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RESEARCH ARTICLE OPEN ACCESS

The TRPM5 Antagonist Triphenylphosphine Oxide Increases Sebaceous Lipogenesis and Modulates Immune Phenotype of Human Sebocytes in a TRPM5-Independent Manner

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Received: 6 June 2024 | **Revised:** 15 April 2025 | **Accepted:** 22 April 2025

Funding: This project was supported by the National Research, Development and Innovation Office (grant numbers: 134235, 134725, EFOP-3.6.3-VEKOP-16-2017-00009, EFOP-3.6.1-16-2016-00022 and GINOP-2.3.2-15-2016-00050). A.O. is the recipient of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (ID: BO/00660/21/5). A.O. was supported by the ÚNKP-22-5 and ÚNKP-23-5 New National Excellence Programs of the Ministry for Culture and Innovation from the source of the National Research, Development and Innovation Fund ('Bolyai+ Scholarship', grant numbers: ÚNKP-22-5-DE-427 and ÚNKP-23-5-DE-477) as well as by the 'Momentum' proof-of-concept fund (Faculty of Medicine, University of Debrecen). Sz.P. was supported by the project TKP2021-NKTA-34, which has been implemented with the support provided by the Ministry for Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-NKTA funding scheme.

Keywords: acne | dry skin | inflammation | sebocyte | sebum | TPPO

ABSTRACT

Transient receptor potential melastatin 5 (TRPM5) ion channel is expressed in human hair follicles, where its spontaneous activity contributes to the maintenance of the growing, anagen phase of the hair cycle. Because adjacent sebaceous glands also exhibited TRPM5 immunopositivity, topically applied TRPM5 modulators administered to influence hair growth may also affect sebaceous glands. Hence, we aimed to assess expression of TRPM5 as well as effects of TRPM5 modulators [activators: 2,5-dimethylpyrazine, 2-heptanone; antagonist: triphenylphosphine oxide (TPPO)] on human SZ95 sebocytes, i.e., on the best available in vitro model to study human sebaceous glands. First, using complementary methods [RNA-Seq, RT-qPCR, western blot, siRNA-mediated gene silencing and fluorescent Na^+- (SBFI AM) and Ca^{2+}-measurements (Fura-2 AM)], we found that TRPM5 is not expressed in human sebocytes in a functionally active form. Importantly, while non-cytotoxic (MTT-assay) concentrations of the activators were ineffective, TPPO promoted sebaceous lipogenesis (Nile Red labelling). This effect was TRPM5-independent and was found to be mediated in an Akt- and epidermal growth factor receptor (EGFR)-dependent manner,

Abbreviations: ABCA1, ATP-binding cassette subfamily A member 1; CSF2, colony-stimulating factor 2, a.k.a. granulocyte-macrophage colony-stimulating factor; CXCL, C–X–Cmotif chemokine ligand; DGAT2, diacylglycerol O-acyltransferase 2; DMP, 2,5-dimethylpyrazine; EGFR, epidermal growth factor receptor; Hept, 2-heptanone; HSP27, heat shock protein 27; IGF, insulin-like growth factor; IL, interleukin; MAPK, mitogen-activated protein kinase; MMP9, matrix metallopeptidase 9; Nefl, Neflamapimod; NFKBIA, NFKB inhibitor alpha; PPIA, peptidylprolyl isomerase A; SCR, scrambled RNA; TNFSF15, tumour necrosis factor superfamily member 15; TPPO, triphenylphosphine oxide; TRPM, transient receptor potential melastatin; Tyr, tyrphostin AG.

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most likely via the Akt-induced up-regulation of diacylglycerol O-acyltransferase (DGAT)-2. Moreover, TPPO up-regulated interleukin (IL)-6 in an EGFR- and p38α MAPK-dependent manner (RT-qPCR), whereas it decreased the release of IL-8 (ELISA), and down-regulated additional pro-inflammatory cytokines [chemokine (C-X-C motif) ligand [CXCL]-1, CXCL2, CXCL6, colony-stimulating factor 2, IL-32; RNA-Seq]. Collectively, specific TRPM5 modulators are unlikely to exert direct sebaceous gland-related side effects, while safe TPPO analogues may induce beneficial moderate lipogenic and anti-inflammatory effects in dry skin dermatoses.

1 | Introduction

Transient receptor potential melastatin 5 (TRPM5) is a Ca^{2+}-activated, thermosensitive (14°C–38°C) cation channel that is mostly permeable for Na^+ and K^+. It is involved in chemosensation and regulates insulin release in pancreatic β-cells [1, 2]. According to a recent study, it is also expressed in human hair follicles [3], and its homeostatic activity appears to play a role in maintaining the anagen (growth) phase of the hair cycle, since both siRNA-mediated silencing of the channel as well as its pharmacological blockade with triphenylphosphine oxide (TPPO) promoted premature entry to the regressive catagen hair cycle phase [3]. Thus, appropriate pharmacological modulation of TRPM5 emerged as a potential novel therapeutic tool in treating certain forms of unwanted hair loss or overgrowth. Importantly, however, according to the immunofluorescent images published in the same article, not only certain regions of the hair follicles but also the adjacent sebaceous glands exhibited TRPM5 positivity [3], highlighting the possibility that TRPM5 modulators administered with the intention of influencing hair growth may also have an unintended impact on sebaceous gland functions. Because such interactions may theoretically give rise to severe side effects (including iatrogenic acneiform eruptions or skin dryness), we aimed to investigate in this study the expression and functional role of TRPM5 as well as the potential biological effects of its known modulators (i.e., TPPO and two agonists, namely 2,5-dimethylpyrazine [DMP] and 2-heptanone [Hept]) [4, 5] on human sebocytes.

2 | Materials and Methods

2.1 | Materials

The TRPM5 modulators triphenylphosphine oxide [TPPO; Cat. No. T84603, solvent: dimethyl sulfoxide (DMSO; Cat. No. 34869; Sigma-Aldrich, St. Louis, MO, USA)], 2-Heptanone [Hept; Cat. No. 8187110100; solvent: phosphate-buffered saline (PBS; 115 mM NaCl, 20 mM Na_2HPO_4, pH 7.4; all from Sigma-Aldrich)], 2,5-dimethylpyrazine (DMP; Cat. No. W327204; solvent: PBS), an ultrapotent TRPV4 agonist [6] GSK1016790A (Cat. No. G0798) and the EGFR antagonist tyrphostin AG 1478 [Tyr; Cat. No. T4182, solvent: absolute ethanol (Cat. No. 1001901000; Scharlab Magyarország Kft., Debrecen, Hungary)] were purchased from Sigma-Aldrich. The HSP27 inhibitor J2 (Cat. No. HY-124653), the IGF-1R antagonist AG1024 (Cat. No. HY-10253) and the p38α MAPK inhibitor Neflamapimod (Nefl; Cat. No. HY-10328) were obtained from MedChem LLC (Sollentuna, Sweden), whereas the Akt1/2/3 inhibitor (S)-4-(2-(4-Amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-(piperidin-3-ylmethoxy)-1H-imidazo[4,5-c]pyridin-4-yl)-2-methylbut-3-yn-2-ol (GSK690693; Cat. No. 4144) was purchased from Tocris Bioscience (Bristol, UK). All inhibitors as well as the IGF-1R antagonist were dissolved in DMSO.

We prepared 1000× concentrated stock solutions of the materials that have been dissolved in the culture medium in 1:1000 to achieve the desired working concentrations. Control cultures were always treated by using identical amount of vehicle(s).

