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The Effect of AhR Agonist Tapinarof on Dendritic Cells and T cells In Vitro

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in Partial Fulfillment of the Requirements

For the degree of Master of Medical Sciences in Immunology

Harvard University

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Abstract

Psoriasis is a chronic inflammatory skin condition mediated by a dysregulated immune system. Patients with psoriasis not only deal with plaques on their skin, they also have an increased risk of developing comorbidities such as obesity, cardiovascular disease, and metabolic syndromes. Current treatments most commonly involve topical corticosteroids for mild disease; however, these treatments do not always work and stopping treatment normally leads to relapse. For more severe cases, physicians often prescribe biologics such as secukinumab and etanercept that target important cytokines (IL-17A and TNFa, respectively) involved in disease pathogenesis and maintenance. While these treatments can be highly effective, long term systemic immunosuppression impacts the ability to fight off infections and other diseases. Additionally, halting treatment can lead to relapse. Tapinarof is a new non-steroidal topical treatment for atopic dermatitis and psoriasis that shows promising clinical trial results with long-lasting effects. While not yet FDA-approved, phase 3 clinical trials showed complete or near-complete lesion resolution in 35-40% of patients with a long remission of 4 months on average after discontinuation. Recent studies have pinned tapinarof as an aryl hydrocarbon receptor (AhR) agonist, a first-in-its-class therapeutic. Due to the novelty of this treatment and the complex behavior of AhR, the mechanism behind tapinarof has yet to be elucidated. Our in vitro studies examined how the drug directly affects important mediators of psoriasis such as dendritic cells and T cells. We investigated tapinarof's effect on dendritic cell development and maturation as well as T cell exhaustion, cytokine production, resident memory T cell generation/survival, and the transition from regulatory T cells to Th17. Our results indicated that tapinarof inhibits formation, cytokine production and persistence of resident memory T cells as well as upregulates immunosuppressive marker CD39 in vitro.

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Chapter 1: Introduction

Psoriasis vulgaris is a common, immune-mediated skin disease that affects around 7.5 million adults in the United States.[1] The most common treatments for psoriasis include topical corticosteroids, topical retinoids and non-steroidal topicals such as calcipotriene. For stubborn bouts of psoriasis, biologic treatments exist that target inflammatory cytokines such as IL-17A (secukinumab), TNFa (etanercept) and IL-23 (guselkumab); however, these drugs systemically suppress the immune system and lead to a greater susceptibility for infection. Tapinarof is a newly formulated non-steroidal topical treatment that acts as an aryl hydrocarbon receptor (AhR) agonist[2] with promising clinical efficacy against psoriasis[3] and atopic dermatitis.[4] While we understand some of the downstream pathways of AhR, questions remain in determining the exact mechanism by which triggering of AhR signaling leads to improvement of psoriatic and eczematous lesions. In these studies, we carried out initial studies of the effect of tapinarof on dendritic cell development, T cell differentiation and resident phenotypes in vitro, and T cell cytokine production.

The initiating event for psoriasis is not entirely understood, but there are some indications that injury, infection, and particular medications can contribute to its pathogenesis.[5] Psoriasis appears to be mediated most directly by the adaptive system with Th17 playing the most prominent role in its immunopathogenesis.[6] Of course, the adaptive immune system receives activation signals from innate cells that cannot be ignored in the initiation and maintenance of inflammatory diseases such as psoriasis. These signals can come in the form of cytokines such as TNFa, IL-23, IL-12, and IL-6[6] or from a variety of chemokines that promote cell migration and inflammation.[7]

Dendritic Cells and Psoriasis

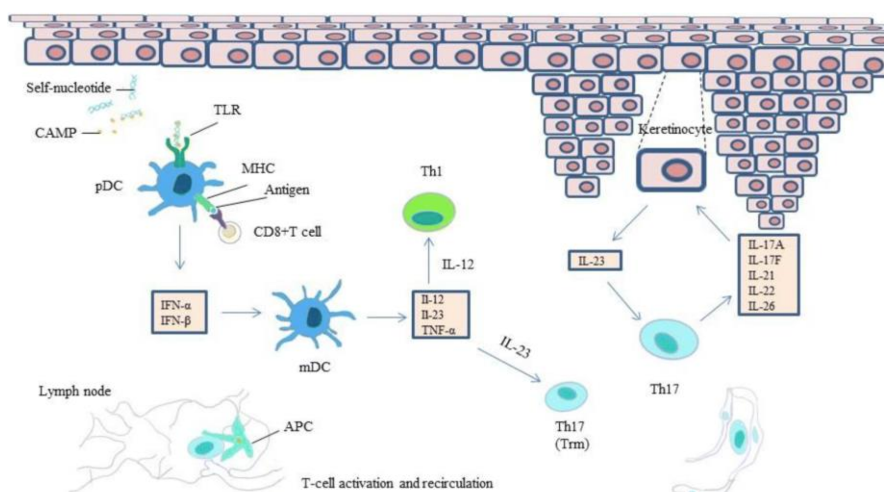
Dendritic cells represent the main bridge between the innate and adaptive immune system and employ multiple mechanisms to promote the attack on self-antigens. First, they act as professional antigen-presenting cells capable of presenting self-antigens to T cells. Genetic analyses have shown that some MHC II alleles are associated with psoriasis risk and could promote the presentation of particular self-antigens by dendritic cells.[8] Secondly, activated dendritic cells produce inflammatory cytokines that promote survival of pathogenic T cells.[9] In psoriasis, dendritic cells have been found to overproduce IL-23 which acts on Th17 cells causing them to survive and proliferate.[10] IL-23 binds to IL-23R, found on Th17 and Th22 cells, where it activates the STAT3 pathway and leads to IL-17 and IL-22 production.[11] Additionally, the IL-23/Th17 axis appears to act as the pivotal pathway for inducing psoriatic lesions in both humans and mice, as evidenced by the fact that biologic therapies targeting IL-17 cause resolution of disease.[12] Lesions in humans contain increased levels of IL-23, IL-22, and IL-17 that determine severity.[12] Meanwhile, the most popular mouse model, the imiquimod-induced psoriasis mouse model, depends on increased IL-23 and IL-17 production for its phenotype.[13] Conventional dendritic cells can be anti-inflammatory by producing IL-10, TGF-beta, and IL-27.[11] This dendritic cell phenotype promotes T_{Reg} homeostasis and demonstrates that dendritic cells may be induced to have heightened inflammatory properties, or reduced anti-inflammatory properties, and could play a pivotal yet delicate role in mediating psoriasis.

T Cell Effects in Psoriasis

The adaptive immune system plays a prominent role in psoriasis and effective biologics target T cell produced cytokines that drive tissue inflammation.[14] Interestingly, the first

indication that T cells mediated psoriasis came from the observation that cyclosporine, a drug that suppresses TCR-mediated activation, cleared psoriatic lesions in patients being treated for arthritis.[15] Originally, Th1cells were thought to participate in lesional formation, given the high levels of IFNγ in psoriatic lesions.[16] However, clinical trials have demonstrated that inhibition of IL-17A causes complete clearance of psoriasis, demonstrating that psoriasis is an IL-17 driven disease.[17–19] Th17 cells produce IL-17A and play a pathogenic role in many autoimmune diseases.[19] IL-17 promotes proliferation of epidermal cells as well as IL-23 production from keratinocytes. In return, IL-23 is thought to promote Th17 proliferation and survival, thus creating a positive feedback loop.[19] Additionally, IL-22 is produced in psoriatic lesions; IL-22 acts on keratinocytes to promote epithelial cell regeneration and production of antimicrobial peptides (AMP) that influence inflammatory conditions.[20] Psoriatic lesions contain high amounts of AMP and some of these peptides may even be the target of pathogenic T cells. One particular AMP, LL37, is overproduced in these lesions and has been shown to be recognized by T cells that have escaped tolerance and promote inflammation.[21]

Figure 1



Li, B. *et al.* The role of Th17 cells in psoriasis. *Immunol. Res.* **68**, 296–309 (2020).

Figure 1. Cells and cytokines that promote keratinization in psoriasis

Once activated, dendritic cells signal to T cells by presenting antigen and producing cytokines. Dendritic cells can produce cytokines including IL-12, IL-23, and TNFα that promote Th17 activation. These Th17 cells produce IL-17A and IL-22 that activate keratinocytes and lead to epidermal hyperplasia and differentiation defects. Keratinocytes also participate in augmenting inflammation by producing IL-23 in response to their activation which further activates Th17 cells.

