Activation of MyD88/IRAK1 axis and downstream proinflammatory signaling in healthy adult and neonatal African American skin

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The increased prevalence of inflammatory skin diseases such as atopic dermatitis, hidradenitis suppurativa, acne, lupus erythematosus in African American (AA) compared to White Non-Hispanic population is well recognized. However, the underlying mechanisms are largely unknown. Here we analyzed proteome in healthy skin biopsies from AA and White Non-Hispanic volunteers using Olink Explore 384 Inflammation biomarker panel. Among proteins with higher expression in AA skin were IRAK1, IL1A, IL4, IL22RA1. It is known that IL1A binding to IL1R1 receptor results in recruitment of signaling adapter MyD88 and IL1R1-associated kinases including IRAK1, a key signal transducer involved in activation of downstream NF-κB and MAPK signaling cascades. We confirmed the increased IRAK1 expression as well as activation of NF-κB and extracellular signal−regulated kinase1/2 signaling in both AA adult and neonatal skin by western blot analysis of relevant proteins (p65/RelA, IKKs, IκBα, extracellular signal−regulated kinase1/2) phosphorylation at specific activating sites. We also confirmed the overexpression of previously reported differentially expressed in AA skin pro-inflammatory genes such as IL1A, TNFα, fold change ER1G in our sets of AA healthy adult and neonatal skin using qRT-PCR. Overall, our study suggests the importance of further analysis of molecular landscape of healthy AA skin to assess how it may contribute to the increased risk of certain inflammatory diseases within the AA population.

Keywords: African American skin, Inflammation, Inflammatory skin diseases, Neonatal skin, Proteomics

INTRODUCTION

Morphological and physiological differences in African American (AA) skin compared to White non-Hispanic (WNH) skin are well-documented. AA skin is characterized by accumulation of eumelanin pigment, numerous convoluted dermal-epidermal junctions, increased epidermal proliferation, thicker stratum corneum, stronger corneocyte cohesion, and enhanced skin barrier function (Brown-Korsah et al, 2022; Halder and Nootheti, 2003; Salminen et al, 2023).

There are also differences in the prevalence and clinical manifestations of many inflammatory skin diseases, abnormal wound healing, and skin cancer. The risk of atopic dermatitis (AD), acne, hidradenitis suppurativa, and lupus erythematosus as well as keloids (abnormal scarring) is significantly higher in AA populations (Agbai et al, 2014; Alexis and Blackcloud, 2014; Eichenfield et al, 2014; Feldman et al, 2019, 2013; Halder and Nootheti, 2003; Mei-Yen Yong and Tay, 2017; Price et al, 2021, 2020). WNH individuals have a higher risk of psoriasis, however, the severity of psoriasis might be greater in AA patients (Abrouk et al, 2017; Yan et al, 2018). This disparity could be even more pronounced, given the challenges in visualizing and scoring erythema in darker skin (Adawi et al, 2023; Kaufman and Alexis, 2018).

The most extensive studies of molecular phenotype of AA diseased skin were done in AA patients with AD. Transcriptomic analysis of full-thickness skin biopsies or tape strips revealed numerous differentially expressed genes (DEG) in AA lesional skin compared to AA non-lesional or healthy skin (Hawkins et al, 2024; Luo et al, 2007; Navrazhina et al, 2024; Nomura et al, 2020; Wu et al,

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Abbreviations: AA, African American; Abs, antibodies; AD, atopic dermatitis; AP1, activator protein-1; DEG, differentially expressed gene; DEP, differentially expressed protein; ERK, extracellular signal-regulated kinase; HSO, human skin organoid; LCE, late cornified envelope; TF, transcription factor; WNH, white non-Hispanic

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2020). In some of these studies RNA-seq was combined with analysis of serum and skin immunostaining, and the results were compared with WNH AD skin phenotype (Brown-Korsah et al, 2022; Brunner and Guttman-Yassky, 2019; Wongvibulsin et al, 2021). These studies revealed that AA patients with AD often exhibit higher serum IgE levels, higher CRP values, ferritin, and blood eosinophils compared to WNH patients with AD along with immune polarization primarily characterized by T helper type 2/T helper type 22 skewing. In addition, it was found that FLG loss-of-function alterations typical for WNH and Asian patients with AD are not prevalent in AA patients. Instead, there are frequent loss-of-function FLG2 alterations and significantly lower levels of ceramides in the stratum corneum in AA patients with AD (Brown-Korsah et al, 2022; Brunner and Guttman-Yassky, 2019; Jamerson et al, 2022). Interestingly, the skin phenotype of Tanzanian patients appeared to be consistent with that of AA patients, exhibiting dominant T helper type 2/T helper type 22 skewing, minimal dysregulation of terminal differentiation with no suppression of epidermal barrier marker genes, such as FLG, loricrin, and periplakin but with significant down-regulation of genes related to lipid metabolism including HAO2, ELOVL3, GAL, FAR2, AWAT1 (Lang et al, 2021).

Despite well-recognized morphological and physiological differences, the molecular landscape of healthy AA skin compared to WNH or other skin types remains underexplored. There is practically no data on healthy AA skin proteome and very limited literature on genomic profiling comparing healthy AA skin to WNH skin, except for some transcriptomic studies with a minimal number of healthy volunteers/group whose skin biopsies were used as a reference for comparison with the same ethnicity diseased skin features/expression status (Sanyal et al, 2019; Walker et al, 2020; Wongvibulsin et al, 2021). In our recent larger study focused exclusively on healthy skin transcriptome, the analysis of gene expression in skin biopsies from healthy AA and WNH volunteers of both sexes identified hundreds of DEGs, (fold change > 1.5; $P < .02$) in AA skin (Klopot et al, 2022). We reported the upregulation of FCER1G - the major IgE receptor; proinflammatory genes such as TNFα and IL-32, and epidermal differentiation cluster (EDC) genes like late cornified envelope (LCE) genes. Notably, the expression of some of these DEGs could be correlated with clinical observations, such as increased IgG and IgE levels in the serum of healthy AA individuals and AA patients with various diseases (Albandar et al, 2002; Lucey et al, 1992; Rinker et al, 2007). Additionally, the elevated expression of EDC genes could be linked to the increased stratum corneum thickness and better barrier function in AA skin (Girardeau-Hubert et al, 2019; Muizzuddin et al, 2010). Notably, only four known pigment-related genes were found among reported DEGs in AA skin (Crawford et al, 2017), indicating that differential gene expression in the skin is not closely associated with melanogenesis.