2.2 | Cell Culturing

There are no adequate animal model systems to study the whole complexity of the (patho)physiology of human sebaceous glands [7–11]. Moreover, primary human sebocytes exhibit rapid spontaneous differentiation, which, due to the holocrine nature of sebum production, makes them inappropriate for extensive in vitro studies [7–11]. Thus, in the current study, we opted to use human immortalised SZ95 sebocytes [9], the best studied and widely accepted model system to investigate the biology of human sebaceous glands in vitro [7–11]. SZ95 sebocytes were originated from human facial sebaceous glands [9]. The cells were provided by Prof. Christos C. Zouboulis (the sole provider of the cell line), and only cells below passage number 65 were used in the study. SZ95 sebocytes were cultured in Sebomed Basal Medium (Cat. No. F8205; Merck KGaA, Darmstadt, Germany) supplemented with 10 (V/V)% foetal bovine serum (Cat. No. 10500064; Gibco, Thermo Fisher Scientific Waltham, MA, USA), 1 mM $CaCl_2$ (Cat. No. C7902; Sigma-Aldrich), 5 ng/mL human epidermal growth factor (Cat. No.: E9644; Sigma-Aldrich), 1.12 μg/mL Amphotericin B (Cat. No. 15290018; Gibco) and MycoZap Plus-CL (1:500; Cat. No.: VZA-2012 Lonza, Basel, Switzerland) [12–19]. The medium was renewed every other day, and cells were subcultured at 60–70% confluence. Cells were regularly checked for *Mycoplasma* contamination by using MycoStrip Mycoplasma Detection Kit (Cat. No. rep-mys-10; Invivogen, San Diego, CA, USA), and every assessment yielded a negative result.

2.3 | RNA Isolation, Reverse Transcription and Quantitative 'Real-Time' PCR (RT-qPCR) [19, 20]

RT-qPCR was performed on a Roche LightCycler 480 System (Roche, Basel, Switzerland) using the 5′ nuclease assay. SZ95 sebocytes were seeded in 500 000 cells/35-mm Petri dish density in 1.5 mL culture medium and were treated as indicated. The total RNA was extracted using TRI Reagent Solution (Cat. No. AM9738; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). DNase treatment was performed according to the manufacturer's protocol

with DNase I and DNase I buffer of DNase I, Amplification Grade (Cat. No. 18068015; Invitrogen) and 50 mM EDTA (Cat. No. 324503; Merck Millipore, Burlington, MA, USA). One microgram of total RNA was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Cat. No. 4374967; Applied Biosystem, Thermo Fisher Scientific).

PCR amplification was performed using the TaqMan Gene Expression Assays (assay IDs: Hs00175822_m1 and Hs05060590_s1 for TRPM5, Hs00174092_m1 for IL-1α, Hs00174097_m1 for IL-1β, Hs00985639_m1 for IL-6, Hs00174103_m1 for IL-8, Hs01059101_m1 for ABCA1 and Hs01045913_m1 for DGAT2, all from Applied Biosystems) and the TaqMan Gene Expression Master Mix (Cat. No.: 4369016; Applied Biosystems) according to the manufacturer's protocol. As internal controls, transcripts of peptidylprolyl isomerase A (PPIA; assay ID: Hs99999904_m1) were determined. The amount of the transcripts was normalised to those of the housekeeping gene using the ΔCT method. Finally, the relative expression values were further normalised to the ones of the vehicle-treated controls (ΔΔCT method).

2.4 | RNA-Seq Method [19, 20]

2.4.1 | Sample Preparation [19, 20]

SZ95 sebocytes were seeded in 500 000 cells/35-mm Petri dish/1.5 mL culture medium. The indicated 24-h treatments [vehicle control, TPPO (300 μM)] were started on the next day using three biological replicates per group. Total RNA was extracted using TRI Reagent Solution (Cat. No. AM9738; Invitrogen, Thermo Fisher Scientific).

To obtain global transcriptome data, high-throughput mRNA sequencing analysis was performed on the Illumina-sequencing platform. Total RNA sample quality was checked on the Agilent BioAnalyzer using the Eukaryotic Total RNA Nano Kit (Cat. No. 5067-1511; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Samples with RNA integrity number (RIN) value >7 were accepted for library preparation process. RNA-Seq libraries were prepared from total RNA using Ultra II RNA Sample Prep kit (Cat. No. E7775S; New England BioLabs; Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, poly-A RNAs were captured by oligo-dT conjugated magnetic beads, then the mRNAs were eluted and fragmented at 94°C. First-strand cDNA was generated by random priming reverse transcription, and after the second-strand synthesis step, double-stranded cDNA was generated. After repairing ends, A-tailing and adapter ligation steps, adapter ligated fragments were amplified in enrichment PCR, and finally, sequencing libraries were generated. Sequencing runs were executed on the Illumina NextSeq 500 instrument (Illumina, San Diego, CA, USA) using single-end 75 cycles sequencing.

2.4.2 | RNA-Seq Data Analysis [19, 20]

Raw-sequencing data (fastq) was aligned to the human reference genome version GRCh38 using the HISAT2 algorithm, and BAM files were generated. Downstream analysis was performed using StrandNGS software (www.strand-ngs.com). BAM files were imported into the software, and the DESeq algorithm was used for normalisation. A moderated *t*-test was used to determine differentially expressed genes between conditions. Raw data of the RNA-seq analysis are accessible in the NCBI SRA database (hyperlink to the data: http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1037731).

2.4.3 | Pathway Analyses [19, 20]

CytoScape v3.4 software with *ClueGo v2.3.5 application* was used for identifying over-represented gene ontology (GO) terms. A two-sided hypergeometric test with Bonferroni step-down correction was performed using the list of differentially expressed genes and GO Biological process database.

2.5 | siRNA Transfection-Mediated Selective Gene Silencing of TRPM5

Human SZ95 sebocytes were cultured in Petri dishes ($d=150$ mm), and cells were transfected with siRNA oligonucleotides targeting human TRPM5 (Stealth RNA_i, assay IDs: HSS120989, HSS120990 and HSS179077, all from Thermo Fisher Scientific) using Lipofectamine RNA_i MAX Transfection Reagent (Cat. No. 13778150; Thermo Fisher Scientific) and serum-free Opti-MEM (Cat. No. 31985-070; Gibco). For control, siRNA Negative Control Duplexes with 'medium' GC ratio (SCR; Cat. No. 12935100; Thermo Fisher Scientific) were employed (3 h at 37°C). Silencing efficiency was monitored at the protein level (Western blot) on post-transfection day 2.

2.6 | Statistical Analysis

Data were analysed by *GraphPad Prism 10.1.0 (316)* (GraphPad Software LLC, San Diego, CA, USA). Outliers were identified by using the ROUT method ($Q=1\%$). Depending on the sample size, Gaussian distribution was tested by Anderson-Darling or Shapiro–Wilk tests. In case of Gaussian distribution, Student's two-tailed, unpaired *t*-test (paired comparisons), one-way ANOVA followed by Šídák's multiple comparisons test (multiple comparisons) or Dunnett's multiple comparisons test (comparison to a single control group) were used, whereas in case of non-Gaussian distribution, two-tailed Mann–Whitney test (paired comparisons) or Kruskal–Wallis test followed by Dunn's multiple comparisons test (multiple comparisons) were used, and $p<0.05$ values were regarded as significant differences. Graphs were plotted using *GraphPad Prism 10.1.0 (316)*.

Description of the methods to assess viability (MTT assay), lipid synthesis (Nile Red labelling), siRNA-mediated silencing of EGFR, Ca^{2+}- and Na^{+}-homeostasis (Fura-2 AM- and SBFI AM-based fluorescent measurements, respectively), as well as expression at the protein level (western blot) and phosphorylation status of selected signalling molecules (phosphokinase array) can be found in the Data S1.