Resident Memory T Cells in Psoriasis

In psoriasis, relapse after treatment often occurs in the same location on the skin. High throughput sequencing and immunostains have demonstrated that αβ T cells are the predominant T cell population in psoriatic lesions.[22] In particular, tissue-resident memory T cells (T_{RM}) are stubborn mediators of psoriasis and contribute to the disease's ability to recur in the same location.[23] T_{RM} cells persist in the same area of the skin by upregulating retention markers such as CD69 that sequesters the sphingosine-1-phosphate receptor which is important for migration out of tissue.[24] CD103 is also an important skin T_{RM} marker that anchors to E-cadherin on epithelial cells.[25] Both of these markers are found on CD4 and CD8 T cells in the skin that persist for decades into old age, although the most dominant T_{RM} population in the skin is the single positive $CD69^{+}$ $CD103^{-}$.[25,26] T_{RM} provide rapid on-site protection against known pathogens in epithelial barrier tissues. However, T_{RM} specific for self-antigens are thought to drive chronic relapsing inflammatory disorders, including psoriasis rheumatoid arthritis and vitiligo. The failure of E-selectin blockade to improve psoriasis was an eloquent argument that the pathogenic T cells driving disease are present within the skin and that recruitment of T cells from the circulation is not required for lesional formation.[27] Elegant studies in which never lesional skin from psoriatic patients were transplanted onto immunodeficient mice showed that

psoriatic lesions could be induced in the absence of T cell recruitment from blood.[28] These studies have sparked interest in not only targeting T_{RM} in inflammatory skin diseases, but also taking advantage of their persistence by inducing them with vaccines to enhance protection.[29,30] Furthermore, studies have also shown that resident memory T cells found in psoriatic lesions are capable of producing IL-17 and IL-22, important cytokines in psoriasis development and maintenance.[31]

The Aryl Hydrocarbon Receptor

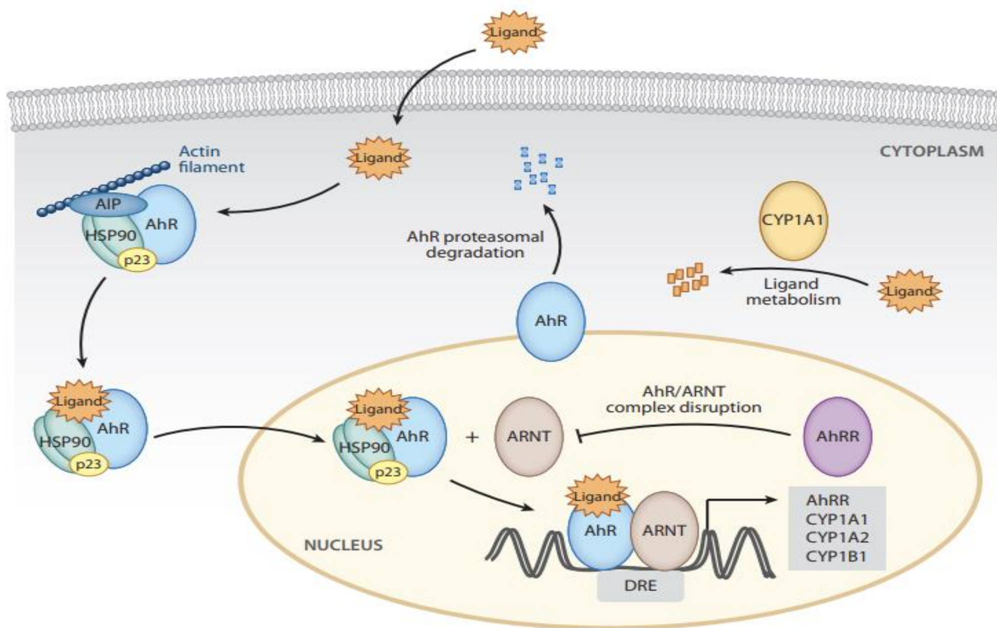
Tapinarof is an aryl hydrocarbon agonist and a first in its class novel topical therapy for psoriasis and atopic dermatitis.[32] AhR has been implicated in both psoriasis and atopic dermatitis because of its role in balancing reactive oxygen species levels and influencing epidermal differentiation.[33] AhR operates as a chemical sensor that recognizes phytochemicals, dioxins, and external and internal ligands. Keratinocytes, dendritic cells, T cells, fibroblasts, and other immune cells express high levels of AhR and signaling is thought to contribute to maintaining homeostasis in the skin.[34] Considering the wide range of ligands that AhR binds to, it is considered a promiscuous receptor that induces a diverse variety of responses after activation. The receptor resides in the cytoplasm where it binds to a ligand and translocates to the nucleus. Once AhR reaches the nucleus, it controls the transcription of many different genes, leading to its wide range of cellular responses.[35]

Exemplary of the extraordinary and complicated nature of this receptor, recent studies looking at serum from patients with multiple sclerosis have shown decreased levels of circulating AhR agonists compared to healthy patients; however, there are increased levels of AhR agonists

during acute CNS inflammation.[36] Additionally, another study concluded that patients with inflammatory bowel syndrome have reduced levels of AhR ligands.[37]

AhR also plays a complicated role in balancing reactive oxygen species (ROS) levels. AhR dimerizes with the AhR nuclear-translocator (ARNT) in the nucleus where it can bind to xenobiotic responsive elements as well as target genes such as CYP1A1 and nuclear factor-erythroid 2-related factor-2 (NRF2), known for its antioxidative capabilities, for transcription.[38,39] 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) is a dioxin that binds to AhR. AhR-induced CYP1A1 tries to break down TCDD and increases ROS levels in the process.[40] Although this pathway demonstrates that AhR exacerbates uncontrolled ROS levels and oxidative stress, AhR simultaneously upregulates anti-oxidative molecules such as NRF2 and NQO1.

Figure 2



Stockinger, B., Di Meglio, P., Gialitakis, M. & Duarte, J. H. The aryl hydrocarbon receptor: multitasking in the immune system. *Annu. Rev. Immunol.* **32**, 403–432 (2014).

Figure 2. Pathway for AhR activation and its subsequent effects

AhR resides in the cytoplasm in a complex with HSP90 before binding to a ligand for activation. The complex translocates to the nucleus where AhR is released from the complex and forms a dimer with Aryl Hydrocarbon Receptor Nuclear Transporter (ARNT). This complex then binds directly to DNA to direct transcription of particular genes including CYP1A1 and its own inhibitor, Aryl Hydrocarbon Receptor Repressor (AhRR). Especially important for inflammatory skin diseases such as psoriasis and atopic dermatitis, AhR plays a role in epidermal differentiation.[38] Studies with ARNT-deficient mice show that AhR signaling disruptions lead to poor epidermal barrier function.[41] AhR plays two contrasting roles in maintaining a healthy epidermis; perpetual AhR activation from dioxins can lead to excessive keratinization, a prominent phenotype in psoriasis,[42] whereas moderate AhR activation from particular ligands promotes healthy barrier maintenance.[38] Thus, AhR can modulate skin health based on the type of ligand it binds, how strong or frequently the ligand binds, and how quickly the ligand is metabolized.

The Aryl Hydrocarbon Receptor in Immune Cells

AhR not only impacts epidermal cell differentiation and growth, it also affects immune cells involved implicated in driving psoriasis. In T cells, studies show that AhR regulates both Th17 and regulatory T cells formation (T_{reg}).[43,44] Studies have also shown that activating AhR in developing Th17 cells leads to an increased proportion of Th17 cells, as well as amplified IL-22 production in fully developed Th17 cells.[44,45] Thus, modulating AhR impacts the development and function of T cells and may exacerbate or attenuate T cell-mediated diseases depending on the ligand.

In AhR-deficient mice, AhR promotes T_{RM} persistence in the epidermis.[46] This indicates that not only does AhR have effects on specific cytokine secretion, it also plays a broad role in influencing T cell residency in at least the epidermis.

AhR activation also has known impacts on dendritic cell function. Kado and colleagues demonstrated that TLR-activated monocyte-derived dendritic cells (Mo-DCs) expressed increased levels of AhR and became more sensitive to AhR ligands than non-activated DCs.[47] Activation of Mo-DCs with 6-Formylindolo[3,2-b]carbazole (FICZ) or TCDD led to increased IL-1β expression and decreased IL-12A expression. Prior studies have shown that IL-1β plays a role in psoriasis by stimulating keratinocytes to produce molecules that attract IL-17-producing gamma-delta T cells.[48] Additionally, IL-12 induces CD8 T cells to produce increased amounts of TNFα a cytokine that contributes to the inflammation in psoriasis.[49] These experiments show that modulating AhR may impact the T cell and dendritic cell responses in psoriasis; however, the type of ligand may affect whether the immune cells are activated or suppressed.

To further illustrate the sensitivity of AhR to particular ligands and their effects on immune cells, researchers have looked at responses to TCDD and FICZ. Exposure to UVB rays from the sun converts tryptophan into FICZ which then binds to AhR and activates its cellular responses.[50,51] In vitro addition of FICZ to T cells in Th17-inducing conditions leads to enhanced IL-17A, IL17-F, and IL-22 production.[45] In an experimental autoimmune encephalomyelitis mouse model, researchers found that injecting FICZ in AhR^{+} mice led to faster disease progression compared to AhR-deficient mice.[45] However, in a separate experiment using graft-versus-host disease mouse models, injection of TCDD into mice led to increased numbers of T_{reg} cells.[52] These contrasting studies indicate that AhR is a versatile receptor that promotes a wide range of responses based on differential responses to distinct AhR agonists. While this versatility complicates our ability to target AhR for therapeutic purposes, its high expression in Th17 cells and potential for T_{reg} differentiation identifies it as a potentially paradigm-shifting target for Th17-mediated diseases.

