Sharma M, et al., J Altern Complement Integr Med 2019, 5: 080
DOI: 10.24966/ACIM-7562/100080

HSOA Journal of Alternative, Complementary & Integrative Medicine

Research Article

East Indian Sandalwood Oil Suppresses IL-17-Dependent and -Independent Inflammatory Psoriasis Characteristics

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Abstract

Psoriasis is a chronic inflammatory skin disease that affects 2-3% of people worldwide. While multiple heritable and environmental factors can cause psoriasis, mechanistically, disease initiation is due to IL-23-mediated IL-17 production by pathogenic T cells that trigger a chronic inflammatory response by the affected epidermis. Appreciation of these events has led to the development of agents that disrupt this IL-23/IL-17 axis. There is, however, evidence that psoriatic characteristics persist in organoids devoid of leukocytic infiltrate, and that the anti-inflammatory, standardized pharmaceutical-grade natural product, East Indian Sandalwood Oil (EISO), to suppress chronic inflammatory chemokine/cytokine production, and psoriatic characteristics, by these organoids. These observations suggest that persistent IL-23/IL-17 axis activity may not be required to sustain disease. We hypothesize that, once activated, the psoriatic epidermal inflammatory program is independent of IL-17, and that EISO can block IL-17-mediated and -independent, inflammatory programs. Using normal human skin organoids, we observed that IL-17 was sufficient to induce loss of epidermal architecture, stratum basal is hyper proliferation and dysregulated expression of psoriasin, and key cytokines/chemokines linked to psoriasis: IL-6, IL-8, ENA-78 and MIP-3α. With the exception of elevated MIP-3α expression, these pathophysiologic characteristics were similarly observed in psoriatic organoids composed of normal keratinocytes and psoriatic dermal fibroblasts that have undetectable Th17 infiltrate or IL-17 levels. MIP-3α expression was robustly elevated in psoriatic organoids by IL-17 stimulation. EISO co-treatment of IL-17-stimulated psoriatic and normal skin organoids suppressed induction of psoriatic characteristics and expression of IL-6, IL-8, ENA-78 and MIP-3α. We conclude that IL-17 can trigger a perpetuated pro-inflammatory cascade giving rise to a psoriatic phenotype in normal skin organoids, and exacerbate disease characteristics in psoriatic organoids and that EISO can suppress both initiation, and chronic presentation, of these disease characteristics by suppressing cytokine/chemokine production.

Keywords: IL-17; Inflammation; Keratinocytes; Normal skin organoid; Psoriasis; Th17

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Citation: Sharma M, Clements I, Castella P, Cox ME (2019) East Indian Sandalwood Oil Suppresses IL-17-Dependent and -Independent Inflammatory Psoriasis Characteristics. J Altern Complement Integr Med 5: 80.

Received: October 24, 2019; **Accepted:** November 04, 2019; **Published:** November 11, 2019

Introduction

Psoriasis (PS) is a chronic inflammatory autoimmune skin disease that affects up to 3% of people worldwide [1,2]. In genetically susceptible individuals, various factors, such as infection, injury and some medications, result in release of self-antigens from stressed or dying cells that activate dendritic cells, which in turn activate Th17, Th22 and Th1, that further stimulate recruitment and activation of macrophages and neutrophils [3-56]. The resulting self-amplifying pro-inflammatory conditions drive the formation of the inflamed psoriatic plaque and epidermal hyperplasia [7-9]. Although conventional treatments with emollients, vitamin D and topical corticosteroids are often effective for symptom management, adverse effects, including susceptibility to infections and skin atrophy limit their use [10,11]. Failure to respond to these topical agents can lead to patients being treated with targeted therapeutics, most commonly an anti-TNFα, and more recently, anti-IL-17 immunotherapies, depending on disease phenotypes and immunogenic status [12,13]. The discovery of biological agents targeting specific components of the inflammatory process offers significant improvements in psoriasis treatment; however, side effect risks, high cost, repeated injections and drug resistance may limit their use [14-18]. Hence, the quest for less expensive alternatives is ongoing.

Due to their structural diversity, natural products are one of the best sources for the development of new therapies [19]. A natural product demonstrated to provide relief for chronic inflammatory diseases such as psoriasis is the essential oil from Indian sandalwood trees, *Santalum album* (*Santalaceae*). The essential oil from the sandalwood tree has been used topically for many years in traditional Indian (Ayurvedic), Chinese and Tibetan medicine,for religious purposes including incense and for carvings, in perfumes, aromatherapy, cosmetics, flavoring agent in variety of food products and is considered safe by FDA [20,21]. Of the more than a dozen sandalwood species sourced for essential oils, International specification is issued only for two species: *Santalum album* (East Indian sandalwood) and *Santalum spicatum* (Western Australian sandalwood). These agents, commonly known as East Indian Sandalwood Oil (EISO) and Western Australian andalwood Oil (WASO), are composed of over 125 chemical compounds including saponin, tannins, terpenoids and phenolics [22,23]. The major constituents, accounting for up to 80% of the oil's

constitutions, are two isomers of the sesquiterpene alcohols; (Z)-α-santalol and (Z)-β-santalol [24,25]. According to the international standard for EISO (ISO3518:2002), the primary active components of essential oil include 41–55% (Z)-α-santalol and 16-24% (Z)-β-santalol [26,27]. We have previously reported active ingredients of the essential oils as (Z)-α-santalol ~50% and ~20% by weight and (Z)-β-santalol ~25% and ~10% by weight for EISO and WASO respectively.

