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Article

Fisetin a 3, 7, 3′, 4′-tetrahydroxyflavone inhibits PI3K/Akt/mTOR and MAPK Pathways and Ameliorates Psoriasis Pathology in 2D and 3D Organotypic Human Inflammatory Skin Models

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Abstract: Psoriasis is a chronic immune-mediated skin disease that involves interaction of both immune and skin cells, and is characterized by cytokine-driven epidermal hyperplasia, deviant differentiation, inflammation and angiogenesis. Because available treatments for psoriasis have significant limitations, dietary products are potential natural sources of therapeutic molecules, which can rescind molecular defects associated with psoriasis and could possibly be developed for its management. Fisetin (3,7,3′,4′- tetrahydroxyflavone), a phytochemical naturally found in pigmented fruits and vegetables, has demonstrated pro-apoptotic and antioxidant effects in several malignancies. This study utilized biochemical, cellular, pharmacological and tissue-engineering tools to characterize the effects of fisetin on normal human epidermal keratinocytes (NHEKs), peripheral blood mononuclear cells (PBMC) and CD4+ T lymphocytes in 2D and 3D psoriasis-like disease models. Fisetin treatment of NHEKs, dose and time-dependently induced differentiation and inhibited interleukin-22-induced proliferation, as well as activation of the PI3K/Akt/mTOR pathway. Fisetin treatment of TNF-α-stimulated NHEKs and significantly inhibited the activation of p38 and JNK, but had no effect on ERK1/2. In addition, fisetin treatment significantly decreased the secretion of Th1/Th-17 pro-inflammatory cytokines, particularly IFN-γ and IL-17A by 12-O-tetradecanolylphorbol 13-acetate (TPA)-stimulated NHEKs and anti-CD3/CD28-activated human PBMCs. Furthermore, we established the *in-vivo* relevance of fisetin functions, using a 3D full-thickness human skin model of psoriasis (FTRHSP) that closely mimics *in-vivo* human psoriatic skin-lesions. Herein, fisetin significantly ameliorated psoriasis-like disease features, and decreased the production of IL-17 by CD4+ T lymphocytes co-cultured with FTRHSP. Collectively, our data identify pro-differentiative, anti-proliferative and anti-inflammatory effects of fisetin, via

modulation of PI3K-Akt-mTOR and p38/JNK pathways and the production of cytokines in 2D and 3D human skin models of psoriasis. These results suggest that fisetin has a great potential to be developed as an effective and inexpensive agent for the treatment of psoriasis and other related inflammatory skin disorders.

Keywords: Fisetin; psoriasis; normal human epidermal keratinocyte; cell signaling; cell differentiation; proliferation; inflammatory cytokine, PBMC; CD4+ T lymphocyte; 3D psoriasis-like skin disease model.

1. Introduction

The physical and immunological protective skin barrier function, mainly executed by the outermost epidermis, is sustained through the tight regulation of epidermal keratinocyte proliferation and differentiation, ultimately resulting in the dense impenetrable stratum corneum [1]. In normal epidermis, keratins are the most abundant structural proteins that form the keratin intermediate filament cytoskeleton [2]. Upon commitment to terminal differentiation, keratinocytes switch from expressing proliferative keratin 5 and 14 (K5 and K14) in the basal epidermis to express the early differentiation keratin 1 and 10 (K1 and K10) and associated induction of various differentiation-related protein markers in the suprabasal epidermis, which uphold the skin barrier's integrity. These **markers** include keratin tonofilaments, the filament aggregating protein (filaggrin) [1, 3, 4], caspase-14 [5], the enzyme transglutaminase, [4] and loricrin, an impenetrable late cornified envelope protein. Increased interactions between these proteins **cause beneficial effects:** enhance**ment of** skin natural moisturizing factor **and** epidermal hydration**;** maintain stratum corneum barrier function and skin integrity; and protects the induction of inflammation by external stimuli. This cognate process is dysregulated in several chronic inflammatory skin diseases including psoriasis and atopic dermatitis, where lesioned skin keratinocytes exhibit hyperproliferation and aberrant differentiation, two crucial disease hallmarks [6]. Moreover, the expression levels of several differentiation-related proteins including caspase-14 [7], filaggrin [7] and loricrin [8], [4] are downregulated in inflamed, porokeratotic, psoriatic skin lesions compared to non-lesioned and normal skin. Skin keratinocytes serve as the principal source of several pro-inflammatory mediators, including the cytokines interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, IL-20, tumor necrosis factor (TNF) [8, 9], and the chemokines CXCL8, CXCL11 and CCL20 [10]. In response to local stimuli, the production of these mediators can initiate an inflammatory process in psoriasis. In addition to keratinocytes, immune cells play a predominant role in chronic inflammatory skin diseases via the production of mediators such as IFN-γ, IL-17, IL-22, IL-23, IL-36 and the chemokine, IP-10 [11-13] [14, 15] [16, 17]. Keratinocytes also possess cytokine receptors that serve as targets for activated T lymphocyte-derived IL-17 and IL-22, resulting in increased proliferation, aberrant differentiation and further cytokine production by keratinocytes [8].

Agents that possess direct anti-proliferative, pro-differentiative and anti-inflammatory effects in epidermal keratinocytes and immune cells are potentially ideal candidates for treating inflammatory skin disorders such as psoriasis. Efforts to develop treatments for psoriasis remain elusive, mostly relying on small molecules and biologics [11-14], [15], [16, 17]. Most of these treatments have significant limitations, including costs, the need for injections, adverse drug reactions, and loss of efficacy over time [18]. Thus, there is a need for discovery and development of new, safe and effective mechanism-based therapeutics.

Dietary botanicals are important natural sources of biologically active products that possess the inherent ability to rescind multiple disease features. Fisetin (3,7,3′,4′-tetrahydroxyflavone) (Figure 1A (inset)), is a bioactive flavonol abundantly found in many dietary botanicals, particularly in pigmented fruits and vegetables, including apples, cucumbers, onions, persimmons, and strawberries [19-21]. Fisetin has been reported to possess pleiotropic effects in diverse disease models, including anticancer, anti-inflammatory and antioxidant activities [22-29].

Recently, we and others, in the quest for defining mechanism-based dietary antioxidants for disease prevention, showed that at higher micromolar concentrations, fisetin treatment causes growth arrest, apoptosis and regression of both melanoma and UVB-induced cutaneous cancers by modulating the activation of components of the PI3K/Akt/mTOR signaling pathway [24, 26, 30]. Furthermore, we and others have recently shown that these pathways, which are frequently deregulated in diverse cancers [31, 32], are also overexpressed in psoriatic and atopic dermatitis skin lesions [33, 34]. There is limited knowledge regarding the role of fisetin on immune cells. In basophils, fisetin suppresses the expression level of the type-2 cytokines [35]. In mice, fisetin reduces the production of type-1 and type-2 cytokines by T lymphocytes [36] and attenuates NF-κB activity and IL17 production in an *in vivo* allergic airway inflammation mouse model [37]. These observations led us to examine the potential of fisetin as an agent to mitigate the three major hallmarks of psoriasis: activation of inflammation, keratinocyte-induced proliferation, and aberrant differentiation [38]. To the best of our knowledge, no study has evaluated the effects of fisetin on psoriasis. In this study, we assessed the effect of fisetin in psoriasis model, and demonstrated that at low-micromolar concentrations fisetin inhibited intracellular PI3K/Akt/mTOR and MAPK signaling and normal human epidermal keratinocyte (NHEK) proliferation, and it promoted NHEK differentiation without inducing apoptosis. Moreover, fisetin reduced the secretion of pro-inflammatory cytokines by keratinocytes; activated peripheral blood mononuclear cells (PBMC) and CD4+ T lymphocytes; and mechanistically, inhibited intracellular PI3K/Akt/mTOR and MAPK pathways. Furthermore, the functional characteristics/roles of fisetin were also examined in an established *in vivo*-relevant 3D full-thickness engineered human psoriasis-like skin model. Our study demonstrates that fisetin acts on both inflamed keratinocytes and immune cells in 2D and reconstituted 3D skin tissue architecture similar to in vivo psoriatic skin lesions, and clarify its mechanism of action on these systems.

2. Materials and Methods

2.1. Chemicals and Reagents

Fisetin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT), propidium iodide (PI) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St Louis, MO). The antibodies for caspases (-3, -8, and -9), PARP, Bak, Bax, Bad, Bcl-2, PathScan® Multiplex (Phospho-p90RSK, Phospho-Akt, Phospho-p44/42 MAPK (Erk1/2), Phospho-S6 Ribosomal Protein, and Rab11) Western Detection Cocktail I; #5301, Phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (D3F9) XP® Rabbit mAb #4511, Phospho-Akt (Ser^{473}) (D9E) XP® Rabbit mAb #4060, Phospho-mTOR (Ser^{2448}) (D9C2) XP® Rabbit mAb #5536, Phospho-mTOR (Ser^{2481}) Antibody #2974, Phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) (81E11) Rabbit mAb #4668, β-Actin (13E5) Rabbit mAb #4970, PI3 Kinase p110α (C73F8) Rabbit mAb #4249, PI3 Kinase p85 (19H8) Rabbit mAb#4257, Phospho-Akt (Thr^{308}) (D25E6) XP® Rabbit mAb #13038, PhosphoPlus® p70 S6 Kinase (Thr^{389}, Thr^{421}/Ser^{424}) Antibody Kit #9430, mTOR (7C10) Rabbit mAb #2983, and Lamin B1 (D4Q4Z) Rabbit mAb #12586 were obtained from Cell

Signaling Technology (Danvers, MA, USA). Recombinant human (rh) IL-22, IL-17A, TNF-α, anti-CD3, anti-CD28, and biotinylated polyclonal goat anti-human IL-17A were from R&D Systems (Minneapolis, MN, USA). Anti-human IL-17A, IFN-γ (clone 2G1) was purchased from Endogen (Pierce/Thermo Scientific), IFN-γ (clone B133.5), IL-4 (clone 8D4-8) and IL-4 (clone MP-25D2) (Pharmingen, Inc.,), p-JNK (clone G-7,sc-6254), p-p38 (clone D-8, sc-7973), filaggrin (clone AKH1,sc-66192), p-p38(sc-7973), cytokeratin-1(sc-65999), cytokeratin-10 (sc-51581), Transglutaminase 1 (sc-25786), Fra-1(sc-605X), c-Fos(sc-52X), Fos B(sc-8013), c-Jun(sc-1694), Jun B(sc-46x), Jun D(sc-74), caspase-14 (sc-5628), were all obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). CELLnTEC progenitor cell culture medium was from ZenBio (ZenBio, Raleigh, NC, USA). Fetal bovine serum (FBS) was obtained from Life Technologies (Grand Island, NY, USA). Transglutaminase Activity Assay Kit (C, K571-100) was from BioVision Inc. (Milpitas, CA, USA). Procarta Plex Mix & Match Human 6-Plex kit was from eBioScience/affymatrix (EPX060-15073; Santa Clara, CA, USA). Horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibody was obtained from Amersham Life Science Inc. (Arlington Height, IL, USA), and BCA Protein assay kit was obtained from Pierce (Rockford, IL , USA). Novex precast Tris-glycine gels were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Human Subjects

The study protocols were approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board (protocol no. 2013-0059-CR004), informed written consent was obtained from subjects prior to participation, and were conducted according to the principles of the Declaration of Helsinki.

2.3. Keratinocytes isolation, culture, activation with IL-22, TNF-α, TPA, and treatments

Primary normal human epidermal keratinocytes were isolated from neonatal foreskin and adult skin biopsies, and primary cultures were established in CELLnTEC progenitor cell culture medium (ZenBio, Raleigh, NC, USA) supplemented with penicillin (100 U/mL), 100 µg/ml streptomycin (100µg/mL) and amphotericin (100µg/mL) (Life Technologies) as previously described [62]. Frozen cells were thawed and maintained for about 2 months (≈8 passages). Human epidermoid carcinoma A431 cells, obtained from ATCC (Manassas, VA, USA), and human immortalized keratinocytes HaCaT cells (ThermoFisher Scientific), were cultured and maintained in DMEM, supplemented with 10% FBS and 1% penicillin–streptomycin. Stock solutions of fisetin were made in dimethyl sulfoxide (DMSO), and further diluted in respective growth media for the treatment of NHEK, A431 and HaCaT cells. Control cells were treated with an equivalent volume of vehicle alone, corresponding to a final maximum concentration of 0.1% (v/v) DMSO for each treatment, at which concentrations had no effect on cell viability. All cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and the growth media were replenished every alternate day until reaching desired confluence (60-80%) prior to experimentation. For rhIL-22 stimulation and drug treatment protocol, near-confluent keratinocytes were pre-treated with or in the absence of varied concentrations of fisetin (10 - 20 µM) for 8 hours (h) followed by co-treatment with or without rhIL-22 (20ng/mL) for 40 h (making a total of 48 h exposure to fisetin). Cells were harvested and lysates were prepared for immunoblot as described below. IL-22-induced cell proliferation and viability analysis in the presence or absence of fisetin as described below.

