Cutaneous manipulation with topical solenopsin formulations of the KC-Tie2 murine model of psoriasis

Undergraduate Research Thesis

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Abstract

Psoriasis is a multifaceted disorder known to primarily affect keratinocytes, blood vessels, and the immune system. The CD1 KC-Tie2 murine model manifests symptoms analogous to those experienced by those 125 million psoriasis patients worldwide. In order to investigate the underlying causes a topical treatment, solenopsin, was applied to the psoriasiform model. After four weeks, decrease in epidermal thickness was noted. Additionally, $CD4^+$ T cells, $CD8^+$ T cells, and $CD11c^+$ dendritic cells numbers were significantly reduced. Measurements of hallmark mRNA transcripts further demonstrate to reduction of T cell numbers and their downstream products. The data collected suggest control of the T-helper17 pathway, via application of topical solenopsin, reinstates the wild type phenotype for the KC-Tie2 model.

Introduction

Psoriasis

Psoriasis is an autoimmune disorder characterized by chronic inflammation [3,11]. Approximately 1.5% of the United States population, over three million individuals, is inflicted with this disease. The precise causes of psoriasis have yet to be elucidated, but the autoimmune disorder is understood to have both genetic and environmental cues [9]. The inheritance pattern is unknown, but frequency of inheritance within familial studies suggests a degree beyond random inheritance. Patients experience alleviated symptoms through the summer months, due to increased sun exposure, yet symptoms aggravate in the winter [3]. A combination of pathogenesis within the integumentary, immune, circulatory, and nervous systems produces the disease's manifestations [9,11].

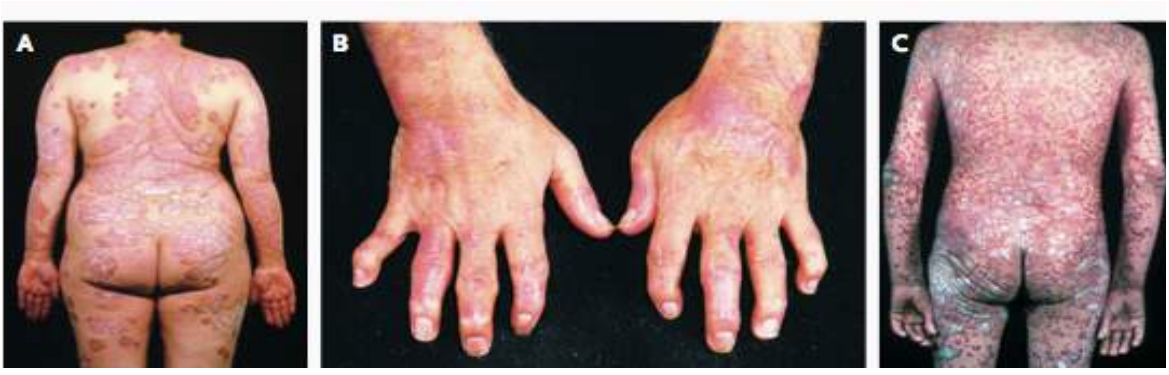

Figure 1: Extracutaneous demarcated, erythematous plaques characteristic of psoriasis (Panel A&C). Psoriatic Arthritis affects 5 – 20% of psoriasis patients (Panel B).[9]

Keratinocytes, the major cell contributing to skin structure, replicate within the stratum basale of the epidermis. These cells mature over thirty days and are forced upward by the newly replicating keratinocytes below. The maturation process involves producing keratin fibers at the stratum spinosum, loss of nuclei and cellular death at the stratum granulosum, and sloughing off of dead cells at the stratum corneum. However, the keratinocytes in psoriatic skin undergo hyperproliferation, taking only three to five days to depart the stratum basale, which obscures the standard phases. The pathogenesis results in parakeratosis, which is the retention of nuclei, and acanthosis, the retention of stratified, squamous keratinocytes in the epidermis. The malformation causes skin thickening and scaling, and excess cells form extracutaneous demarcated silvery plaques (Figure 1). The vasculature of the body undergoes an increased level of angiogenesis and capillaries at the skin surface have markedly thinner endothelium. The changes in the microvasculature generate the erythematous coloration of the plaques [(9)].

The main immune cells leading to the pathogenesis of the disease are $CD4^+$, $CD8^+$ T cells, and dendritic cells. Tissue necrosis factor alpha (TNFα) has been correlated as a main cytokine in the inflammation of disease, and Interferon gamma (IFNγ) plays a similar inflammatory role [(9)]. Thelper1 (Th1) cytokines, IL-23, IL-12, and INFγ are elevated, while Thelper17 (Th17) IL-17 and IL-22 exhibit similar increases [(9)]. Thelper2 (Th2) cytokines remain stagnant in comparing the healthy and the psoriatic model. Regulatory T cells (Treg) release less anti-inflammatory IL-10, further allowing the hallmark inflammatory response in psoriasis [(3,9)].