The goals of this study were to (i) analyze protein expression in AA and WNH adult healthy skin using Olink proteomics (Uppsala, Sweden) validated by western blotting; (ii) identify potential upstream pathways driving proinflammatory signaling in healthy AA skin; and (iii) confirm previously found overexpression of selected proinflammatory genes in AA healthy skin biopsies. We also included in our studies neonatal skin, that differs from adult skin in gene/protein expression and barrier function (Visscher et al, 2022, 2021) to assess whether intrinsic proinflammatory signaling in AA skin develops earlier in life.

RESULTS

Olink proteomics analysis of inflammation-related proteins expression in adult skin

To extend our previous findings on intrinsic proinflammatory signaling in AA skin we used Olink proteomics (Uppsala, Sweden) (Supplementary Table S1). Olink proteomics is a proximity extension multiplex assay that allows for precise detection of multiple proteins in the sample. The test uses two antibodies (Abs) bound to single strand nucleotides ("barcode") to recognize each protein antigen by qRT-PCR.

Proteins were isolated from 4 mm full-thickness skin biopsies from 12 healthy volunteers (5 AA and 7 WNH women, aged 24−41 years) grouped by self-identification combined with Fitzpatrick skin score. Published data indicate that self-reported AA ancestry correlates with genotyping at approximately 90% (Yaeger et al, 2008).

The analysis using the *Explore Inflammation* 384 biomarker panel revealed 11 differentially expressed proteins (DEP) (fold change > 1.5; $P < .05$). Nine out of these DEPs were upregulated in AA skin including known mediators of skin inflammation IL4, IL1A, IL22RA1 (a component of the receptor for IL20, 22 and 24) and IRAK1 (IL1R1 Associated Kinase 1) whose expression was 2−4 folds higher in AA skin (Figure 1a).

Activation of MyD88/IRAK1 axis and downstream NF-κB and MAPK signaling in adult and neonatal AA skin

Binding of IL1A to IL1R1 receptor results in the recruitment of adapter protein MyD88 which in turn recruits IRAK kinases including IRAK1, a key signal transducer involved in activation of downstream NF-κB and MAPK signaling cascades (Cohen et al, 2015; Di Paolo and Shayakhmetov, 2016; Jain et al, 2014; Malik and Kanneganti, 2018). Thus, we first focused on validating IL1A and IRAK1 overexpression in AA skin. We added neonatal foreskin to our studies (samples were grouped by mothers' self-identification) to include male skin for the analysis and to assess whether proinflammatory signaling in AA skin develops earlier in life when skin has different features, not completely established barrier function and a markedly dissimilar array of protein biomarkers (Visscher et al, 2021).

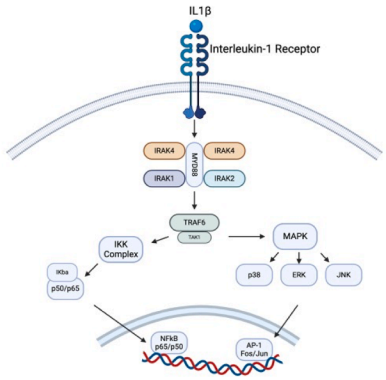
Even though the differences in morphology of adult AA and WNH skin have been documented (Girardeau et al, 2009; Montagna and Carlisle, 1991), the comparative analysis of neonatal AA and WNH skin is lacking. Thus, we first performed morphological analysis of AA and WNH neonatal foreskin focusing on rete ridges (convoluted dermal-epidermal junctions), total thickness of epidermis, thickness of stratum corneum (cornified layer).

We found that the features of neonatal AA skin were very similar to those previously described in AA adult skin including statistically significant increased frequency and depth of rete ridges and increased stratum corneum thickness compared to WNH neonatal skin (Figure 2).

a

Protein	Average Protein Expression (mean) AA	Average Protein Expression (Mean) WNH	P Value	Fold Change
IRAK1	0.34	0.167	0.001	2.041
NELL2	0.01	0.01	0.60	0.00
RABGAP1L	0.56	0.39	1.45	0.02
ICA1	0.56	0.29	1.93	0.02
FST	0.082	0.03	0.019	2.731
IL1A	8.39	2.103	0.037	3.99
IL4	1.041	0.32	0.037	3.252
CLSTN2	0.00	0.00	0.27	0.04
IL22RA1	0.261	0.152	0.042	1.716
YTHDF3	0.525	0.159	0.045	3.295
AOC1	0.147	0.054	0.053	2.734

b



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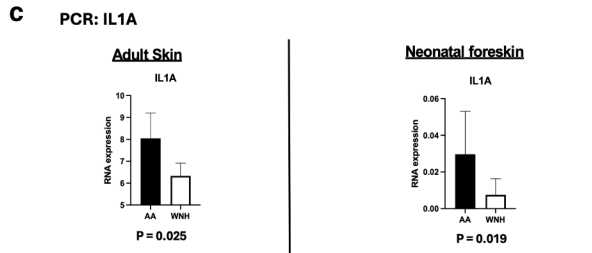

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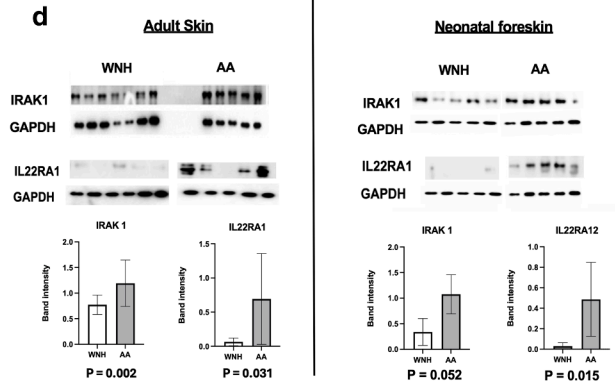

Figure 1. Activation of proinflammatory MyD88/IRAK1 axis in AA skin. Proteins and RNA were extracted from 4 mm full-thickness skin biopsies from the relatively sun-protected inner upper arm skin in AA and WNH healthy volunteers and from neonatal foreskin. **(a)** Table of DEPs, ($P < .05$, FC > 1.5) in AA adult skin revealed by Olink proteomics. The average expression data for each protein is presented as Mean of NPX values. The DEPs in AA skin compared to WNH skin were identified by unpaired two-tailed Welch's *t*-test. using criteria of FC > 1.5 and *P*-value $< .05$. **(b)** Diagram of IL1R1/MyD88/IRAK1 signaling**. (c)** IL1A expression was assessed by qRT-PCR in individual AA (black bars) and WNH (white bars) adult skin RNA samples using delta-delta Ct method. RPL27 was used as a cDNA loading control. **(d)** Western blot analysis of IRAK1 and IL22RA1 expressions. GAPDH was used as a loading control. In **a, c** and **d** Statistical analyses were performed using unpaired two-tailed Welch's *t*-test. The error bars represent Mean ± SD. AA, African American; DEP, differentially expressed protein; FC, fold change; NPX, Normalized Protein Expression; WNH, White non-Hispanic.