For TNF-α treatment, cells were cultured and pretreated with fisetin over night and were stimulated with or without rhTNF-α (10ng/ml) for 10 to 60 min prior to harvest for western blotting analysis as described below. For 12-O-tetradecanoyl-phorbol-13-acetate (TPA) stimulation, near-confluent keratinocytes were pre-treated with or without altered concentrations of fisetin (10 - 20 µM)

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for 20 h, followed by co-treatment with or without 100 nM/mL of TPA for the final 6 h (for a total of 26 h of exposure to fisetin). Cultured supernatants were centrifuged and centrifugates were collected and stored at -80°C until used. The supernatants were only subjected to a single freeze-thaw cycle and used for analysis of TPA-induced keratinocytes secreted pro-inflammatory mediators (IL-1α, IL-1β, IL-6, IL-8 (CXCL8), TGF-α and TNF-α) according to manufacturer's protocol and as previously described [90], and as described below.

2.4. Determination of cell viability by MTT assay

The effect of fisetin on the viability of cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay. NHEK, A431 and HaCaT cells were plated at $5x10^3$ cells per well in 200 µl of respective culture media in 96-well microtiter plates. After cells were adherent and proliferating, media containing fisetin (0-80 µM) were replenished and cultured for 24 and 48 h. After incubating for specified times, MTT solution (0.5 mg/ml in phosphate-buffered saline (PBS)) was added to each well and incubated for 3 h, after which the plate was centrifuged at 2000 rpm for 5 min at 4°C.

Additionally, the effect of fisetin treatment was assessed on the viability of NHEK in relation to the proliferative effect of pre-stimulated with or without rhIL-22 (20ng/ml) and was determined by MTT assay. Briefly, NHEKs were seeded at a density of $2×10^4$ cells/well in 24-well poly(D)lysine (0.1 mg/mL; Sigma-Aldrich, USA) pre-coated plates in 1 ml complete culture medium and incubated at 37°C and 5% CO_2. At 80% confluent, cells were treated for 6 h with/without fisetin 10-20 µM, after which rhIL-22 (20ng/ml) was added and further cultured. At 48 h of incubation, medium was removed and cells were rinsed with PBS and incubated for 3 h with 150 µL of MTT solution (0.5 mg/ml in medium). In either cases, MTT solution was removed and the formazan crystals were then solubilized in DMSO (200 µL) by shaking, and absorbance was spectrophotometrically recorded at 570 nm on a Bio-Tek microplate reader (Bio-TEK Instruments Inc., Winooski, VT, USA). The experiment was repeated three times each in sextuplicate with similar results. The effect of fisetin on normal and IL-22 stimulated growth inhibition was assessed as percentage cell viability, where DMSO-treated cells (untreated controls) were considered as 100% viable. DMSO at the concentrations used has no effect on cell viability.

2.5. Cell cycle analysis

NHEKs were treated with fisetin (1-120 µM) for 24 h, harvested and fixed in chilled 70% alcohol overnight. Cells were then washed twice with PBS, digested with DNase-free RNase (10 µg/ml) at 37°C for 1 h, stained with PI (5 µg/ml) for 3 h at 4°C in the dark and were analyzed by FACS Calibur (Becton Dickinson) for cell cycle phase distribution.

2.6. Preparation, culture, activation and treatment of peripheral blood mononuclear cells (PBMC)

Peripheral blood was obtained by venipuncture from volunteer healthy donors with appropriate University of Wisconsin-Madison Health Sciences Institutional Review Board approval, as previously described with some modifications[91]. Briefly, heparinized whole blood was centrifuged (700×g, 20 min) over a Percoll density gradient (density 1.090 g/ml; Pharmacia Biotech) to separate mononuclear cells from granulocytes. PBMCs were then washed, layered over new calf serum and centrifuged at 800 rpm for 15 min to eliminate platelets. PBMCs were cultured in 24-well dishes at $1.0 × 10^6$ cells/ml with RPMI 1640, 10% FBS, and antibiotics. PBMCs were treated with fisetin (10 µM)

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for 10 min, and then activated with anti-CD3 (R&D Systems; coated on plates at 1 µg/ml) plus soluble anti-CD28 (R&D systems; 1 µg/ml). After 6 h of culture, RLT buffer (Qiagen, Valencia, CA) was added on cells for RNA extraction and PCR analysis. After 48 h of culture, cells supernatant fluids were harvested for measurement of proteins by ELISA.

2.7. *Preparation and activation of blood CD4+ T lymphocytes*

The CD4+ T lymphocytes were prepared as previously described [60]. Briefly, heparinized blood was diluted 1:1 in HBSS, and overlaid above Percoll (1.090 g/ml). After centrifugation at 700x*g* for 20 min at room temperature, the mononuclear cells were recovered from the plasma/Percoll interface, and CD4+ cells were prepared by negative selection using the Miltenyi Biotec $CD4^+$ T Cell Isolation Kit II. Using this method, purity was typically >98% as determined by flow cytometry [60]. For activation, $CD4^+$ T cells (2×10^6/ml) were cultured in 1 ml of complete medium (RPMI plus 10% FBS) with 1µg/ml of plate-bound anti-CD3 plus 1 µg/ml of soluble anti-CD28 (clones 37407 and UCHT1, respectively; R&D Systems, Minneapolis, MN, USA) in a 24-well plate (Corning Costar, Lowell, MA, USA). In this condition, after 48 h activation, $CD4^+$ T cells had a memory/effector phenotype (CD45RO+ CD25+) as previously shown [60] and were placed with the tissue engineered reconstructs over it. CD4+ T cell subset purification was performed by positive selection with magnetic bead separation (Miltenyi), and cell and FTRHSP free supernatants cytokine levels were detected by ELISA as above.

2.8. *ProcartaPlex™ multiplex bead-based immunoassays for cytokines and chemokines*

Cultured conditioned supernatant from TPA-stimulated and control keratinocyte cultures in the presence or absence of varied concentration of fisetin were used to determine the levels of produced cytokine and chemokine levels by human 6-Plex Procarta Multiplex Beads. A commercially available human Procarta 6-Plex Bead immunoassay kit (Affymetrix/eBioscience, Santa Clara, CA) was used to determine cytokine and chemokine levels in conditioned media from TPA-stimulated NHEKs as earlier described [90]. The assay includes a range of customized mix-matched human six pro-inflammatory cytokine and chemokine panels that reflect key processes and responses relating to the activation of keratinocyte inflammation. Beads of defined spectral properties conjugated to analyte-specific capture antibodies were combined with 50 µl of cultured supernatant samples to be tested in separate wells of a 96-well black side/transparent bottom microplate and incubated at room temperature (RT) for 120 min following the manufacturer's instructions and as previously described [90]. All cell culture supernatant samples were analyzed in triplicate along with serial standards (7-point dilutions). After each analyte was allowed to bind to the captured antibodies on the beads, washes were performed to remove non-specifically bound proteins, and analyte-specific biotinylated detection antibodies were added and incubated with the beads at RT for 30-60 min. During this incubation, the analyte-specific biotinylated detection antibodies bind to specific epitopes on the immobilized analytes. Following the binding incubation and washes, streptavidin conjugated to Phycoerythrin (SA-PE or Streptavidin-PE), a pigment complex that serve as a fluorescent tag was added and samples were incubated at RT with slow shaking for 30 min. During this final incubation, Streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich. After washing cycles to remove unbound SA-PE, excess reading buffer was added and incubated at RT for a minimum of 5

min in the dark with shaking and stored at 4°C overnight. Fluorescently tagged beads were analyzed with the xMAP Luminex reader (Luminex instrument, Austin, TX) for quantitative analysis. The spectral properties of the beads and the amounts of associated PE fluorescence were monitored to determine the concentration of the following analytes: IL-1α, IL-1β, IL-6, CXCL8 (IL-8), TNFα and TGF-α in cultured supernatants from different NHEKs treatment groups. The analyte concentrations were determined by importing data into Procarta Plex™ Multiplex Analyst software v.1.0 (Affymetrix/eBioscience, Santa Clara, CA) for analysis as earlier described [90]. Cytokine/chemokine values obtained by immunoassay were referenced against established standards to allow for comparisons between study groups.

2.9. ELISA analysis of Th1-Th2-and-Th17 cytokines, IL-4, IL-17A, and IFN-γ

Supernatant cultures from antiCD3/antiCD28-stimulated PBMC in the presence or absence of varied concentrations of fisetin were used to determine the levels of produced pro-inflammatory cytokine levels by a sandwich ELISA immunoassay. Cytokines were measured in the 48 h PBMC and CD4+ T cells supernatant fluids. IL-17 A, IFN-γ, and IL-4 levels were measured utilizing an "in-house" sandwich ELISA [60, 93]. Coating antibodies included anti-human IL-17A (clone 41809.111, R&D Systems), IFN-γ (clone 2G1, Endogen/Pierce/Thermo Scientific), and IL-4 (clone 8D4-8, Pharmingen, Inc.). Detection antibodies included biotinylated polyclonal goat anti-human IL-17A (R&D Systems), IFN-γ (clone B133.5, Pharmingen, Inc), and IL-4 (clone MP-25D2, Pharmingen, Inc). The ELISA assay sensitivities were < 5 pg/ml for others and <3 pg/ml for IL-17A.

2.10. RNA preparation and real-time-quantitative (q)-PCR

Total RNA was extracted from PBMC using the RNeasy Mini Kit (Qiagen). The reverse transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY, USA). Gene expression levels were determined by qPCR using SYBR Green Master Mix (SABiosciences, Frederick, MD, USA). Forward and reverse specific primers ((IFNG), forward: gaaacgagatgacttcgaaaagct, reverse: catgtattgctttgcgttgga, and (IL17A), forward: cgatccacctcaccttgga, reverse: tcccagatcacagagggatatctctc) were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA, USA) to span an exon-exon junction, and blasted against the human genome to determine specificity using http://www.ncbi.nlm.nih.gov/tools/primer-blast. The reference gene primers, ß-glucuronidase ((GUSB), forward: caggacctgcgcacaagag, reverse: agcgtgtcgacccattc), were used to normalize the samples. Standard curves were performed and efficiencies were determined for each set of primers. Efficiencies ranged between 91 and 96%. Data are expressed as fold change using the comparative cycle threshold (ΔΔCT) method as described previously [60]. The values presented are fold change = ($2^{-\Delta\Delta Ct}$) compared to expression in resting PBMC.

2.11. Preparation of protein lysates from cultured cells and western blotting

Following the treatment of NHEK cells with fisetin (10-20 μM; for a maximum of 48 h prior to harvest), in the presence or absence of TNF-α or rhIL-22, the media was aspirated, and the cells were washed with cold PBS (pH 7.4). Cells were incubated in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA), 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4), with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) on ice for 30 min. The cells were scraped and cell lysates collected in microfuge tubes, and passed through 22.5-gauge syringe needles to break up the cell aggregates. The lysates were cleared by centrifugation at 14,000 g for 30 min at 4°C, and the supernatant (whole cell lysate) protein concentrations were determined using a BCA protein assay kit (Pierce) according to the manufacturer's protocol. Lysates were used or immediately aliquoted and stored at −80 °C for further analysis. Western blotting was performed as previously described [94],[95] [33, 95]. Briefly, 10-20 μg protein was resolved on 4–12% polyacrylamide gels and transferred to a nitrocellulose

membrane. The blots were blocked in blocking buffer (5% non-fat dry milk or BSA/1% Tween 20; in 20 mM TBS, pH 7.6) for 45 min at room temperature. Membranes were incubated with appropriate monoclonal or polyclonal primary antibody in the blocking buffer for 2 h at RT, to overnight at 4°C, followed by 3x5min washes, and incubated with anti-mouse or anti-rabbit horseradish-peroxidase conjugated (HRP) secondary antibody obtained from Amersham Life Science Inc. (Arlington Height, IL, USA). Chemiluminescence and autoradiography was detected using Bio-Rad detection and analysis systems as earlier described [94],[95]. Equal loading of protein was confirmed by stripping the immunoblot and re-probing for β-actin, vinculin or lamin B1 as loading controls. The immunoblot results displayed herein are representative of three independent experiments.