3 | Results

3.1 | Expression of TRPM5 Is Dubious in Human Sebocytes

First, by using pre-confluent (actively proliferating) as well as post-confluent (hence spontaneously differentiated) sebocytes, we intended to confirm the presence of TRPM5 on these cells, and therefore, we assessed the expression of TRPM5 at the mRNA level. Interestingly, although we tested our samples by using two different TaqMan assays, mRNA expression level of TRPM5 was found to be below/around the detection limit in all experiments (RT-qPCR Ct values > 40 in all experiments; Table S1). Next, we continued our investigation at the protein level by using the same antibody that was applied in the aforementioned publication [3]. Because this attempt yielded several (seemingly non-specific) bands at unexpected molecular weights on the western blot (Figure S1), we tried another antibody as well. Importantly, this antibody resulted in clearer, specific-looking bands; however, immunopositivity did not appear at the molecular weight predicted by the manufacturer (i.e., at ~100 kDa) but was located at a somewhat higher molecular weight (~110 kDa; Figure 1A). In order to challenge the specificity of the bands mentioned above, we transfected the sebocytes by using TRPM5-specific siRNA constructs and found that none of the three constructs were efficient in significantly decreasing the relative optical density of the western blot bands compared to the non-sense RNA-transfected scrambled control (SCR) group (western blot; Figure 1B,C). Because together with the negative RT-qPCR data, these western blot and gene silencing findings raised suspicion regarding the specificity of the labelling, we tried to investigate the functional activity of TRPM5 using fluorescent Na^+- and Ca^{2+}-sensitive dyes and found no evidence of measurable TRPM5 activity in response to non-cytotoxic (MTT assay; Figure S2) concentrations of TRPM5 modulators (for further details, see Results section in Data S1 as well as Figure S3). These functional data, together with the aforementioned RT-qPCR, western blot and siRNA transfection findings strongly argued that, in contrast to hair follicles [3], human sebocytes most likely do not express TRPM5 in a functionally active form, and even if they do, the applied modulators cannot measurably influence its activity.

3.2 | The TRPM5 Antagonist TPPO Promotes Sebaceous Lipogenesis and Influences Immune Phenotype of Human Sebocytes

As mentioned above, our data rendered the functional expression of TRPM5 in human sebocytes rather unlikely. However,

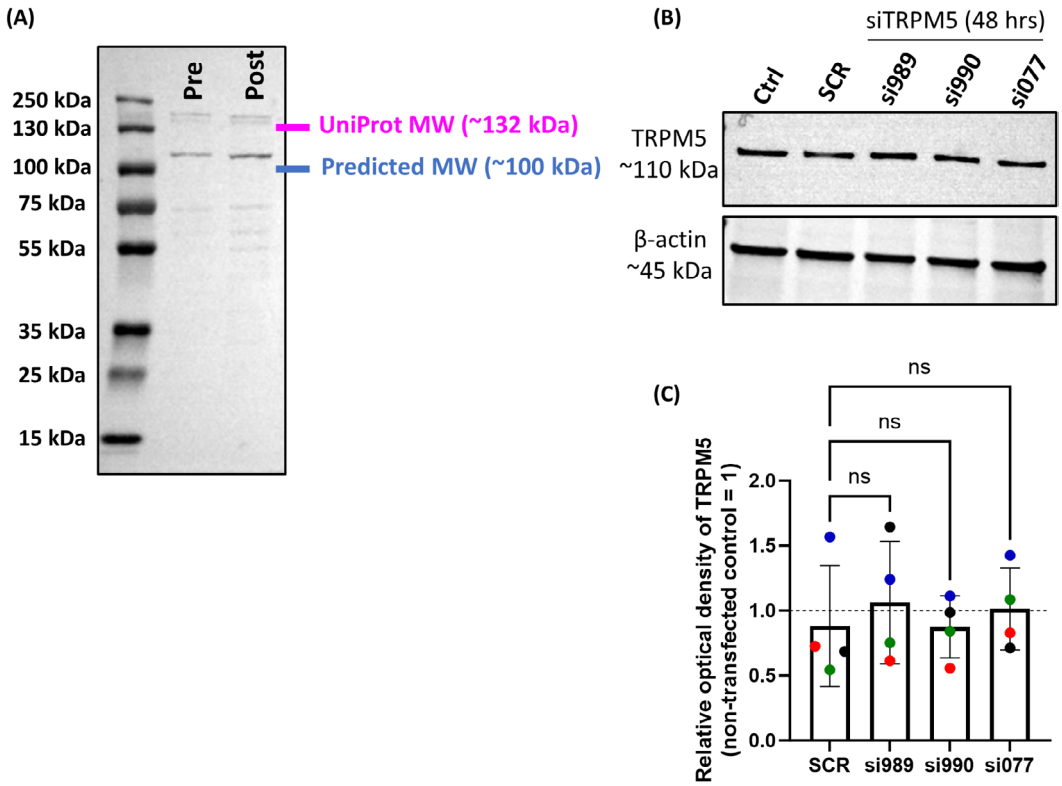

FIGURE 1 | Expression of TRPM5 is dubious in human sebocytes. Western blot. (A) Human sebocytes were cultured and harvested at pre-confluent (non-differentiated, actively proliferating) and post-confluent (spontaneously differentiated) stages, and western blot analysis was performed (primary antibody: Abcam [ab154788]). The strongest bands were observed at ca. 110 kDa. Note that the molecular weight of TRPM5 is ca. 132 kDa according to the UniProt database (pink marker), and that the manufacturer predicted the appearance of the TRPM5-specific bands at ca. 100 kDa (blue marker). (B, C) siRNA-mediated silencing of TRPM5. Samples were harvested 2 days after the transfection. Panel (B) shows representative immunoblots of TRPM5 and β-actin (loading control), whereas results of the densitometry analysis are shown on panel (C). Mean of the non-transfected control cultures is regarded as 1 (dashed line). Data are expressed as mean ± SD of four biological replicates (from four independent transfections). Each colour represents data from an independent experiment. Ctrl, non-transfected control; ns, not significant; SCR, non-sense RNA-transfected ('scrambled' control); si989/si990/si077: TRPM5-specific siRNA constructs; TRPM, transient receptor potential melastatin.

because topically applied TRPM5-targeting hair growth modulators may theoretically influence sebaceous gland biology in a TRPM5-independent manner as well, we decided to investigate the effects of the TRPM5 antagonist TPPO and the agonist DMP by using human sebocytes. To this end, first, we confirmed our aforementioned viability data (Figure S2A–F) by using Nile Red labelling to assess polar (i.e., mostly membrane) lipids, whose levels usually correlate with the cell count [21, 22]. The assay revealed that while up to 300 μM, both DMP and TPPO exerted only negligible effects, 1000 μM TPPO (but not DMP) significantly decreased the level of polar lipids following 24- and 48-h treatments (Figure S4A–D). These data argued that both DMP and TPPO can most likely be used without the risk of any cytotoxic effects up to 300 μM.

Next, we assessed the level of neutral (sebaceous) lipids. We found that DMP did not influence the lipid synthesis (Nile Red; 24- and 48-h treatments; Figure S5A,B). Interestingly, however, TPPO could significantly and concentration-dependently increase sebaceous lipogenesis (Nile Red; Figure 2A,B) over the course of 24- and 48-h treatments. As expected, neither Hept nor DMP could prevent this action (Nile Red; 48-h treatments; Figure S6A,B), confirming that it indeed develops in a TRPM5-independent manner. Moreover, TPPO could also significantly increase the expression of the key pro-inflammatory and sebaceous lineage-indicating cytokine interleukin (IL)-6 [23], while it differentially influenced the expression of IL-1α and IL-1β, and it had no significant effect on the expression of IL-8 (RT-qPCR; 3- and 24-h treatments; Figure 2C–J). Interestingly, TPPO did not increase IL-6 release over the course of 3- and 24-h treatments but significantly suppressed the release of IL-8 (24-h treatments) (ELISA; Figure S7A–D).

