. While we observed a global change of 63% in the AA scalp profile, we detected a nearly complete reversal toward non-lesional state (98% improvement) in a previously reported hair keratin gene-subset[38] at week 48.

At week 24, the last timepoint in which the control arm was still treated with placebo, only dupilumab-treated patients presented suppression of cellular infiltrates including T-cells, atopic DCs presenting markers related to IgE/Th2 ($FcER1^+$ and $OX40^+$), and eosinophils.

Suppression of Th2-related markers (CCL13/MCP-4, CCL18/PARC, CCL26/eotaxin-3, CCL24/Eotaxin-2), but also general inflammation (MMP12), T-cell and DCs (CD1B, ITGAX/CD11c, CXCR1/IL-8RA), and other products were also exclusively detected in the dupilumab arm, starting at week 12. Further, while clinical responses to dupilumab were primarily evident at week 48, significant upregulation in the hair keratin gene-set[39] was already evident by week 24, only in dupilumab-treated patients. These results were more pronounced in atopic AA patients (which showed better clinical response to dupilumab),[28] who presented with deeper, broader Th2 attenuation when compared with non-atopic patients, along with greater upregulations of hair keratins. Also at week 24, only Th2-related markers were significantly correlated with increases in hair keratins, including CCL26/eotaxin-3 (associated with dupilumab response in skin of AD patients treated with dupilumab)[40] and eosinophil scalp infiltrates. On the other hand, at week 24, placebo-treated patients showed a 54% worsening of hair keratins when compared with baseline. The suppression of Th1 immune pathway was only evident at week 48 (with no changes in this pathway seen at week 24), primarily in atopic individuals. The inhibition beyond the Th2 pathway may likely be explained by the fact that although dupilumab is targeting only the IL-4Rα, this receptor can be found on multiple cell types and thus allows wider inhibition of immune cells, including DCs, T-cells, eosinophils, and keratinocytes.[41] Taken together, the increase in hair keratins at 24 weeks in dupilumab-treated patients in the setting of a selective Th2 inhibition, along with the exclusive correlations between Th2-related markers and increase in hair keratins and hair regrowth, suggest that Th2 cytokines are likely pathogenic in AA and may contribute to the suppression of hair keratins in AA.[16,39]

At week 48, robust correlations were found between SALT improvement and changes in multiple scalp markers, including both Th2- (IL-10, CCL18/PARC, CCL13/MCP-4) and Th1-related (IFNγ and CXCL9) markers. These observations also support the role for Th1/IFNγ in perpetuating the AA phenotype,[11,12] with a possible scenario where early Th2 inhibition allows restoration of the follicular immune privilege and thus prevents further follicular damage, which is contributed by Th1 activation.

The JAK–STAT family, including JAK1/2/3 and tyrosine kinase 2 (TYK2), had been increasingly studied in AA.[42,43] Within this class, JAK1/JAK3 mediate signaling for γ-common cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.[39,44] While JAK inhibition presents promising clinical results in AA, safety concerns were raised due to broad effects on the immune system, especially in the setting of long-term treatment, as needed in AA.[45] Recently, a JAK3 inhibitor (ritlecitinib) showed pronounced Th2 suppression, including CCL13/MCP-4, CCL26/eotaxin-3, and CCL18/PARC in an AA clinical trial.[39] Furthermore, Th2 markers, including CCL17/TARC, CCL18/PARC, IL-5, and IL-13, showed the strongest correlations with SALT improvement in ritlecitinib-treated AA patients, in both scalp and serum. Interestingly, JAK–S TAT markers, including JAK1, JAK2, JAK3, and TYK2, did not show significant downregulations with dupilumab in our cohort. However, given the mechanism of action of JAK inhibition, blocking γ-common cytokines, it is possible that JAK1/3 inhibition will block IL-4R-related signaling in a way that may be comparable with dupilumab, explaining the similar results obtained across both therapeutics.

The role of Tregs in AA is unknown, with some data showing Treg deficiency,[46,47] while others demonstrate mild increase in scalp Tregs in AA,[48] perhaps indicating some Treg dysfunction instead of absence, similar to AD.[49] Moreover, the lack of efficacy of low-dose IL-2 treatment (which increases Treg population) in AA may support the latter.[50] Indeed, the response to dupilumab treatment was not associated with increased Tregs, with no significant changes observed in FOXP3 and other regulatory markers (except IL-10 that is also a Th2-related cytokine).