These adaptive immunity signatures recruit adhesion molecules on the surface of endothelial cells and keratinocytes, leading to heightened numbers of innate immune cells (neutrophils, dendritic cells, and macrophages) to the dermis [(3)]. Leukocytes then migrate from the skin's dermis layer and chronically persist in the epidermal layer [(3)]. This autoimmune response causes keratinocytes to release increased amounts of antimicrobial peptides s100a8 and s100a9, as the body believes a foreign substance is in the body [(3,9)].

KC-Tie2 murine model

The KC-Tie2 model is a CD1 murine system exhibiting the phenotype characteristic of human psoriasis (Figure 2). Tie2 is an angiopoietin (a vascular growth factor) receptor, and it is localized within the keratinocytes, of the namesake KC, within epidermis [(11)]. Overexpression of this protein only in keratinocytes, not endothelial cells, leads to the classical manifestations of psoriasis [(11)]. Extensive acanthosis, infiltrating and increasing $CD8^+$ and $CD4^+$ T cells, dendritic

cells, and macrophages is evident in the model [11]. Furthermore, the psoriatic cytokine signature of human psoriasis is represented by increases in proinflammatory TNFα, IFNγ, interleukins 1α, 6, 12, 22, 23, and 17, and host defense molecules β-defensin, s100A8, and s100A9 amplification [11]. The murine model also shows phenotype relief when common psoriatic treatment drugs are applied. The model is suitable for studying he mechanisms of psoriasis and potential treatments as it meets clinical, histological, immunophenotypic, biochemical, and pharmacological criteria for an animal model of psoriasis [11].

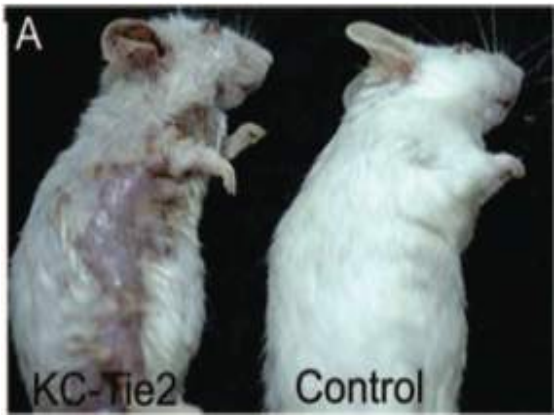

Figure 2: Phenotype comparison of psoriasis model, KC-Tie2 and Control mouse [11].

Current Treatments

Treatments generally vary depending on the severity of the disease. Less severe cases are treated with topical medicines. Vitamin D_3 analogs slow the growth of skin cells, topical retinoid tazarotene normalizes the DNA movement of keratinocytes, and topical corticosteroids are commonly prescribed for moderate cases [3]. This drug combination affects both the keratinocyte and immune cells malfunctioning, while the corticosteroids relieve the drug's common side effects: inflammation. More severe cases require the use of phototherapy, also known as artificial tanning, and systematic drugs [3]. As these drugs are toxic to the human body, long-term use is not recommended. While psoriasis manifests within the skin, psoriatic patients have morbidities impairing normal life comparable to diabetes, cancer, and rheumatoid arthritis [9]. Physicians are trained to address this psychosocial element by appropriately touching their skin to comfort the patient and show them that the disease is neither contagious nor repulsive. There is currently no cure for psoriasis [4]. The aspects of the disease that are currently the common targets for developing drugs are decreasing the number of pathogenic T cells, blocking T cell migration into the higher cutaneous layers, and inhibiting cytokines via

blocking substances [3,9]. This paper pertains to the application and investigation of a topical psoriasis treatment named solenopsin.

Solenopsin

Solenopsin is naturally found in the venom of fire ant *Solenopsis invicta*. Solenopsin, $C_{17}H_{35}N$, is an alkaloid molecule, containing a heterocyclic nitrogen ring structure (Figure 3). It acts in the regulatory pathway of angiogenesis. The compound does not cause the swelling characteristic to fire ant bites, but rather it acts as a cardiorespiratory depressant. Application can cause seizures in rats, and may be the cause of cardiorespiratory failure in human models that have experienced excessive bodily stinging [6]. The compound was found to inhibit angiogenesis both in vivo, embryonic zebrafish, and in vitro, via SVR angiogenesis assay [1]. Later studies showed similarities to ceramide, a lipid common within the plasma membranes of cells, which regulates cell apoptosis. Ceramides are correlated to a decrease in cellular proliferation and death of tumor cells within human melanoma models [7]. Solenopsin's effects on hyperproliferating tumor cells demonstrate a potentially interesting treatment option against the hallmark hyperproliferating keratinocytes of psoriasis. Solenopsin-12 [S12] (Figure 4) and Solenopsin-14 [S14] (Figure 5) have carbon chains of twelve and fourteen carbons respectively, instead of the eleven carbon chain in Figure 3. The ingredients within the cream: 400 µl ethanol, 40 g Lipoderm, and 200 mg S12 or S14.