Tapinarof

Tapinarof was identified as a metabolite produced by *Photorhabdus luminescens,* a gram-negative bacterium.[53] This bacterium lives within parasitic nematodes that infect insects without damaging the tissue. Studies found that this metabolite, 3,5-dihydroxy-4-isopropylstilbene, was released upon entering the insect and utilized anti-microbial properties to preserve the tissue and allow the nematode to grow within.[54,55] Scientists have since isolated this molecule and formulated it into a novel topical therapeutic. Early trials showed high efficacy against mild to moderate psoriasis with minor side effects.[56] Mechanistic studies have demonstrated that tapinarof acts as an AhR agonist.[2]

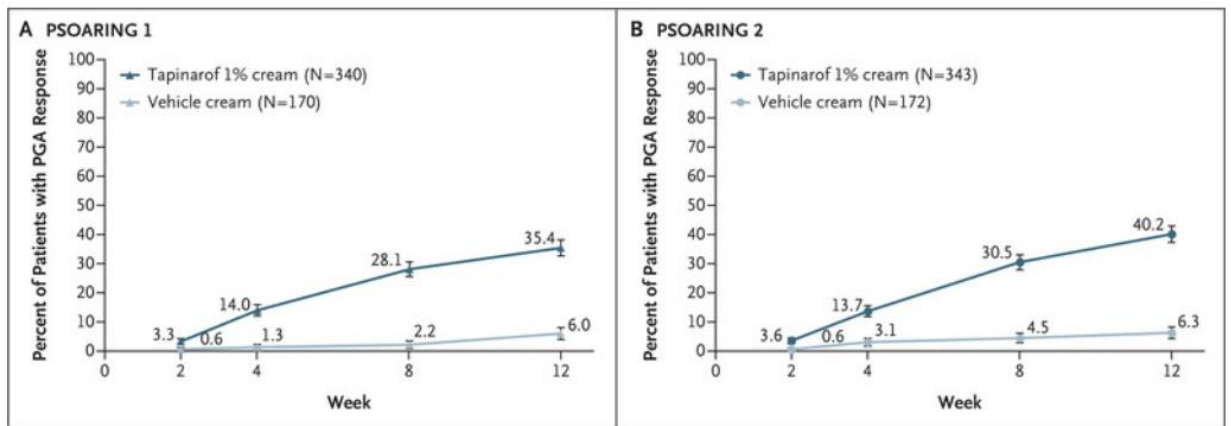
Different AhR ligands activate diverse pathways and lead to different downstream responses. Thus, tapinarof may have distinct effects on immune cells compared to TCDD or FICZ. Competition binding experiments showed that tapinarof does not compete with TCDD to bind to AhR, demonstrating that these two agonists have separate binding sites.[57] TCDD promotes T_{reg} proliferation and immunosuppressive effects. Hence, tapinarof could exploit an alternative AhR signaling pathway to induce immunosuppression. In the topical imiquimod induced model of psoriasiform dermatitis, topical tapinarof reduced levels of IL-17A, IL-17F, IL-22, IL-23, and IL-1β in skin.[2] Additionally, tapinarof directly scavenges ROS and upregulates the NRF2 pathway to promote antioxidative conditions and combat ROS production.[2,32]

Tapinarof acts on primary human keratinocytes to upregulate CYP1A1 and induce transcription of barrier-related genes including filaggrin, hornerin, and involucrin in vitro.[2] These studies suggest that tapinarof may have effects on barrier function, which could explain some of its efficacy in atopic dermatitis.

Clinical Exploration of Tapinarof

In phase 3 trials, researchers followed over 600 patients and found that 35-40% of them had clear or almost clear lesions after 12 weeks of tapinarof treatment compared to 6% of placebo controls.[3] This was an impressive level of clearance for a topical once a day medication. Patients that discontinued the treatment remained clear for an average of four months after discontinuation. This length of remission suggests that tapinarof has lasting effects on immune responses within the skin. Tapinarof is the first in its class as an AhR agonist and promises to be a safe and effective treatment for plaque psoriasis and atopic dermatitis.

Figure 3



Lebwohl, M. G. *et al.* Phase 3 Trials of Tapinarof Cream for Plaque Psoriasis. *N. Engl. J. Med.* **385**, 2219–2229 (2021).

Figure 3. Tapinarof "Cleared" or "Almost Cleared" Psoriatic Lesions in 35-40% of Patients Within Two Phase 3 Tapinarof Trials

Tapinarof successfully cleared lesions in 35% of patients in the first trial (PSOARING 1) and 40% of patients in the second (PSOARING 2) phase 3 clinical trials. A Physician's Global Assessment (PGA) "response" is defined as when the lesion is totally or almost cleared based on observation and scoring

guide. Lesions were graded from 0 to 4 where 4 is most severe. A score of 0 or 1 counted as a PGA response

Experimental Plan

Tapinarof has effects in vitro on IL-17/IL-23 cytokine production but little else is known about how tapinarof suppresses skin inflammation. Further research is needed to determine the specific cells that this drug targets and determine how it impacts the key players in psoriasis, including dendritic cells, keratinocytes, and T cells. In these studies, we evaluated the effect of tapinarof in vitro on human immune cells.

We first evaluated the effect of tapinarof on the generation of immature dendritic cells from PBMC-derived monocytes and dendritic cell maturation. We also evaluated tapinarof's effect on T cells. We tested tapinarof for its ability to inhibit bead stimulated T cell activation, to cause skewing of cytokine production after one week of exposure and to induce markers of T cell exhaustion. We tested the ability of tapinarof to affect T_{RM} differentiation and cytokine production using an in vitro T_{RM} differentiation assay, its ability to affect survival and cytokine driven expansion of T_{RM} from healthy skin.

Chapter 2: Methods and Results

Brief Introduction

The following experiments aimed to characterize how tapinarof directly affects cell types critical in the pathogenesis of psoriasis: dendritic cells and T cells. Considering the importance of resident memory T cells in developing and maintaining psoriatic lesions, we hypothesized that tapinarof induces changes in these T cells and possibly in the dendritic cells that activate them. Although these studies will not replicate the effect of the drug in living human skin, they are a valuable first step towards understanding the effects of tapinarof on these critical cell types.

Methods and Materials

PBMC Isolation

Leukoreduction collars from blood donations were received from the Brigham and Women's Hospital with isolation of PBMCs occurring the same day or stored overnight at room temperature with isolation occurring the following morning. The PBMCs were first separated with a SepMate tube and Lympholyte density gradient medium. The PBMC layer was isolated and washed twice with HHE medium (HBSS 1X without calcium and magnesium, 10 mM Hepes buffer, 5 mM EDTA). The cells were resuspended in SkinT medium (Iscove's DMEM, 20% FBS, 1% L-glutamine penicillin-streptomycin (PSG) mix, 3.5 uL/L beta-mercaptoethanol) and counted for further cell isolation or freezing.

Tapinarof Preparation

Lyophilized tapinarof was obtained from Dermavant Sciences. It was resuspended in sterile DMSO to generate a 10mM stock and frozen in single use aliquots. For culture, the stock was diluted further in culture medium to produce a 1 mM concentration and then was added at 1uL/mL to a final concentration of 1 μM in culture.

Monocyte Isolation

For isolating monocytes, we used magnetic CD14 MACS microbeads (Miltenyi Biotec # 130-050-201) with the autoMACS Separator. The $CD14^{+}$ cells were positively selected from healthy donor PBMCs and then added to 6 well plates for DC culturing. Some of these cells were also stained for flow cytometry to ensure quality isolation.

T Cell Isolation

To isolate T cells, we used the MACS Pan T Cell Isolation Kit (Miltenyi Biotec # 130-096-535) with the autoMACS Separator. T cells were negatively selected from healthy donor PBMCs and added to 24 well plates for the PBMC-derived T cell experiments.

Human Skin Explant Culture

Abdominal skin samples obtained from elective cosmetic surgeries were minced in a petri dish with SkinT medium. Cellfoam matrices (Part # CY-903, Cytomatrix Pty Ltd, Australia) were coated with type 1 collagen (BD Biosciences cat # 354236) mixed in a solution with sterile PBS. The minced skin was then pressed on the grids and added to a 24 well plate with SkinT or SkinT with IL-2 (100 I.U/mL from the NCI) and IL-15 (10ng/mL, PeproTech #200-15). Cultures were

then incubated (37°C, 5% CO_2) for 3 weeks and fed every M, W, F. To harvest, the medium was transferred through a 70 um cell strainer and rinsed with Hank's Buffered Saline solution (Fisher #MT-21-021-CV) with 10 mM Hepes.

Dendritic Cell Culture

Monocytes were cultured for 5 days in X-VIVO 15 medium (Lonza cat #04-418Q) with 1000 U/mL GM-CSF (PeproTech cat # 300-03) and 250 U/mL IL-4 (PeproTech cat # 200-04) for 5 days and harvested 5 days after. $2x10^6$ monocytes per well were incubated at 37°C and 5% CO_2. To mature the dendritic cells, 1ug/mL LPS (Purified from Salmonella minnesota R595, Invivogen: tlrl-smlps) was added to the culture and cells were harvested 24 hours later.

T_{RM} Generation Experiment

T cells were isolated from PBMC, counted, and then added to wells in SkinT medium. They were then activated with anti-CD2/CD3/CD28 T cell activation/expansion beads (Miltenyi Biotec # 130-091-441). These beads were added in a bead:T cell ratio of 1:4. One well of T cells was given tapinarof (1uM) to form the "T cell at activation" group while the other was only given SkinT medium. The T cells were stimulated for 24 hours before being transferred to a confluent keratinocyte monolayer. These keratinocytes had been grown to confluence using keratinocyte medium (KFSM w/ calcium (ThermoFisher 17005-042), 1% PSG, bovine pituitary extract, and epidermal growth factor). At the time of co-culture, one group of T cells was given tapinarof to generate the "Tap at co-culture" group. The T cells and keratinocytes were co-cultured for 7 days. At the time of harvest, the T cells were stimulated with 50 ng/mL PMA (Fluka Biochemica cat # 79346) and 750 ng/mL ionomycin (Invitrogen cat # I-24222). Brefeldin

A at a concentration of 10 ug/mL (BD Biosciences cat # 347688) was used as a golgi stop. After 4 hours the T cells were harvested and stained for flow cytometry.