Limitations of this study include the following: lack of placebo arm after week 24, lack of tissue collections after week 48, and inability to fully dissect the roles of Th2 vs. Th1 immunity to AA pathogenesis.

In conclusion, the results of this study provide insights into the effects of Th2 modulation with dupilumab on the immune and hair keratin dysregulation in AA scalp, particularly in patients with an atopic background. In addition to broad attenuation of Th2 immunity, dupilumab also inhibits additional immune cells and pathways, including inhibition of Th1-related markers as a delayed event. Larger and longer clinical trials with dupilumab in both adults and children with AA are needed to help determine the role of specific Th2 inhibition in the therapeutic paradigm of AA. These studies will also help to fully understand the relative contributions of Th1 and Th2 immune pathways to hair keratin differentiation and synthesis in AA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AA	Alopecia areata
AD	Atopic dermatitis
DC	Dendritic cell

IgE	Immunoglobulin E
JAK	Janus kinase
SALT	Severity of alopecia tool
TYK2	tyrosine kinase 2

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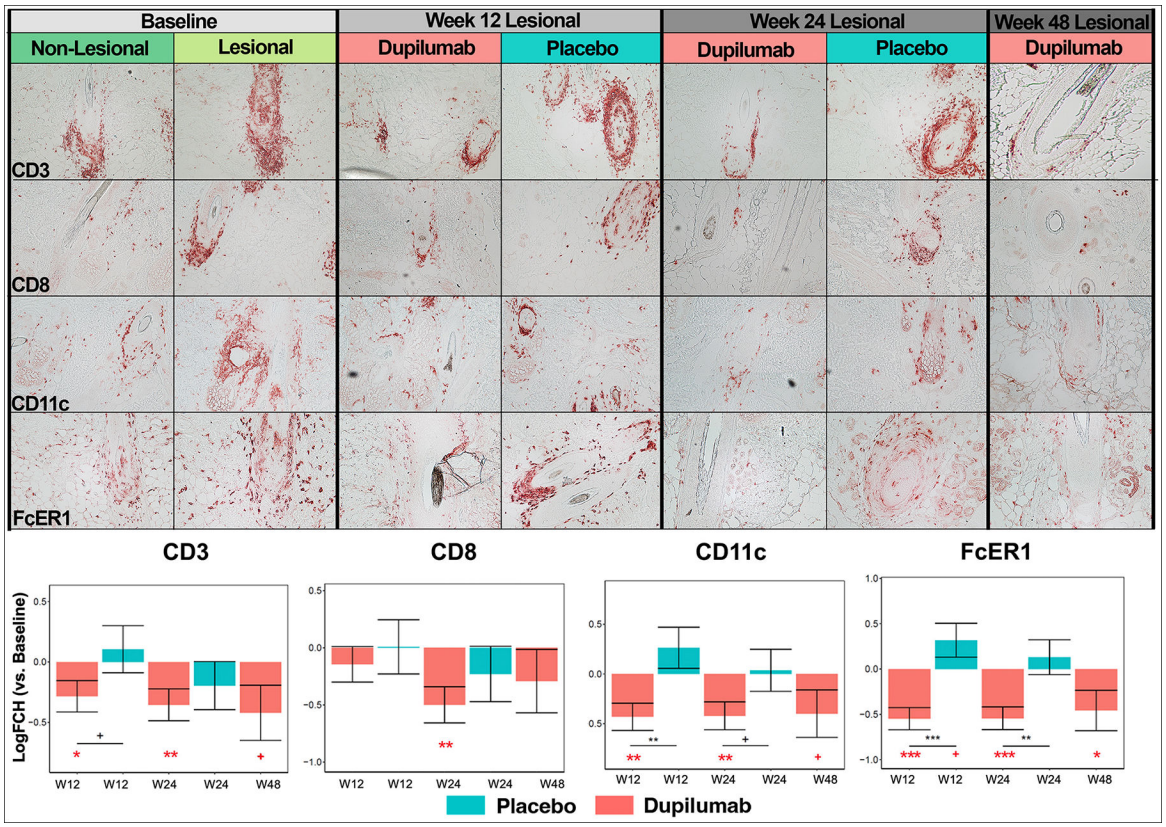


FIGURE 1.