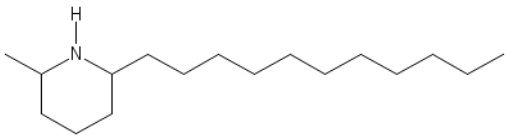

Figure 3: Solenopsin structure ($C_{17}H_{35}N$)

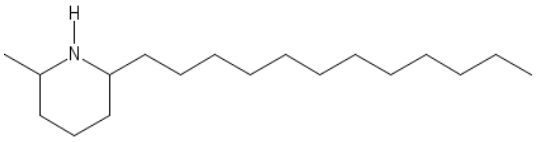

Figure 4: Solenopsin 12 structure ($C_{18}H_{37}N$)

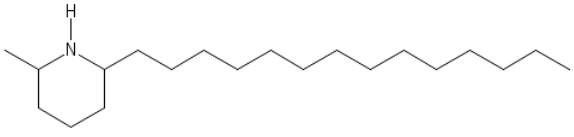

Figure 5: Solenopsin 14 structure $C_{20}H_{41}N$

Materials and Methods

Mouse

Experimental S12 and S14 creams were obtained from Dr. Jack Arbiser at Emory University. Twenty-six adult KC-Tie2 background mice underwent daily treatment for the duration of four weeks. S12 mice (n=9) were shaved as needed and approximately 125 mg of topical S12 cream was applied dorsally in an upward motion, against the natural hair grain, resulting in a thin layer. S14 mice (n=9) underwent the same type of treatment with its namesake cream. Control mice (n=8) were only shaved as needed. Application occurred daily near 10 am, and gloves were changed in between groups to avoid cross-contamination. Mice were euthanized via Avertin injection. Dorsal hair was shaved off and skin was treated for either frozen or paraffin sectioning. For paraffin sectioning, skin was placed in 10% buffered formalin (Surgipath Medical Industries, Richmond, IL), overnight at 4 °C prior to dehydration and embedding (Sakura Finetech, Torrance, CA). For frozen sectioning, skin was placed in tissue freezing medium (TFM) and flash frozen in liquid nitrogen.

Acanthosis

Hematoxylin and eosin (H&E) staining was completed on paraffin sections. Images were captured using a Leica DM L82 microscope with an attached Q Imaging MicroPublisher 3.3 Mega Pixel camera and Q-capture Pro software. Epidermal thickness was quantified using Image Pro Plus software (MediaCybernetics, Bethesda, MD). For each mouse, six to ten measurements were taken from at least five different fields of view from one section. Epidermal thickness was measured from the stratum basale to stratum granulosum, and excluded the stratum corneum and hair follicles.

Immunohistochemistry

Immunohistochemistry against CD4 ($CD4^+$ T cell marker), CD8 ($CD8^+$ T cell marker), CD11c ($CD11c^+$ dendritic cell marker), and F4/80 ($F4/80^+$ macrophage marker) was performed on TFM-embedded frozen skin sectioned, using specific anti-CD4, anti-CD8, anti-CD11c (BD Biosciences, San Jose, CA), and anti-F4/80 (eBioscience, San Diego, CA) antibodies. Antibodies were detected using either rabbit anti-rat IgG biotinylated (CD4, F4/80; Vector labs, Burlingame, CA) or goat anti-hamster IgG biotinylated (CD11c; Jackson Immunoresearch labs, West Grove, PA), amplified with Avidin/Biotinylated Enzyme Complex (Vector labs) and were visualized using the enzyme substrate diaminobenzidine (Vector labs). The slides were counterstained with hematoxylin.

Images were captured as aforementioned. For quantification of $CD11c^+$ and $F4/80^+$ cells, image analyses was completed in a blinded fashion using an automated Metamorph software program (Molecular Devices, Sunnyvale, CA) from at least five fields of view per animal. Quantification of $CD4^+$ and $CD8^+$ cells were hand counted in blinded fashion from at least five fields of view per animal.

MECA

Blood vessel analyses were completed on flash frozen tissue using a specific primary antibody targeting the pan mouse endothelial cell antigen (MECA; Developmental Studies Hybridoma Bank, Iowa City, IA), followed by detection using a rabbit anti-rat IgG biotinylated antibody, amplified with Avidin/Biotinylated Enzyme Complex, and visualized using the enzyme substrate diaminobenzidine. The slides were counterstained with hematoxylin. Photographs were taken and image analyses completed in a blinded fashion using an automated Metamorph software program (Molecular Devices). Background staining was minimal therefore color thresholds were not altered between samples. Four fields of view from about six individual sections were analyzed per animal.