2.12. Generation of three-dimensional (3D) Full-Thickness Reconstituted Human Skin Model of Psoriasis (FTRHSP)

Metabolically active 3D full-thickness Reconstructed Human Skin model of Psoriasis (FTRHSP) was generated as described previously [40, 62] with slight modifications. The FTRHSP consisted of a multilayered NHEK in a highly differentiated epidermis developed on a fibroblasts contracted-collagen dermal substratum, and a suspension of activated CD4+ T cells underneath the 3D reconstructed epithelium exposed at air-liquid interface (ALI) for 12-14 days as described previously [40, 62] with slight modifications. The dermal component was prepared by contracting collagen gels populated with fibroblasts seeded into in 3.0 μm Millicell-PCF inserts (Millipore Corporation, Billerica, MA, USA) each placed in wells of a 24-well plate as described previously, and the epidermal component was generated on top [62, 96]. For epidermal components, 2nd-to-3rd passage NHEKs established in low calcium Epi-Life medium were seeded, synchronized and maintained in Progenitor Cell targeted Culture Media (CnT-02-07; CELLnTEC, ZenBio, Research Triangle Park, NC, USA) prior to harvest and used to generate the 3D FTRHSP cultures as earlier described [62, 96]. Briefly, after trypsinization, 3×10^5 cells per insert in 250 μl of CnT-02-07, were seeded into the surface of the generated dermal substratum, each placed in wells of 6 well-tissue culture plates containing 2.5 ml of CnT-02-07 medium in a humidified atmosphere with 5% CO_2 and maintained at 37°C. After 48 h of culture, CnT-02-07 was switched to CnT-02-3DP5 differentiation medium (2 ml outside the insert, and 0.4 ml inside the inserts (day 2), and further grown for 48-72 h to initiate differentiation (day 5). The medium outside and inside the inserts were then removed, and medium outside the insert was replenished with 1.2 ml of CnT-02-3DP5 complete medium, and cultured at ALI from this time point onwards. After 5 days of cultures at ALI, the medium outside was replenished with pre-warmed CnT-02-3DP5 medium in plates containing coated anti-CD3 and soluble anti-CD28 stimulated CD4+ T cells ($3x10^5$/0.9ml) (herein referred to as activated T cells) prepared as above (day 10) to initiate inflammation. Anti-CD3/CD28 CD4+ T cells in 0.9 ml of medium in the trans-wells at Day 10 were overlaid by placing the insert on top, exposing the underneath dermal side of the 3D skin equivalents on it. Memory T cells were activated, while control or unactivated CD4+ T cells were employed in the control reconstructs. On the same day of co-culture of 3D and activated T cells, topical treatment with or without different concentrations of the test compounds (fisetin or Vit-D_3) was initiated. Fresh media as above were replenished after every alternate days and continued prior to harvest at indicated harvest day (day 15). After 5 days of co-culture in the presence or absence of test compounds, and at harvest, 3-4 mm diameter punch biopsies from each insert **were** obtained, formalin-fixed and processed for either H&E or immunostaining. We then analyzed the morphology and protein expression levels of markers of inflammation, proliferation and differentiation as well as

activation of the PI3K/Akt/mTOR cascade. Additionally, cell-free supernatants were collected for pro-inflammatory cytokine analysis using sandwitch ELISA as described above.

2.13. Fisetin and Vit-D3 treatment of the 3D FTRHSP model system

Fisetin and Vit-D_3 were dissolved in DMSO and stored as 80 mM, and 0.1 mM aliquots, respectively, at - 20°C protected from light. At the time of use, test compounds, fisetin (1-80 μM), or vit-D_3 (0.1 μM) were diluted in culture medium containing 0.1% BSA to stabilize the drugs, and treatment was started at 80% confluence (for monolayer). For the 3D FTRHSP studies exposed to ALI, 10μl of different concentration of test agents, fisetin (10-40μM) and vit-D_3 (1×10^{-7} μM) that are known to control psoriasis, and used as a positive control were added in the developing tissue.

2.14. Histology, morphometry and immunostaining analyses of the FTRHSP

Formalin fixed paraffin embedded (FFPE) 5 μM cross-sections were de-paraffinized by incubation in xylene (soaking twice for 10 minute each), rehydrated with ethanol (twice in 100% ethanol for 10 min, twice in 95% ethanol for 10 min and then in 70% ethanol for 10 min, and finally in distilled water for 10 min). Antigen retrieval was performed by treatment with fresh sodium citrate buffer with 0.05% Tween 20 at pH 6.0, for 30 min at 100°C, and sections were stained with H&E and evaluated for gross morphology, epidermal and horny layer thickness and immunostained for the specified markers as previously described [62, 75, 97]. Briefly, endogenous peroxidase was blocked with 3% H_2O_2 in methanol and **was** incubated with blocking solution containing 3% BSA/3% normal goat serum (NGS)/0.4% Triton X-100 in PBS for 60 min at RT, followed by overnight incubation at 4°C with monoclonal antibodies **(m**ouse cytokeratin 10 (RKSE 60) (1:75, sc-23877), mouse anti-Desmoglein-1 (1:75, Invitrogen #326000), rabbit anti-filaggrin (1:100; ab34584); rabbit anti-involucrin (1:75; 2215)). After three washes, samples were incubated for 2 h with Texas Red@-X Goat Anti-mouse (1: 600; Invitrogen T6390) and Alexa fluor Goat Anti-rabbit, (1:600; Life technology A11008), followed by three washes of 10min each and were mounted in situ mounting media with 4′,6-diamidino-2-phenylindole (DAPI) (#DUO82040, Sigma). Slides were visualized on an Olympus IX71 system microscope (Olympus/HuntOptic & Imaging, Inc., Pittsburg, PA, USA), and digital images at 20x magnification were captured with an Olympus U-CMAD3 attached Olympus DP71 camera (Olympus/HuntOptic & Imaging, Inc.,) linked to a high resolution computer screen and connected to an Olympus U-RFL-T Mercury Burner. Images were processed by using CellSens dimension software v1.6. The thickness in terms of the area of the entire epidermis and the stratum corneum were determined as earlier described [62].

2.15. Statistical Analysis

Statistical analyses were carried out with GraphPad Prism version 7.1 (San Diego, CA), except for qPCR and ELISA, analyses which were performed using the SigmaPlot 11.0 software package (Systat Software, Inc., San Jose, CA, USA). All quantitative data are expressed as means ± SD or SEM, and significant differences were determined by the Student t-test or ANOVA with Bonferroni and/or turkey post hoc testing, and p values <0.05 were considered significant.

3. Results

3.1. Fisetin inhibits cell proliferation and viability, but does not affect apoptosis of keratinocytes at doses ≤20 μM

We first examined the effect of fisetin on the growth and viability of primary NHEK and compared to HaCaT, a precancerous immortalized human keratinocytes and A431, an epidermoid carcinoma cell line. Employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, dose-and-time course analyses revealed that after 24 and 48 h, fisetin at doses ≤20 μM had modest effect on NHEK, while exerting significant inhibition of cell growth and viability of HaCaT and A431. The IC_{50} on NHEK were calculated to be 59.8 μM and 40.9 μM, as compared to HaCaT of 39.6 μM and 26.8 μM and A431 of 23.4 μM and 20.6 μM at 24 h and 48 h, respectively (**Figure 1A-B**).

To examine whether the effect of fisetin on viable cell number is related to cell cycle arrest or apoptosis, the proportion of cells containing diploid (sub-G_1) levels of DNA were quantified using propidium iodide (PI) staining and flow cytometric analysis. Treatment with 90 and 120 μM of fisetin as positive control for apoptosis [39], significantly increased the proportion of NHEK in the sub-G_1 population (6.9% and 8.24 %), respectively compared to lower doses of fisetin (30-60μM) that only expanded the G_1 and increased the S-phase compartments (0% and 0.22%), but did not induce any increases in keratinocyte apoptosis (**Figure 1C-D**). Fisetin at 5-30 μM did not exhibit toxicity, but cell death was evident at higher doses (**Figure 1C-D**). Western blotting, revealed that at concentrations less than or equal to 20μM, fisetin did not induce apoptosis at the time points investigated as evidenced by the absence of activation of caspases 3, 8 and 9 and/or changes in PARP, Bak, Bax and Bcl2 protein levels **(Figure 1E-F)**. These observations led us to select doses of 0 to 20 μM over 24-48 h for further mechanistic studies.

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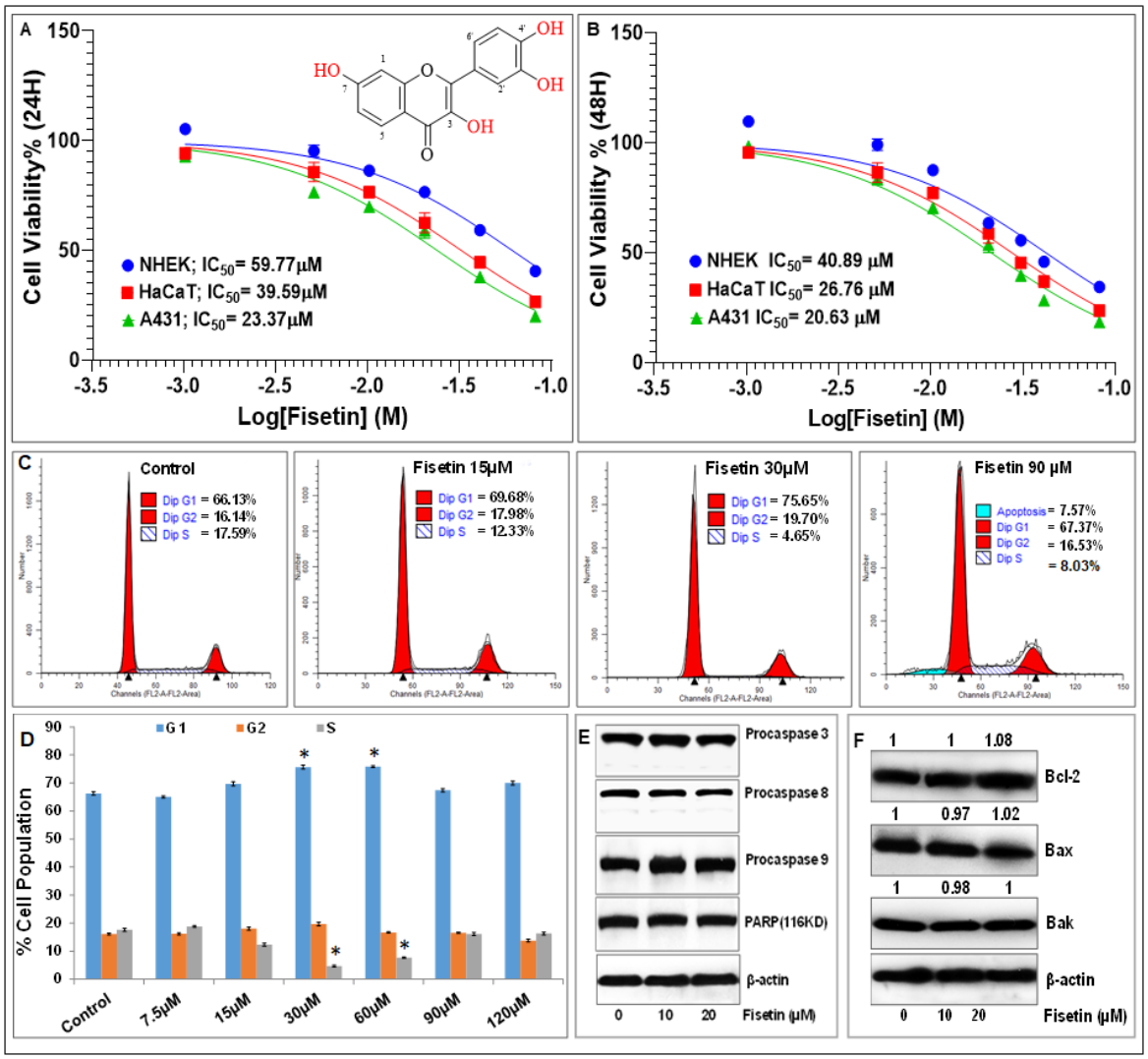

Figure 1: Fisetin at low doses (< 20 µM) does not significantly affect primary normal human epidermal keratinocyte (NHEK) growth and viability and does not induce apoptosis. (A/B) Relative number of viable NHEK, immortalized keratinocytes (HaCaT), and A431 cancer cells treated with or without fisetin (1-80 µM) for 24 h (A) and 48 h as determined by MTT assay. Mean of percentage viability of NHEK, HaCaT and A431 cell lines plotted against the indicated doses of fisetin. Experiments were performed three times with each concentration done in octoplicate wells, and IC_{50} values calculated from these plots are shown. (C) Effects of fisetin on the percentage of cells population in the different phases of cell cycle, and indication of late apoptosis or necrosis (i.e. PI positive) cells were only seen in cells treated with higher fisetin concentration. (D) Levels or percent of cell cycle distribution in fisetin treated cells as assessed by flow cytometry analysis. Bar graphs represent mean ± SD of results from three independent experiments performed in triplicate. Statistical significance was determined using one-way ANOVA and Dunn's multiple comparison test and significance was considered when $p<0.05$ (*), as compared with control. (E) and (F) Effect of different concentrations of fisetin on the expression of markers of apoptosis including caspase-3, -8 and -9, PARP (85 kDa and 116 kDa) and Bcl-2 family of proteins (Bcl2, Bax and Bak) on cells harvested after 48 h of treatment as analyzed by western blotting. Equal protein loading was confirmed using β-actin as loading control. (F) Numerical data above the blots represent relative quantitative density values for the blots normalized with internal loading control. The western blot data shown are representative immunoblots of two-to-three independent experiments with similar results.