Immunohistochemistry (IHC) of scalp cellular infiltrates in alopecia areata (AA) at baseline and during placebo and dupilumab treatment. Representative images of $CD3^+$ T-cells, $CD8^+$ T-cells, $CD11c^+$ dendritic cells, $FcER1^+$ cells, $OX40^+$ cells, and major basic protein $(MBP)^+$ eosinophils are presented along with cell count plots at the bottom of the figure. W12, week 12; W24, week 24; W48, week 48. +/*/**/*** denote significance ($p < .1/.05/.01/.001$, respectively) of difference in comparison with baseline (when in *red*) and in dupilumab versus placebo at a specific timepoint (when in *black*).

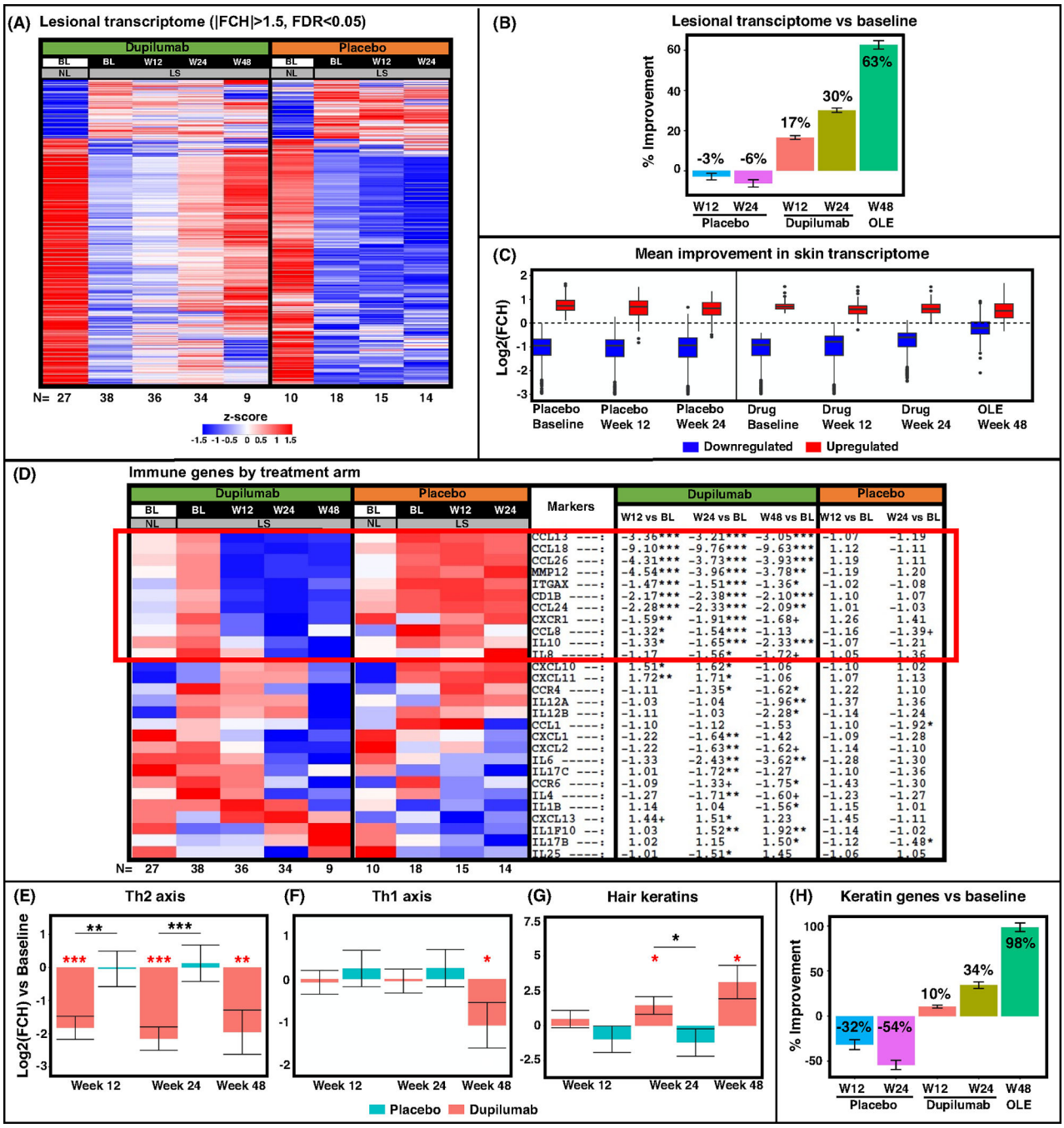


FIGURE 2.

RNA-sequencing results of scalp samples in alopecia areata (AA) at baseline and during placebo and dupilumab treatment. Lesional transcriptome of all differentially expressed genes (DEGs) presented as a heatmap with respective number of participants at the bottom (A), and as a plot indicating percent of improvement in DEGs during treatment (B). *Blue* and *Red* boxes denote down- and upregulated DEGs, respectively, across treatment arms during the trial, with a dashed line representing non-lesional scalp at baseline (C). Immune and keratin pathway-specific results are presented as a heatmap of preselected immune

genes, including a *red* box highlighting genes related with dupilumab's mechanism of action (D), gene-set variation analysis (E, F, G), and a plot of keratin gene-set percent improvement (H). BL, baseline; LS, lesional; NL, non-lesional; OLE, open label extension; W12, week 12; W24, week 24; W48, week 48. +/*/**/*** denote significance ($p < .1/.05/.01/.001$, respectively) of difference in comparison with baseline (when in *red*) and in dupilumab versus placebo at a specific timepoint (when in *black*).