mRNA

mRNA transcript analyses were completed on flash frozen tissue. Tissue was homogenized using Mikro Dismembrator S (Sartorius) and mRNA was isolated via standard manufacturer protocol using a Rneasy Mini Kit (Qiagen) and underwent reverse transcription to form stable cDNA. Primers and cDNA were measured with qPCR in triplicate using Stepone realtime PCR (Applied Biosystem, Foster City, CA). The thermocycler used the following protocol: Step 1 50 °C; 2min, Step 2 95 °C; 10min, Step 3 95 °C; 15sec, Step 4 60 °C; 1min, Step 5 go to Step 3 for 40cycles. Transcripts and respective primers tested: TNFα; Mm01336295_m1, IFNγ; Mm00802029_m1, IL-22; Mm01226722_g1, IL-22RA2; Mm01192969_m1, IL-1F5; Mm01333586_m1, IL-1F6; Mm00457645_m1, IL-1F8; Mm01337546_g1, IL-1F9; Mm00463327_m1, IL-34; Mm01243248_m1, IL17a; Mm00439618_m1, IL-17c; Mm00521397_m1, IL-17f; Mm00521423_m1, KLK5; Mm01203811_m1, s100a8; Mm00496696_g1, s100a9; Mm00656925_m1, defb3; Mm04214158, IL12B/P40; Mm01288989_m1, and IL23a; Mm00518984_m1. These transcripts were quantified and compared to those of GAPDH. Glyceraldehyde 3-phosphate dehydrogenase is constitutively expressed. Markers were selected due to prevalence in psoriasis, and others were chosen as a result of microarray analysis performed by a collaborator, Dr. Arbiser, at Emory University.

Statistical analysis

All data sets are represented as mean ± standard error of the mean (SEM). At least seven animals for each group were used for statistical analyses. Between group comparisons were analyzed using an unpaired, two-tailed Student's t-test and one-tailed Student's t-test. Figures with one asterisk (*) denote significance with a one-tailed t-test, while two asterisks (**) signify statistical significance with a two-tailed t-test. Statistical significance was defined as $P < 0.05$.

Results and Discussion

Solenopsin reduces psoriasiform skin phenotype in KC-Tie2 mice

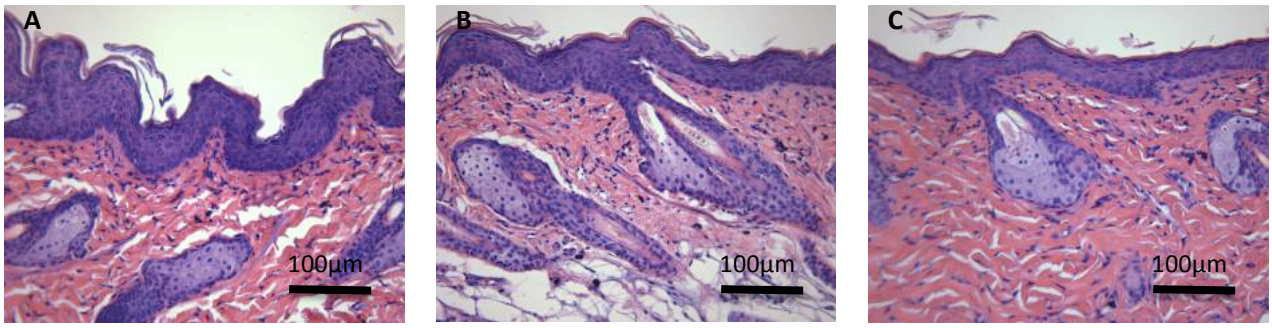

Figure 6: Representative H&E staining for KC-Tie2 control group (Panel A). S12 (Panel B), and S14 (Panel C) representative pictures displaying decreased acanthosis.

KC-Tie2 mice treated with the solenopsin analogs S12 and S14 show significant reduction in disease severity. Both creams resulted in significant decreases in epidermal thickness (Figure 6,7) and dermis infiltrating $CD4^+$ T cell numbers (Figure 8,9). The S14 treated mice had significantly reduced $CD8^+$ T cells (Figure 10,11) and $CD11c^+$ dendritic cells (Figure 12,13). The

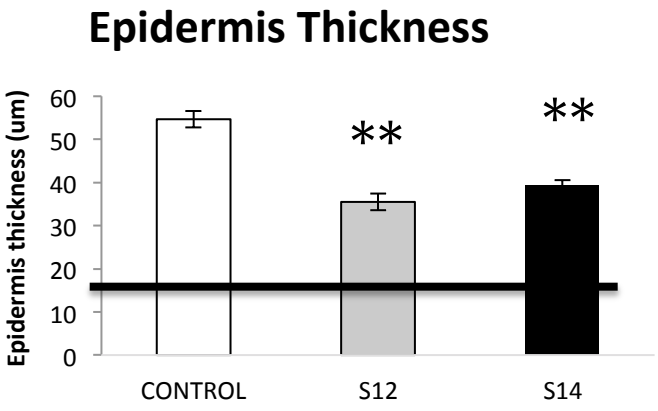

Figure 7: Acanthosis measurements show a marked decrease in S12 and S14 versus control. The black line represents standard CD1 background mouse epidermal thickness (10-15um).