3.2. Fisetin treatment promotes human primary epidermal keratinocyte differentiation and upregulates the expression of AP-1 transcription factor proteins

Since psoriasis is characterized by aberrant keratinocyte differentiation, a key requirement for epidermal homeostasis [10], we examined whether the anti-proliferative effect of fisetin on NHEK paralleled differentiation. The ability of different concentrations of fisetin to induce terminal differentiation was analyzed by monitoring transglutaminase (TGase) enzyme activity, a marker of terminal keratinocyte differentiation. Significant dose-dependent induction of TGase activity was observed in NHEK (**Figure 2A and B** (graph for linear regression used to plot TGase activity)). In addition, the ability of fisetin to stimulate differentiation was also examined by western blot analysis of the expression levels of early (K1 and K10), intermediate (caspase-14) and late (filaggrin and TGase-1) keratinocyte differentiation markers [40-42]. Following 24-48 h exposure of near confluent NHEK, fisetin dose-and-time-dependently increased the expression of these differentiation markers (**Figure 2C**), indicating that fisetin induce terminal differentiation of keratinocytes at higher concentrations.

Activator protein-1 (AP-1), a dimeric protein complex comprising members of the Jun and Fos proto-oncogene subfamilies, is a transcription factor known to play an important role in the regulation of epidermal keratinocytes proliferation, terminal differentiation, cytokine production and inflammation [43],[44, 45]. AP-1 subunits expression levels are downregulated in lesioned compared with non-lesioned psoriatic skin [46, 47]. We determined the effect of fisetin on expression of AP-1 factors in NHEKs and observed that fisetin increased the nuclear expression of members of AP-1 factor subunits, including Fos (Fra-1/2, c-Fos, Fos B) and Jun (c-Jun, JunB, JunD), as compared to control cultures (**Figure 2D-E**).

3.3. Fisetin regulates IL-22-induced keratinocyte proliferation by inhibiting the PI3K/AKT and mTOR pathway components

It is known that type-17 cytokines such as IL-17 and IL-22 play critical roles in psoriasiform pathology [48, 49], including Akt/mTOR activation [50, 51]. In this light, modulation of the PI3K/Akt/mTOR activity is known to promote epidermal hyperplasia, and exert distinct regulatory roles in the same innate immune cells that are implicated in the immunopathogenesis of psoriasis [34,52, 53]. Here, we employed recombinant human (rh) IL-22-activated keratinocytes *in vitro* to investigate the mechanistic role of fisetin in psoriasis-like changes driven by epidermal keratinocytes hyper-proliferation. We observed by MTT analysis that IL-22 treatment of NHEK significantly induced proliferation (117±0.16 %) compared to control (100±0.11 %) (**Supplementary Figure 1**, $p<0.01$). When NHEK cultures were treated with 10 and 20 μM fisetin for 24 h before stimulating with 20 ng/ml of rhIL-22, IL-22-induced proliferation (117 ± 0.16 %) was significantly reduced to 75 ± 0.54 %, and 65 ± 1.09 %, respectively (**Supplementary Figure 1**, $p<0.01$).

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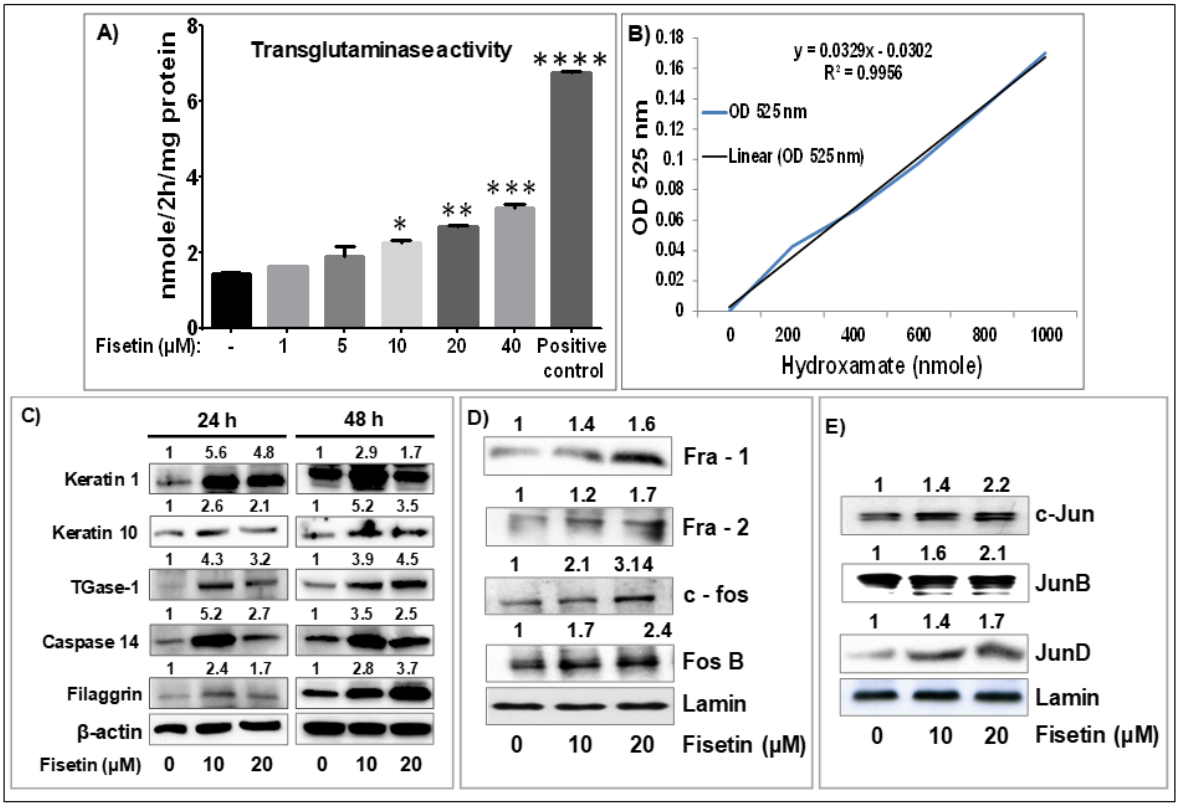

Figure 2: Fisetin treatment increased the expression of markers of epidermal keratinocytes differentiation and AP-1 transcription factor in NHEK. (A - B) The bar graphs represent dose-dependent induction of the differentiation marker transglutaminase (TGase) (a terminal differentiation marker) activity in NHEK treated with or without different doses of fisetin for 24 h, and the linear plot of the hydroxamate analysis of TGase activity. Data represent means ± SEM of three independent experiments each performed in quadruplicate. Significance of comparisons were made for control cells versus fisetin-treated cells or positive control, determined one-way analysis of variance as denoted by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Dose-and-time dependent increases in the protein expression of (C) early and late differentiation markers, and (D-E) nuclear protein expression of members of AP-1 factors subunits including Jun (C- Jun, Jun B, Jun D) and Fos (C-Fos, Fra-2, Fos-B) in NHEKs when compared to control cultures after 48 h, as analyzed by western blotting. Panel C-E are representative of 2-3 experiments with similar results. Equal protein loading was confirmed using ß-actin and Lamin as respective loading controls, and the numerical data above the blots represent the quantitative relative densitometry values for the blots normalized to their respective loading controls.

Studies have demonstrated that IL-22-induced proliferation of NHEK is mediated through PI3K/AKT/mTOR signaling pathway [50, 54, 55]. Therefore, to mechanistically elucidate how fisetin inhibits the proliferative responses, we examined the effects of fisetin on IL-22-induced activation of PI3K, Akt, mTOR and p70S6K after 12 h. Our western blot analysis showed that fisetin treatment of NHEK significantly and dose-dependently suppressed IL-22-induced increases in protein expression of PI3Ks (p110α and p85), the phosphorylation of Akt (at Ser^{473} and Thr^{308}), and of mTOR (at Ser^{2448} and Ser^{2481}), as well as its downstream effector p-p70S6K (Thr^{389}), as compared with control (**Figure 3**).

3.4. Fisetin regulates TNF-α-induced activation of the PI3K/Akt/mTOR and MAPK signaling pathways in NHEK

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Given that fisetin inhibited IL-22-induced responses that are dependent on PI3K/AKT/mTOR and MAPK signaling, we next examined the effect of fisetin on TNF-α-induced PI3K/AKT/mTOR and MAPK pathway responses. NHEK cultures were pre-treated with 10μM fisetin for 24 h before stimulating with 10 ng/ml of rhTNF-α, and the ensuing effect on TNF-α-induced activation of PI3K/AKT/mTOR and MAPK- signaling was examined by western blotting after 30 and 60 min (**Figure 4**). At 30 min, TNF-α treatment increased the levels of p-p38, p-JNK, PI3K (p110a and p85), p-p90RSK, p-Akt, p-ERK and p-S6 (**Figure 4**). These TNF-α-induced effects were significantly inhibited upon pre-treatment with 10-μM fisetin, except for the expression of p-p90RSK and p-ERK, which latter was enhanced by fisetin treatment (**Figure 4**).

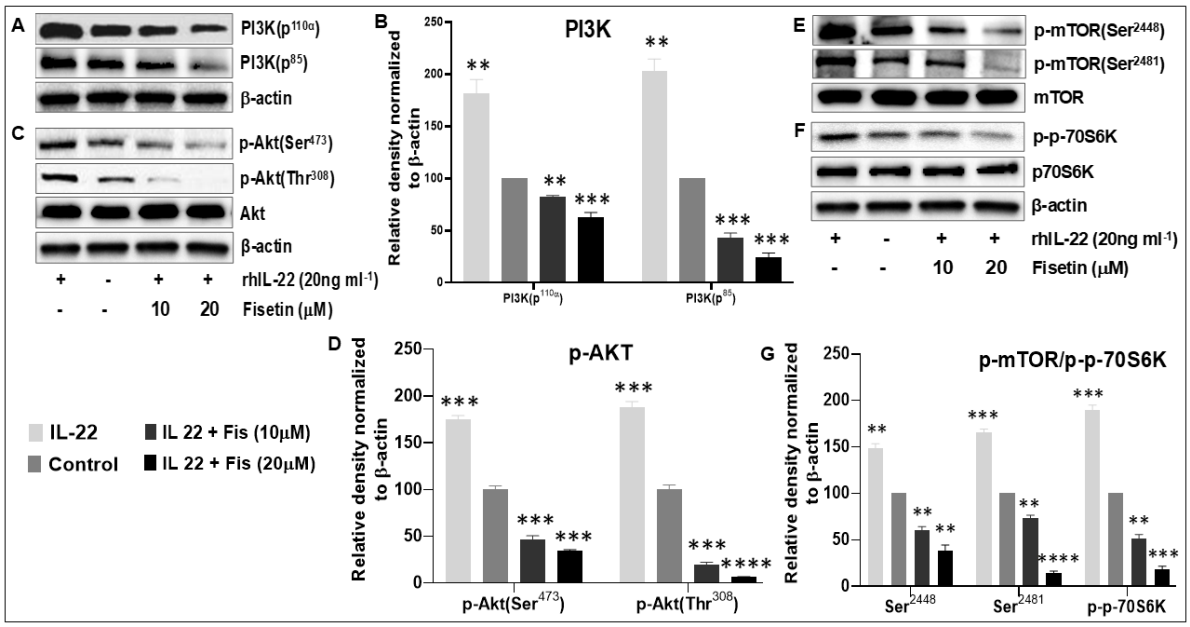

Figure 3. Figure 3: Fisetin significantly suppresses the IL-22-induced activation of the PI3K/Akt/mTOR signaling in NHEK. Western blot determined protein expression levels of (A-B) PI3-K (p110 and p85), and (C-D) phosphorylation of Akt (at Ser473 and Thr308), and (E) phosphorylation of mTOR (at Ser2448 and Ser2481), and (F) phosphorylation of p-p70S6K (Thr389), as compared with untreated cells (control) and IL-22-treated cells only. (B, D and G) graphs of relative intensity of normalized protein components, and each bar depict means +/-SD of three different experiments. *, ** and *** indicate p<0.05, p<0.01 and p<0.001 vs control for IL-22 treated only or vs IL-22 for fisetin treated cells.