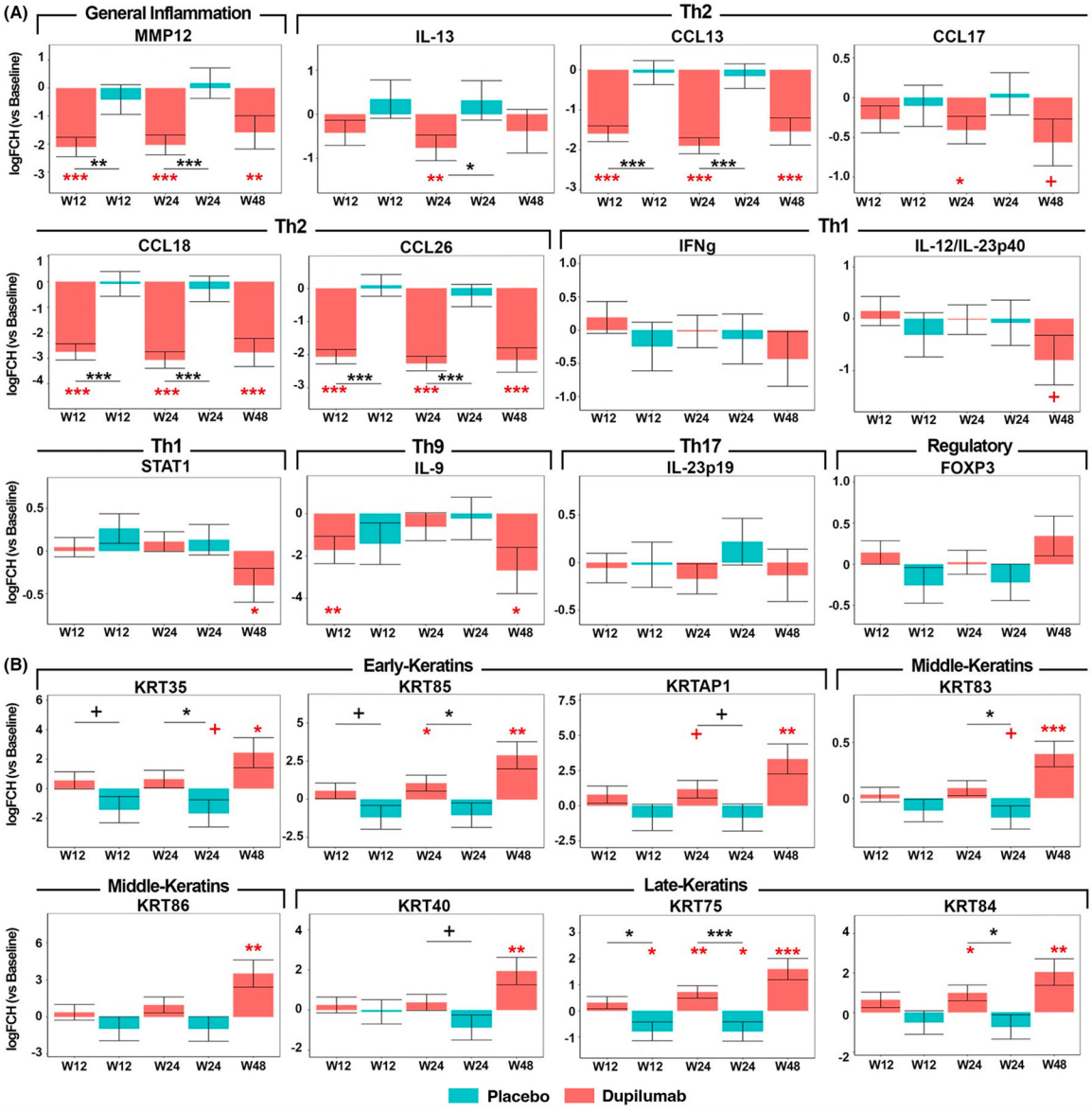
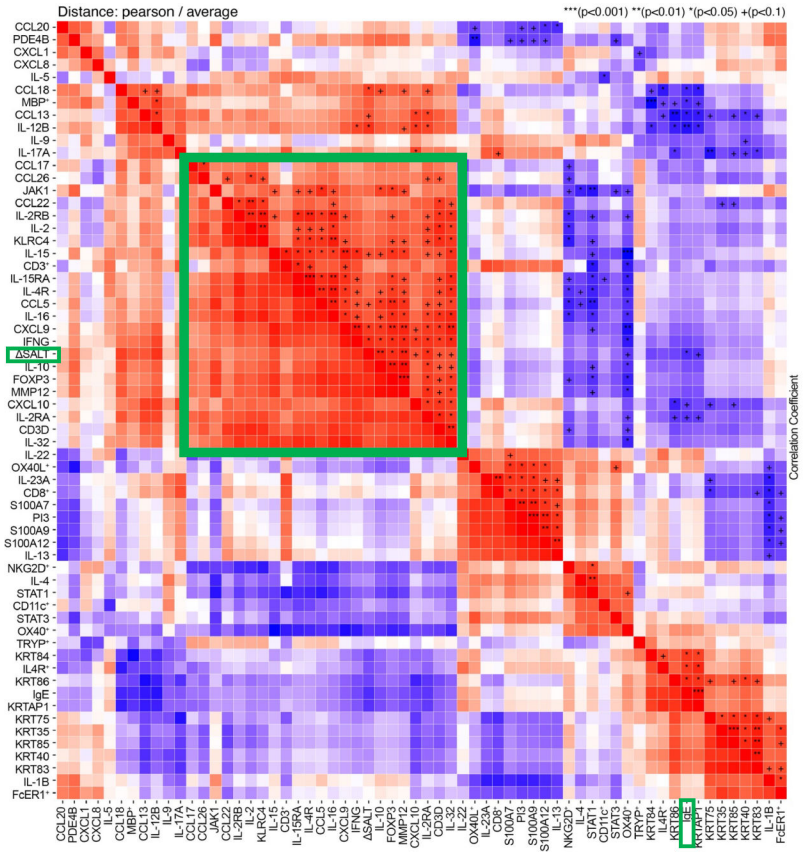


FIGURE 3.

qRT-PCR analysis of selected immune (A) and hair keratin (B) genes in alopecia areata (AA) across treatment arms. Values show log2 FCH expression and are presented as means ± SEMs. W12, week 12; W24, week 24; and W48, week 48. +/*/**/*** denote significance (p < .1/.05/.01/.001, respectively) of difference in comparison with baseline (when in *red*) and in dupilumab versus placebo at a specific timepoint (when in *black*).