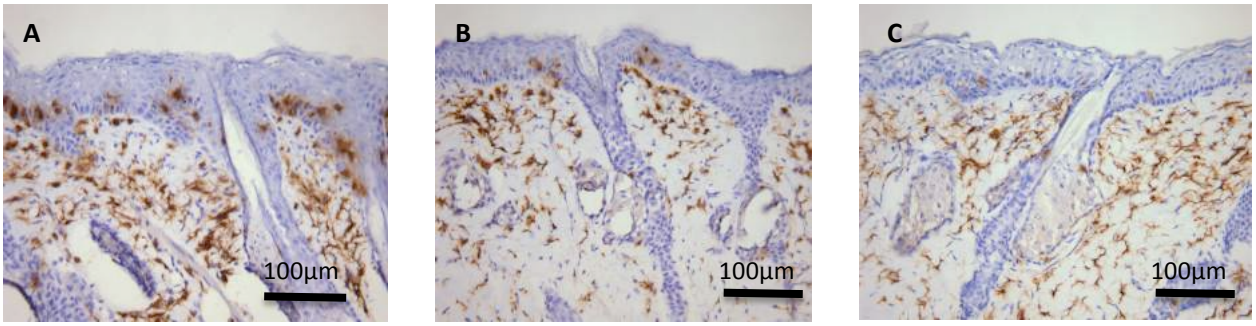

Figure 8: CD4$^+$ Staining of KC-Tie2 control (Panel A), S12 (Panel B), and S14 (Panel C) demonstrate reduced numbers of cells after treatment

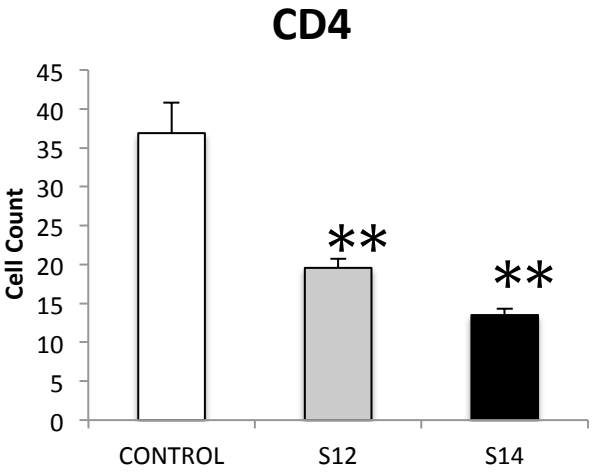

Figure 9: CD4$^+$ T cells are significantly reduced in both S12 and S14 treatment groups.

corresponding counts in the S12 group showed significantly decreased CD11c$^+$ and a downward trend of CD8$^+$. Decrease in dermal infiltrating F4/80 macrophages was not observed (Figure 14). Dermal angiogenesis via MECA endothelial cell staining did not display increase of blood vessel area (data not shown), length (data not shown), or number (Figure 15).

Histological H&E staining examination revealed the S12 experimental group epidermis thickness (acanthosis) experienced significant decrease (n=9, 35.509+/-1.91 µm, P<0.001), reduced by 19.168 µm when compared to the KC-Tie2 control group. Similarly, the S14 experimental group epidermis thickness experienced significant decrease (n=9, 39.275+/-1.318 µm, P<0.001), reduced by 15.402 µm when compared to the KC-Tie2 control littermates. Psoriasis characteristic acanthosis was reduced in the S12 and S14 KC-Tie2 mice, but not to the extent of simply CD1 mice. S12 CD4$^+$ T cells were significantly reduced nearly 2-fold (19.564+/-1.15 cells,

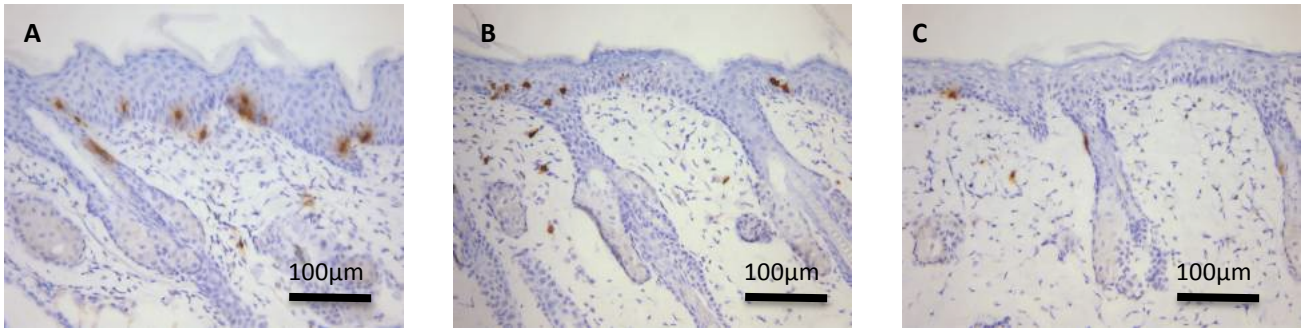

Figure 10: CD8+ Stain displays reduction in the S14 treated mice. Control Panel A, S12 Panel B, S14 Panel C.