T Cell Exhaustion Experiment

T cells isolated from PBMCs were stimulated for 7 days with 1:4 MACS activation beads in SkinT medium and fed every other day by removing half of the medium and replacing it with fresh medium with or without 1 μM tapinarof. On day 7, the cells were stimulated with PMA/ionomycin along with Brefeldin A at the same concentrations in prior experiments. Four hours after stimulation, the T cells were harvested and stained with a viability dye. After the viability stain, cells were stained for surface markers, intracellular cytokines, and the intranuclear transcription factor FoxP3. For collection, counting beads (ThermoFisher cat # C36950) were added to count the absolute number of cells.

Flow Cytometry Staining

For surface stains, the cells were harvested, centrifuged at 1400 RPM for 5 minutes, then resuspended in FACS buffer (DPBS, 0.5% BSA, 0.1% sodium azide) and added to a staining plate. The cells were then Fc blocked with human IgG (1 ug/mL)and stained with conjugated antibodies for 30 minutes. The cells were then washed twice with FACS buffer and followed by fixation in 1.0% paraformaldehyde (PFA).

For intracellular staining, the cells underwent the surface stain as above and were placed in the fridge for 20 minutes in PFA before continuing. The cells were washed twice with BD Perm/Wash Buffer (BD Biosciences cat # 554723) and then resuspended in BD Perm/Wash with

the addition of conjugated antibodies for 30 minutes. The cells were washed twice with BD Perm/Wash and resuspended in FACS buffer for analysis.

For intranuclear staining, we used the FOXP3/Transcription Factor Staining Buffer Set (Invitrogen # 00-5523-00). The cells were fixed in fix/perm buffer for 30 minutes on ice and then washed twice with perm buffer. The antibodies were then added and incubated for 30 minutes on ice. Cells were then washed twice with perm buffer and resuspended in FACS buffer for analysis.

Antibodies (5 uL unless otherwise stated)

To stain the cells we used (3 uL) PerCp conjugated anti-CD3 (Biolegend), (1 uL) APC-Cy7 conjugated anti-CD8 (Biolegend), PE-Cy7 conjugated anti-CD69 (Biolegend), AF647 conjugated anti-CD103 (Biolegend), AF488 conjugated anti-IL17a (Biolegend), FITC conjugated anti-IFNg (Biolegend), PE conjugated anti-IL22 (Biolegend), (1 uL) APC conjugated anti-TNFa (Biolegend), PE conjugated anti-IL10 (Biolegend), PE-Cy7 conjugated anti-IL22 (Biolegend), FITC conjugated anti-CD86 (Biolegend), APC-Cy7 conjugated anti-CD14 (Biolegend), PE conjugated anti-CD11c (Biolegend), PerCP-Cy5.5 conjugated anti-CD209 (Biolegend), APC conjugated anti-CD83 (Biolegend), PE-Cy7 conjugated anti-HLA-DR (Biolegend). APC conjugated anti-FOXP3 (Biolegend), AF488 conjugated anti-ICOS (Biolegend), BV421 conjugated anti-CTLA4 (Biolegend), PE conjugated anti-LAG-3 (Biolegend), BV750 conjugated anti-TIM3 (Biolegend), BV605 conjugated anti-CD39 (Biolegend), PE-Cy7 conjugated anti-PD-1 (Biolegend), PE conjugated anti-CTLA4 (Biolegend), 1:250 Zombie NIR viability dye (Biolegend), 1:250 Zombie Aqua viability dye (Biolegend).

Results

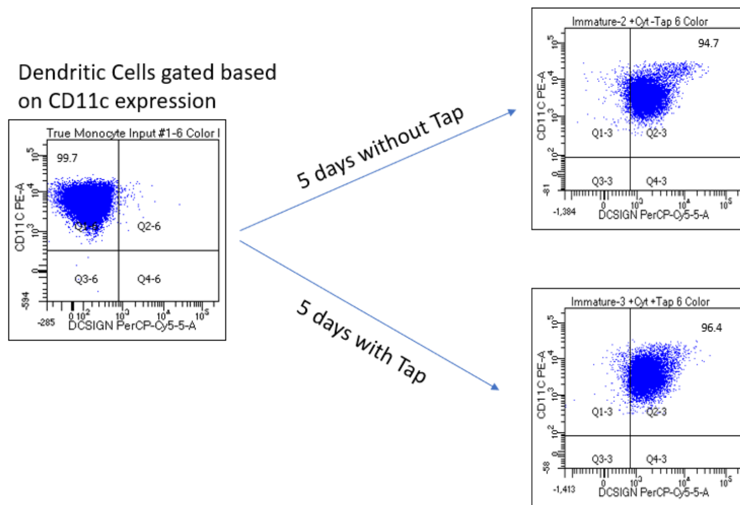
The Effect of Tapinarof on the Generation of Immature Dendritic Cells from Monocytes

Circulating monocytes from the bone marrow eventually undergo diapedesis and travel into tissue where they differentiate into macrophages or dendritic cells.[58] Different types of dendritic cells reside in the skin with the main types consisting of Langerhans cells (LCs) and dermal dendritic cells (DDCs).[59] We focused on monocyte-derived dendritic cells (Mo-DCs) due to their ease of generation in vitro. $CD14^+$ monocytes isolated from PBMCs were cultured for 5 days in the presence of GM-CSF and IL-4 to generate immature dendritic cells and analyzed by flow cytometry.

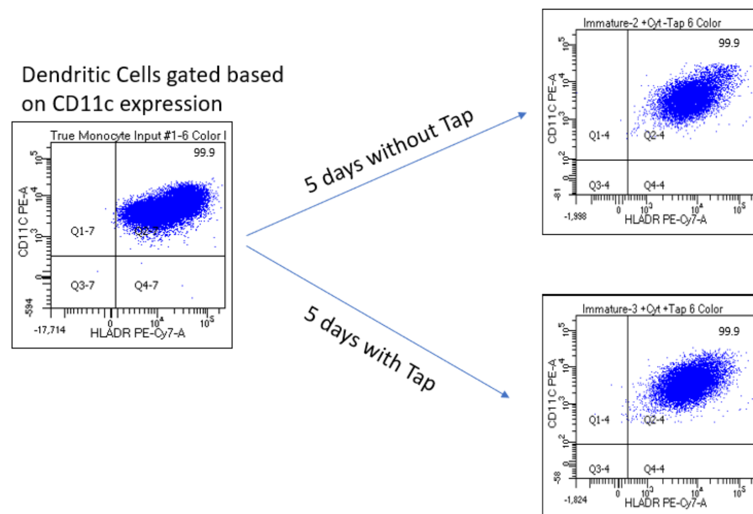
To identify the immature dendritic cells, we looked at key identifiers including CD11c, CD209 (DC-SIGN), HLA-DR, and CD14. We also monitored their maturation markers, CD83 and CD86, to ensure we were only measuring the transition from monocytes to immature dendritic cells. CD11c was our most robust marker for monocytes and dendritic cells and thus all other markers were analyzed in the $CD11c^+$ population. First, we determined that we developed immature dendritic cells due to the low expression of CD86 and CD83 (Fig. S1a, S1b). Monocytes given cytokines in the presence of tapinarof did not have a reduced ability to differentiate into immature dendritic cells, as measured by CD11c and DC-SIGN expression (Fig. 4a, 4c). Additionally, the lack of any change in the expression of HLA-DR in the presence of tapinarof indicates that this drug may have minimal effects on antigen presentation (Fig. 4b, 4c).

Figure 4

A



B



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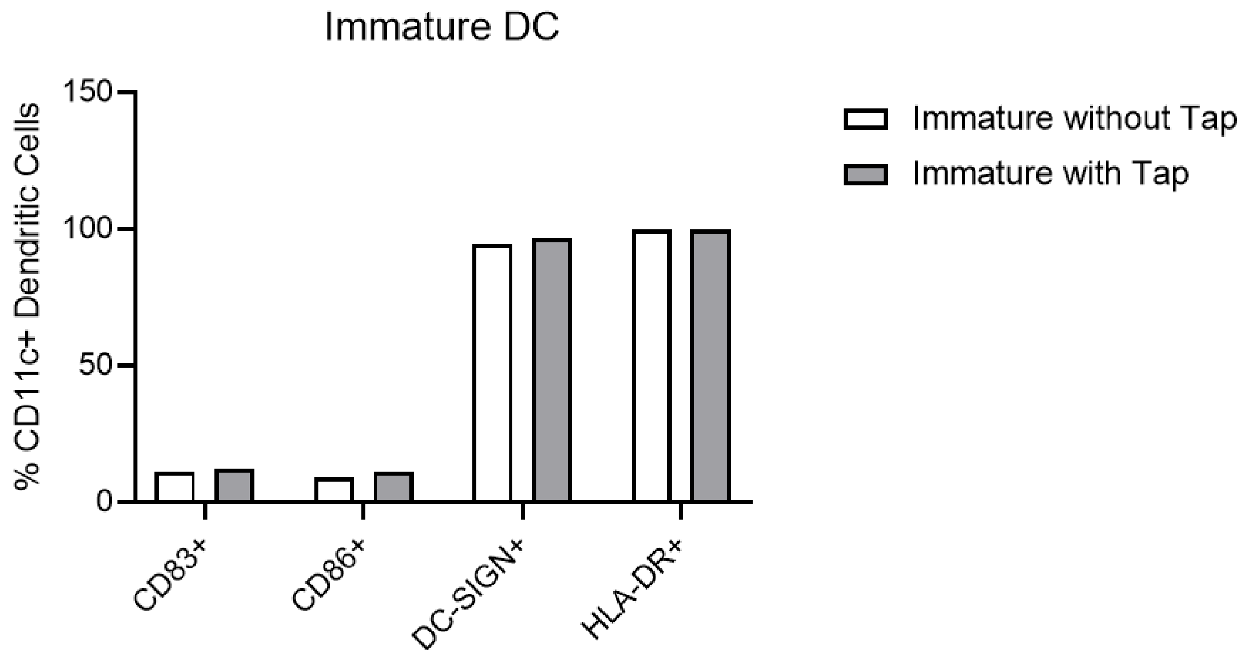


Figure 4. Tapinarof Did Not Affect Generation of Dendritic Cells in First Donor

a) Flow plots of CD11c and DC-SIGN expression on input monocytes (left) and monocytes cultured with IL-4 and GM-CSF for 5 days to generate immature dendritic cells (right). **b)** HLA-DR expression on the input monocytes (left) and immature dendritic cells (right). **c)** Flow analysis of DC-SIGN and HLA-DR expression along with maturation markers CD83 and CD86 in immature dendritic cells with and without tapinarof.