ΔSALT correlations

	r	P-value
MMP12	0.97	0.01
IL10	0.96	0.01
IFNγ	0.94	0.02
FOXP3	0.94	0.02
IL2RA	0.94	0.02
IL12B	0.93	0.02
CXCL9	0.91	0.03
CCL18	0.89	0.04
IL15	0.84	0.08
CXCL10	0.84	0.08
IL32	0.83	0.08
CD3D	0.83	0.08
CCL13	0.82	0.09
CCL5	0.81	0.10
IL16	0.80	0.10
KRT86	-0.81	0.10
KRTAP1	-0.87	0.05
IgE	-0.90	0.04

IgE correlations

	r	P-value
KRTAP1	0.99	<0.001
KRT86	0.95	0.01
KRT84	0.92	0.03
Δ SALT	0.90	0.04
CXCL10	-0.84	0.08
IL2RA	-0.86	0.06
MBP⁺ cells	-0.90	0.03
CCL18	-0.92	0.03
CCL13	-0.96	0.01
IL12B	-0.97	0.01

FIGURE 4.

Correlation analysis between qRT-PCR markers, immunohistochemistry (IHC) cell counts, baseline serum IgE levels, and severity of alopecia tool (SALT) change at week 48. Presented as a heatmap (*left*) with a cluster of tissue markers correlating with SALT highlighted in a *green* box, and as tables (*right*) of top correlations with SALT change and baseline serum IgE levels ($r \geq |.8|$, $p \leq .1$). +/*/**/*** denote significance ($p <$ .1/.05/.01/.001, respectively).