P=0.001). S14 $CD4^+$ T cells were significantly reduced approximately 2.75-fold (13.500+/-0.795 cells, P<0.001). $CD8^+$ T cells were significantly reduced nearly 2-fold in the S14 treatment (3.957+/-0.552, P=0.012), while S12 mice $CD8^+$ T cells demonstrated a slightly negative trend versus control mice (6.343+/-1.46, P=0.56). S14 $CD11c^+$ dendritic cells were significantly

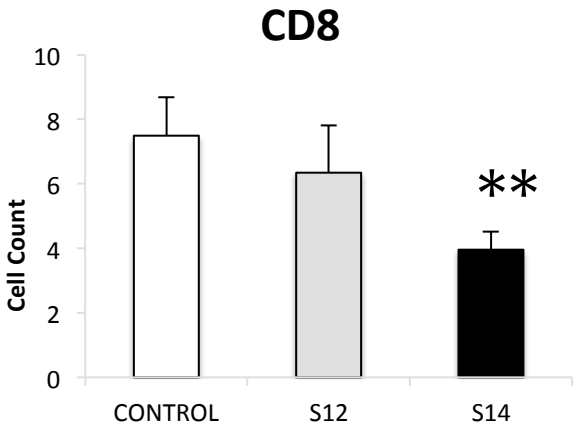

Figure 11: $CD8^+$ cells reduced in S14.

reduced over 1-fold (45.20+/-2.956, P=0.044). S12 $CD11c^+$ dendritic cells were significantly reduced versus control mice (46.415+/-1.458 cells, P=0.56). S14 MECA stained blood vessel numbers exhibited minimal differences from control (31.443+/-1.73, P=0.80), length (15.519+/-0.654, P=0.18) (data not shown), and area (65.654+/-5.76, P=0.11) (data not shown). S12 blood vessel MECA staining exhibited similar trends as both S14 and control groups, with blood vessel enumeration (32.151+/-2.216, P=0.90), length (16.729+/-1.25, P=0.836) (data not shown), and area (79.159+/-11.32, P=0.92) (data not shown). F4/80 macrophages were not reduced in either experimental group. S14 macrophages cell numbers (113.433+/-9.57, P=0.87) and S12 (123.518+/-4.35, P=0.29).

Treatment with S14 significant decreases versus control models in DEFB3 (0.018+/-0.004, P=0.046 1-tailed t-test) (Figure 16) and IL-22 (0.00002+/-0.000003, P=0.0317 2-tailed t-test)

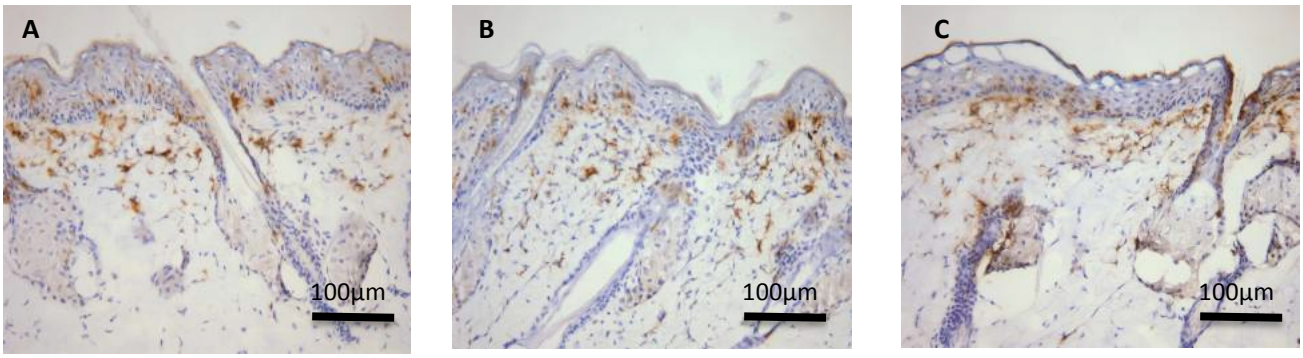

Figure 12: CD11c Stain shows reduction in both S12 and S14 groups. Control Panel A, S12 Panel B, S14 Panel C