3.5. Fisetin pre-treatment inhibits human epidermal keratinocytes, peripheral blood mononuclear cells (PBMC) and CD4+ T-lymphocytes-induced inflammatory responses

Next, we examined the role of fisetin on inflammatory responses induced by 12-O-tetradecanoylphorbol 13-acetate (TPA) on NHEK and induced by anti-CD3 plus anti-CD28 on PBMCs, and CD4+ T lymphocytes. Using Procarta-based Multiplex cytokine immunoassay, we first examined the effects of fisetin (10 and 20 μM) pretreatment on TPA (100 ng/ml)-induced production of pro-inflammatory mediators IL-1α, IL-1β, IL-6, IL-8, TGF-α and TNF-α by NHEK (**Figure 5**). It has been shown that treatment of NHEK with TPA can induce inflammatory response mimicking the initial keratinocyte activation phase of the psoriatic process [10, 56]. Here, we observed that fisetin pretreatment significantly decreased unactivated and TPA-induced NHEK secretion of the

proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, TNF-α, and the pro-fibrotic mediator TGF-α. Of note, fisetin's effects were highly similar to the effects elicited by 1, 25 hydroxyvitamin-D3 (Vit-D3) treatment upon TPA-stimulation (**Figure 5**).

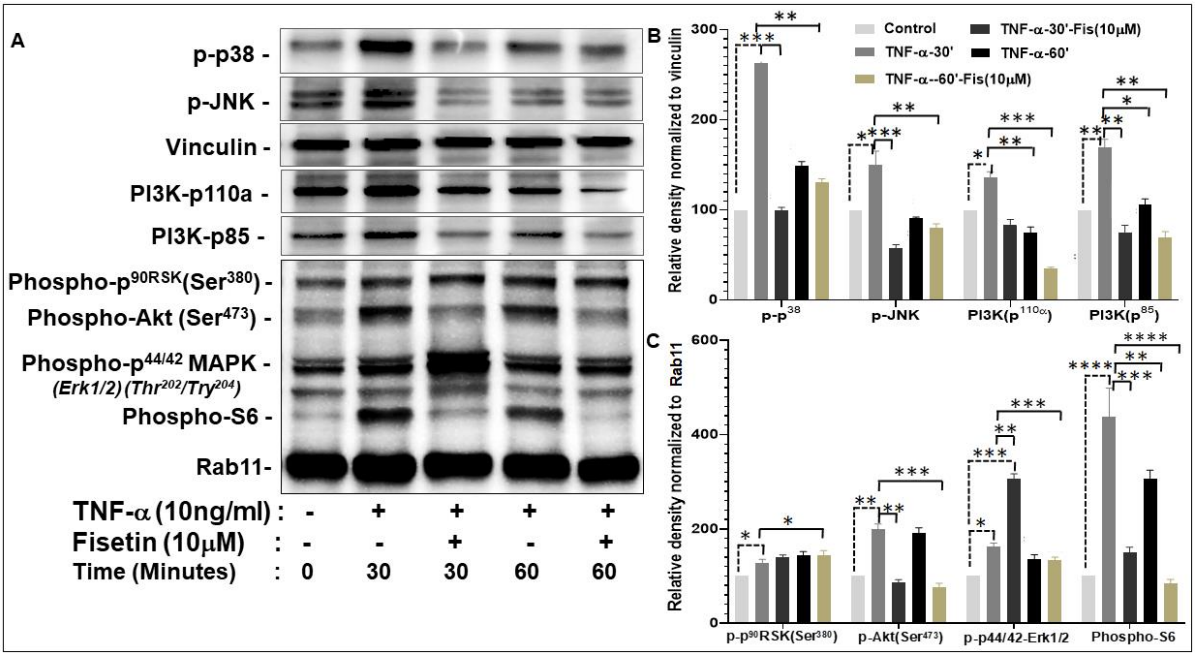

Figure 4: Fisetin regulates the PI3K/Akt/mTOR and MAPK signaling pathways in TNF-α-activated NHEK. NHEK cultures were pre-treated for 24 h with/without 10 μM fisetin and then stimulated with 10 ng/ml TNF-α for 30 and 60 min prior **to** immunoblot analysis and quantification of the expression levels of; p-p38, p-JNK, PI3K (p110a and p85), p-p90RSK, p-Akt(Ser473), p-ERK(p44/42) and (phospho-S6).. Proteins were quantitated using the Bio-Rad Image Lab v6 software compared with untreated and TNF-α-activated control cells and were normalized to Rab11 or vinculin as loading controls. (B-C) Quantitative analysis of normalized target protein expressions, and the plotted values are mean ± SD of each data set from three independent experiments performed in triplicates. Significance of comparisons were made for TNF-α-stimulated control cells versus fisetin-treated at 30 and 60 minutes time points, corresponding to the solid lines, and also between unstimulated control cells versus TNF-α-stimulated cells indicated by the broken lines as denoted by *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

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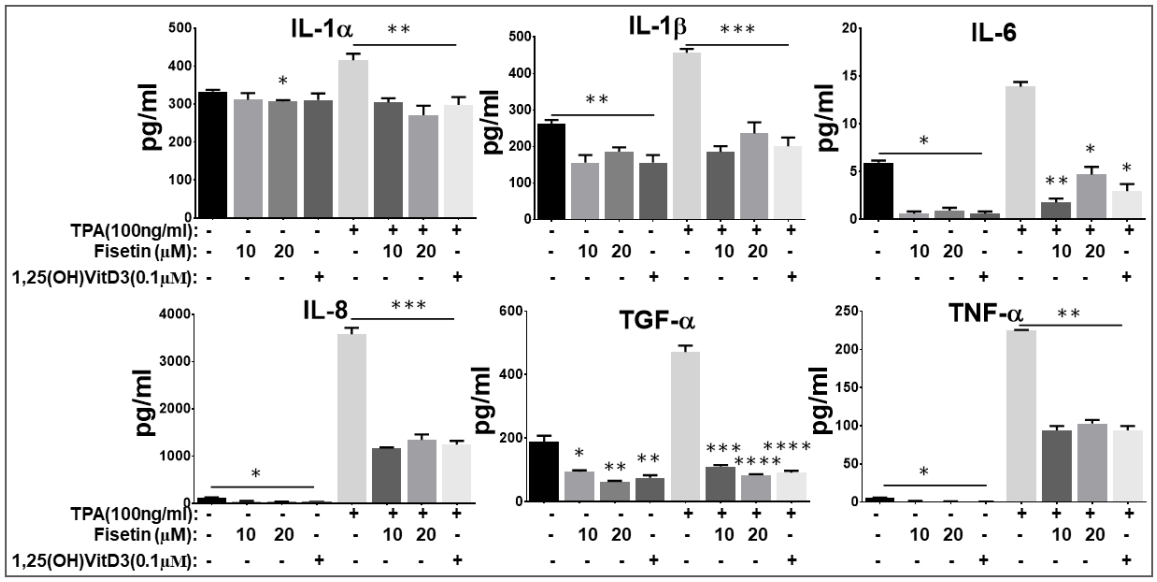

Figure 5: Fisetin reduces the inflammatory response in 12-O-tetradecanoyl-phorbol-13- acetate (TPA)-activated NHEK. NHEKs were pretreated with or without different concentration of fisetin (10 and 20 μM) for 24 h, before stimulation with or without 100 ng/mL of TPA. The **secretion** and release of pro-inflammatory cytokines (IL-1 α, IL-1 β, IL-6, IL-8(CXCL8) and TNF-α, and TGF-α) in condition **culture** media was measured by procarta based multiplex immunoassay. Fisetin pretreatment suppressed TPA-induced secretion of these proinflammatory mediators to values similar to that of **1,** 25 dihydroxyvitamin D3 (Vitamin D3) pretreatment. Values are mean ± SD of results from three independent experiments each performed in quadruplicate. Statistical significance was determined using one-way ANOVA and Turkey's multiple comparison test. Significance of comparisons were made for TPA-stimulated alone cells versus TPA-stimulated and fisetin/vitamin D3-treated cells, and also between unstimulated control cells versus fisetin/vitamin D3-treated cells as denoted by *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Because both keratinocyte and immune cell activation are involved in the initiation and chronicity of psoriasis and other inflammatory skin diseases [57-59], we next examined the effect of fisetin on anti-CD3/anti-CD28-activated PBMC secretion of inflammatory mediators. Subsequently, the mRNA expression level of type-1 and type-17 pro-inflammatory cytokines (*IFNG* and *IL-17A*), were analyzed when PBMCs were pretreated with fisetin (10 μM) for 10 min followed by activation with anti-CD3/CD28 for 6 h. As shown in **Figure 6A**, pretreatment of PBMC with fisetin resulted in significant inhibition of *IFNG* and *IL17A* mRNA accumulations. Under these conditions, PBMC were cultured for 48 h and analyzed for the cytokine protein production and release by ELISA. Altogether, these results demonstrated a significant inhibitory effect of fisetin on IFN-γ and IL-17 secretion, in spite of no obvious effect on the type-2 cytokine IL-4 (**Figure 6B**), and thus inhibition of inflammatory cells induced responses associated with modulation of the expression of critical pro-inflammatory cytokines in psoriasis.

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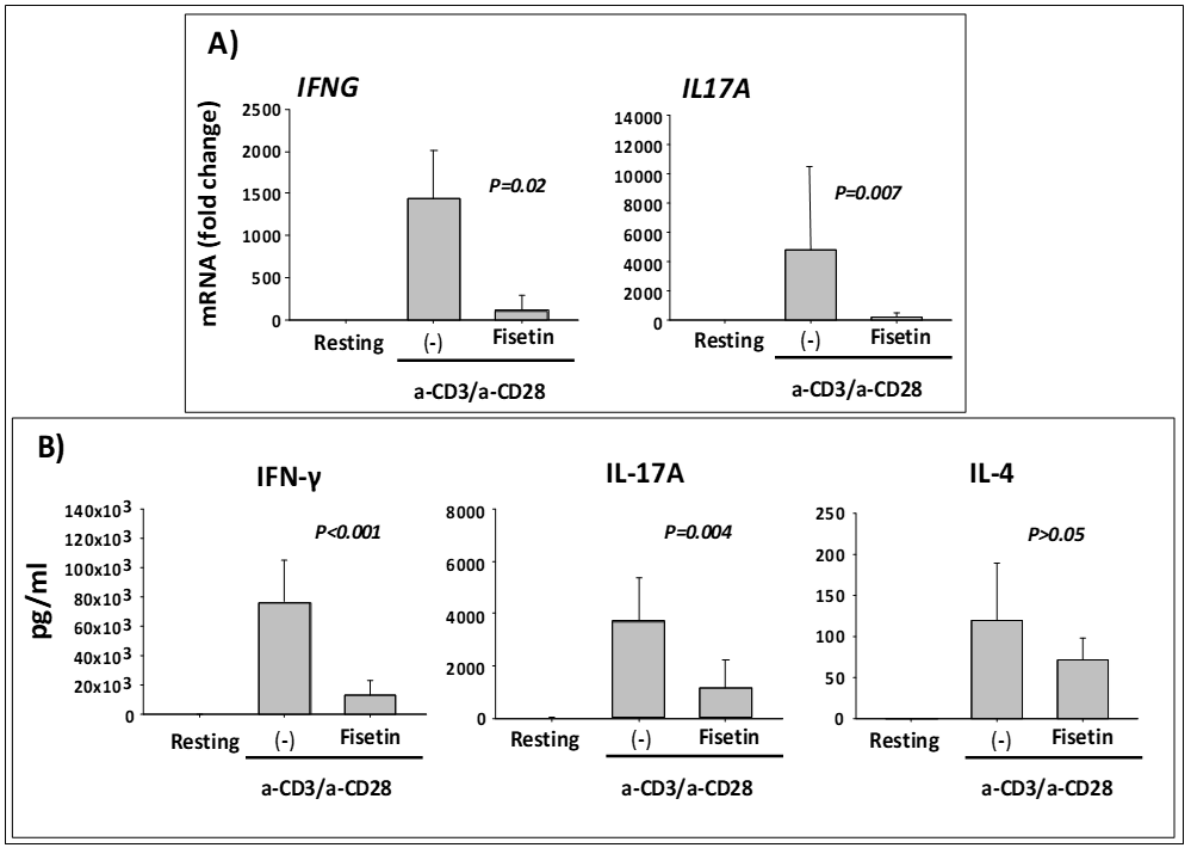

Figure 6: Fisetin suppresses the expression and secretion of IFN-γ and IL-17 in activated-human peripheral blood mononuclear cells (PBMC). PBMC were prepared from circulating blood from three different healthy blood donors, and were cultured without (resting) or with anti-CD3 plus anti-CD8 (a-CD3/a-CD28), either treated with 10 μM of fisetin or vehicle alone (-) for 10 min before activation. (**A)** PBMC were activated for 6 h before measurement of the type-1 and type-17 proinflammatory cytokines, IFN-γ (***IFNG***) and IL-17A (***IL17A***) mRNA expression levels. Fold change in mRNA levels compared to resting cells, which expression level was fixed at one, were determined. Means ±SE are shown, and log10 transformation followed by paired t test was used to compare mRNA levels in a-CD3/a-CD28-activated PBMC with or without fisetin. **(B)** PBMC were activated for 48 h in the indicated conditions and secreted cytokines (IFN-γ, IL-17A and IL-4) present in the cell culture media were measured by ELISA. Means ±SD are shown and the paired t test was used to compare values between fisetin and no fisetin (-) for a-CD3/a-CD28-activated PBMC.