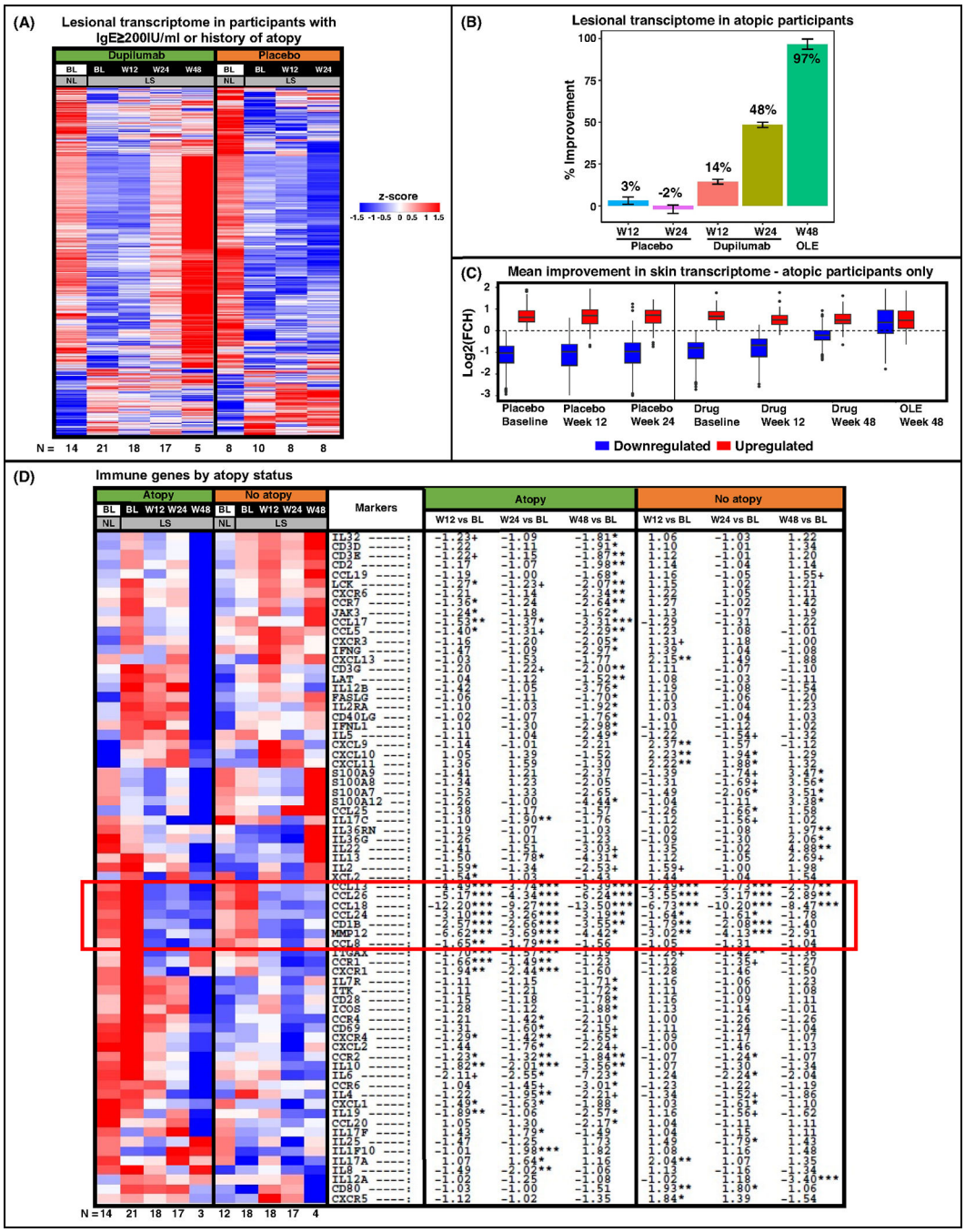


FIGURE 5.

RNA-sequencing results of scalp samples in alopecia areata (AA) by atopic background (determined as personal/family history of atopy or elevated serum IgE levels ≥200 IU/ml). Lesional transcriptome of all differentially expressed genes (DEGs) in atopic participants only presented as a heatmap with respective number of participants at the bottom (A), and as a plot indicating percent of improvement in DEGs during treatment (B). *Blue* and *Red* boxes denote down- and upregulated DEGs, respectively, across treatment arms during the trial in atopic participants, with a dashed line representing non-lesional scalp at baseline (C).

Immune gene-set presented as a heatmap stratified by atopic background, including a *red* box highlighting genes related with dupilumab's mechanism of action (D). BL, baseline; LS, lesional; NL, non-lesional; OLE, open label extension; W12, week 12; W24, week 24; W48, week 48. +/*/**/*** denote significance ($p < .1/.05/.01/.001$, respectively).