3.3 | Activation of Akt- and EGFR-Signalling Is Involved in the Lipogenic Effect of TPPO

Next, in order to identify the signalling pathways involved in mediating the effects of TPPO, we employed a phosphokinase array enabling the simultaneous investigation of several relevant signalling pathways. We found that short-term (10-, 30- and 60-min) treatments with 300 μM TPPO increased phosphorylation of several potentially 'acne-relevant' signalling molecules, including Akt [13, 24, 25], p38α mitogen-activated protein kinase (MAPK) [26], epidermal growth factor receptor (EGFR) [27] and the p38α MAPK down-stream molecule heat shock protein (HSP)-27[28] in a time-dependent manner (Figure 3A,B). Next, using appropriate pharmacological inhibitors, we showed that inhibition of Akt, p38α MAPK and EGFR by GSK690693 (1–10 μM), Neflamapimod (Nefl 0.1–10 μM) and tyrphostin AG (1–10 μM), respectively, could significantly suppress TPPO-induced sebaceous lipogenesis (Nile Red; 48-h treatments; Figure 3C–E). Importantly, GSK690693 and tyrphostin AG did not influence the level of polar lipids in the samples, indicating that inhibition of Akt and antagonism of EGFR might have selectively interfered with the lipogenic effect of TPPO, whereas the p38α MAPK-inhibitor Nefl significantly and concentration-dependently suppressed the level of the polar lipids as well, suggesting that its apparent lipostatic activity was at least in part the consequence of a moderate anti-proliferative effect (Nile Red; 48-h treatments; Figure S8A–C). It is noteworthy that this concept was further supported by the observation that the moderate decrease of TPPO's lipogenic effect seen in case of HSP27 inhibition (one of p38α MAPK's down-stream effector molecules) [28] by using J2 (0.1–1 μM) was also accompanied by a significant, concentration-dependent (0.1–10 μM) suppression of the level of polar lipids and hence, was most likely the consequence of the reduction of the cell count (Nile Red; 48-h treatments; Figure S9A,B).

Finally, in order to further confirm the role of EGFR in mediating the lipogenic action of TPPO, we used siRNA transfection to silence its expression. In line with our pharmacological data, we found that successful silencing (western blot; Figure 3F,G) of EGFR could efficiently abrogate the lipogenic effect of TPPO (Nile Red; 48-h treatments; Figure 3H).

3.4 | TPPO-Induced Up-Regulation of IL-6 Appears to Be Independent of the Lipogenic Akt-Signalling and Is Rather Mediated by EGFR and the p38α MAPK Pathway

Next, by using the most potent lipostatic concentrations of Nefl (1 μM), GSK690693 (10 μM) and tyrphostin AG (10 μM), we assessed the involvement of the respective signalling pathways in the TPPO-induced up-regulation of IL-6. We found that co-administration of Nefl and tyrphostin AG, but, interestingly, not of GSK690693, could significantly suppress TPPO-induced up-regulation of IL-6 (RT-qPCR; 24-h treatments; Figure 3F). These data strongly argued that, unlike sebaceous lipogenesis, up-regulation of IL-6 is most likely independent of the lipogenic Akt-signalling and is rather mediated by EGFR and the p38α MAPK pathway, which is essential to maintain sebocyte proliferation in vitro and in vivo [29–31].

3.5 | RNA-Seq Analysis Reveals That TPPO Deeply Influences Immune Phenotype of Human Sebocytes and Identifies Several Clinically Relevant, Putative Target Genes

Next, in order to get a deeper insight to the mechanism of action of TPPO, we subjected TPPO-treated sebocytes as well as appropriate vehicle-treated controls to RNA-Seq analysis (24-h treatments). In line with our RT-qPCR findings (Table S1), no TRPM5-specific sequences were found in either of the investigated samples, indicating that human sebocytes indeed do not express this channel. On the other hand, pathway analysis revealed that TPPO-treatment modulated several 'sebocyte-relevant' signalling pathways, such as 'CXCR chemokine receptor binding', 'acylglycerol-O-acyltransferase activity' or 'insulin-like growth factor binding' (Figure 4A). These pathway analysis data together with the facts that insulin-like growth factor-1 receptor (IGF-1R) is a positive regulator of sebaceous lipogenesis [25, 32] and it is one of the possible up-stream regulators of the Akt-signalling in human sebocytes [25] collectively highlighted the possibility that TPPO may directly or indirectly activate IGF-1R. Thus, we investigated whether an IGF-1R antagonist (AG1024) influenced lipogenic effect of TPPO. Importantly, we found that co-administration of AG1024 did not decrease TPPO-induced lipogenesis (Nile Red; 48-h treatments; Figure S10), indicating that IGF-1R is most likely not targeted by TPPO.