The effects of Tapinarof on Dendritic Cell Maturation

The transition from monocytes to dendritic cells only accounts for one stage in which tapinarof could impair dendritic cells in inflammatory conditions. Tapinarof may affect dendritic cell maturation to reduce inflammation. Maturation occurs as a result of the dendritic cells sensing pathogens or inflammatory cytokines. To stimulate maturation of the Mo-DCs, we added LPS to the immature DC culture on day 5 and harvested 24 hours later. We first compared CD83 and CD86 expression between the immature and mature dendritic cells to ensure we achieved maturation in these dendritic cells (Fig. 5a). The dendritic cells exposed to tapinarof did not downregulate activation/maturation markers compared to the control group (Fig. 5b). Additionally, we did not see a decrease in HLA-DR that would indicate reduced antigen presentation in these mature dendritic cells (Fig. 5b).

Figure 5

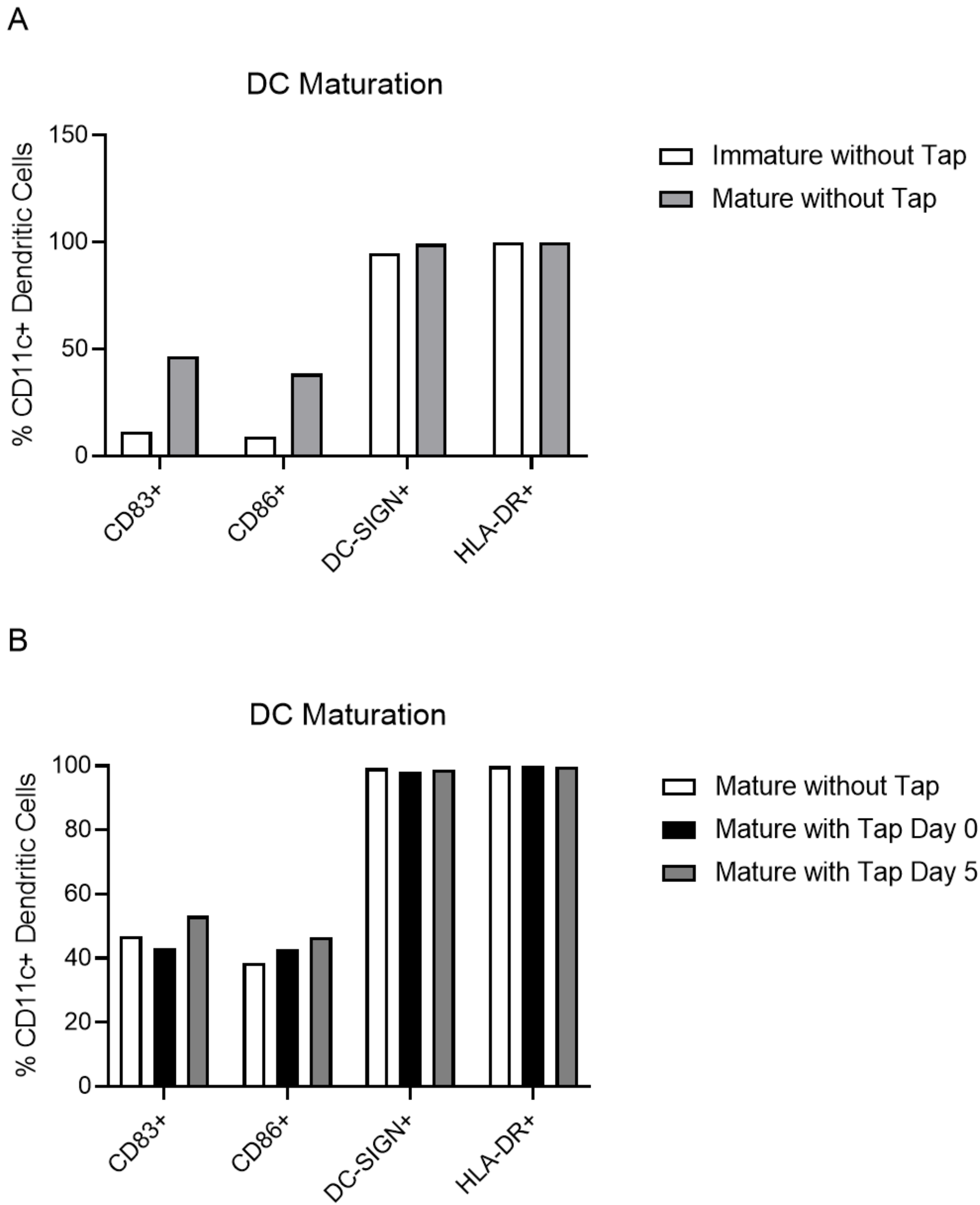


Figure 5. Tapinarof Did Not Affect the Maturation of Dendritic Cells in the First Donor

a) Flow analysis of LPS-induced dendritic cell maturation. **b)** Flow analysis of dendritic cell maturation comparing the control group with the addition of tapinarof at day 0 or day 5.

The Effects of Tapinarof on T Cell Activation and Tissue-Resident Memory T Cell Generation

We hypothesized that tapinarof may inhibit T cell activation as one mechanism for its ability to treat both psoriasis and atopic dermatitis. To test how tapinarof affects early events in T cell activation, we stimulated PBMC-derived T cells with anti-CD2/CD3/CD28 beads for 24 hours with and without tapinarof and evaluated CD69 expression as an early activation marker. We found that tapinarof reduced both CD4 and CD8 T cell expression of CD69 (Fig. 6a,b) T_{RM} play a critical role in the development and recurrence of psoriatic skin lesions. To test tapinarof's effect on T_{RM}, we tested the ability of tapinarof to inhibit in vitro generation of T_{RM} from peripheral blood T cells, using a validated assay.[60] In this assay, T cells are stimulated with anti-CD2/CD3/CD28 beads for 24 hours and then cultured for one to two weeks on monolayers of human keratinocytes. T cells were harvested after one week of culture and analyzed by flow cytometry for the expression of resident memory T cell markers (CD69, CD103) and for cytokine production after stimulation with PMA and ionomycin. Tapinarof reduced the generation of both $CD69^{+}CD103^{-}$ (single positive or SP) and $CD69^{+}CD103^{+}$ (Double Positive or DP) CD4 T_{RM} (Fig. 7a, b). There were no significant differences in CD8 T cells (Fig. 7c,d).

We next evaluated cytokine production of CD4 and CD8 T cells in the presence of tapinarof. We observed a decrease in TNFα production in DN and SP $CD4^{+}$ T cells (Fig. 8a) as well as a reduction in the percentage of IL-17A-producing CD4 T cells for T_{RM} and non-T_{RM} cells (Fig. 8a,d). There were no effects on IL-22 and IFNγ production by CD4 T cells (Fig. 8a). Finally, there was no impact of tapinarof on TNFα or IFNγ production by CD8 T cells (Fig. 8b).

Figure 6

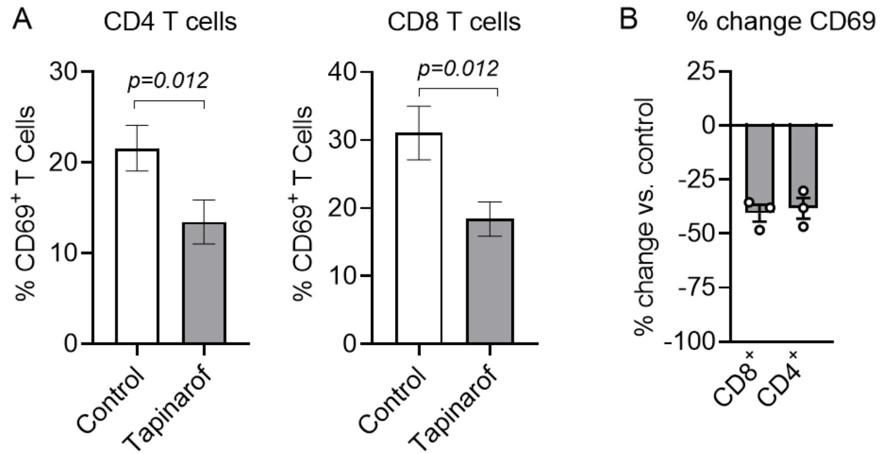


Figure 6. Tapinarof Reduced CD4 and CD8 T Cell Activation After 24 Hour Bead Stimulation

a) Percent of CD4 or CD8 T cells expressing CD69 after 24 hours of bead stimulation. **b)** Percent change in CD69 expression for CD4 and CD8 T cells with tapinarof compared to the control. Expression was assessed by flow cytometry analysis.