Western blotting revealed the increased expression of IRAK1 and IL22RA1 proteins in AA adult and neonatal skin (Figure 1c), The expression of IL1A and IL4 were below western blot detection. However, IL1A expression at mRNA was significantly increased in both adult and neonatal AA skin (Figure 1b).

Next, we assessed the activation of downstream NF-κB and MAPK signaling. NF-κB activation is a complex multi-step process. The primary mechanism for canonical NF-κB activation is the inducible degradation of IκBα triggered through its site-specific phosphorylation (at Ser32 and Ser36) by a multi-subunit IκB kinase (IKK) complex composed of two catalytic subunits, IKKα and IKKβ. In addition, IKKβ phosphorylates major NF-κB protein RelA/p65 at Ser536 and Ser468 which facilitate RelA/p65 nuclear translocation and enhance NF-κB transcriptional activity (Christian et al, 2016; Motolani et al, 2020).

MAPKs including extracellular signal-regulated kinase (ERK1/2), Jun kinase (c-Jun N-terminal kinase/stress-activated protein kinase) and p38, are activated via phosphorylation cascade induced by extracellular stimuli (growth factors, hormones, and stress) and transmitted first to MAPKKKs then to MAPKKs and finally to MAPKs via sequential phosphorylation of these kinases at specific amino acids (Seger and Krebs, 1995).

We assessed activation of IKKs, IκBα, p65/RelA and ERK1/2 by western blotting using Abs against specific activating phosphorylation sites in these proteins. We indeed observed the constitutive activation of NF-κB in healthy adult (Figure 3) and neonatal (Figure 4) AA skin as determined by the statistically significant increased phosphorylation of IKKα/β, RelA/p65 at Ser536 and Ser468 and IκBα at Ser32 that targets IκBα for ubiquitination and proteolysis. Analysis of major MAPK signaling branch ERK1/2 by western blotting with respective phospho-Abs revealed significant increase in ERK1/2 activation in both AA skin types (Figures 3 and 4). Despite the substantial individual variability in phosphorylation patterns, the increase of phosphorylation in NFκB-related proteins and ERK in AA skin was in most cases statistically significant (bar graphs in Figures 3 and 4).

Analysis of MyD88/IRAK1 axis and downstream signaling in AA and WNH human skin organoids

The skin consists of multiple cell types. Thus, to address the role of keratinocytes in the activation of MyD88/IRAK1 axis and downstream signaling in human skin we compared the expression of IRAK1, NF-κB and MAPK phosphoproteins in AA and WNH human skin organoids (HSO) made from the individual primary neonatal keratinocyte lines. The overall difference in protein expression/phosphorylation between

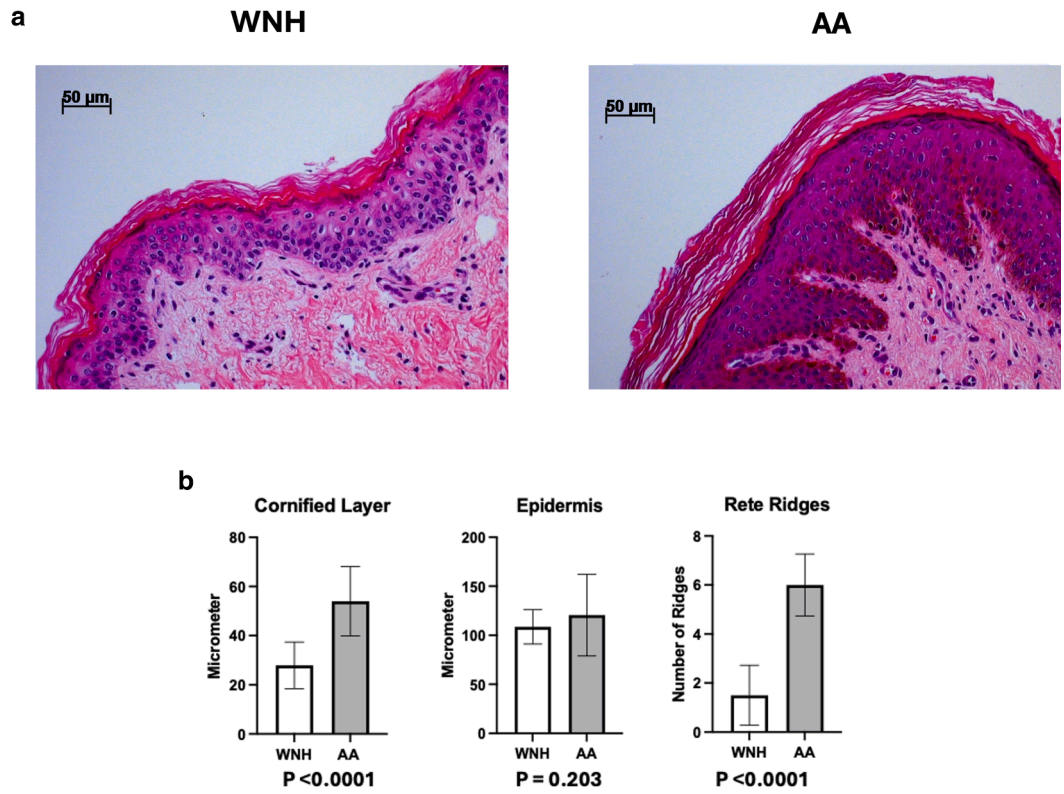

Figure 2. Differences in AA and WNH neonatal skin morphology. (a) H&E staining was done in formalin-fixed, paraffin-embedded samples of neonatal skin. **(b)** Total epidermal thickness, thickness of cornified layer and number of rete ridges/field of view were determined by Zeiss Axio Vision software. At least three individual fields of view/slide in 7 AA and 7 WNH individual skin samples (~20 images/group) were analyzed. Statistical analysis was done by unpaired two-tailed Welch's *t*-test. The error bars represent Mean ± SD. AA, African American; WNH, White non-Hispanic.