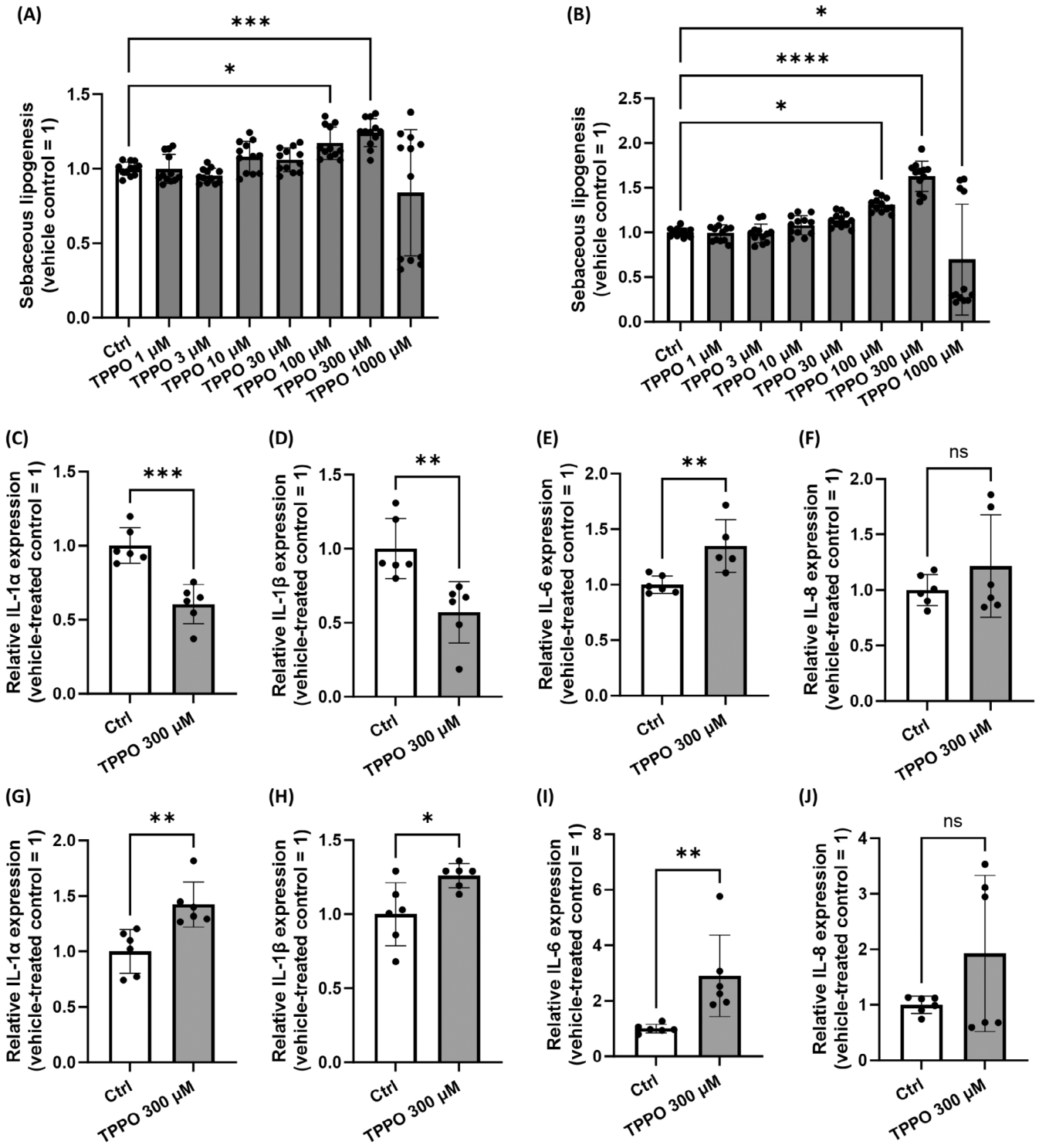

FIGURE 2 | TPPO concentration-dependently enhances sebaceous lipogenesis and differentially modulates expression of various pro-inflammatory cytokines. (A, B) Nile Red labelling for neutral (sebaceous) lipids. Lipogenesis of SZ95 sebocytes was assessed following the indicated 24- (A) and 48-h (B) treatments. Mean of the vehicle-treated control group is regarded as 1. Data are presented as mean ± SD of $N = 12$ biological replicates. (C–J) RT-qPCR. mRNA expression of IL-1α (C, G), IL-1β (D, H), IL-6 (E, I) and IL-8 (F, J) was assessed by RT-qPCR following the indicated 3- (C–F) or 24-h (G–J) treatments of SZ95 sebocytes. Data are presented by using the ΔΔCT method regarding PPIA-normalised mRNA expressions of the vehicle-treated control group as 1. Data are expressed as mean ± SD of $N = 5–6$ biological replicates (each determined as mean of three technical replicates). *, **, *** and **** $p < 0.05$, 0.01, 0.001 and 0.0001, respectively, as indicated. IL, interleukin; ns, not significant; PPIA, peptidylprolyl isomerase A (internal control); TPPO, triphenylphosphine oxide; TRPM, transient receptor potential melastatin.

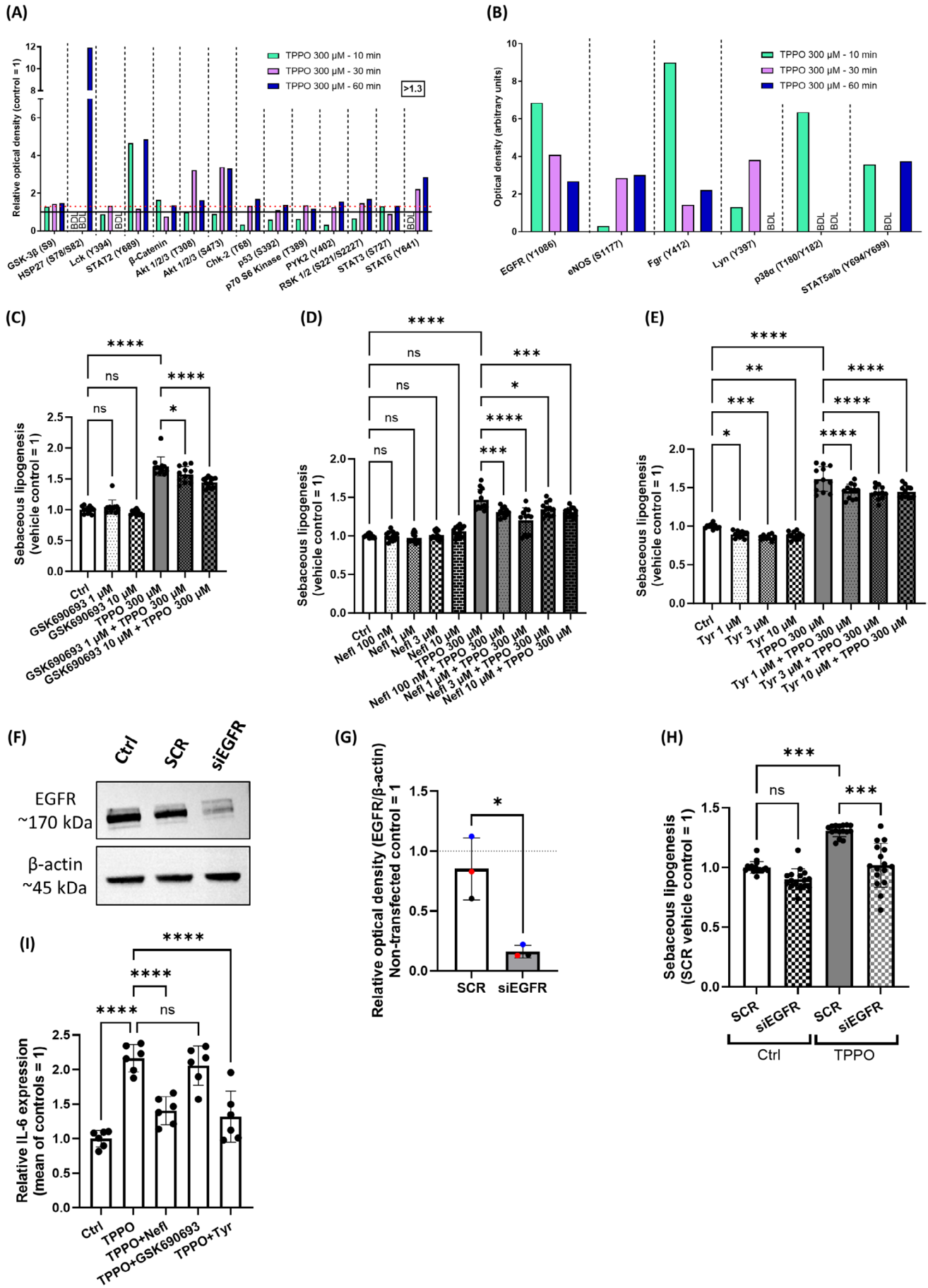

FIGURE 3 | Legend on next page.