Figure 7

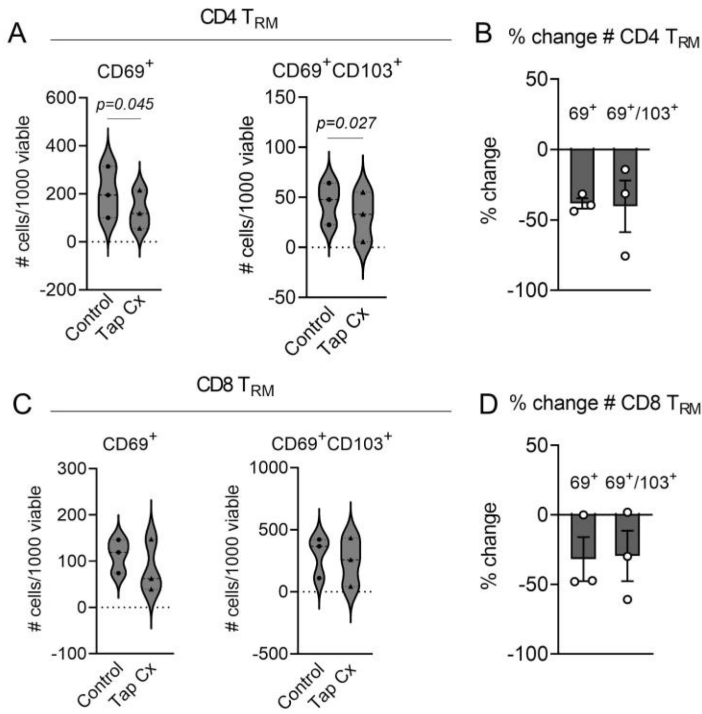


Figure 7. Tapinarof Reduced Formation of Resident Memory T Cells In Vitro

a) n=3 Flow cytometry analysis of SP and DP CD4 T_{RM} populations. **b)** Percent change in SP and DP CD4 T_{RM} generation with tapinarof compared to the control. **c)** Analysis of SP and DP CD8 T_{RM} populations. **d)** Percent change in SP and DP CD8 T_{RM} generation with tapinarof compared to the control.

Figure 8

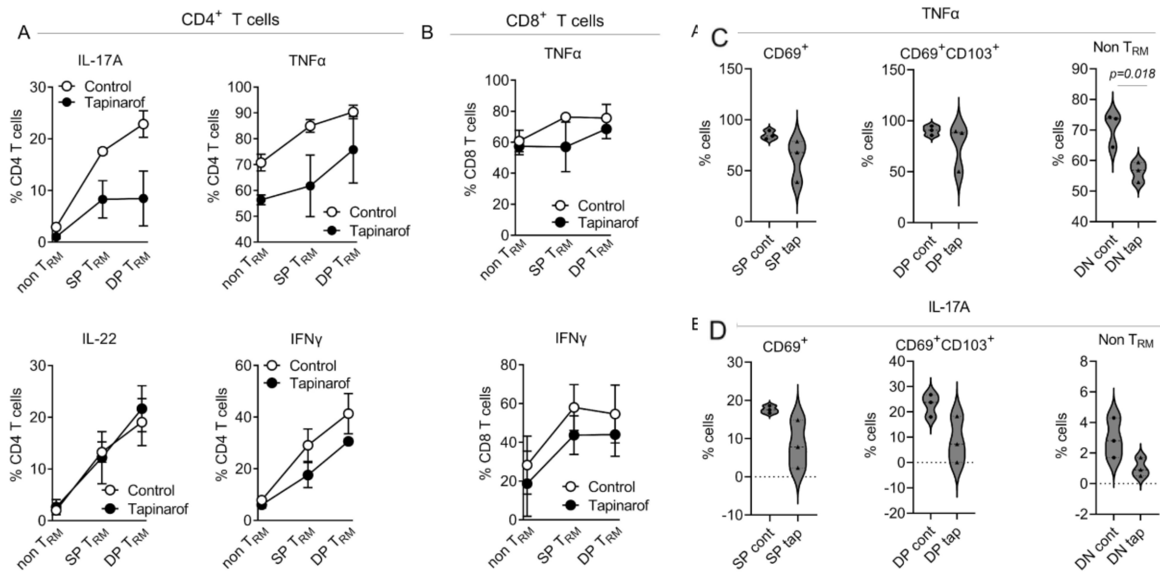


Figure 8. Tapinarof Reduced IL-17A and TNFa production in CD4 T Cells

a) n=3 Flow cytometry analysis of IL-17A, TNFa, IL-22, and IFNg in CD4 T_{RM} and non-T_{RM} cells. **b)** TNFa and IFNg production in CD8 T_{RM} and non-T_{RM} cells. **c)** Individual data points for TNFa production in CD4 T_{RM} and non-T_{RM} cells. **d)** Individual data points for IL-17A production in CD4 T_{RM} and non-T_{RM} cells.

The Effects Of Tapinarof On Skin T_{RM} Survival And Expansion

We next investigated if tapinarof affects the survival and expansion of T_{RM} from human skin. We received healthy human skin samples from patients undergoing abdominoplasty procedures. After removing the fatty and subcutaneous tissues, skin was minced and adhered to Cellfoam matrices then cultured with or without IL-2 and IL-15 for three weeks. In the absence of exogenous cytokines, this assay tests the ability of T_{RM} to survive in vitro. IL-2 and IL-15

induce a 5-10 fold expansion of T_{RM} in culture.[61] Furthermore, unpublished observations in the noted an interesting $Foxp3^{+}IL\text{-}17^{+}$ double positive T cell population developed in the presence of IL-2 and IL-15. This may represent conversion of T_{Reg} into IL-17A-producing cells given previous studies showing that IL-2/IL-15 treatment differentiates T_{Reg} into $Foxp3^{+}IL\text{-}17^{+}$ cells that may act as an intermediate stage between T_{Regs} and Th17 cells.[62]

We quantified T_{RM} survival by harvesting T cells from the explant cultures at three weeks, immunostaining for CD69 and CD103 and analyzing by flow cytometry in the presence of counting beads. We observed some reductions DN, SP, and DP CD4 T cell populations in the first donor tested. However, the non-T_{RM} CD4 T cells were not reduced in the second donor (Fig. 9a). There were no effects on the survival of CD8 T cells (Fig. S2a,b). This data suggests that tapinarof might reduce CD4 T_{RM} survival. Next, we investigated tapinarof's effect on T cell expansion in the presence of IL-2 and IL-15. Tapinarof significantly reduced expansion of both CD4 T_{RM} and non-T_{RM} cells in donor 1 (Fig. 9c,d) but had no effect on the expansion of CD8 T cells. In fact, there was an slight increase in non-T_{RM} CD8 cells (Fig. S2c,d). T cells from the second donor did not expand well; we are replicating this experiment. We also assayed cytokine production by CD4 and CD8 T cells isolated from human skin explants. We did not observe the same reduction in IL-17A and TNFα observed in the in vitro T_{RM} generation assay (Fig. S2e). In fact, there was an increase in IL-17A production by SP CD4 T cells. Finally, looking at the T_{Reg} to Th17 transition, we did not see observable changes in the $CD4^{+}Foxp3^{+}IL\text{-}17^{+}$ or $CD4^{+}Foxp3^{-}IL\text{-}17^{+}$ populations with tapinarof treatment (Fig. 10).