AA and WNH HSO was slightly less pronounced compared to full-thickness skin (Figure 5). However, the increase in phosphorylation of p65/RelA, IκBα, ERK1/2 and in AA HSO was statistically significant. (Figure 5). The increase in IRAK1 expression and IKKα/β phosphorylation at Ser536 in AA HSO was revealed as a trend (Figure 5).

Differential gene expression in adult and neonatal AA skin compared to WNH skin

We previously reported significant differences in AA and WNH skin transcriptome with DEGs in AA skin enriched by inflammation and formation of cornified envelope functions (Klopot et al, 2022) when we used skin biopsies obtained from volunteers of both sexes aged 25–64 years. In this work, we used skin samples obtained from a more focused group of volunteers, all females, aged 24–41 years. This provided us with the opportunity to determine whether the previously reported expression differences in proinflammatory genes and keratinocyte differentiation markers such as LCEs are reproducible. qRT-PCR analysis of RNA extracted from the same adult skin biopsies that were used for Olink proteomics, confirmed the increased expression of TNFα, FCER1G (major Receptor for IgE), CCL2, several LCE genes (LCE1E, 2B, 5A) along with IL1A and MAPK11/p38-β in AA adult skin (Figure 6a). Importantly, TNFα, FCER1G and IL1A mRNAs were also expressed higher in AA versus WNH neonatal skin (Figure 6b). However, we did not reveal significant differences in LCE genes expression in AA compared to WNH neonatal skin possibly due to incomplete maturation of skin in newborns (Fluhr et al, 2010; Rahma and Lane, 2022). The previously published data suggest that FLG2 expression inversely varies with skin inflammation in AA patients with AD and in the presence of inflammatory mediators (Seykora et al, 2015).We did not observe differences in basal FLG and FLG2 mRNA expression between AA and WNH healthy adult or neonatal skin (Figure 6).

Analysis of NF-κB and Activator Protein-1 putative binding sites in AA skin DEG promoters

The major transcription factors (TF) activated by signaling cascades related to IL1A/IRAK1 axis are NF-κB downstream from IKK complex and Activator Protein-1 (AP-1) downstream from MAPKs. We used previously published human skin bulk RNA-seq data (Klopot et al, 2022; GSE120783) to assess the role of these TFs in the regulation of AA DEGs expression. We searched for putative NF-κB (RelA) and AP-1 (c-Fos and c-Jun separately) binding sites in DEG promoters using gene transcription regulation database — the database that combines the results of ~23,000 chromatin immunoprecipitation-Seq experiments performed in 30 cell types, and reports site count information for 65,000 transcribed DNA regions with ENSEMBL IDs representing coding and noncoding RNAs. Importantly, the gene transcription regulation database is not tissue/cell type specific. For TF binding sites, we selected meta-cluster analysis and a maximum gene distance of 5000 nucleotides to define

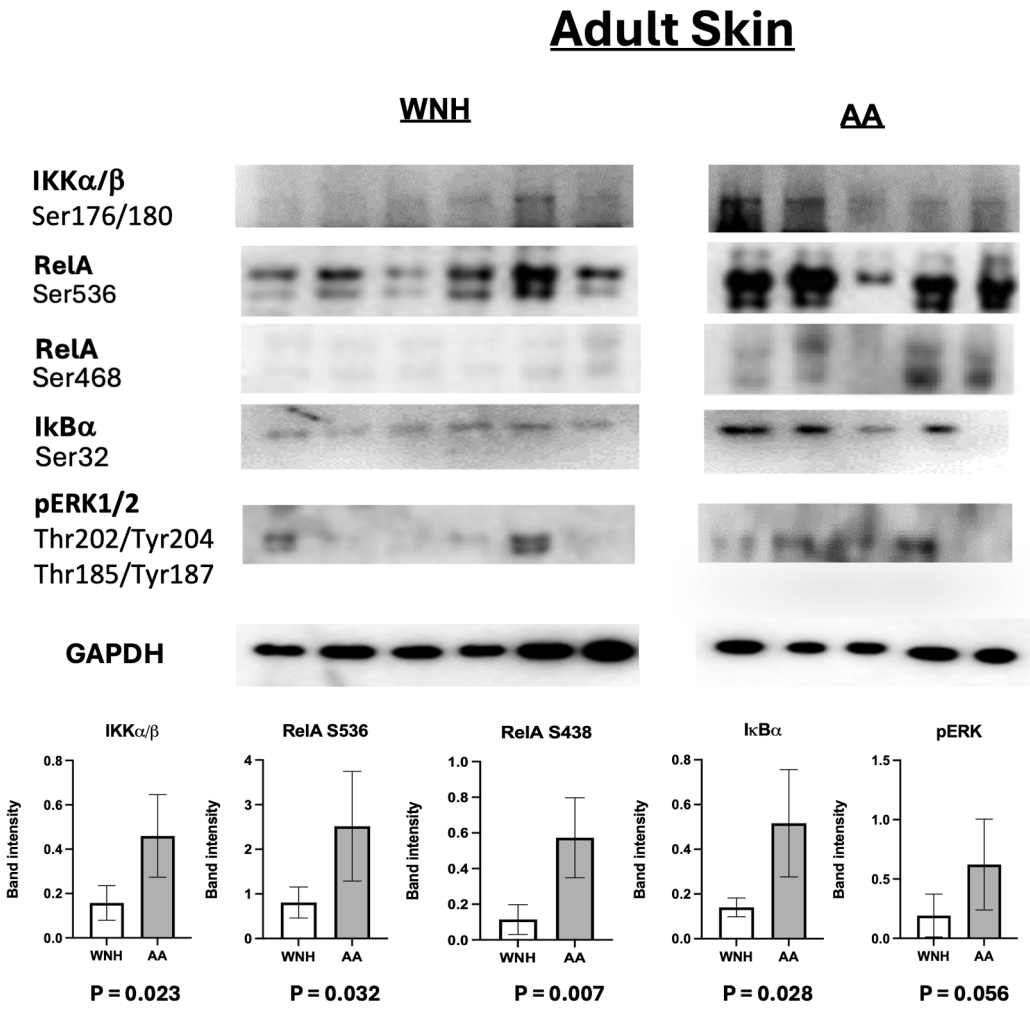

Figure 3. Activation of proinflammatory NF-κB and MAPK signaling pathways in adult AA skin. Protein extracts from AA and WNH adult skin biopsies were used for western blotting. GAPDH was used as a loading control. Western blots were quantified by normalizing protein bands to GAPDH. Statistical analysis was performed using unpaired two-tailed Welch's *t*-test. The error bars represent Mean ±SD. NB: Due to the limited protein amount, we were able to use protein extracts from only four AA samples for Ser32 IκBα analysis. Samples match those seen in Supplementary Figure S1. AA, African American; WNH, White non-Hispanic.