FIGURE 3 | Lipogenic and inflammatory effects of TPPO are most likely mediated via the activation of partially overlapping signalling pathways. (A, B) Phosphokinase array (when relevant, phosphorylated sites are shown in parenthesis). SZ95 sebocytes were treated as indicated. Samples were then harvested and processed as described in the (Data S1) Supplementary Materials and Methods section. (A) Following appropriate background subtraction, mean signal intensities (calculated from data of 2 technical replicates) were normalised to the mean signal intensity of the same molecule measured in case of the vehicle-treated (10 min) control culture (control = 1; solid line). ≥ 1.3-fold TPPO-induced increase of the signal intensity was considered relevant alteration (indicated by red dashed line). When mean signal intensity in the control group was found to be at or below the background level, we plotted raw optical density data of those molecules that appeared following the given treatment (B). (C–E) Nile Red labelling for neutral (sebaceous) lipids. Lipogenesis of SZ95 sebocytes was assessed following the indicated 48-h treatments. Mean of the vehicle-treated control group is regarded as 1. Data are presented as mean ± SD of $N = 12$ biological replicates. (F–H) siRNA-transfection-mediated silencing of EGFR. Samples were harvested 4 days after the transfection. Panel (F) shows representative immunoblots of EGFR and β-actin (loading control), while results of the densitometry analysis are shown on panel (G). Mean of the non-transfected control cultures is regarded as 1 (dashed line). Data are expressed as mean ± SD of 3 biological replicates (from three independent transfections). Each colour represents data from an independent transfection. (H) Nile Red labelling for neutral (sebaceous) lipids. Lipogenesis of SZ95 sebocytes was assessed following the indicated 48-h treatments (TPPO: 300 μM). Treatments were started on post-transfection Day 4 (i.e., when EGFR expression was the lowest). Mean of the vehicle-treated SCR control group is regarded as 1. Data are presented as mean ± SD of $N = 16$ biological replicates. (I) RT-qPCR. mRNA expression of IL-6 was assessed by RT-qPCR following the indicated 24-h treatments (GSK690693: 10 μM; Nefl: 1 μM; TPPO: 300 μM; Tyr: 10 μM). Data are presented by using the ΔΔCT method regarding PPIA-normalised mRNA expressions of the vehicle-treated control group as 1. Data are expressed as mean ± SD of $N = 6$ biological replicates (each determined as mean of three technical replicates). *, **, *** and **** $p < 0.05$, 0.01, 0.001 and 0.0001, respectively, as indicated. BDL, below detection limit; Chk, checkpoint kinase; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; Fgr, FGR proto-oncogene (src family tyrosine kinase); GSK, glycogen synthase kinase (inhibitory phosphorylation); GSK690693, (S)-4-(2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-(piperidin-3-ylmethoxy)-1H-imidazo[4,5-c]pyridin-4-yl)-2-methylbut-3-yn-2-ol (Akt-inhibitor); HSP, heat shock protein; IL, interleukin; Lck, lymphocyte protein tyrosine kinase; Lyn, LYN proto-oncogene (src family tyrosine kinase); MAPK, mitogen-activated protein kinase; Nefl, Neflamapimod (p38α MAPK inhibitor); ns, not significant; PPIA, peptidylprolyl isomerase A (internal control); PYK, proline-rich tyrosine kinase; RSK, ribosomal protein S6 kinase; SCR, scrambled RNA-transfected group; siEGFR, EGFR-silenced group; STAT, signal transducer and activator of transcription; TPPO, triphenylphosphine oxide; TRPM: transient receptor potential melastatin; Tyr, tyrphostin AG 1478 (EGFR antagonist).

Besides the above pathway analysis, our RNA-Seq also identified a number of important target genes that were significantly (≥ 1.5 fold-change, $p < 0.05$) up- or down-regulated by TPPO. Indeed, as shown in Figure 4B, TPPO down-regulated multiple potentially 'acne-relevant' inflammatory (colony-stimulating factor 2 [CSF2 also know as granulocyte-macrophage colony-stimulating factor], C-X-C motif chemokine ligand [CXCL]-1 [also known as GRO-α], CXCL2, CXCL6, IL-32, NFKB inhibitor alpha [NFKBIA], tumour necrosis factor superfamily member 15 [TNFSF15]) and other (matrix metallopeptidase 9 [MMP9] [33], ATP-binding cassette subfamily A member 1 [ABCA1] [34]) molecules, while increased the expression of diacylglycerol O-acyltransferase 2 (DGAT2) [35, 36] (Figure 4B). Because ABCA1$^{-/-}$ mice were shown to have sebaceous gland hyperplasia, and higher cholesterol levels in the skin [34], and DGAT2 is known to be a key enzyme in triglyceride synthesis [35, 36], finally, we asked whether up-regulation (DGAT2) and down-regulation (ABCA1) of these genes were mediated by the activation of Akt-, p38α MAPK- or EGFR-coupled signalling pathway. To this end, we repeated our 24-h treatments and found that TPPO could indeed up-regulate (DGAT2) and down-regulate (ABCA1) the above mentioned genes over the course of 24-h treatments (RT-qPCR; Figure 4C,D). Moreover, although down-regulation of ABCA1 was not affected by either of the pharmacons (RT-qPCR; Figure 4C), up-regulation of DGAT2 could be prevented by the co-administration of the Akt-inhibitor GSK690693 (10 μM), whereas the p38α MAPK-inhibitor Nefl (1 μM) and the EGFR-antagonist tyrphostin AG (10 μM) did not influence the effect (RT-qPCR; Figure 4D), indicating that the mechanism of TPPO-induced lipogenesis may involve the Akt-dependent up-regulation of DGAT2 as well as alternative signalling pathways that suppress ABCA1 expression.

4 | Discussion

Over the past years, several members of the TRP ion channel superfamily were shown to be expressed on human sebocytes, including TRPV1 [16, 37–39], TRPV2 [16], TRPV3 [17] and TRPV4 [16], whereas mRNA expression of TRPA1 and TRPM8 was found to be below the detection limit [16]. Regarding their functional role, the mostly Ca^{2+}-permeable TRPV1, TRPV3 and TRPV4 were all shown to negatively regulate sebaceous lipogenesis [16, 17, 38], and activation of TRPV3 was also linked to pro-inflammatory effects [17]. Moreover, it has recently been reported that another member of the TRP family, namely TRPM5, may also be expressed on human sebaceous glands [3]. TRPM5 is a Ca^{2+}-activated, thermosensitive ion channel that is mostly permeable for monovalent cations [1, 2]. Importantly, TRPM5 was recently proven to be a potent 'pro-anagen' (i.e., hair growth promoting) regulator of the hair follicle cycle, and hence, it emerged as a possible novel therapeutic target in different hair growth disorders [3]. Because during the most desired topical treatment of hair follicle diseases TRPM5 modulators would most likely be able to reach the adjacent sebaceous glands as well, in the current study, we aimed to investigate the expression of TRPM5 and effects of its modulators in human sebocytes.

4.1 | TRPM5 Is Most Likely Not Expressed in Human Sebocytes in a Functionally Active Form

The above claim is supported by several lines of evidence. First, TRPM5 mRNA transcripts could not be identified in the cells either by RNA-seq (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1037731) or by RT-qPCR (even though we