Sandalwood oils have demonstrated anti-inflammatory, antiseptic and astringent properties that make them effective topical remedies for epidermal maladies [21,28]. Importantly, the ability of EISO to alleviate symptoms in acne and PS patients has been attributed to its anti-inflammatory property in skin models [26,29-31]. EISO has also been reported for its anti-cancer activity in skin cancer and bladder cancer, oral cancer, breast cancer, prostate cancer and hepatocellular carcinoma [32-46]. EISO was commercially available over-the-counter acne product in the United States sold by Galderma (Fort Worth, Texas) under the Benzac® name.

Although there is strong evidence for Th17 cell activation and production of IL-17 in psoriasis pathogenesis, the relative contribution of the Th17 cytokine IL-17 to the chronic disease condition is unknown. Here, we use normal and psoriatic skin organoids known to have undetectable Th17 infiltrate or IL-17 protein to assess the relative impact of IL-17 on inflammatory and pathohistologic features. We demonstrate that skin organoid composed of normal dermal fibroblasts and keratinocytes, and to exhibit normal epidermal characteristics, acquire inflammatory and histologic characteristics of psoriasis when exposed to IL-17 alone, and that treatment with EISO can suppress the response of normal skin organoids to IL-17 exposure. Furthermore, we demonstrate that EISO can antagonize production of pro-inflammatory cytokines/chemokines and markers of proliferation in organoids composed of psoriatic dermal fibroblasts and normal keratinocytes that retain psoriatic characteristics in the absence of detectable undetectable Th17 infiltrate or IL-17 protein. Our results indicate that psoriatic organoids chronically express pro-inflammatory chemokines and cytokines, as demonstrated by ENA-78, IL-8, and IL-6 expression, in the absence of IL-17, and that exogenous IL-17 exacerbates an inflammatory feedback loop by driving MIP-3α expression. The ability of EISO to reduce proliferation and psoriasin expression by psoriatic keratinocyte and expression of key inflammation markers (MIP-3α, ENA-78, IL-8 and IL-6) in normal and psoriatic organoids suggests that once induced, the psoriatic phenotype can be sustained by the lesional fibroblasts and keratinocytes, and that EISO can effectively suppress production of cytokines and chemokines that drive psoriasis pathogenesis.

Materials & Methods

East Indian Sandalwood oil

EISO (lot: APISO-150308SD/SA), an essential oil obtained by steam and vacuum distillation from the heartwood of TFS plantation-grown Indian sandalwood (*Santalum album*) trees, was obtained from Santalis Pharmaceuticals (San Antonio, TX, USA) and was in compliance with the (26,27). The chemical composition of the principal components of EISO is shown in table 1. The operating parameters (Shimadzu GC17A with an AOC-20i Auto sampler), the physico-chemical elucidation, pesticide and heavy metal analysis are shown in the certificate of analysis from TFS Corporation Ltd. (ABN 97 092 200 854) (Supplemental Figure, S1). EISO was Diluted in Dimethyl Sulfoxide (DMSO) as a 10% (v/v) stock. The equivalent volumes of DMSO used as vehicle controls had no adverse effects on the cells.

Sesquiterpenes	EISO (%)[b]			
(Z)-α-Santalol	41-55%			
(Z)-β-Santalol	16-24%			
(Z)-Nuciferol	0.8-3.0%			
epi-β-Santalol	3.5-4.1%			
(Z)-α-trans-Bergamotol	5.0-6.7%			
(Z)-β-curcumen-12-ol	0.5-1.9%			
β-Santalal	1.0-2.4%			
(Z)-lanceol	1.4-5.6%			
(E)-β-Santalol	3.5-4.1%			
β-Santalene	0.7-1.5%			
[a]EISO component analysis was performed by TFS corporation Ltd, Albany, WA.				
[b]% concentration of sesquiterpenes mass per volume				

Table 1: Chemical composition of East Indian Sandalwood Oil (EISO)[a].

Human full-thickness skin model

Reconstituted full-thickness normal human skin and psoriatic phenotype organoids (MatTek Corp., Ashland, MA) have been demonstrated to lack Th17 cells and to have undetectable levels of IL-17 [47,48]. These normal skin and psoriatic organoid cultures were incubated in the manufacturer's assay media supplemented with or without EISO at 0.001 or 0.002% (v/v) and rhIL-17 (100 ng/ml; Cedarlane labs. Canada: CL101-17-5UG) [49,50]. Media with or without supplementation were changed every second day. On day 4, specimens were collected for histology and Immunohistochemistry (IHC) analysis.