transcription initiation sites in DEGs. To estimate the number of putative binding sites for the TFs NF-κB (RelA), and AP-1 (c-Fos and c-Jun separately) in DEG promoters, we searched within (−100, 1000 bases) from the start codon. DEGs with ≥ 2 binding sites for specific TF were considered TF targets. About 32% of all AA skin DEGs emerged as putative RelA targets, 21% were putative cJUN and 11 % cFOS targets with at least two chromatin immunoprecipitation-Seq peaks in their promoters (Figure 6c).

DISCUSSION

The major goal of this work was to explore previously reported proinflammatory bias in healthy AA skin by proteomics and limited qRT-PCR analysis of previously identified inflammatory and skin barrier DEGs (Klopot et al, 2022). The Olink proteomics analysis of skin biopsies followed by western blot validation revealed the activation of IL1R1/MyD88/IRAK1 axis and the activation of downstream proinflammatory NF-κB and MAPKs signaling. In addition to their leading role in inflammation and cutaneous immunity, NF-κB and MAPKs together with downstream AP-1 TFs are involved in control of skin morphogenesis, affect skin cells proliferation, survival, differentiation and regulate skin barrier function (Eckert et al, 2002; Pasparakis, 2012). Therefore, it is possible that there are some differences in morphology (for example the increased thickness of cornified layer in AA neonatal epidermis, Figure 2b) and physiology of healthy AA skin may result from heightened activation of NF-κB and MAPKs/AP-1.

We acknowledge that our analysis of differential protein expression in adult skin has some limitations due to the small size of volunteer cohort which included only female participants and also due to the semi-quantitative nature of WB analysis used for validation. Nevertheless, we detected a similar increase of IRAK1 expression at protein level and IL1A at mRNA level along with the activation of NF-κB and ERK1/2 in male neonatal AA skin further supporting our results. Those are, to our knowledge, previously unreported findings as comparative analysis of gene/protein expression in neonatal skin of color has not been done previously.

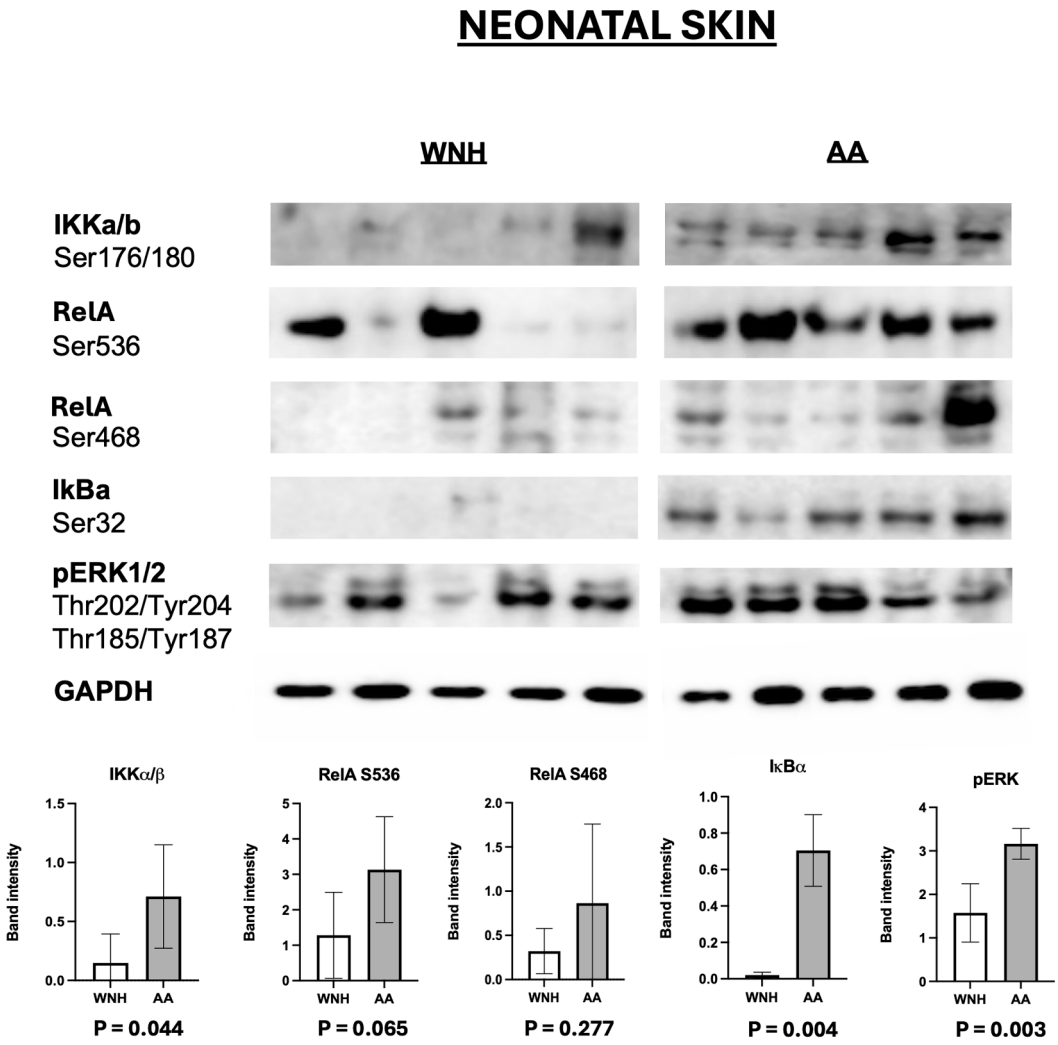

Figure 4. Activation of proinflammatory NF-κB and MAPK signaling pathways in AA neonatal foreskin. Proteins extract from AA and WNH neonatal foreskin were used for western blotting. GAPDH was used as a loading control. Western blots were quantified by normalizing protein bands to GAPDH. Statistical analysis was performed using unpaired two-tailed Welch's *t*-test. The error bars represent Mean ± SD. AA, African American; WNH, White non-Hispanic.