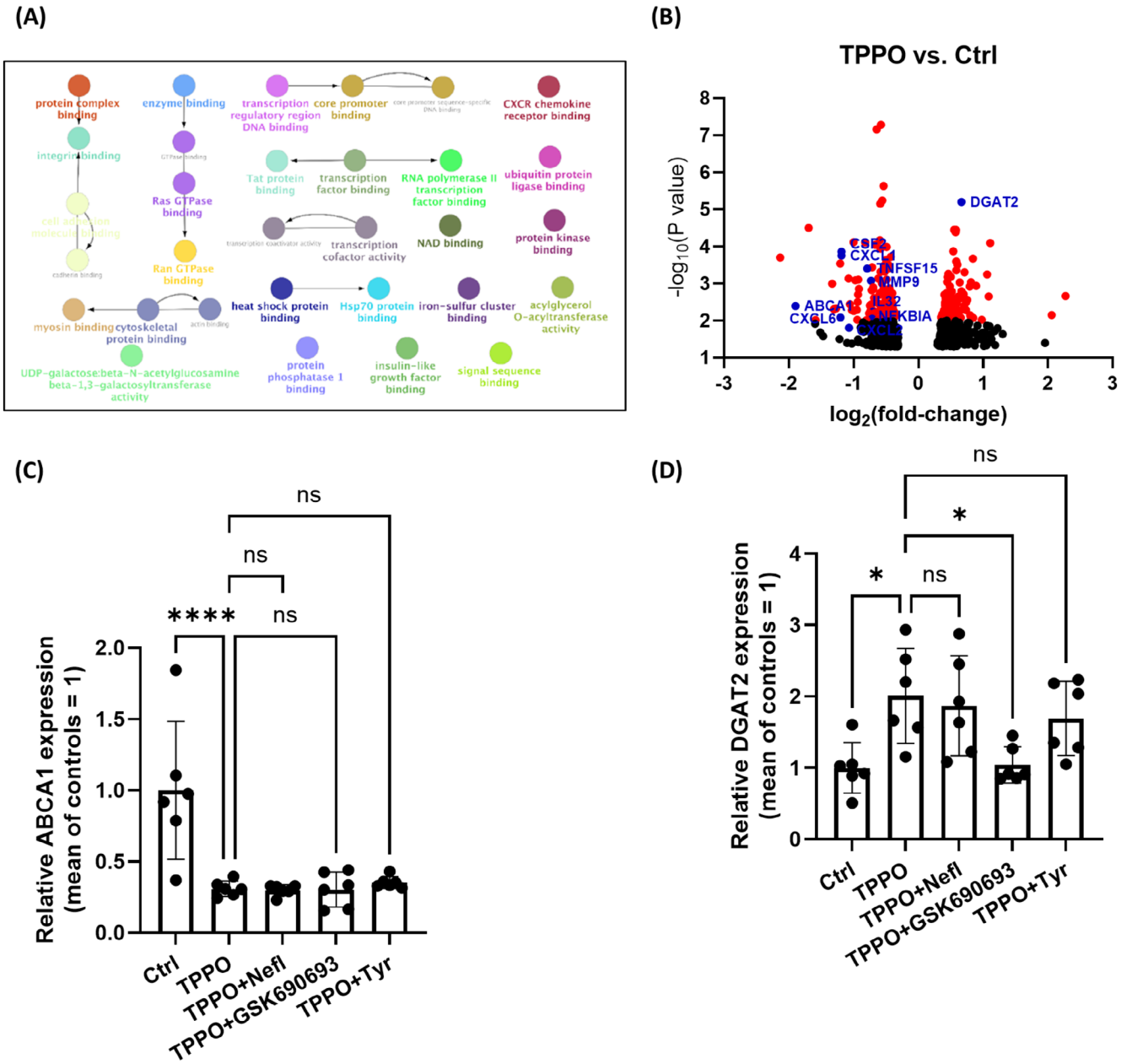

FIGURE 4 | TPPO profoundly influences immune phenotype of human sebocytes, and activates the Akt–DGAT2 lipogenic signalling pathway. (A, B) RNA-seq analysis. SZ95 sebocytes were treated as indicated for 24 h. (A) Pathway analysis of the significantly (≥1.5 fold-change; $p < 0.05$) regulated genes. Enrichment analysis was performed against the GO: Molecular function pathways database. (B) Volcano plots created by using all genes exhibiting significant ($p < 0.05$) alterations. Red dots highlight genes exhibiting more significant ($p < 0.01$) alterations. The most 'acne-' and 'inflammation-relevant' genes are highlighted as blue and are named next to the corresponding dots. (C, D) Confirmatory RT-qPCRs. mRNA expression of ABCA1 (C) and DGAT2 (D) was assessed by RT-qPCR following the indicated 24-h treatments (GSK690693: 10 μM; Nefl: 1 μM; Tyr: 10 μM). Data are presented by using the ΔΔCT method regarding PPIA-normalised mRNA expressions of the vehicle-treated control group as 1. Data are expressed as mean ± SD of $N = 6$ biological replicates (each determined as mean of three technical replicates). * and **** $p < 0.05$ and 0.0001, respectively, as indicated. ABCA, ATP-binding cassette subfamily A; DGAT, diacylglycerol O-acyltransferase 2; EGFR, epidermal growth factor receptor; GSK690693, (S)-4-(2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-(piperidin-3-ylmethoxy)-1H-imidazo[4,5-c]pyridin-4-yl)-2-methylbut-3-yn-2-ol (Akt-inhibitor); MAPK, mitogen-activated protein kinase; Nefl: Neflamapimod (p38α MAPK inhibitor); ns, not significant; PPIA, peptidylprolyl isomerase A (internal control); TPPO, triphenylphosphine oxide; Tyr, tyrphostin AG 1478 (EGFR antagonist).

probed our samples by using two different TaqMan assays, expression levels were found to be below/around detection limit; Table S1). Second, albeit we tried two different TRPM5-specific antibodies, none of them resulted in specific bands at the predicted molecular weights on western blot (Figure S1 and Figure 1A). Third, unlike in the case of human hair follicles [3], siRNA-transfection-mediated selective gene silencing of TRPM5 failed to significantly alter the intensity of the supposedly TRPM5-specific bands on western blot in the case of human sebocytes (Figure 1B,C). Fourth, neither SBFI AM-based fluorescent Na^+- nor Fura-2 AM-based fluorescent Ca^{2+}-measurements revealed any alterations in the Na^+- or Ca^{2+}-homeostasis of

the sebocytes following their treatment by using a high (but clearly non-cytotoxic; Figure S2A–F) concentration of characteristic TRPM5 modulators Hept, DMP and TPPO (300 μM in all cases; Figure S3A–F). Thus, our data indicate that TRPM5 is most likely not expressed in human sebocytes in a functionally active form and highlight that, similar to many other TRP channels [40], antibody-based identification of TRPM5 may be misleading in certain cases. Thus, the use of rigorous control experiments and appropriate complementary methods assessing mRNA-level expression as well as functional activity of the channel are strongly recommended.

4.2 | TPPO Increases Sebaceous Lipogenesis and Modulates Immune Phenotype of Human Sebocytes in a TRPM5-Independent Manner

Obviously, topically applied TRPM5 modulators administered with the intention of influencing hair growth may theoretically have non-specific, TRPM5-independent effects as well. Thus, although our findings indicated that selective modulation of TRPM5 activity will most likely be devoid of direct actions on the sebaceous glands, we decided to investigate the effects of TRPM5 modulators on human sebocytes. Because DMP was found to outperform Hept in prolonging the anagen phase in human hair follicles [3], we opted to use TPPO and DMP in most of our subsequent experiments. While up to 1000 μM, DMP had no effect on sebaceous lipogenesis (Figure S5A,B), non-cytotoxic concentrations (Figures S2 and S4) of TPPO were found to dose-dependently enhance the lipogenesis of the cells over the course of 24- and 48-h treatments (Figure 2A,B). As expected, neither DMP nor Hept (100 μM both) could prevent this effect (Figure S6A,B), confirming that it indeed developed in a TRPM5-independent manner.

Excessive sebum production is one of the key steps in the pathogenesis of acne. However, a normalisation of impaired sebaceous lipogenesis (especially, if accompanied by anti-inflammatory effects) could be highly desirable in case of dry skin dermatoses, for example, atopic dermatitis or psoriasis [41–45]. Thus, next, we intended to unveil the effects of TPPO on the immune phenotype of human sebocytes. We found that TPPO treatment can differentially influence the cytokine profile of the cells (Figure 2C–J). Indeed, potent lipogenic concentration of TPPO (300 μM) up-regulated the sebaceous lineage-indicating cytokine IL-6 [23], time-dependently affected expression of IL-1α and IL-1β, and had no significant impact on the mRNA expression of IL-8 (3- and 24-h treatments; RT-qPCR; Figure 2C–J). Interestingly, although it tended to increase the release of IL-6, the alteration did not reach the level of statistical significance ($p = 0.1356$ after 24-h treatments; ELISA; Figure S7), and it suppressed release of IL-8 after 24-h treatments (ELISA; Figure S7D), indicating that its 'net' effect may be a rather complex modulation of the immune phenotype.