IHC antibodies and reagents

Monoclonal Antibodies (mAb) for Ki67 (8D5) was purchased from Cell Signaling Tech (Danvers, MA, USA) and psoriasin (47C1068) from abcam (Cambridge, UK). Recombinant human IL-17 (IL-17A) (rhIL-17) (CL101-17-1MG) was purchased from Cedarlanelabs, Burlington, ON, Canada (CL101-17-5UG; Lot: L10117102). IHC staining of psoriatic and normal skin organoids was conducted using deparaffinized sections subjected to antigen retrieval, followed by incubation with primary antibodies (1:500 dilution for anti-Ki67 mAb and 1:200 dilution for anti-psoriasinm Ab). Bound antibodies were detected by DAB staining using Ventana universal secondary antibody (Ventana Medical System, Tuscan, Arizona). All stained slides were digitally imaged at magnification equivalent to 20X and representative fields are shown.

ELISAs

Sandwich ELISAs for CCL20 (MIP 3-α), CXCL5 (ENA-78), IL-8 (CXCL8) and IL-6 (RayBiotech, Norcross, GA, USA) were performed on culture media from control, mock-, or rhIL-17-stimulated normal and psoriatic organoids (with and without treatment with EISO) according to manufacturer's instructions. Standard curves were constructed with supplied standards to allow conversion of Abs

OD450 nm readings of experimental samples to pg/ml of the respective factors.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA.). For immune-histochemical analysis of cell proliferation, Ki67 counts were compared using a one-way analysis of variance (ANOVA) followed by a Bonferroni post-test comparing only the pairs of interest if ANOVA p-values were significant. The post-test results are shown as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ versus vehicle-treated controls; † = $p < 0.05$, †† = $p < 0.01$, ††† = $p < 0.001$ versus rhIL-17-stimulated controls (Table 2). Cytokines/chemokines ELISA assay results were compared using a one-way Analysis of Variance (ANOVA) followed by a Bonferroni post-test comparing only the pairs of interest if ANOVA p-values were significant. The post-test results are shown as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ versus vehicle-treated controls; † = $p < 0.05$, †† = $p < 0.01$, ††† = $p < 0.001$ versus rhIL-17-stimulated controls (Tables 3a & 3b).

Bonferroni's Mnltiple Comparison Test	P value
N Cvs N + rhIL-17	P < 0.01
N Cvs N + rhIL-17 + EISO 0.002%	P > 0.05
N rhIL-17 vs N + rhIL-17 + EISO 0.002%	P < 0.01
PS Cvs PS + rhli.-17	p > 0.05
PS Cvs PS + rhli.-17 + EISO 0.002%	P < 0.01
PS + rhIL-17 VS PS + rhIL-17 +EISO 0.002	p < 0.001

Table 2: Ki67 in normal skin (N) and psoriasis (PS) organoids ± rhIL-17/EISO: ANOVA and Bonferroni post-hoc test of treatment pairs of interest.

Bonferroni's Mnltiple Comparison Test	P value
MIP-3α N C VS N + IL- 17	P < 0.001
N CVS N + IL-17 + EISO 0.001%	P < 0.001
N C VS N + IL-17 + EISO 0.002%	P < 0.05
N + IL-17 VS N + IL- 17 + EISO 0.001%	P < 0.001
N + IL-17 VS N+ IL-17 + EISO 0.002%	P < 0.001
E NA-78 N C VS N + IL-17	P < 0.001
N CVS N + IL-17 + EISO 0.001%	P < 0.001
N C VS N + IL-17 + EISO 0.002%	P < 0.05
N + IL-17 VS N + IL-17 + EISO 0.001%	P < 0.001
N + IL-17 VS N + IL- 17 + EISO 0.002%	P < 0.001
IL-8 EPI cVS EPI + IL- 17	P < 0.001
N C VS N + IL-17 + EISO 0.001%	P < 0.001
N C VS N + IL-17 + EISO 0.002%	P > 0.05
N + IL-17 VS N + IL- 17 + EISO 0.001%	P < 0.01
N + IL-17 VS N + IL-17 + EISO 0.002%	P < 0.001
IL-6 N C VS N + IL- 17	P < 0.001
N CVS N + IL-17 + EISO 0.001%	P < 0.05
N C VS N + IL-17 + EISO 0.002%	P > 0.05
N + IL-17 VS N + IL-17 + EISO 0.001%	P < 0.001
N + IL-17 VS N + IL-17 + EISO 0.002%	P < 0.001

Table 3a: Cytokines/chemokines in normal skin organoids (N) ± rhIL-17/EISO: ANOVA and Bonferroni post-hoc test of treatment pairs of interest.