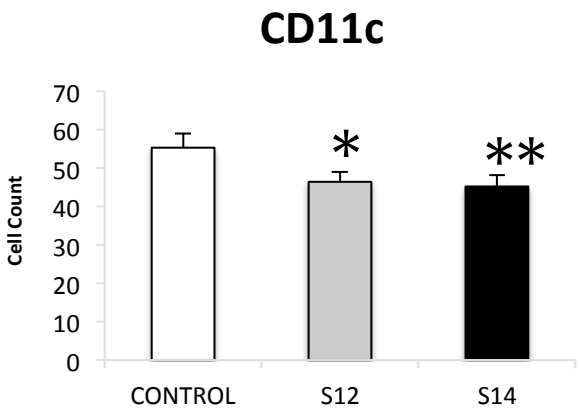

Figure 13: CD11c^{+} cells are significantly reduced in S14 and S12.

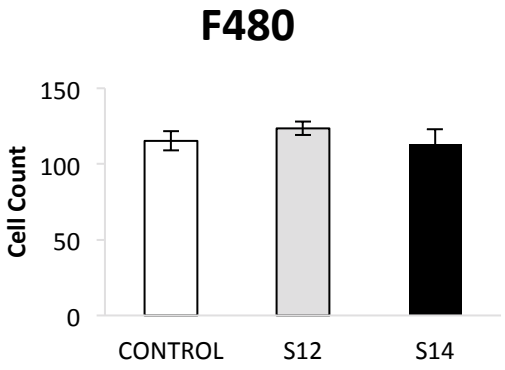

Figure 14: F480^{+} macrophages did not vary significantly between control and experimental groups.

(Figure 17). Juxtaposed, IFN*y* (0.0001+/-0.00002, P=0.073 1-tailed t-test) (data not shown) and IL-17c (0.002+/-0.0005, P=0.037 1-tailed t-test) (data not shown) both increased after S14 treatment. The S12 treatment group presented near significant decreases in IL-17F (0.0003+/-0.00008, P=0.069 1-tailed t-test) (Figure 18), significant DEFB3 (0.021+/-0.004, P=0.0463 1-tailed t-test), and significant IL-22 (0.00007+/-0.00002, P=0.061 2-tailed t-test). The messenger RNA transcripts did not significantly fluctuate substantially amongst KLK5, IL-34, IL1F5, IL1F6, IL1F8, IL1F9, TNF*a*, s100a8, s100a9, IL-17a, IL-12b/p40, IL22RA2, and IL-23a (data not shown).

The cream's molecular signature was analyzed via enumeration of immune cells, typical cytokines, chemokines, and antimicrobial peptides typically detected in psoriasis patients [(11)]. Daily treatment with solenopsin over four weeks upon KC-Tie2 background mice, mainly affects T cells, both $CD4^+$ (classification includes Th1, Th2, and Th17 cells) $CD8^+$, and $CD11c^+$ dendritic cells. These results suggest the possibility of regulation within the bridging of the innate to adaptive immune response. Reducing the quantity of dendritic cells lowers to amount of antigen presenting cells, thus less self-antigens can be presented to T cells, and less proliferation of activated T cells would occur. So, less T cells infiltrate the dermis and epidermis. This suppression would cause a decrease in leukocyte recruiting chemokines, and help explain the lowered numbers of dendritic cells in the skin. However, this is only one possible location of regulation, and further study need be performed to isolate the mechanism of solenopsin.

By targeting the keratinocytes directly with topical solenopsin, it is possible that the cream is

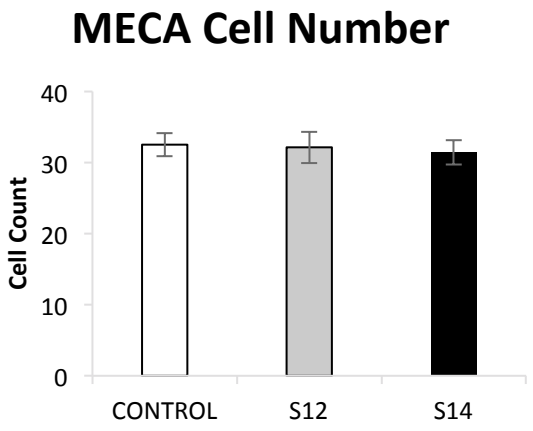

Figure 15: $MECA^+$ cells staining blood vessels did not vary among groups

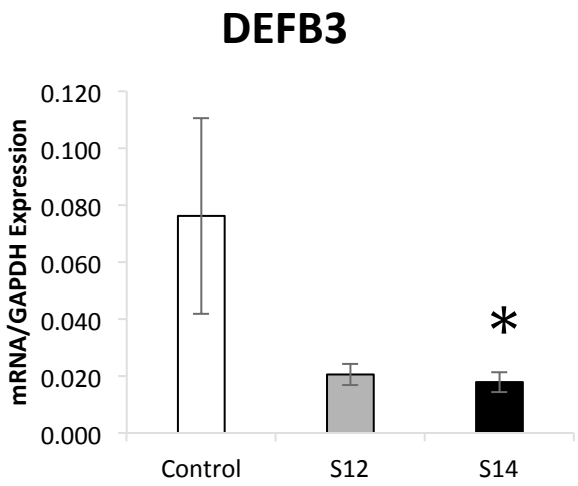

Figure 16: S14 mice had significantly reduced Defb3.