Experiments with three-dimensional HSO cultures further confirmed our major observations and the role of keratinocytes in proinflammatory bias in AA healthy skin.

An important question that requires further investigation is why NF-κB and MAPK signaling pathways are activated in healthy AA skin. A recent analysis of the literature suggests that stress-induced signaling pathways contribute to ethnic heterogeneity in inflammatory skin diseases such as psoriasis and AD through modulation of immune pathways and may influence disease susceptibility (Jamerson et al, 2022). Thus, the environmental and social factors may play a significant role in shaping the molecular landscape of healthy skin. In this work we focused only on two out of 11 DEP proteins, IL1A and IRAK1, as they act cooperatively within the same major pro-inflammation signaling network activated by IL1R1. In addition, IL1A and IRAK1 have other important functions and may activate inflammatory signaling via different mechanisms. The truncated IL1A can enter the nucleus to induce the expression of proinflammatory cytokines such as IL6 and IL8 and some chemokines independently of IL1R1 (Cohen et al, 2015; Malik and Kanneganti, 2018). In turn, IRAK1, a serine/threonine-protein kinase that can phosphorylate multiple targets including E3 ubiquitin ligases, TIRAP — an adaptor protein involved in the toll-like receptor 4 signaling pathway; the interferon regulatory factor 7 (IRF7) resulting in transcriptional activation of type I IFN genes and signal transducer and activator of transcription 3 (Kim et al, 2024).

Other important DEPs revealed by proteomics, include IL22RA1 and IL4 that could contribute to activation of proinflammatory signaling in healthy AA skin. IL22RA1 is a component of the receptor for IL22, IL20 and IL24. IL22RA1 enables IL22 to activate ERK1/2, protein kinase B, JAK/signal transducer and activator of transcription pathways. Activation of IL-22 has been associated with keratinocyte proliferation, epidermal hyperplasia, hyperkeratosis, and other barrier abnormalities and contributes to the development of AD, allergic contact dermatitis, and psoriasis (Laska et al, 2024; Wongvibulsin et al, 2021). As discussed above, Th22 skewing is typical for AD skin in AA and in Tanzanian African

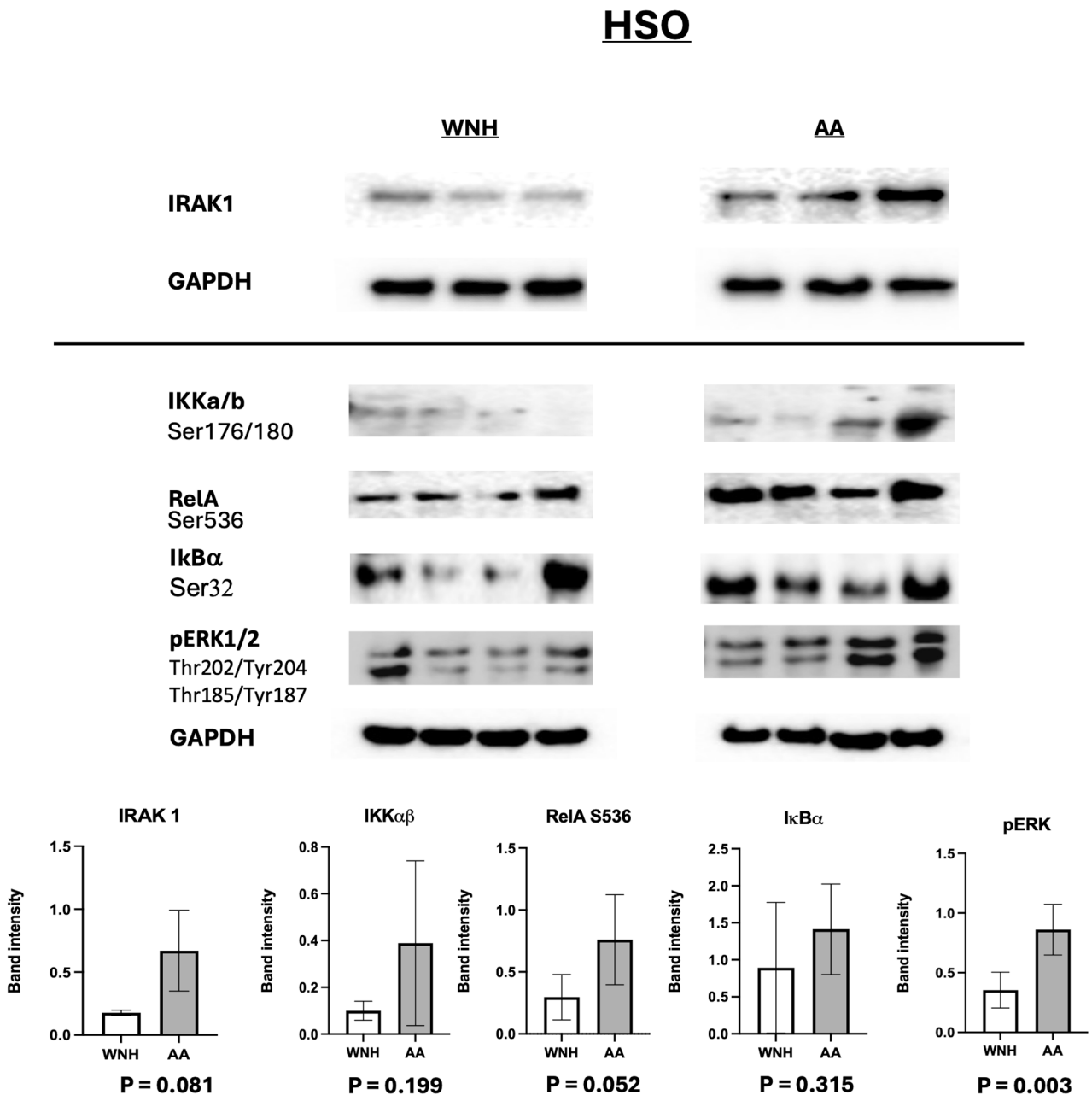

Figure 5. Activation of proinflammatory NF-κB and MAPK signaling pathways in AA HSO. Proteins extract from AA and WNH HSO were used for western blotting. GAPDH was used as a loading control. Western blots were quantified by normalizing protein bands to GAPDH. Statistical analysis was performed using unpaired two-tailed Welch's *t*-test. The error bars represent Mean ± SD. AA, African American; HSO, human skin organoid; WNH, White non-Hispanic.

patients (Lang et al, 2021). Thus, the analysis of T helper 22 and JAK/signal transducer and activator of transcription signaling in healthy AA skin could be an important future focus of research.