Bonferroni's Mnltiple Comparison Test	P value
MIP-3α P C VS PS + IL- 17	P < 0.001
P VS P + IL- 17 + EISO 0.001%	P < 0.001
P C VS P + IL-17 + EISO 0.002%	P > 0.05
P + IL- 17 VS P + IL- 17 +EISO 0.001%	P < 0.001
P + IL- 17 VS P + IL-17 + EISO 0.002%	P < 0.001
ENA-78 P C VS P +IL- 17	P < 0.001
P VS P + IL- 17 + EISO 0.001%	P < 0.01
P C VS P + IL-17 + EISO 0.002%	P < 0.001
P + IL- 17 VS P + IL- 17 + EISO 0.001%	P < 0.001
P + IL- 17 VS P + IL- 17 + EISO 0.002%	P < 0.001
IL-8 P C VS P + IL- 17	P < 0.001
P VS P + IL- 17 + EISO 0.001%	P < 0.001
P C VS P + IL-17 + EISO 0.002%	P < 0.001
P + IL- 17 VS P + IL- 17 + EISO 0.001%	P < 0.001
P + IL- 17 VS P + IL- 17 + EISO 0.002%	P < 0.001
IL-6 P C VS P + IL- 17	P < 0.001
P VS P + IL- 17 +EISO 0.001%	P < 0.001
P C VS P + IL-17 + EISO 0.002%	P < 0.001
P + IL- 17 VS P + IL- 17 + EISO 0.001%	P < 0.001
P + IL-17 VS P + IL-17 + EISO 0.002%	P < 0.001

Table 3b: Cytokine/chemokines in psoriasis skin organoids (PS) ± rhIL-17/ EISO: ANOVA and Bonferroni post-hoc test of treatment pairs of interest.

Results

EISO suppresses acquisition of rhIL-17-induced psoriatic characteristics in skin organoids

Although Th17 cells are considered drivers of PS pathogenesis, whether they are sufficient for disease initiation or required for disease persistence, remain to be fully clarified. To determine if rhIL-17 is sufficient to promote psoriasis-related inflammatory responses, we performed histologic analysis of H&E-stained normal skin organoids defined to have an undetectable TH17 infiltrate or IL-17 level [47]. The epidermis of the normal skin organoids exhibited well-differentiated characteristics of a defined Stratum basale (Sb) layer under a Stratum spinosum (Ss) and Stratum granulosum (Sg) layer topped by discrete Stratum corneum (Sc) layer (Figure 1). In contrast, when exposed to rhIL-17, the epidermis developed hallmark psoriatic characteristics, including parakeratosis (persistent nucleation in the Sc; indicative of impaired terminal differentiation) and partial loss of the Sg resulting in relative thinning of the epidermis (Figure 1). We have previously used normal skin and psoriatic equivalent, organoids to assess how EISO modulates inflammation and affects disease pathologies [30,31]. Normal skin organoids simultaneously treated with rhIL-17 and either 0.001 or 0.002% EISO (Figures 1C and D, respectively) for 4 days retained a normalized epidermal architecture, most notably observed by the reduced thickness, and decreased nucleation of the Sc.

A key characteristic of psoriasis is a hyper-proliferative Sb, and using Ki67 as an immunohistological marker of cell proliferation, and consistent with the psoriatic morphological characteristics presented in figure 2, we observed > 2-fold increase in the fraction of

proliferative cells in the Sb of rhIL-17-stimulated normal skin organoids (Figures 2 & 3) (Table 2). In contrast, EISO-co-treatment reduced the proliferative index of the Sb of rhIL-17-stimulated normal skin organoids to levels indistinguishable from that of the vehicle-treated normal skin organoids (Figures 2 & 3). These observations indicate that IL-17 is sufficient to stimulate hyper proliferation of normal keratinocytes in the normal skin organoids, and that the anti-inflammatory natural product, EISO can suppress acquisition of these characteristics. In addition, while rhIL-17-treatment resulted in little, if any, further increase in the mitotic index of psoriatic skin organoids, co-treatment with EISO profoundly suppressed the mitotic index of rhIL-17-treated psoriatic organoids (Figure 2) to levels comparable to that of the normal skin organoid (Figures 2 & 3). These results indicate that while rhIL-17 can stimulate proliferation of Sb cells, this activity persists in the absence of detectable Th17 infiltrate or IL-17 protein in psoriatic organoids and that EISO is able to suppress IL-17-induced and -independent hyperproliferation of the Sb cells.

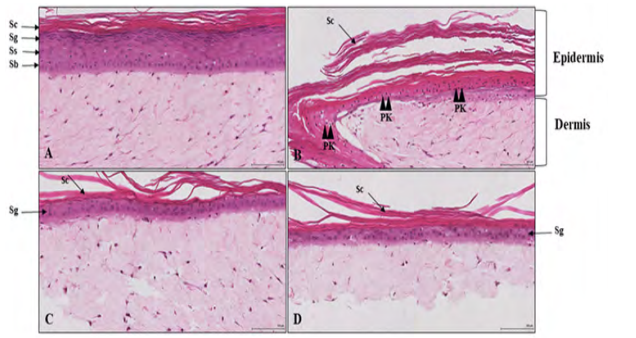

Figure 1: Hematoxylin and eosin staining of the normal skin organoids incubated with (B) or without rhIL-17 (100 ng/ml) (A)/ EISO 0.001-0.002% (v/v) (C-D) for 4 days. Contrasting to normal skin, hallmarks of psoriasis like parakeratosis (purple nuclei retainment of corneocytes), and reduction of the stratum granulosum in the epidermis are distinct. Lines represent the level of the different epidermal layers. Sc: Stratum corneum. Sg: Stratum granulosum. Ss: Stratum spinosum. E: Epidermis. D: Dermis. Scale bars = 100 µm.