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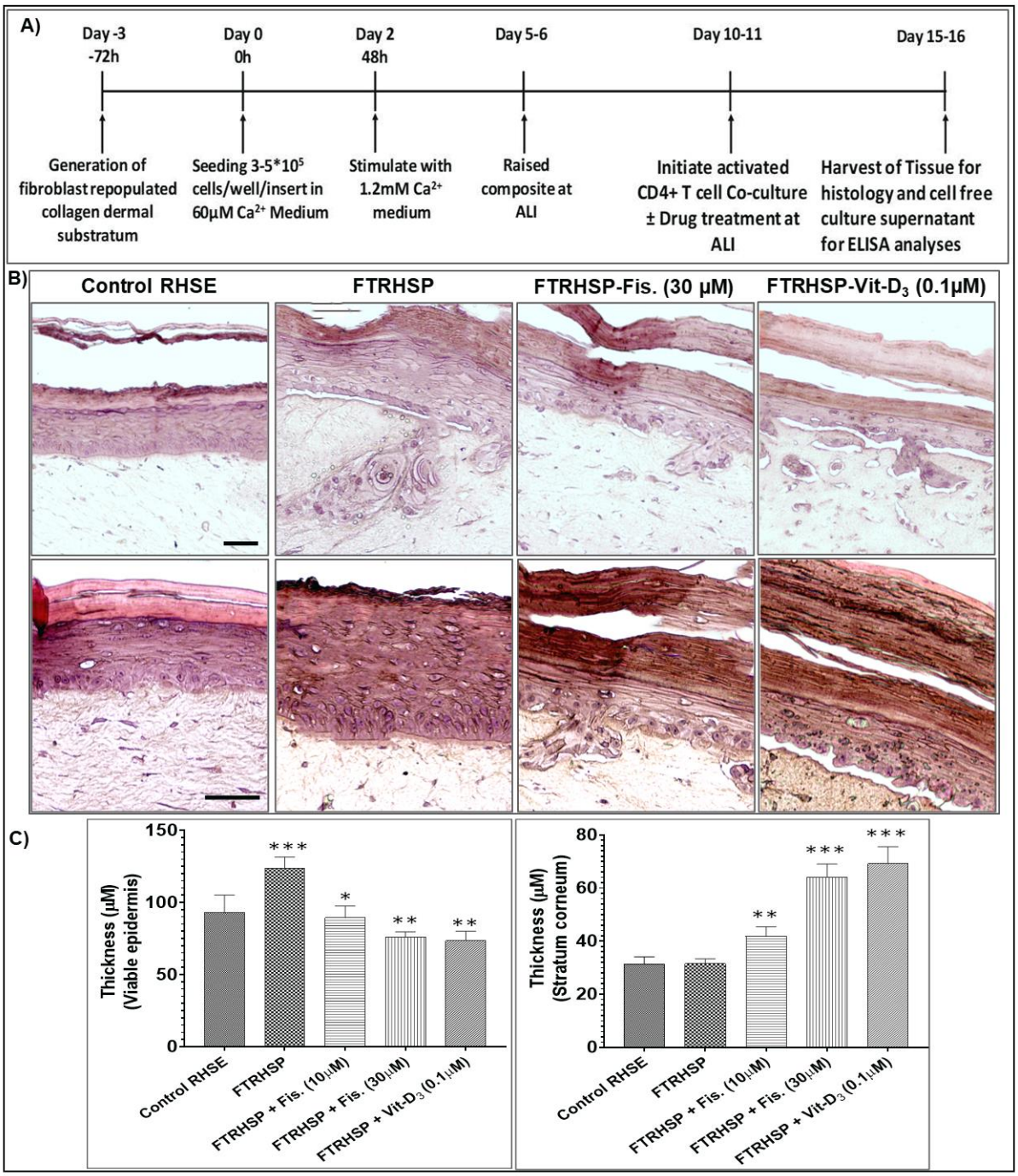


Figure 7: Fisetin modulated psoriasis-like features in three-dimensional (3D) full-thickness reconstituted human skin model of psoriasis (FTRHSP). (**A**) Protocol for the establishment/generation of the 3D full-thickness reconstituted human skin model of psoriasis (FTRHSP), and for evaluation of the therapeutic effects of compounds (fisetin or known control agent vitamin D_3 (Vit-D_3)) on psoriasis-like pathologic features. Candidate compounds were added on top of the tissue after 5 days of lifting to air-liquid interface and at the same day in wells containing pre-activated or not T lymphocytes at the base of the engineered reconstructs and co-cultured. These were continually cultivated for additional 5 days in the presence or absence of these agents prior to harvest, with media replenishment every alternate day. (**B**) Photomicrographs of Modified Mayers's Hematoxylin and Eosin stained control RHSE, FTRHSP and FTRHSP treated with/without fisetin/Vit-D_3 reconstructs, scale bar; top panel 20µM, and bottom panel 50µM. (**C**)

Bar graphs showing quantification of changes in the thickness of: A) the viable epidermis (left panel), significant decreased thickness in treated group vs FTRHSP and B) stratum corneum (right panel), significant increased thickness in treated group vs FTRHSP. The FTRHSP were generated and treated under the different conditions (including with or without test agents fisetin or Vit-D_3), and analyzed as detailed methods section. Significant differences between means ± standard deviation were determined as denoted by *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$ vs. control RHSE.

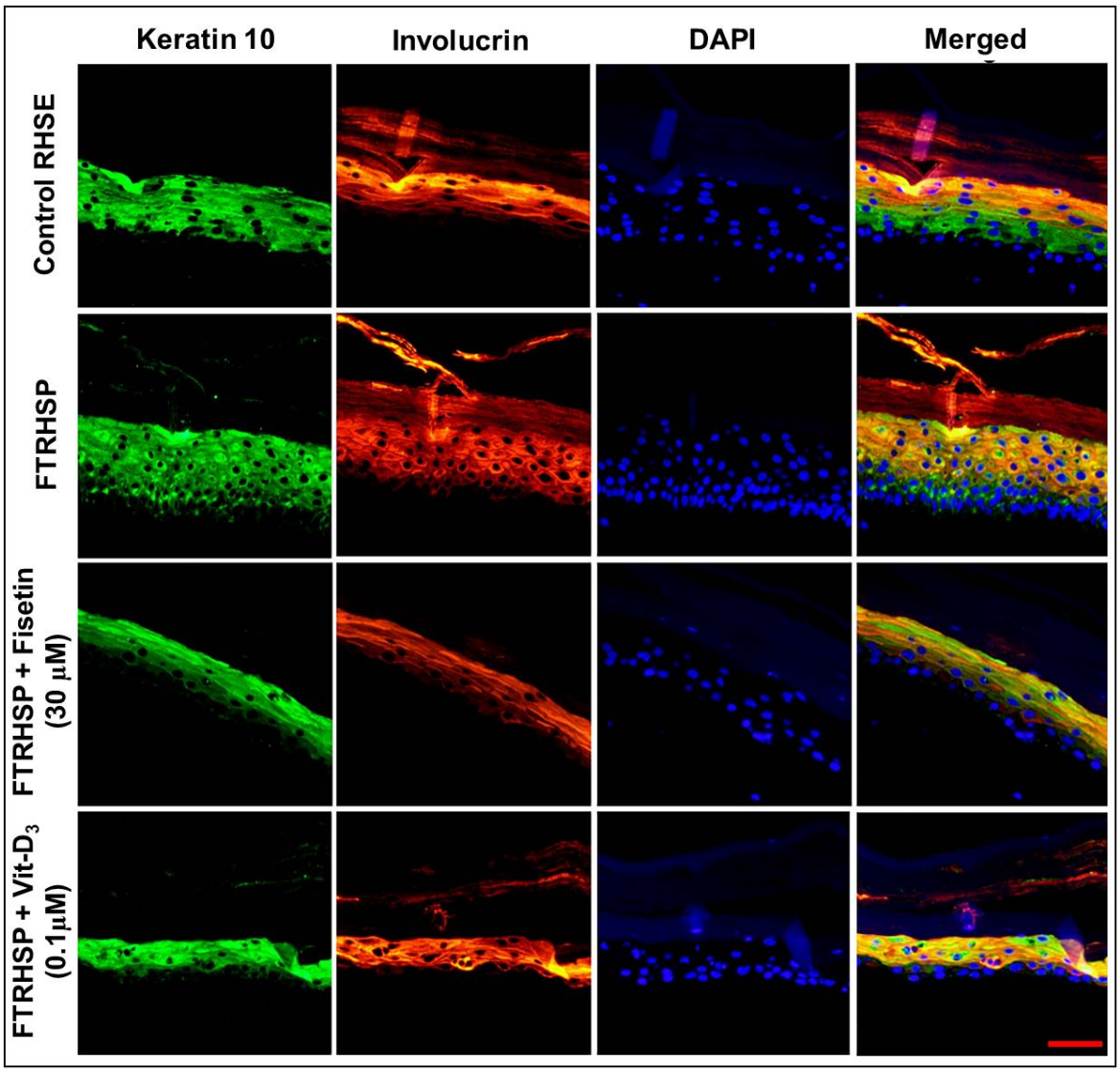


Figure 8. Fisetin modulates psoriasis-like features in the 3D full-thickness reconstituted human skin model of psoriasis (FTRHSP). Immunofluorescence staining was performed on FTRHSP sections by incubating with primary antibody against targets overnight at 4 °C followed by incubation with specific Alexa Flour 488-labelled secondary antibodies for 2 h at room temperature in the dark. Samples were mounted in mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) and analyzed microscopically. Immunofluorescence staining analysis of differential staining showing the protein expressions of early (K10) and late (involucrin) differentiation markers in control RHSE and FTRHSP under different treatment conditions. Stainings are shown in red and green respectively, and DAPI in blue and results are representative images of 2 independent experiments each performed in quadruplicate in comparison to control and treated FTRHSP. Scale bar = 20µm.