Several other DEPs whose function in skin/skin inflammation has been studied less include Follistatin, an inhibitor of follicle-stimulating hormone release. It was shown that Follistatin is highly expressed in keratinocytes and skin fibroblasts, where it negatively regulates the signaling of TGF-β family members activin and bone morphogenic proteins (BMP) and is involved in skin and appendages development, wound healing, and inflammation (Antsiferova et al, 2009). Another important DEP is Copper-containing Diamine Oxidase 1 (AOC1/DAO) - a membrane glycoprotein that deaminates histamine and related molecules and is involved in allergic reactions in skin and other organs (Comas-Basté et al, 2020; Okutan et al, 2023). RABGAP1L (RAB GTPase Activating Protein) and RNA binding protein YTHDF3 have been linked to the development of skin cancer (Shi et al, 2022). Unexpectedly, the expression of NELL2 protein, a marker for AD (Yu and Li, 2022) was lower in AA healthy skin.

In conclusion, this study highlights the importance of continued investigation of healthy AA skin molecular characteristics to better understand their potential relevance to the increased risk of certain inflammatory skin diseases in the AA population.

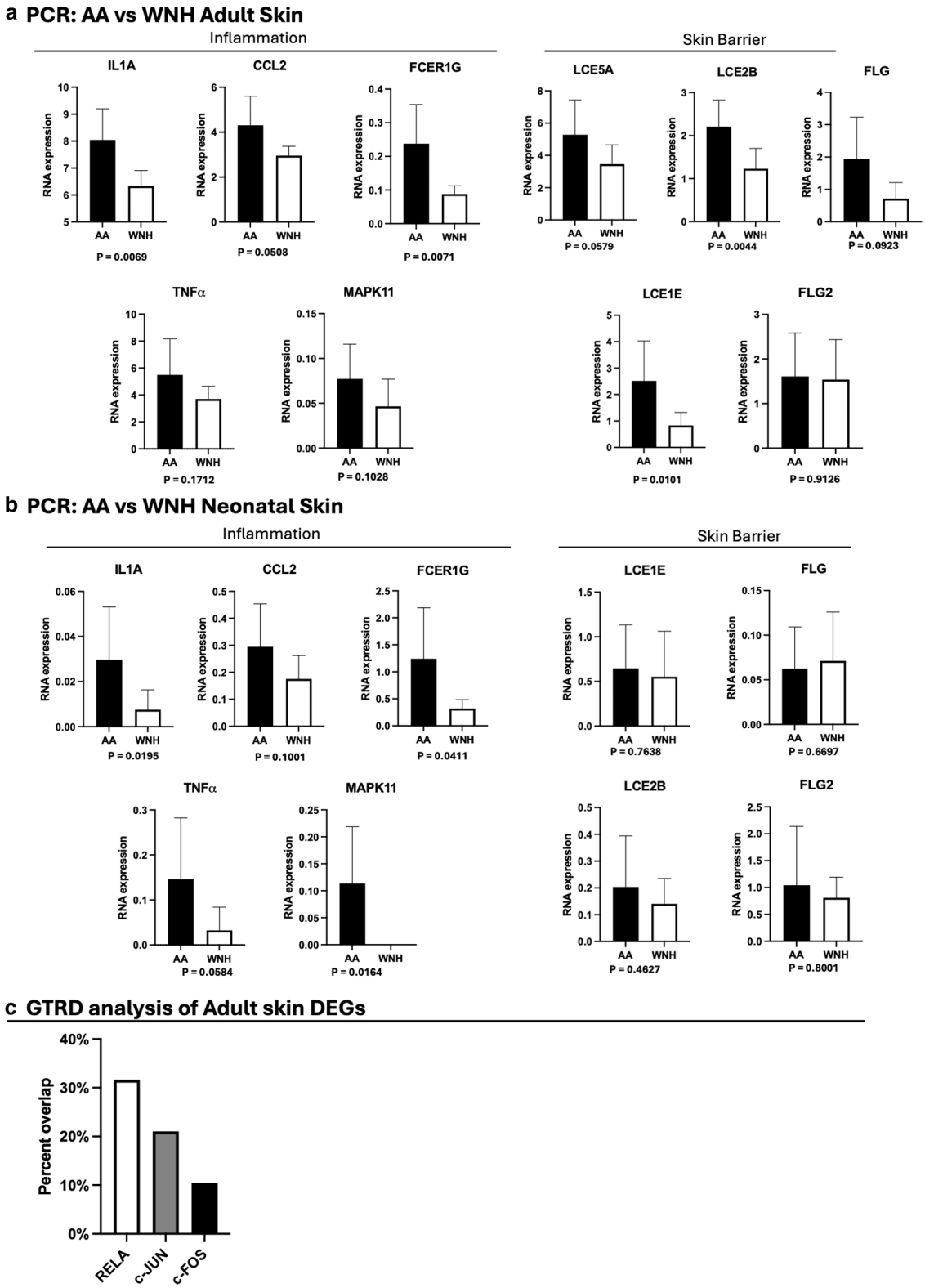

Figure 6. Increased inflammatory and skin barrier gene expression in AA adult and neonatal skin. RNA was extracted from full-thickness skin biopsies from the upper arm skin in AA and WNH healthy volunteers **(a)** and AA and WNH neonatal foreskin (**b**). Gene expression was assessed by qRT-PCR in individual AA versus WNH adult and neonatal skin RNA samples using delta-delta Ct method to detect the amount of target nucleic acid in the sample. RPL27 was used as a cDNA normalization control. Statistical analysis was performed using unpaired two-tailed Welch's *t*-test. The error bars represent Mean ± SD. **(c)** GTRD analysis reveals significant overlap of DEGs in AA compared to WNH skin samples, FC > 1.5; *P*-value < .05) with RelA, cJUN, cFOS target genes. AA, African American; DEG, differentially expressed gene; FC, fold change; WNH, White non-Hispanic.