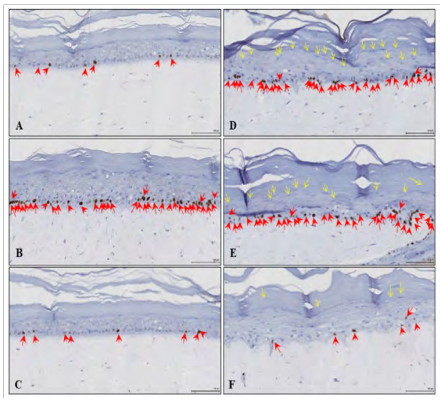

Figure 2: EISO treatment (4 days) reverses epidermal inflammation and hyper proliferation in the human skin organoids by rhIL-17: Reconstituted normal and psoriatic skin organoids were cultured in the presence or absence of rhIL-17 (100 ng/ml) and EISO at 0.002% (v/) for 4 days. Immunohistochemical staining of epidermal and psoriasis tissue model showing Ki67 as marker of proliferation restricted to the stratum basale layer (red arrows). (A) normal skin 3D cultures, (B) normal skin + rhIL-17, (C) normal skin + rhIL-17 and 0.002% EISO, (D) untreated psoriatic skin, (E) psoriatic skin + rhIL-17, (F) psoriatic skin + rhIL-17 and 0.002% EISO. In addition, the increased keratinocyte nuclei (indicative of impaired terminal differentiation) observed in stratum corneum of psoriatic epidermis (yellow arrows) is decreased in EISO treated psoriatic skin cultures. Scale bar = 100 µm.

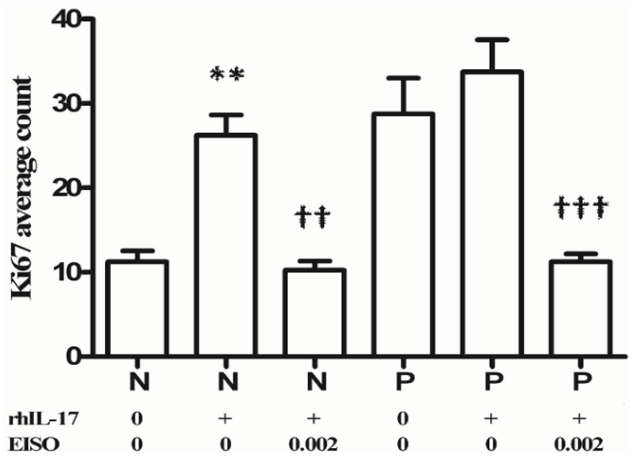

Figure 3: Epidermal proliferation and up-regulation of Ki67 in the human skin organoids by rhIL-17: For immune-histochemical analysis of cell proliferation, immune-staining with anti-Ki67 antibody was used. Normal (N) and psoriasis (P) organoids were maintained in the presence or absence of rhIL-17 (100 ng/ml) and EISO at 0.002% (v/) for 4 days and Ki67 data represent 4 different regions of the two specimens' ± SEM expressed as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ vs. vehicle-treated controls and † = $p < 0.05$, †† = $p < 0.01$, ††† = $p < 0.001$versus rhIL-17 stimulated controls.

Psoriasin is an Antimicrobial Peptide (AMP) and its elevated expression is a diagnostic marker of psoriatic skin lesions [51,52]. Here we assessed whether psoriasin expression and/or distribution might also be affected by IL-17 treatment of normal skin organoids (Figure 4). We observed psoriasin expression in normal skin organoids to be restricted to a discrete layer in the Sg and at the interface of the Sg with the Sc. In contrast, normal skin organoids stimulated with rhIL-17 exhibited pronounced psoriasin staining throughout the epidermal layer (Figure 4). This included strong staining of the normally negative Sb throughout the thickened morphology and clearly defined Rete ridges in psoriatic organoids (Figure 4). When co-treated with EISO, the rhIL-17-stimulated normal (Figure 4) and psoriatic organoids (Figure 4) exhibited epidermal layers with psoriasin expression predominantly localized to the Ss/Sg layers while being suppressed in the Sb. These results suggest that IL-17 is sufficient to promote psoriasin expression. The decreased psoriasin expression as well as the reduction in size and number of Rete ridges in the EISO-treated psoriatic organoids, supports the previous observations indicating that EISO can suppress acquisition of IL-17-mediated psoriatic characteristics.