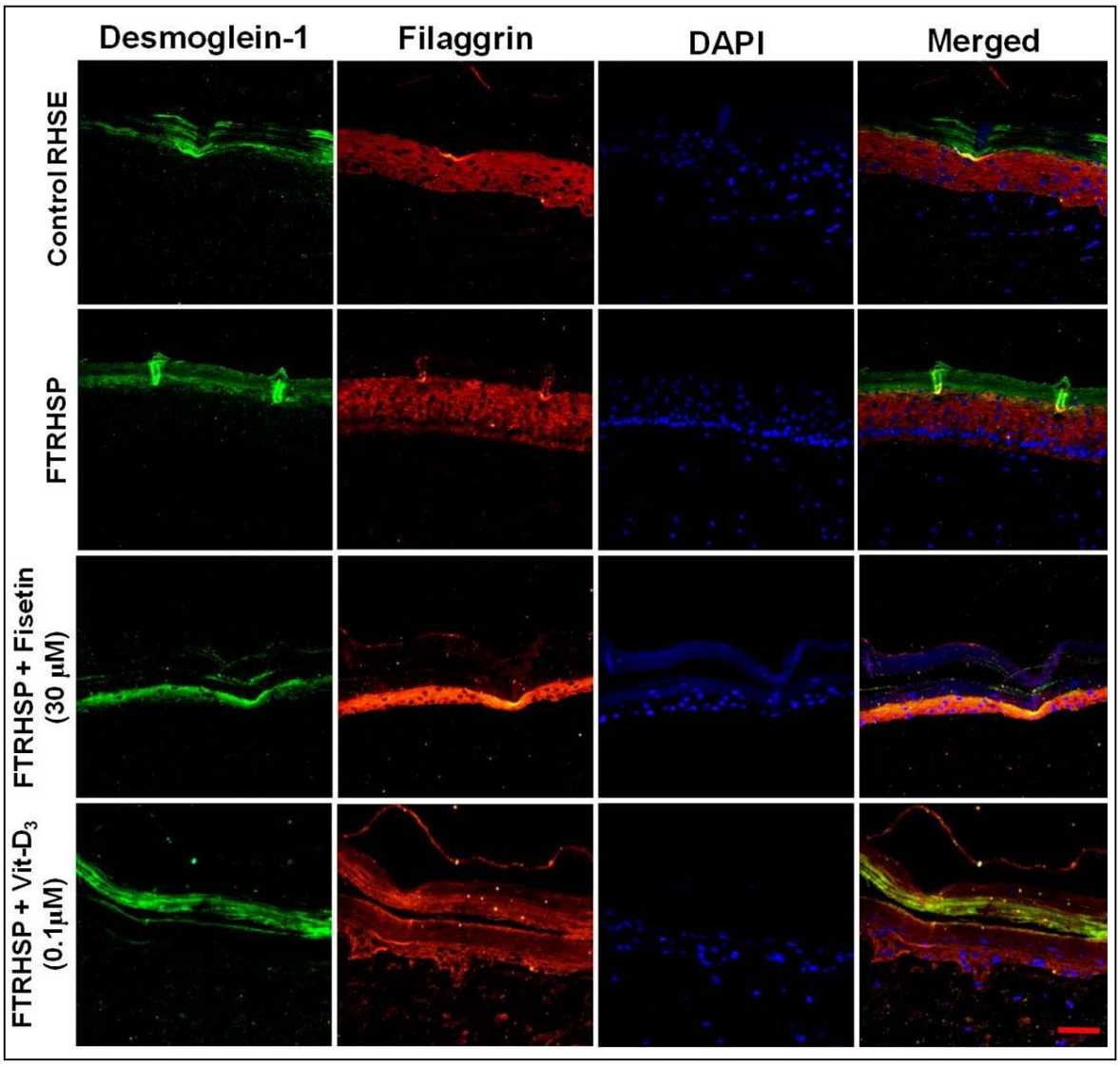
3.6. Topical application of fisetin modulates psoriasis-like features, suppresses proliferation, and modulates differentiation in T cell-induced three-dimensional (3D) full-thickness reconstituted human skin model of psoriasis (FTRHSP)

Since human psoriatic skin lesion is phenotypically characterized by increases in epidermal hyperplasia, antimicrobial peptides, inflammation, and aberrant differentiation and signaling cascades [40, 62-64], we determined the effect of fisetin on the established FTRHSP model which mimic *in vivo* features of psoriatic skin lesions and compared with Vit-D_3. After 5 days of administering T cells to the 3D human skin equivalents (day 15 from start), tissues were harvested as above and molecular markers of epidermal differentiation, inflammation and proliferation were determined as indicated in protocol in **Figure 7A**. We observed that control FTRHSP (RHSE) tissues generated by co-culture with non-activated CD4+ T cells, showed more condensed epithelium. In contrast, the regenerated 3D FTRHSP incorporating activated CD4+ T cells, recapitulated several features of human psoriatic lesion with lesser cornification including increases in epidermal thickness, proliferation and other changes in the aforementioned features (**Figure 2 B-C;** and data not shown). Furthermore, as previously reported [40, 62], fisetin-treated FTRSHP tissues co-cultured with activated CD4+ T cells had much prominently thinner viable epidermis similar to control tissues. These observations were comparable to the effects elicited by a single dose of Vit-D_3 (0.1μM) (**Figure 7 B-C**)

Additionally, by analysis of immunostained FTRHSP tissues for the expression of psoriasis-related skin proteins markers, we observed that compared with control tissues, terminal differentiation process was perturbed, as demonstrated by increased expression of Ki67 (proliferation **(data not shown)**), and aberrant increase and expression of differentiation markers (filaggrin, K-10, involucrin, TGase-1 and junctional protein, desmoglein-1) **(Figure 8 - 9, and Supplementary Figure 2**). Interestingly, we observed that topical application of fisetin or single dose of Vit-D_3 after 5 days markedly suppressed **proliferation**, accompanied by normalization/induction of the expressions of differentiation markers including K-10, involucrin, filaggrin, TGase-1 and desmoglein 1, in the spinous via granular epidermal cell layers of the FTRHSP **(Figure 8 -9, and Supplementary Figure 2)**.

3.7. *Topical application of fisetin suppresses mTOR activation, and inflammation in T cell- induced 3D FTRHSP model.*

Next, we examined the effect of fisetin on the mTOR pathway, the pro-inflammatory alarmin, psoriasin (that acts as antimicrobial peptide) and the secretion of pro-inflammatory cytokine, IL-17A in the FTRHSP tissues and conditioned media respectively, and compared with Vit-D_3 by employing immunofluorescence and ELISA analyses. Deregulated expression of these markers was associated with psoriatic skin lesion in the FRTHSP tissues and supernatants. We observed that compared with control tissues, the activated T cells co-cultured 3D FTRHSP tissues showed increased staining/induction expression of psoriasin and p-p70S6K **(Figure 10A-G)**, as well as increased secretion of IL-17A **(Figure 10H)**. Topical application of fisetin or Vit-D_3 prominently suppressed the observed increases in protein expression of psoriasin, p-p70S6K as well as IL-17A production in the established FTRHSP **(Figure 10)**.



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653 **Figure 9.** Representative immunofluorescent photomicrographs showing protein expression levels of
654 differentiation (filaggrin) and desmosomal (desmoglein-1) protein markers in control RHSE and
655 FTRHSP under different treatment conditions versus fisetin or Vit-D_3-treated FTRHSP tissues. Results
656 are representative of three independent experiments each performed in quadruplicate and comparing
657 control RHSE vs. FTRHSP treated groups. Data green (Dsgl-1); red (filaggrin), blue (DAPI) and mixed
658 is merged representation. Dsgl-1=desmoglein-1. Scale bar = 20µm.

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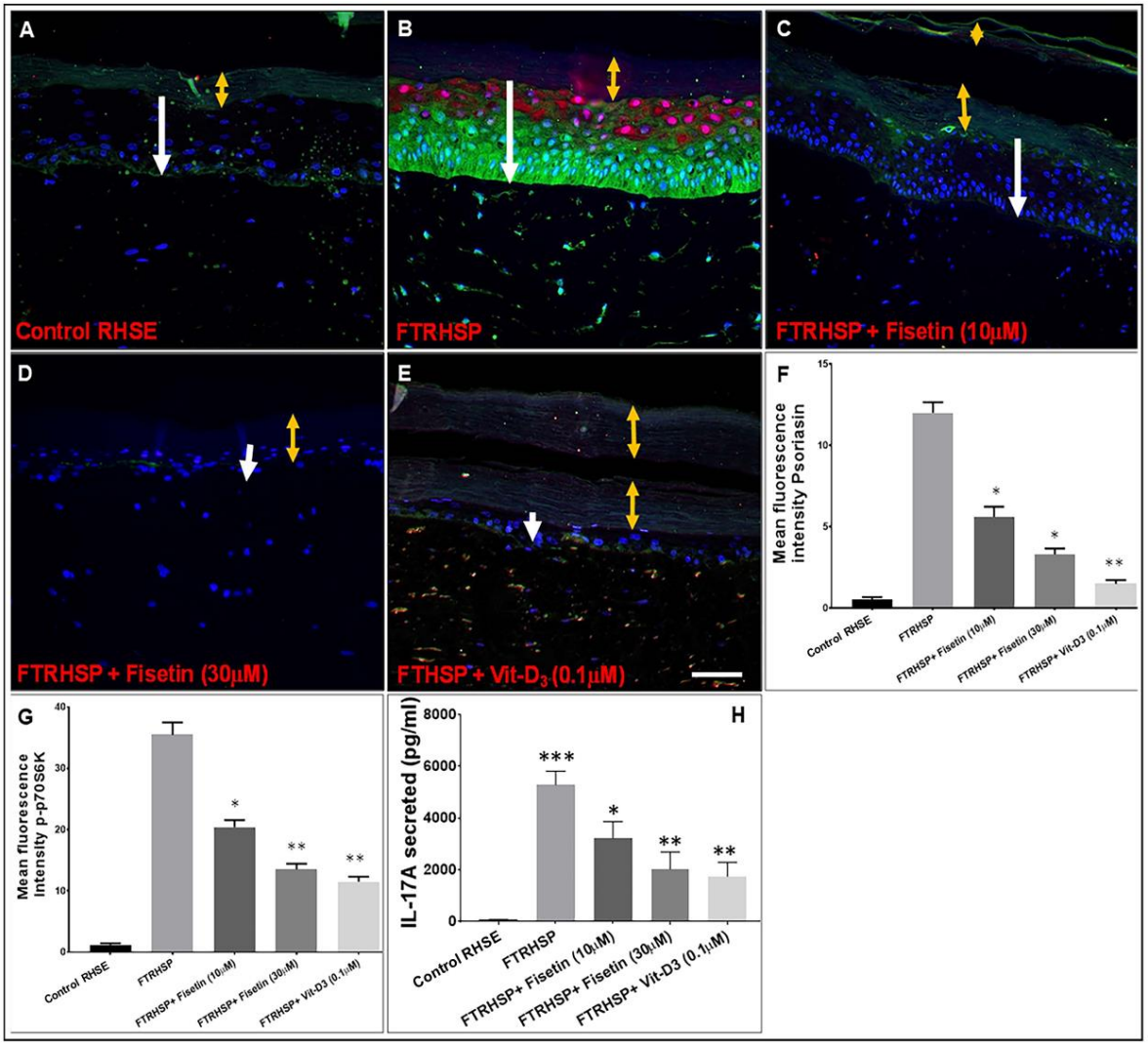

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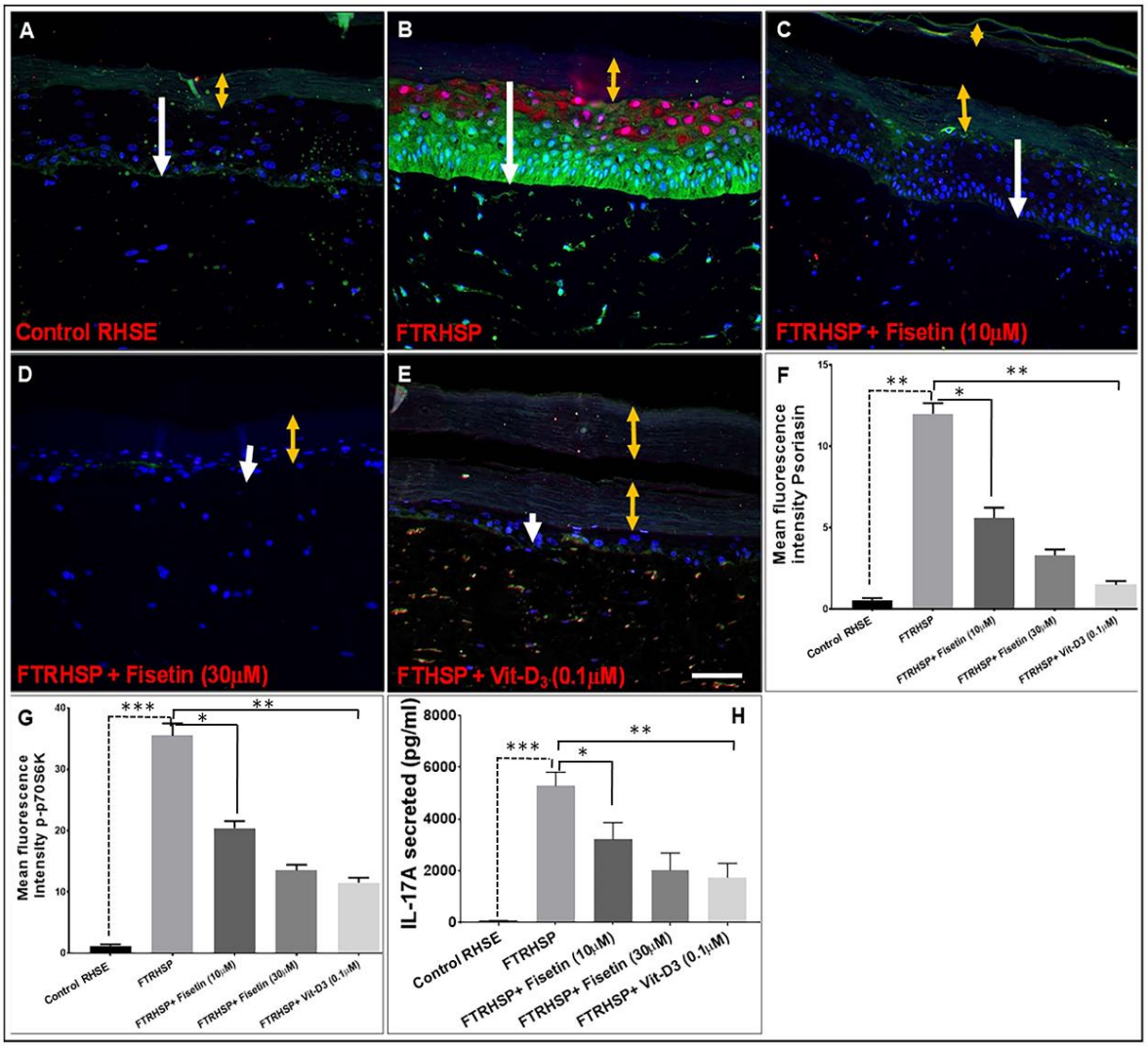