MATERIALS AND METHODS

Human skin samples

Four mm full-thickness skin biopsies from the volar aspect of upper arm and neonatal foreskin skin were provided by institutional review board-approved tissue acquisition and repository services. Skin biopsies were from healthy volunteers without history of skin diseases. 5 AA and 7 WNH volunteers, all females, aged 24−41 years, were grouped by self-identification combined with Fitzpatrick score. Informed consent was obtained in writing, following the principles of the World Medical Association Declaration of Helsinki and the National Institutes of Health Belmont report. The grouping of neonatal skin samples was based on mothers self-reported ethnicity.

Three-dimensional HSO

HSO were prepared using normal human epidermal keratinocytes isolated from AA and WNH neonatal foreskins seeded on collagen matrix infused with fibroblasts and cultured as described (Simpson et al, 2010).

Morphometric analysis of neonatal skin

Total epidermal thickness, thickness of cornified layer and number of rete ridges/field of view were analyzed by Zeiss Axio Vision software in formalin-fixed paraffin-embedded neonatal skin. At least three individual fields of view/slide in seven AA and seven WNH individual skin samples (totally 20 images per group) were analyzed independently by 2 observers. Fields of view for analysis were randomly selected.

Olink proteomics

Part of snap-frozen adult skin biopsies were homogenized in a Qiagen Tissue PowerLyzer at 3500 rpm in TRIzol according to the manufacturer's protocol (Thermo Fisher Scientific). Total protein was isolated according to manufacturer's protocol and eluted in 1X PBS. 50 μl protein extracts diluted to 1 mg/ml/sample were loaded on a 96-well PCR plate and submitted to Olink for quantification of 384 proteins according to their *Explore* 384 *Inflammation* panel manual. The relative expression data for each protein was provided by Olink as Normalized Protein Expression values. Differential protein expression (DEP) analyses were performed using OlinkAnalyze package in R (https://cran.r-project.org/web/packages/OlinkAnalyze/index.html). We used a nominal *P*-value of .05 as the statistical significance cutoff for the comparison of mean abundance between AA and WNH skin sample groups by an unpaired two-tailed Welch's *t*-test. The DEPs in AA skin compared to WNH skin were identified using criteria of fold change > 1.5 and *P*-value $< .05$.

Protein extraction and western blotting

Proteins from the other part of adult skin biopsies, neonatal foreskin and epidermal compartment of HSO were extracted with Urea Sample Buffer. Protein concentration analysis was determined by amido black assay.

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with TBST: 5% non-fat milk (Bio-Rad) membranes were incubated overnight at 4 °C with primary Abs following incubation with secondary Abs at room temperature. Membranes were imaged using AZURE ECL reagents and the AZURE Chemidoc digital imager after universally increasing images contrast and background correction. The equal loading was normalized to the expression of housekeeping protein GAPDH. Statistical analyses of the data were performed by unpaired two-tailed Welch's *t*-test using Prism 10 software (GraphPad). A *P*-value $< .05$ was considered statistically significant.

Antibodies

We used following Abs: IRAK1 (Cell Sig #4504), NF_kB S536 (Cell Sig #3033), NF_kB S468 (Cell Sig #3039), IKKα/β Ser176/180 (Cell Sig #2697), IκBα S32 (Cell Sig # 2859), pERK1/2 (Cell Sig #4695) pERK1/2 Ab is specific for Thr202/Tyr204 in ERK1 and Thr185/Tyr187 in ERK2, GAPDH (Cell Sig #2118), anti-Rabbit IgG (Cell Sig #7074P2).

RNA extraction and qRT-PCR

The residual part of adult and neonatal skin samples was disrupted and homogenized in QIAzol buffer, and RNA was extracted by Qiagen miRNeasy kit and QIAzol reagent. qRT-PCR analysis of target genes was done using a LightCycler96 RT-PCR machine. We used delta-delta Ct method to detect the amount of target nucleic acid in the sample. Primers were designed using NCBI BLAST or publicly available primer sequences. qRT-PCR was run in duplicates for all samples and equal loading was normalized to the expression of housekeeping gene RPL27.

Gene transcription regulation database analysis

To analyze the potential role of NF-κB and AP-1 TFs in the AA skin DEG regulation [DEGs are from our published GSE120783 study], we employed gene transcription regulation databases webtool (http://gtrd.biouml.org/). We selected meta-clusters and maximum gene distance of 5000 nucleotides to define transcription initiation sites in DEGs and searched for putative binding sites for the human TFs RelA (P65), cFOS and cJUN within -100 - +1000 bases from DEG promoters.

Statistical analysis

Data visualization and statistical analysis was conducted using GraphPad PRISM. Western blot and Microscopy images were analyzed using FIJI (ImageJ). *P*-values were assessed by unpaired two-tailed Welch's *t*-test. *P*-values $< .05$ were considered significant. Error bars are represented as standard deviation of the mean.

ETHICS STATEMENT

Northwestern University Institutional Review Board provided approvals following all local and federal regulations. Informed consent for human biospecimens was obtained in writing (STU00009443) and neonatal foreskin tissue was approved for collection under STU00009443 with a waiver of consent.

DATA AVAILABILITY STATEMENT

All raw and preprocessed RNA-Seq data used in this study were previously published and are deposited in the GEO database with the GEO accession number GSE120783 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120783). All other data generated or analyzed during this study are included in this article and in appendix files.

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CONFLICT OF INTEREST

LCT has received support from Galderma and Janssen. Other authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: IB; Formal Analysis: DT, LCT; Funding Acquisition: IB; Investigation: DT, PG, RK, AK; Methodology: IB, BPW; Validation: DT, PG;

Visualization: PG, BPW; Writing — Original Draft Preparation: IB; Writing — Review and Editing: IB, BPW, PG

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) OR LARGE LANGUAGE MODELS (LLMs)

The author(s) did not use AI/LLM in any part of the research process and/or manuscript preparation.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at 10.1016/j.xjidi.2026.100488.

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