EISO suppresses rhIL-17-induced inflammatory cytokine/chemokine production by skin organoids

Based on the above observations that IL-17 was sufficient to induce several key psoriatic characteristics, but that organoids composed of normal keratinocytes and psoriatic dermal fibroblasts exhibit psoriatic characteristics in the absence of Th17 infiltrate or detectable levels of IL-17 [48,s53]. We next assessed how IL-17 alone affected the expression of 4 key psoriasis-associated cytokines and chemokines: ENA-78, IL-8, IL-6 and MIP-3α (Figure 5 & Table 3). Cytokine/chemokines expression was assessed by ELISA from organoid conditioned medium for comparison of mock- or rhIL-17-treated normal skin and psoriatic organoids. Basal expression of these four factors by normal skin organoids was the lowest of all the tested conditions. Relative to the normal organoid cultures, psoriatic organoids produced significantly elevated levels of ENA-78 (> 40-fold),

IL-6 (5-fold) and IL-8 (> 5-fold), however MIP-3α levels in psoriatic organoid cultures (35 pg/ml) was comparable with that detected in normal skin organoid cultures (20 pg/ml). When rhIL-17-treated, normal skin organoids significantly increased the production of ENA-78 (15-fold), IL-8 (> 4-fold), IL-6 (5-fold) and MIP-3α (> 45-fold), to levels in general agreement with those detected in the basal psoriatic organoid cultures. Similarly, rhIL-17 stimulated increased production of ENA-78 (2-fold), IL-8 (> 1.5-fold) and IL-6 (> 2-fold) in the psoriatic organoids. While these fold increases were relatively modest, they were to levels substantially higher than what has observed in the rhIL-17-stimulated normal skin organoid cultures (ENA-78, > 5-fold; IL-8, > 2-fold; IL-6, > 2-fold). Notable, however, was the substantially increased production of MIP-3α by the psoriatic organoids in response to rhIL-17 treatment. Its production was increased > 30-fold to 1.25 ng/ml; a level comparable to that observed in the rhIL-17-treated normal skin organoids (0.95 ng/ml). These results indicate that IL-17 can activate an inflammatory response by normal skin organoids that is consistent with what is observed chronically in the basal psoriatic organoid cultures. The exception that MIP-3α is not chronically produced by the psoriatic organoids, and is equivalently induced by IL-17 implies that its expression requires IL-17 stimulation and that while it may participate in driving acquisition of psoriatic characteristics, is not necessary, over basal levels, to sustain those characteristics.

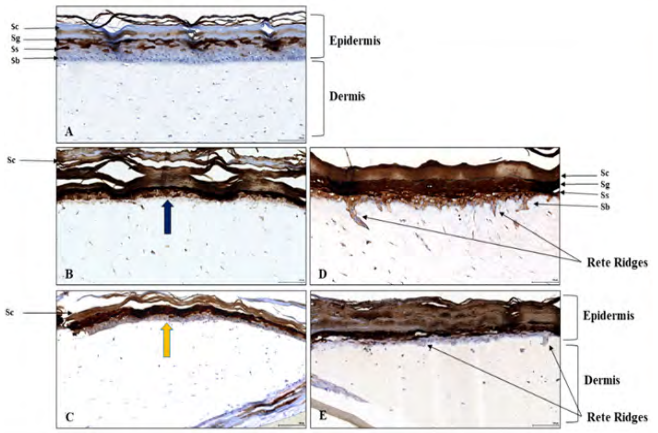

Figure 4: Psoriasin (S100A7) IHC: Immunohistochemical analysis of the cellular distribution and pattern of expression of psoriasin within normal skin organoids (A), Normal skin stimulated with rhIL-17 100 ng/ml (blue arrow) (B), EISO-treated rhIL-17 induced normal skin organoid at 0.002% (v/v) for 4 days (yellow arrow) (C), Psoriatic organoid (D) and EISO-treated rhIL-17 induced psoriatic organoid at 0.002% (v/v) (E). Scale bars = 100 µm.

Based on the observations here that EISO could suppress psoriatic characteristics in IL-17-stimulated normal skin organoids, and in basal, and IL-17-stimulated, psoriatic organoids, and our previous demonstration that EISO can potently suppress proinflammatory responses in skin models we assessed whether EISO co-treatment might affect rhIL-17-mediated expression of ENA-78, IL-8, IL-6 and MIP-3α in these organoids (Figure 5) [28,30,31]. The elevated production of these cytokines/chemokines by IL-17-stimulated normal skin organoids was dose-dependently suppressed by co-treatment with EISO by ~75%, to levels approaching what was detected in the basal cultures. EISO also significantly suppressed the production of these cytokines/chemokines by the psoriatic organoid by >75% of the rhIL-17-stimulated cultures. For ENA-78, IL-8 and IL-6, 0.002% EISO reduced production by > 2-fold below their respective levels in the basal psoriatic organoid cultures. While EISO also significantly suppressed MIP-3α production by rhIL-17-stimulated psoriatic organoid cultures, as was observed in the normal skin organoid cultures, this was still significantly higher than the basal condition level. Overall, our data indicate that IL-17 can trigger a perpetuated pro-inflammatory cascade giving rise to a psoriatic phenotype in normal skin organoids, and exacerbate disease characteristics in psoriatic organoids, and that EISO can suppress both initiation of, and chronic presentation of, these disease characteristics.