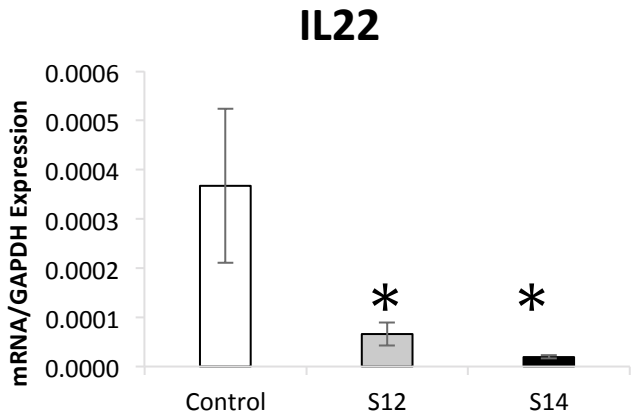

Figure 17: IL-22 mRNA transcripts are significantly down in the S14 and in the S12 group

lowering the amount of antimicrobial peptides, such as β -defensin and s100a9. This reduction may lead to the decreased autoimmune response, by sending negative feedback signals to the immune system. The lower antimicrobial peptides cause the leukocytes to stop inflammatory signals, as the "infection" of the autoimmune disease is thought to be cleared.

Furthermore, psoriasis is known to affect the T helper cellular pathways, specifically up-regulating both the Th1 and Th17 cascades [(9)]. Data suggests that solenopsin may affect the Th17 pathway. IL-1 and IL-6 cause Th1 differentiation to Th17 and IL-23a causes proliferation of Th17 cells [(2)]. Investigation into IL-23a and IL-1 derivatives in the experimental groups displayed

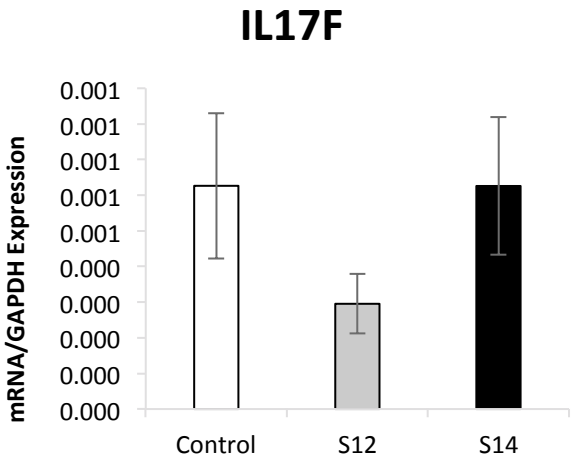

Figure 18: IL-17f transcripts were significantly decreased in S12 mice

a level constant with that of the control cytokines (IL-6 was not studied). However, further downstream investigation displays regulation in IL-17 and IL-22.

Previous research as been performed on a Janus Kinase (JAK) inhibitor called tofacitinib [(10)]. JAKs play a role in signal transduction of inflammatory diseases. It was determined that this molecule is able to reduce psoriasis when applied as a cream [(10)]. It functions by blocking IL-23, and thus the Th17 pathway of inflammation. The topical significantly reduced human psoriasis using the Target Plaque Severity Score [(10)]. However, this topical may differ from solenopsin as it targets the immune system first. It is hypothesized that solenopsin targets the keratinocytes expression of proinflammatory molecules, and then causes downstream effects through the immune system.

In the solenopsin trials, three members of the IL-17 family, released from Th17 cells, were investigated: IL-17a/c/f. In the S12 model, IL-17a trended downward versus control, IL-17c remained near control levels, and IL-17f was significantly reduced. In S14 mice, IL-17a remained near control concentration, yet IL-17c was found at higher levels than control mice, and IL-17f remained at control levels. IL-22 has been considered the key cytokine regulating the proliferation in psoriasis [(2)], and mRNA analysis revealed a significant reduction of the S14 litter's transcription of IL-22, while the S12 litter displayed the same negative trend. TNFα, another Th17 proinflammatory cytokine, had relatively stable transcription in all groups. Further analysis showed trends of IL-22 downstream reduction in antimicrobial s100a9 and β -defensin. IL-1f8, also known as IL-36b, expression is induced via IL-22 [(3)], explaining its downward trend in both

experimental groups. Previous studies have shown that IL-22 and IL-17a/f cooperatively induce the release of antimicrobial peptides, explaining why there was reduction in s100a9 as well as β-defensin in solenopsin treated groups [8].

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