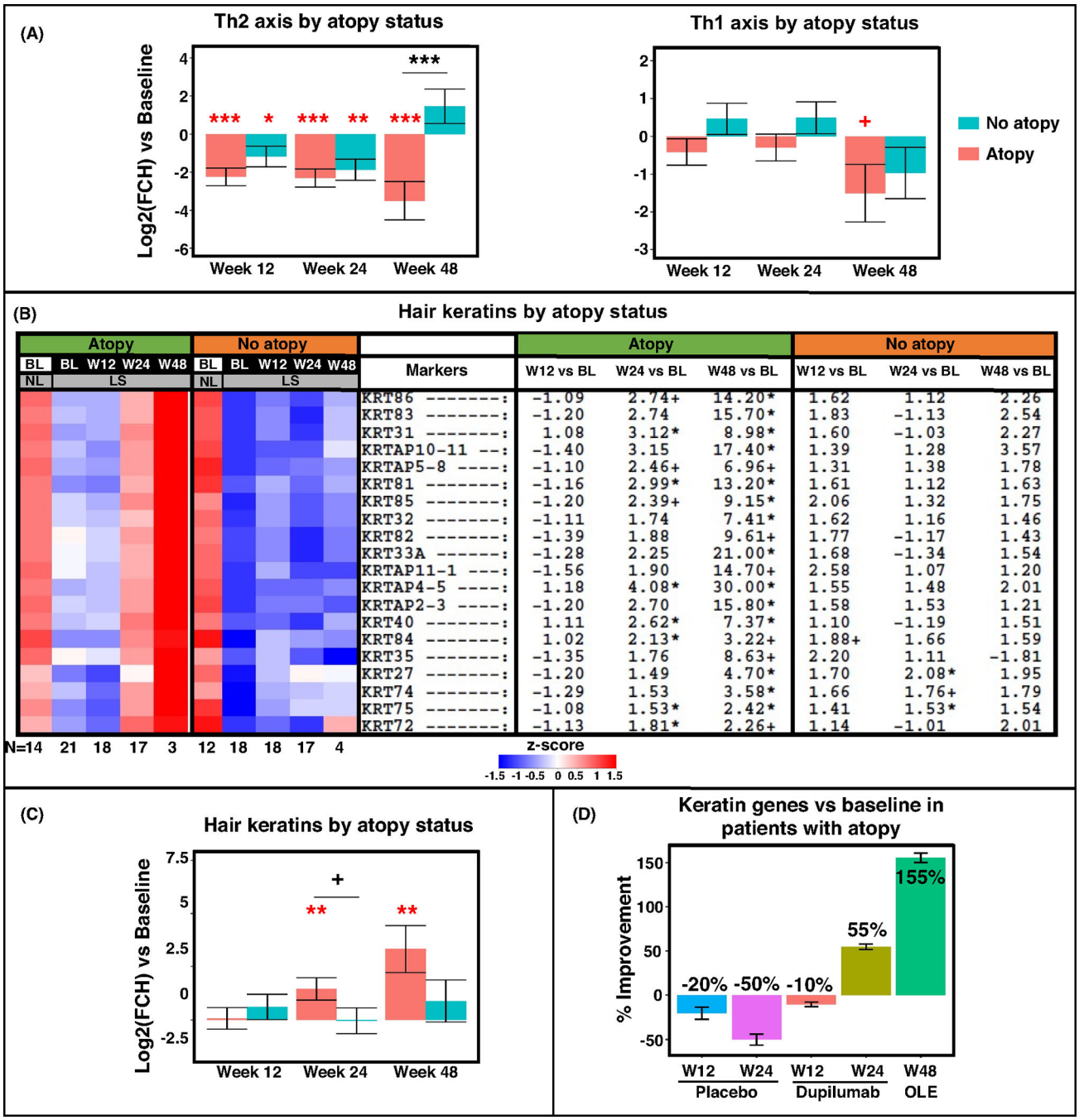


FIGURE 6.

RNA-sequencing results by gene-set pathways from scalp samples by atopic background (determined as personal/family history of atopy or elevated serum IgE levels ≥200 IU/ml). Presented as a gene-set variation analysis (GSVA) of Th2 and Th1 pathways (A), a heatmap and GSVA of hair keratin genes (B, C), and a plot of keratin gene-set percent improvement in atopic participant only (D). BL, baseline; LS, lesional; NL, non-lesional; OLE, open label extension; W12, week 12; W24, week 24; W48, week 48. +/*/**/*** denote significance (*p*

< .1/.05/.01/.001, respectively) of difference in comparison with baseline (when in *red*) and in dupilumab versus placebo at a specific timepoint (when in *black*).

TABLE 1

Characteristics of patients with baseline lesional scalp biopsies

	Placebo (*N* = 18)	**Dupilumab (*N* = 38)**	***p*-value**
Mean age, years (SD)	46.3 (12.5)	41.7 (14.0)	.24
Female sex, *N* (%)	11 (61.1)	28 (73.7)	.34
Race, *N* (%)			
White	13 (72.2)	29 (76.3)	.66
African American	2 (10.5)	3 (7.9)	
Asian	2 (10.5)	6 (15.8)	
Other	1 (5.3)	0 (0)	
Mean duration since last hair regrowth, years (SD)	3.4 (3.1)	3.8 (2.9)	.7
Mean SALT (SD)	76.3 (24.4)	68.9 (27.1)	.34
Patients with BL SALT > 75, *N* (%)	10 (55.6)	18 (47.4)	.57
Patients with AD history, *N* (%)	4 (22.2)	16 (42.1)	.15
Patients with family history of atopy, *N* (%)	8 (44.4)	17 (44.7)	.98
Mean IgE, IU/ml (SD)	179.3 (309.8)	508.4 (1209.7)	.27
Patients with IgE > 200 IU/ml, *N* (%)	6 (33.3)	12 (31.6)	.9
Any atopic background (personal/family/ high IgE)	10 (55.6)	21 (55.3)	.98
Additional biopsies			
Baseline–non-lesional	10	27	
Week 12–lesional	36	15	
Week 24–lesional	34	14	
Week 48–lesional	9		