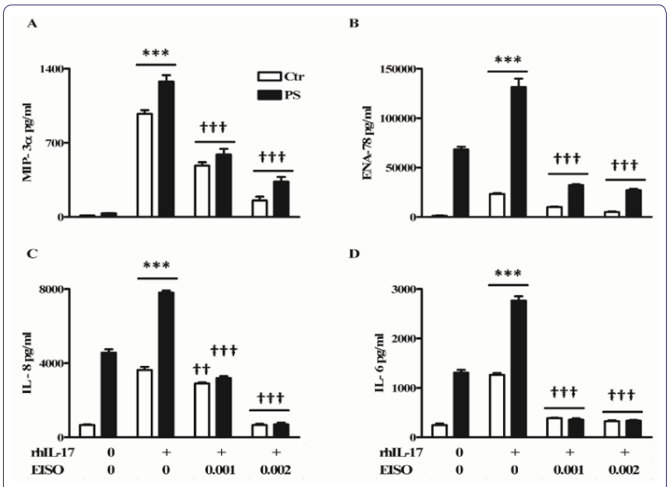

Figure 5: Suppression of MIP-3α, ENA-78, IL-8 and IL-6 expression by EISO in rhIL-17 induced normal and psoriatic skin organoids. Normal human skin and psoriatic organoids were treated with rhIL-17+/-EISO for96 h. The levels of all the four cytokines/chemokines were elevated in normal skin organoids after stimulation with rhIL-17 and were further elevated in psoriatic organoid as compared to psoriatic organoids. EISO 0.001-0.002% (v/v) treatment of rhIL-17 induced normal and psoriatic organoids down-regulated levels of indicator cytokines, expressed as pg/ml, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ vs. vehicle-treated controls and †$p < 0.01$, ††$p < 0.001$ versus rhIL-17 stimulated controls.

Discussion

Psoriasis is an immune-mediated chronic inflammatory skin disease characterized by hyper proliferation of keratinocytes, dilated blood vessels, infiltration of inflammatory leucocytes into the dermis and high relapse rate [54-56]. Pathogenesis of psoriasis involve interplay between various factors like genetics, skin barrier disruption, environmental triggers, Dendritic Cells (DCs), T cells and cytokines/chemokines which result in a cellular and cytokine imbalance [57-62]. The IL-23/Th17 axis plays an important role in the immunopathogenesis of psoriasis. Antigen-presenting cells produce IL-23 that stimulate the development of Th17 cells and increase IL-17 and other TH17 related cytokines (IL-22 and TNF-α) [63-65].

IL-17-associated cytokines and chemokines, including IL-8, IL-6, ENA-78 and MIP-3α are thought to play a central chemotactic role for inflammatory T cells and DCs, however, elevated levels of these and other factors in psoriatic lesions cannot be explained by the effect of one cytokine on skin cells [66]. Our results support the previous contention that the lack of IL-17-producing cells in the psoriatic organoids suggests that IL-17 is not required for maintenance of the lesion phenotype by the afflicted dermal fibroblasts [48]. Nevertheless, this biology may be due to persistent IL-17 effects, and our results indicate that IL-17 is sufficient to initiate acquisition of psoriatic characteristics in the normal skin organoids.

Botanical therapies have been used for many years as traditional medicines for treating wide variety of skin diseases including psoriasis [67]. Herbal products have many advantages with less side effects, low cost, easily available and ability to target multiple signaling pathways with varied biological targets. Thus, natural products are potential alternative candidate for the synthetic drugs. We and others have demonstrated that EISO may be "a useful botanical therapeutic" [21,24,27,28,30,31,39,40,42]. Our previous and current work demonstrate that psoriatic organoids exhibit persistent disease characteristics despite being devoid of immune infiltrate and that IL-17 induces expression of pro-inflammatory cytokines and chemokines, and psoriasis proliferation markers [30]. This supports the hypothesis that IL-17 is a primary driver of inflammation in psoriasis, but is not required to maintain the psoriatic phenotype [48]. IL-17 promotes anti-microbial functions and inflammation, in part, by activating neutrophils, requires cooperativity with other cytokines/chemokines, including those monitored here: IL-6, IL-8, ENA-78 and MIP-3α to promote psoriasis pathophysiology [68,69].

Clinically, dermal induration, erythema, and scaling are chronic inflammatory features of psoriasis. This chronic condition has been described to be due to the ability of Th17 cells to maintain their presence in inflamed tissue by secreting cytokines that stimulate epithelial cells to produce chemokines, such as MIP-3α, that promote infiltration of additional Th17 cells [70,71]. While we conclude that MIP-3α expression is not necessary for the maintenance of the psoriatic phenotype in dermal fibroblast/keratinocyte organoids, it is evident that IL-17 is sufficient to promote MIP-3α expression in both normal and psoriatic skin organoids and potentially exacerbate the inflammatory state of the lesion.