Figure 9

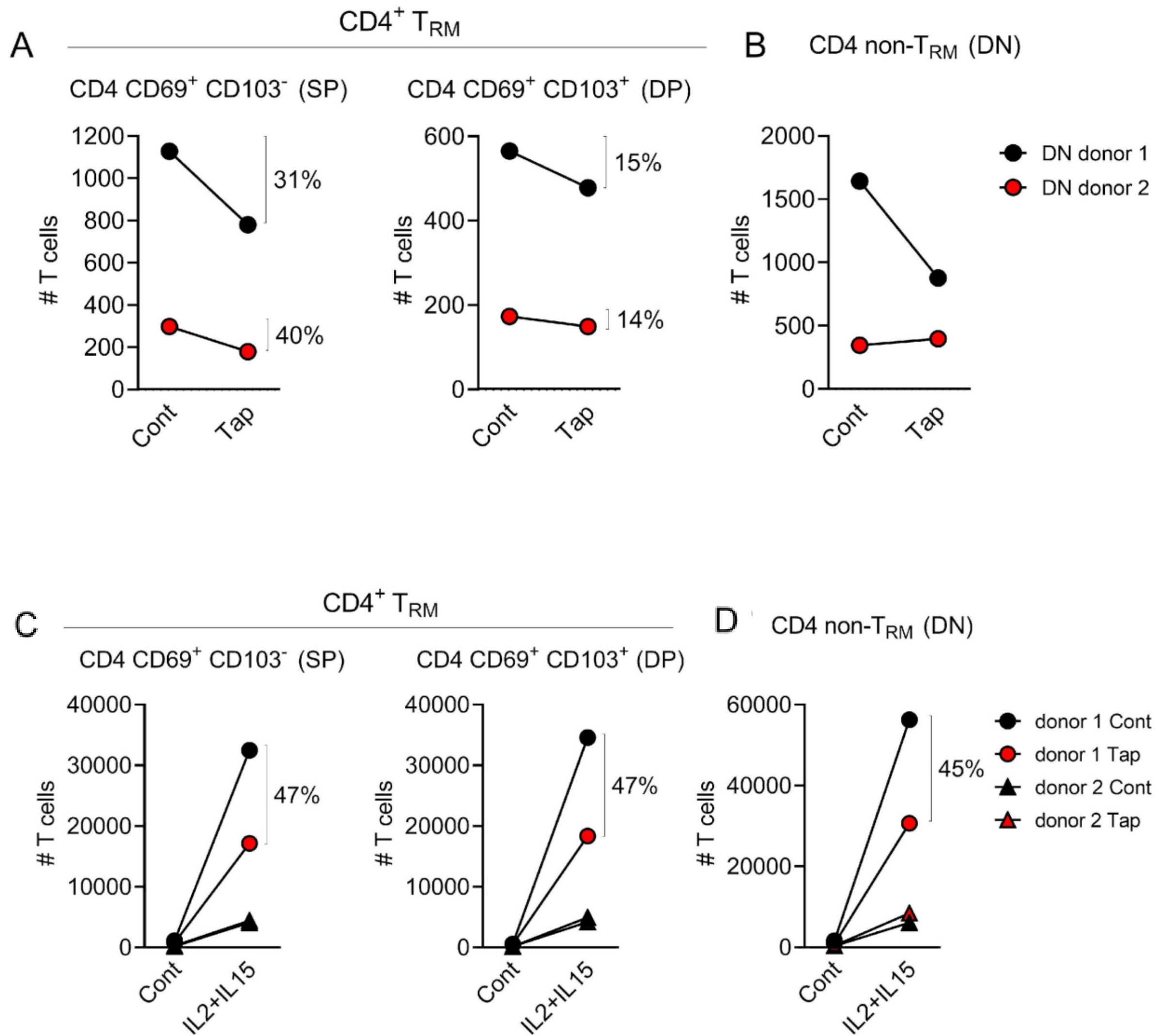


Figure 9. Tapinarof Reduced T_{RM} persistence and Expansion in Human Skin Explants

a) Absolute number of surviving SP and DP CD4 T_{RM} cells cultured without cytokines. **b)** Absolute number of surviving non-T_{RM} CD4 T cells cultured without cytokines. **c)** Absolute number of expanded

SP and DP CD4 T_{RM} cells cultured with IL-2 and IL-15. **d)** Absolute number of expanded non-T_{RM} CD4 T cells cultured with IL-2 and IL-15.

Figure 10

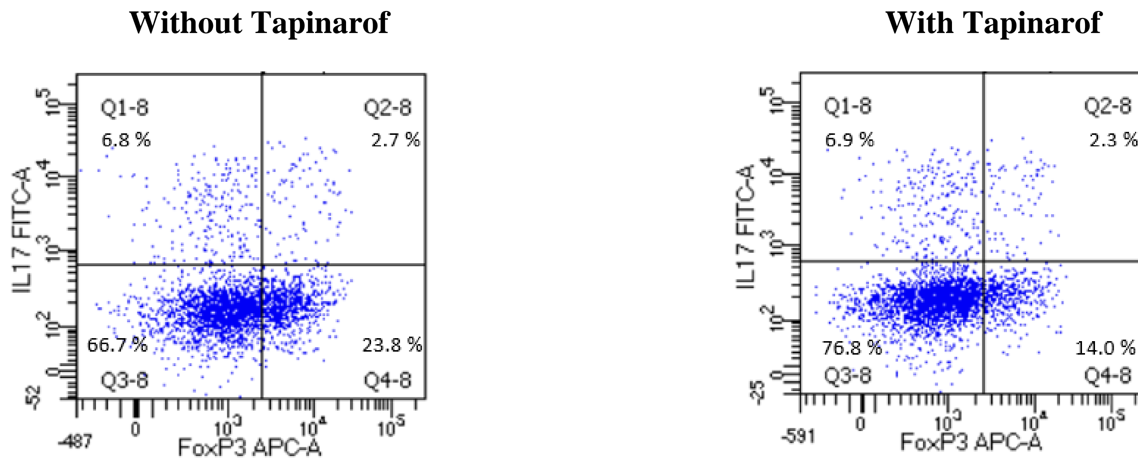


Figure 10. Tapinarof Did Not Affect FoxP3 or IL-17A Expression in CD4$^+$ T Cells

Flow plots showing percentages of IL-17A and FoxP3-expressing CD4 T cells in human skin explant culture with IL-2 and IL-15 cytokines.

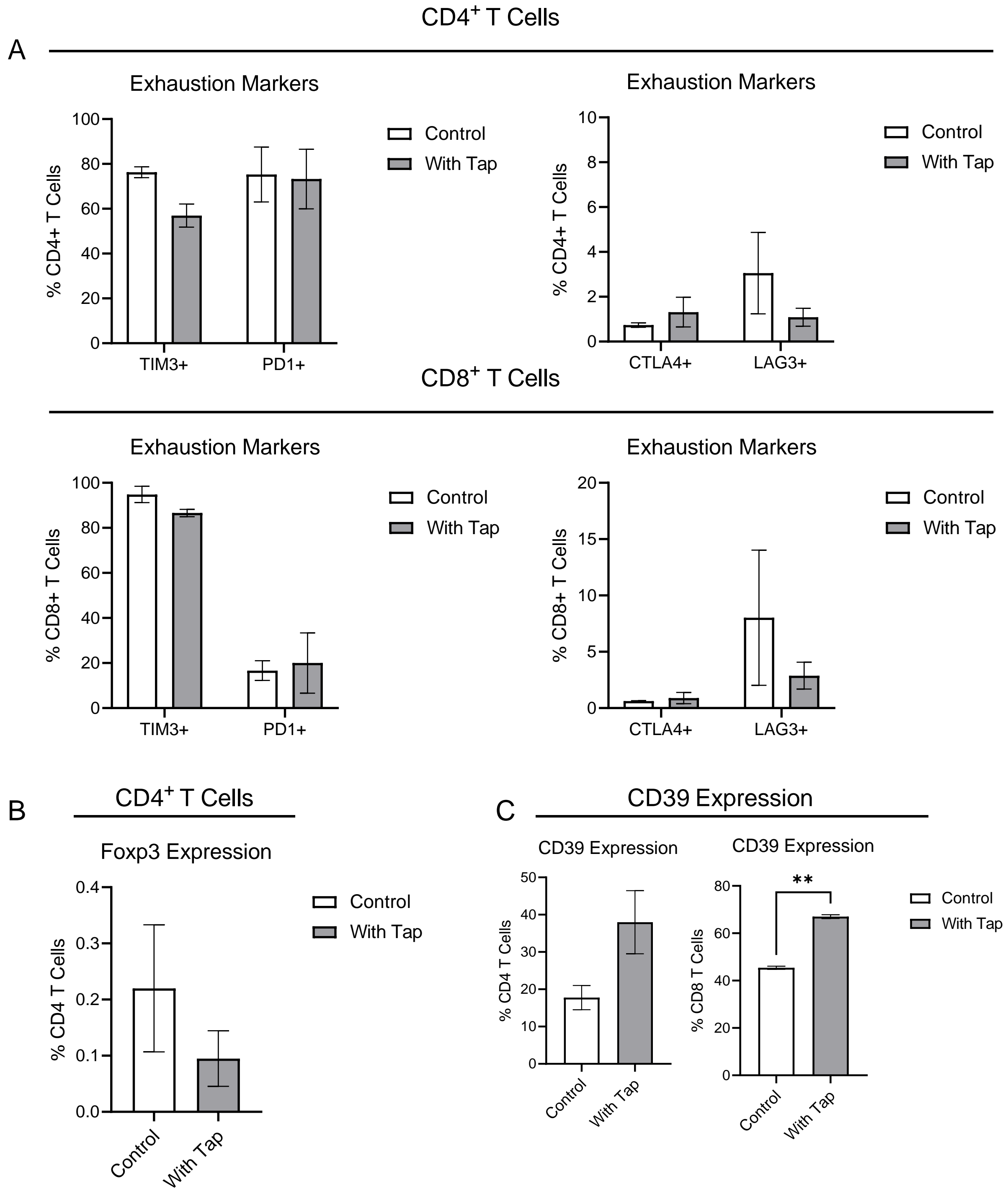
The Effect of Tapinarof on T Cell Exhaustion

T cell exhaustion plays a prominent role in cancer immune evasion. Whereas cancer treatment often attempts to reactivate immune cells by blocking inhibitory receptors such as PD-1, PD-L1, or CTLA4, autoimmune diseases require the opposite approach. Therapies such as abatacept (CTLA4-Ig) aim to mimic the effects of immune checkpoint molecules and inhibit T cell activation.[63] We next studied the ability of tapinarof to induce the expression of T cell

immune checkpoints.

To assay the effect of tapinarof on expression of T cell exhaustion markers, we cultured T cells isolated from PBMCs for 7 days in the presence of anti-CD2/CD3/CD28 T cell stimulation beads with or without tapinarof and then assayed by flow cytometry. We observed no effect of tapinarof on CD4 and CD8 T cell expression of CTLA4, TIM3, Lag3, or PD-1 (Fig. 11a). In fact, tapinarof decreased TIM3 and Lag3 expression in CD4 T cells (Fig. 11a). There was also no impact on Foxp3 expression, suggesting no change in the percentage of T_{Reg} (Fig. 11b). We did observe a significant increase in CD39 expression on both CD4 and CD8 T cells in the presence of tapinarof (Fig. 11c). CD39 is an enzyme that hydrolyzes ATP and ADP and is upregulated in response to chronic inflammation and T cell exhaustion.[64] Additionally, studies show that AhR upregulates CD39 expression in tumor-associated macrophages and CD4 T cells.[64-66] Finally, we assayed cytokine production via flow cytometry and found no differences in TNFα, IL-17A, IL-10, IL-22, or IFNγ production by CD4 T cells (Fig. 11d) or CD8 T cells (Fig. 11e).