Figure 10. Fisetin modulated the expression of makers of psoriasis-like inflammation and mTOR activity in 3D human skin model of psoriasis. Immunofluorescence staining for p-p70S6K and Psoriasin was performed on FTRHSP sections by incubating with primary antibody against targets overnight at 4 °C followed by incubation with specific Alexa Flour 488-labelled secondary antibodies for 2 h at room temperature in the dark. Samples were mounted in mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) and analyzed microscopically. (A-E) the antimicrobial peptide (psoriasin) and mTOR activation effector, p-p70S6K stainings are shown in red and green respectively, and DAPI in blue. Representative pictures are shown. Scale bar = 20 µm. (F-G) For densitometric quantification each color image was separated into its green, blue, and red channel component using ImageJ software (National Institutes of Health), and green and red channels were used to analyze mean fluorescent intensity. Data shown here are mean fluorescence intensity ± SEM. (H) Differential protein expression levels of IL-17A secreted in the conditioned media of FTRHSP cultures. Pro-inflammatory cytokines IL-17A in conditioned media, were analyzed when 3D FTRHSP were treated without and with fisetin (10-30µM) treatment or 0.1µM Vit-D_3 for 5 days following activation by co-culturing with anti-CD3/CD28 activated CD4+ T cells for 7 days as described in method section. Significant differences were determined and significance of comparisons were made for the expression levels of the target proteins of FTRHSP tissues versus fisetin or Vitamin D_3-treated FTRHSP, corresponding to the solid lines, and also between non-activated T cell-exposed control RHSE versus FTRHSP tissues indicated by the broken lines as denoted by *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4. Discussion

In spite of recent progresses in the detailed understanding of the underlying molecular basis of psoriasis pathogenesis, existing treatment strategies still yield fluctuating and often-elusive outcomes; hence, some effective treatments are generally associated with serious side effects. Also, recent efficacious synthetic agents are unrealistically expensive. Therefore, it is necessary to look into cheaper, safe and cost effective anti-psoriatic agents. In this respect, medicinal plants have long been used as traditional remedies and are still used by over 75% of the world's population to meet healthcare needs (World Health Organization, 2013). Bioactive natural ingredients hold promise and remain a source of potential new therapeutics, because the identification of their constituent bioactive phytochemicals provide for the development of novel, clinically-useful, cheaper and relatively safe drugs [65].

In this study, we demonstrated that fisetin has direct pro-differentiative, anti-proliferative, and anti-inflammatory effects on both 2D and 3D cultures of keratinocytes and immune cells. To our knowledge, this is the first report indicating a direct effect in simplified and complex 3D systems of psoriasis-like disease. To investigate the effect of fisetin on NHEK differentiation, we selected differentiation markers relevant to psoriatic skin lesions, reflecting different stages of differentiation. K1 and K10 "often regarded as keratinization markers" are among the first proteins expressed by differentiating keratinocytes indicative of the fundamental switch from the basal proliferative to the post-mitotic phenotype. This switch is dysregulated in human and murine psoriatic skin lesions, and psoriatic keratinocytes maintain their proliferative capacity into the suprabasal and granular layers [4, 54, 66, 67]. We report that fisetin significantly induced protein expression of the early, intermediate and late markers of keratinocytes differentiation after 24 - 48 h of treatment. The bi-phasic effect of fisetin on K10 and other early markers, with concentrations greater than 10 μM inducing less expression levels of K10 and caspase-14 is likely because higher concentrations of fisetin drive keratinocytes to late differentiation. At later stages of differentiation, a decline in K10 and caspase-14 expression is expected, accompanied and as evident by dose-dependent increase in TGase activity and protein expression, a late marker of differentiation that facilitates terminal differentiation and formation of the cornified envelope [42]. Thus, increased expression of K10 and TGase-1 delineates the effect of fisetin not only to initiate keratinocyte differentiation but also to complete the barrier integrity stage. Furthermore, psoriatic lesions exhibit aberrant and reduced distribution and expression of epidermal TGase [68], and other differentiation markers including caspase-14, filaggrin, and involucrin, etc., resulting in hyperproliferation and parakeratotic skin [54, 69, 70]. Cutaneous inflammation in hyperplastic psoriasis skin lesions is often associated with increased expression of markers of proliferation, inflammation and proinflammatory factors. These major characteristic hallmarks of psoriasiform disease including the antimicrobial peptides, psoriasin (S100A7) and koebnerisin (S100A15), two epidermal S100 calcium-binding proteins proteins [64, 71, 72], with distinct roles but both serving as proinflammatory alarmins and chemoattractants[73], and cytokines. Moreover, the overexpression of the components of mTOR/PI3K/Akt and associated signaling has been associated with psoriatic disease [33, 34, 74]. In this study, fisetin (1-40 μM) treatment of NHEKs significantly increased TGase enzyme activity by 13-120%, as well as increased the protein expressions of TGase-1 and other differentiation markers often observed to be downregulated in lesioned psoriatic skin [54, 75]. Therefore, the antiproliferative and prodifferentiative effects of fisetin

on keratinocytes suggest that fisetin may correct proliferative and differentiation imbalances observed in psoriatic keratinocytes.

Importantly, the effect of fisetin was examined on inflammation, another major hallmark of psoriasis by analyzing the expression and secretion of key psoriatic cytokines induced by known activators of human keratinocytes and immune cells. These immune cell-derived cytokines are capable of activating keratinocytes, thereby exacerbating the flares [10]. Therefore, the role of inflammatory and type-17 cytokines in the psoriatic cytokine network has led to the development of antipsoriatic biologic drugs targeting TNF-α and IL-17A. Examples include etanercept (Enbrel®), a TNF-α antagonist and secukinumab, an antibody anti-IL-17A antagonist for psoriasis treatment [16, 17, 76]. Furthermore, high serum levels of TNFα and IL-6 are observed in psoriasis patients compared with healthy controls[77], and IL-1α, which is primarily produced by skin keratinocytes, regulates proliferation, differentiation, as well as dictates immune function [78], via priming IL-23-induced modulation of IL-17A production by T cells[79-82]. Overexpression of IL-1α in basal epidermal layers of transgenic mouse model leads to psoriasis-like phenotype with keratinocyte hyperplasia and immune cell infiltrates [83-85]. Here, inflammatory cytokine production in keratinocytes, was stimulated by a known inducer of keratinocyte inflammatory response, TPA [56], while PBMCs were activated by anti-CD3/anti-CD28 treatment, which mimics antigen presentation to lymphocytes [60]. Since keratinocyte is the major source of a spectrum of different cytokines including IL-1, IL-6 and TNFα in skin inflammatory responses [8, 10, 86], by stimulating NHEKs with TPA, we presumably mimicked the initiation phase of the psoriatic process [54, 87]. Our data on both, multiplexed and sandwich ELISAs, showed that fisetin was capable of efficiently inhibiting the production of inflammatory cytokine induced in keratinocytes, PBMCs and activated-CD4+ T cells co-cultured with 3D FTRHSP. Among the cytokines down-regulated by fisetin were Th-1/Th-17 cytokines, including IL-17A and IFN-γ. Therefore, we propose that fisetin, by inhibiting proliferation, inducing differentiation, and downregulating the psoriatic cytokine network in keratinocytes and immune cells, could restore keratinocyte and skin homeostasis, and may act as a possible anti-psoriatic agent as suggested by the 3D psoriatic skin model studies. Furthermore, these findings suggest that fisetin exerts inhibitory functions on the two major cell types involved in psoriasis pathogenesis, keratinocytes, and immune cells by inhibiting the activation of PI3K/AKT/mTOR and MAPK pathway components. The induction of ERK1/2 activation by fisetin is a mechanistic phenomenon observed in several other studies by our team and others. This is reflected in relation to activation of activation of cutaneous keratinocyte differentiation (specialized program cell death in keratinocytes) rather than the much expected induction of proliferation PMID: 30356079; PMID: 31438640, and the result is not much surprising as constitutive activation of ERK is related to apoptosis. It is admitted that targeting immune cells and their mediators is the ultimate approach to treat psoriasis. Moreover, considering the essential role of keratinocyte cross-talk with immune cells in inflammatory skin diseases like psoriasis, targeting inflammatory mediator network from both keratinocytes and immune cells, as well as the anti-proliferative and pro-differentiation potential may enhance the anti-psoriatic effect of a compound such as fisetin. Considering the fact that fisetin exerts a pleiotropic effect, targeting the three major hallmarks of psoriasis as a single, natural agent, and not only is it safe and effective, fisetin may also be less compromising to the patient's immune system. The observed increased expression of psoriasin (S100A7), an antimicrobial peptides in the generated CD4+ T cells activated 3D FTRHSP compared to normal FTRHSP, was suppressed by topical fisetin

treatment to values comparable to suppression induced by physiologic Vit-D_3 [73, 88, 89]. We earlier showed increased expression of keratinocyte-induced immune mediator secretion without immune cells derived cytokines in psoriatic skin equivalents [62], which are critical facets in the pathogenesis of psoriasis [89]. Herein, we observed that fisetin inhibited the increased release of both keratinocyte and activated immune cell-associated Th1/Th-17 pro-inflammatory cytokines production including (IL-17A, TNF-a, IFN-γ etc.). It is important to note that the inculcated keratinocytes and activated immune cells in the currently established FTRHSP model elicits and mimics the feedback loop often induced by specific immune cells in the immunopathogenesis of psoriatic skin lesions. In view of the intricacy of psoriasis etiology, it is noteworthy that agents like fisetin that **are** capable of rescinding numerous critical endpoints in psoriasiform disease, are prospective therapeutic agent**s** for the control of psoriasis.

To the best of our knowledge, this study demonstrates for the first time in 2D and 3D psoriasis-like systems the direct effects of fisetin on psoriasis-like features and thus highlights its likely multifactorial prospect as an agent from natural botanical sources consumed on daily basis. This could peak interest to be developed for preventing, slowing the progression or treatment of psoriasis and other inflammatory diseases. Furthermore, granting the current model is promising, additional studies employing complimentary animal, and humanized murine psoriasis-like skin models that integrate more of the immune axis (e.g. activated dendritic cells) are needed to further corroborate the detailed mode of action and efficacy of fisetin without involving too much into animal experimentation. Taken together, the current study provides a rationale for future preclinical proof-of-concepts leading to clinical studies to evaluate fisetin for the control of psoriasis in human patients.

Author Contributions:

JCC conceived and designed the research, conducted experiments, acquisition, analysis and interpretation of data, and wrote the first draft of the manuscript. SE and VMA contributed to the design, experiments, acquisition, analysis and interpretation of data and writing the manuscript. ALN, SBM, TR, SSS, contributed in performing research and collected the data and initial analysis. HM provided initial lab space, and together with SH, and KGK provided guidance and key insights into the planning of the project, interpretation of the data, and placing the conclusions into the broader context. All authors revised the manuscript critically for important intellectual content, and approved the final version of the manuscript to be published.

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Conflicts of Interest:

The authors declare no conflict of interest

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