As keratinocytes protect the body from external insults, MIP-3α could be an initial trigger activating Th17 signaling pathway in psoriasis. CC Chemokine Receptor 6 (CCR6) is the sole receptor for MIP-3α, and an important functional marker for Th17 cells; recruit Th17 cells to inflamed sites and may provide a positive feedback loop for amplification of IL-17 [72]. MIP-3α is expressed at very low levels in normal human skin, however, increased expression of MIP-3α recruits CCR6+ cells including Th17, DCs and γdT into target sites, and there is increased levels of IL-17 resulting in inflammation and formation of psoriatic lesions [73,74]. Here we demonstrate that IL-17 induced elevated expression of MIP-3α: a chemoattractant for both Th17 and dendritic cells. While it remains to be determined precisely how the psoriasis organoids retain a pro-inflammatory profile as indicated by increased levels of ENA-78, IL-6 and -8 but not MIP-3α. It would appear that IL-17 drives increased expression of all, but that it has the biggest influence on MIP-3α as Th17 cells share the same gene signature including IL-17, MIP-3α, and IL-23R [75]. Our results are consistent with reports indicating that the local production of Th17 cytokines within the plaques appears to contribute to the increased production of chemokine MIP-3α, a key chemokine necessary for the migration of Th17 cells [71,76].

IL-17 and IL-22 are known to modulate the immune response by increasing expression of proinflammatory cytokines/chemokines and AMPs [77-79]. AMPs are released as a result of cell death due to injury to the skin, have direct antimicrobial activity and modulate immune cells by upregulating pro-inflammatory cytokine/chemokines including IL-6 and IL-8 that further recruit immune cells like macrophages and neutrophils in the epidermis resulting in a pathogenic loop and pro-inflammatory properties [55,78]. Psoriasin (S100A7) is the most highly secreted AMP and is a key player in epidermal-dermal cross-talk inflammatory response in psoriasis [80,81]. Our demonstration that cellular distribution and expression of psoriasin up-regulated by IL-17 and reverted by EISO in normal and psoriatic organoids is consistent with the anti-inflammatory and anti-proliferative properties of EISO [30,31].

Conclusion

Psoriasis is an immune-mediated, common cutaneous disease that often relapses and is associated with multiple co morbidities. Although IL-17, produced by (Th17) T helper cells, is implicated in inflammatory skin diseases sustaining T-cells activation and the psoriatic phenotype, increased levels of IL-17 alone are insufficient to produce inflammation. Hypothesizing that IL-17 acts synergistically with other cytokines to affect the psoriatic phenotype, we investigated the effects of IL-17 on the expression of MIP-3α, ENA-78, IL-8 and IL-6 in normal human skin and psoriatic organoids. We observed that IL-17 induced elevated expression of pro-inflammatory cytokines/chemokines in normal skin by increasing levels of MIP-3α: a chemoattractant for both Th17 and dendritic cells, ENA-78 and IL-8: chemo attractants for neutrophils, IL-6: a cytokine important for the survival of Th17 cells, and psoriasin and Ki67 as markers of proliferation. In psoriatic organoids, IL-17 induced increased expression of MIP-3α and further increased elevated levels of ENA-78, IL-8 and IL-6. We conclude that T-cell-derived IL-17 and skin organoid-derived MIP-3α, ENA-78, IL-8 and IL-6 help sustain an inflammatory feedback loop. Further, IL-17 can trigger a perpetuated pro-inflammatory cascade giving rise to a psoriatic phenotype in normal skin organoids, and exacerbate disease characteristics in psoriatic organoids. Our demonstration that EISO- treated normal and psoriatic organoids induced with rhIL-17, reverted psoriatic pathology including expression of psoriasin, reduced size and number of rete ridges is again consistent with anti-proliferative properties of EISO. EISO is anti-inflammatory, suppressing NFkB and COX activity in inflammatory models; that EISO can suppress both initiation, and chronic presentation, of these disease characteristics by suppressing cytokine/chemokine production demonstrates anti-inflammatory property being consistent with the anti-proliferative properties of EISO. EISO may therefore, be an attractive natural therapeutic for chronic inflammatory skin disease like psoriasis.

Acknowledgment

Histology support was provided by the Terry Fox/Vancouver Prostate Centre pathology core. Dr. Ladan Fazli provided pathology analysis.

Author Contributions

MC and MS are responsible for study design. MS and MC performed all in vitro studies. Santalis Pharmaceuticals, Inc. (IC and PC) provided EISO for the experimental use. Manuscript was written by MC and MS, and all other authors provided editorial advice.

Funding

This work was supported by a collaborative research agreement between MC and Santalis Pharmaceuticals, Inc. (UBC-F1502441).

Conflict of Interest

IC and PC are paid employees of Santalis Pharmaceutical Inc.

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