Indeed, it is noteworthy that our RNA-Seq analysis also provided some important details regarding the effects of TPPO on the immune phenotype of the cells. More precisely, we found that TPPO significantly down-regulated CSF2 (also known as granulocyte–macrophage colony-stimulating factor), CXCL1 (also known as GRO-α), CXCL2, CXCL6, IL-32, NFKBIA, TNFSF15 and MMP9 (Figure 4B). Importantly, CS2, IL-6, IL-8, CXCL1, CXCL2, and CXCL6 are up-regulated at the mRNA level in acne lesions [46–48], whereas IL-32 may have a role in the pathogenesis of atopic dermatitis [49]. NFKBIA was upregulated in *Cutibacterium (Propionibacterium) acnes*-infected prostate epithelial cells [50], and certain alleles of TNFSF15 are thought to be involved in the pathogenesis of psoriasis [51], whereas MMP9 was down-regulated in acne patients in response to isotretinoin treatment [33]. This, together with the significant suppression of IL-8 release (Figure S7D) highlight that TPPO-treatment most likely exerts profound and complex effects on the immune phenotype of human sebocytes. Because the majority of the effects mentioned above appear to be rather anti-inflammatory, our data raise the possibility that, by inducing a moderate elevation of sebaceous lipogenesis and dominantly anti-inflammatory actions, safe functional analogues of TPPO may exert beneficial effects in inflammatory dry skin dermatoses.

4.3 | TPPO Modulates Activity of Multiple, Partially Overlapping Signalling Pathways

Next, we aimed to unveil the mechanism of the above actions. Phosphokinase array revealed that a potent lipogenic concentration of TPPO (300 μM) could time-dependently modulate the activity of several signalling molecules, including Akt, EGFR, p38α MAPK, and HSP27, a known downstream effector of p38α MAPK [28] (Figure 3A,B). Using selective pharmacological inhibitors and siRNA-transfection-mediated silencing of EGFR expression, we showed that the lipogenic effect of TPPO most likely involved the activation of Akt and EGFR (Figure 3C,E–H). Interestingly, while the administration of p38α MAPK- and HSP27-inhibitors (Nefl and J2, respectively) could also decrease sebaceous lipogenesis (Figure 3D and Figure S9A), their lipostatic concentrations significantly suppressed the level of polar (membrane) lipids as well (Figures S8B and S9B), indicating that, unlike the pharmacological blockade of Akt and EGFR (Figure S8A,C), they most likely primarily suppress proliferation and not sebaceous lipogenesis of the cells.

Next, we probed whether the above signalling pathways are involved in the TPPO-induced up-regulation of IL-6. We found that the lipogenic Akt signalling is most likely not involved in the process that rather depends on the activation of p38α MAPK and EGFR signalling (Figure 3F). These findings are in a perfect agreement with some recently reported data. Indeed, EGF was shown to increase IL-6 release from human sebocytes (72-h treatments) compared to those cells that have been cultured in the lack of EGF [27], and the p38α MAPK cascade was also demonstrated to exert pro-inflammatory actions in these cells [31, 52, 53].

In order to get a deeper insight to the biological effects of TPPO, we performed RNA-Seq experiment and compared transcriptomes of TPPO- (300 μM) and vehicle-treated sebocytes (24-h treatments). This revealed that multiple relevant signalling pathways (including, e.g., *insulin-like growth factor binding*) were significantly regulated by TPPO (Figure 4A). Because IGF-1 was already shown to stimulate sebaceous lipogenesis via activating Akt signalling [25, 32], we decided to investigate whether IGF-1R was involved in mediating lipogenic effect of

TPPO. Interestingly, pharmacological blockade of this receptor did not prevent the TPPO-induced elevation of sebaceous lipogenesis (Figure S10), indicating that TPPO most likely activated Akt in an IGF-1R-independent manner.

Importantly, careful analysis of the RNA-seq and subsequent confirmatory RT-qPCR data revealed that TPPO significantly down-regulated ABCA1 and up-regulated DGAT2 (Figure 4B–D). Because ABCA1$^{-/-}$ mice have been reported to have sebaceous gland hyperplasia, and higher cholesterol levels in the skin [34], and DGAT2 is a key enzyme in triglyceride synthesis, next we asked if their regulation is linked to the previously identified Akt-, p38α MAPK- or EGFR-coupled signalling pathways. We found that, while down-regulation of ABCA1 was not affected by the inhibition of the above pathways (Figure 4C), up-regulation of DGAT2 was specifically linked to Akt-activity (Figure 4D). Further discussion of the mechanism of action can be found in the '(Data S1) Supplementary Discussion 1'.

4.4 | Conclusions and Limitations of the Study

Finally, as for all in vitro studies, it is important to consider to what extent can these data predict clinical effects. Importantly, SZ95 sebocytes are generally considered to be the best in vitro model system to study human sebaceous gland (patho)physiology [7–11]. Thus, our preclinical data strongly argue that administration of specific TRPM5 modulators is unlikely to result in direct sebaceous gland-related side effects, but use of TPPO or structurally similar, safe analogues may influence sebaceous gland biology in a TRPM5-independent manner (Figure S11). Thus, over the course of future clinical studies exploring the therapeutic effects of TRPM5 modulators, special attention should be paid to sebaceous gland-related (side) effects. On the other hand, possible use of safe functional analogues of TPPO to treat inflammatory dry skin dermatoses should also be investigated. Further discussion of the potential side effects of such treatments can be found in '(Data S1) Supplementary Discussion 2'.

Author Contributions

A.O.: conceptualization. D.Á., J.A., K.F.T., O.P., T.N., Sz.P. and A.O.: methodology. Sz.P. and A.O.: validation. D.Á., J.A., K.F.T., Sz.P. and A.O.: formal analysis. D.Á., J.A., K.F.T., O.P., T.N., Sz.P. and A.O.: investigation. Sz.P., B.I.T., C.C.Z. and A.O.: resources. D.Á., Sz.P. and A.O.: data curation. D.Á. and A.O.: writing – original draft preparation. D.Á., J.A., K.F.T., O.P., T.N., B.I.T., Sz.P., C.C.Z. and A.O.: writing – review and editing. D.Á., J.A., Sz.P. and A.O.: visualisation. A.O.: supervision. D.Á., J.A., K.F.T., O.P., T.N., B.I.T., Sz.P. and A.O.: project administration. B.I.T., Sz.P. and A.O.: funding acquisition. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

This project was supported by the National Research, Development and Innovation Office (grant numbers: 134235, 134725, EFOP-3.6.3-VEKOP-16-2017-00009, EFOP-3.6.1-16-2016-00022 and GINOP-2.3.2-15-2016-00050). A.O. is the recipient of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (ID: BO/00660/21/5). A.O. was supported by the ÚNKP-22-5 and ÚNKP-23-5 New National Excellence Programs of the Ministry for Culture and Innovation from the source of the National Research, Development and Innovation Fund ('Bolyai+ Scholarship', grant numbers: ÚNKP-22-5-DE-427 and ÚNKP-23-5-DE-477) as well as by the 'Momentum' proof-of-concept fund (Faculty of Medicine, University of Debrecen). Sz.P. was supported by the project TKP2021-NKTA-34, which has been implemented with the support provided by the Ministry for Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-NKTA funding scheme. The authors are grateful to Mónika Barotáné Kovács and Beatrix Tolvaj for their excellent technical assistance and contribution.

Conflicts of Interest

C.C.Z. owns an international patent on the SZ95 sebaceous gland cell line (WO2000046353). A.O. provides consultancy services to Monasterium Laboratory Skin & Hair Research Solutions GmbH. Neither Monasterium Laboratory Skin & Hair Research Solutions GmbH, nor the founding sponsors listed in the Acknowledgements section had a role in conceiving the study, designing the experiments, writing the manuscript, or in the decision to publish it.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw data of the RNA-Seq analysis are accessible in the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1037731).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.