Figure 11



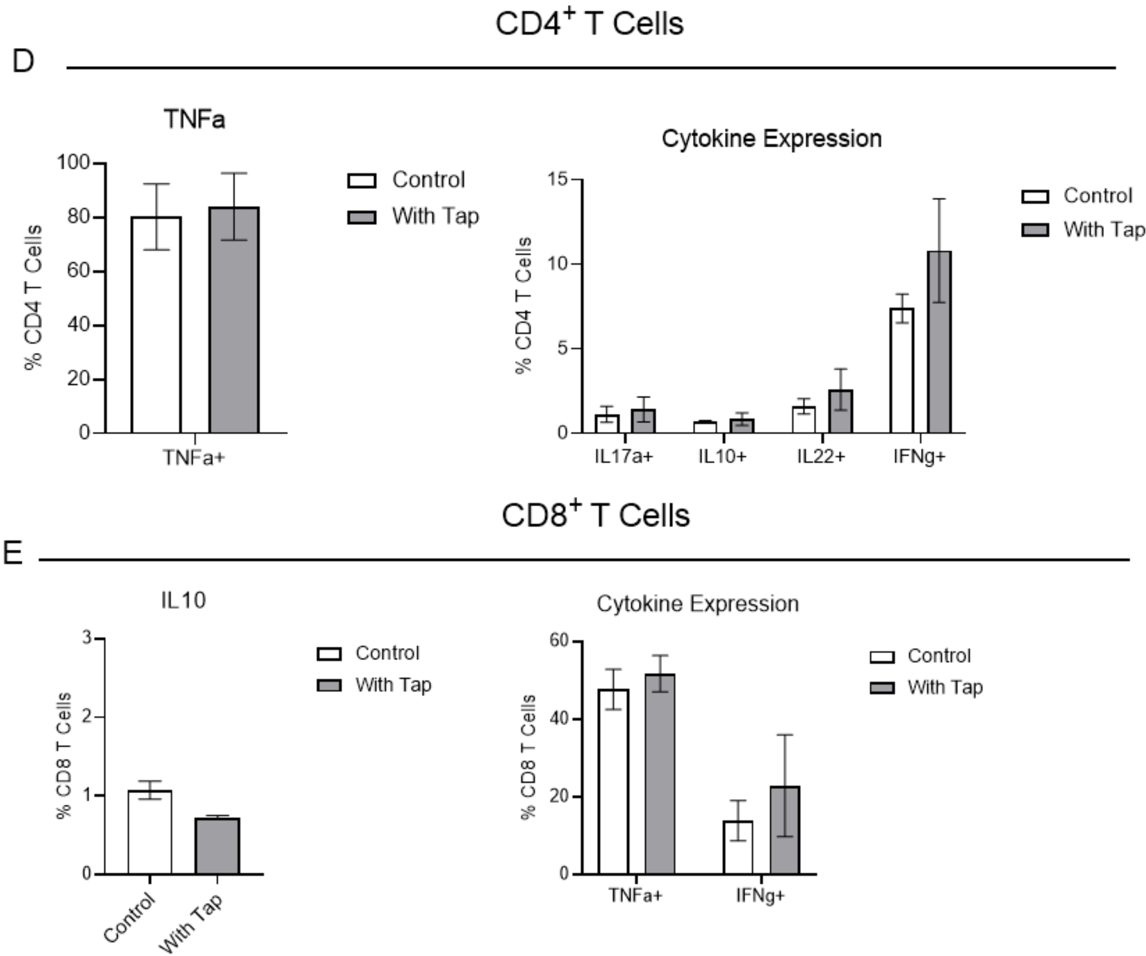


Figure 11. Tapinarof Upregulated CD39 in CD4 and CD8 T Cells

a) n=2 Flow cytometry analysis showing the percentage of CD4 (top) and CD8 (bottom) T cells expressing different exhaustion markers. **b)** Flow analysis of Foxp3 expression in CD4 T Cells. **c)** Percent expression of CD39 in CD4 (left) and CD8 (right) T cells. **d)** Cytokine profile of CD4 T cells using flow. **e)** Cytokine profile of CD8 T cells using flow.

Chapter 3: Discussion

In these studies, we used in vitro assays to investigate the effects of tapinarof on human immune cells with the goal of taking the first steps towards elucidating how tapinarof improves both psoriasis and atopic dermatitis, two very different inflammatory skin diseases. Given time, tissue, and supply line challenges, many of our experiments were carried out on limited numbers of patient samples. These studies are therefore preliminary, but we did observe intriguing evidence that tapinarof has potentially important effects on T cells, particularly $CD4^+$ T cells from skin.

T Cell Exhaustion and Immunoregulation

Tapinarof significantly reduced the activation of PBMC-derived T cells after 24 hours of CD2/CD3/CD28-bead stimulation and significantly increased expression of CD39 after one week of treatment in vitro. This broad inhibition of early T cell activation combined with induction of CD39, which can reduce local immune activation by metabolizing extracellular ATP into adenosine, may both contribute to the immunosuppressive effects of tapinarof.[67] T cells express reduced levels of CD39 in psoriasis, which may increase inflammation within psoriatic lesions.[68-70] We did not observe any consistent changes in PBMC derived T cell cytokine production or exhaustion markers in the limited number of donors we were able to study.

Future experiments will investigate the effect of tapinarof on the transcriptome and epigenetics of T cells in vivo using NSG mice grafted with human skin. The human skin graft will be topically treated with tapinarof versus vehicle control and the mouse will be infused with allogeneic PBMC derived from a second unrelated human donor.[60] in this model, infused T cells

migrate specifically into the human skin graft, encounter allogeneic APC, generate inflammation and undergo resident memory T cell differentiation.[60] This is a useful model to study the effect of topical or systemic therapies on T cell homing to skin, T cell mediated skin inflammation, and resident memory T cell generation and persistence. We hypothesize that tapinarof therapy may upregulate the expression of immunoregulatory genes and pathways in immune and stromal cells. We will utilize RNA sequencing and digital spatial profiling of tapinarof treated skin as well as ATAC sequencing T cells purified from tapinarof treated skin to evaluate the effects of tapinarof on T cell gene expression and chromatin structure.

Resident Memory T Cell Generation, Survival and Cytokine Production

Tapinarof significantly reduced $CD4^+$ T_{RM} generation in an in vitro T_{RM} generation assay. Tapinarof also reduced $CD8^+$ T_{RM} generation, although results did not reach significance because only three donors were studied. T_{RM} are of central importance in the establishment and recurrence of psoriatic lesions and also drive disease recurrence in vitiligo, rheumatoid arthritis and contact dermatitis.[71] Tapinarof reduced the increase in IL-17A and TNFα production that normally accompanies CD4 T_{RM} formation, suggesting it may reduce the Th17 inflammatory capacity of T_{RM} generated in skin. Planned follow up studies include confirming these intriguing findings in vivo, using human skin grafted NSG mice injected with allogeneic PBMC, a useful model to study the development of T_{RM} in human skin.

Tapinarof modestly reduced the survival and IL-2/IL-15 driven expansion of CD4 T_{RM} derived from human skin in explant cultures. These results were from a limited number of human donors and will need to be confirmed in additional studies. If these results are replicated, an inhibition of T_{RM} proliferation and survival may contribute to the ability of tapinarof to reduce

inflammation and delay recurrence of lesions in psoriasis.

Effects of Tapinarof on Antigen Presenting Cells

We did not observe any effects on DC generation or activation in vitro; however, we were able to fully evaluate only one donor. Future experiments will evaluate DC development, activation, and cytokine production in multiple donors. Additionally, tapinarof may also affect the interaction between dendritic cells and T cells. To evaluate this possibility, the effect of tapinarof on T cell activation, proliferation, and cytokine production will be evaluated in mixed leukocyte reactions consisting of dendritic cells cocultured with allogeneic T cells.

Tapinarof could also conceivably impair dendritic cell migration. In vitro, dendritic cells migrate out of human skin explants within 24 hours. 24-hour culture of skin explants in the presence of tapinarof versus vehicle control, followed by enumeration of the DC that have migrated of the skin, will be used to study this possibility.

Dendritic cells play a critical role in psoriasis pathogenesis, by producing inflammatory cytokines such as IL-23 and directly stimulating T cell activation, proliferation, and cytokine production. Additional studies are needed to determine if tapinarof affects cytokine production by dendritic cells or induces a tolerogenic phenotype characterized by increased expression of TGFβ and IL-10.

There would also be value to repeating our experiments using a variety of tapinarof concentrations and including AhR inhibitors to ensure that the effects we observe are a result of AhR signaling and not a result of tapinarof's known antioxidant effects. These experiments will also incorporate RT-PCR based quantification of *CYP1A1*, a gene reliably increased as a result

of AhR signaling.

Tapinarof's Clinical Potential

Tapinarof is a first in its class, novel topical therapeutic that is effective in the treatment of both psoriasis and atopic dermatitis, two chronic inflammatory skin diseases with distinct immunological etiologies. This suggests that tapinarof targets inflammatory or immunomodulatory pathways that are common to both diseases. A better understanding of its mechanism and effects on immune and stromal cells in skin could lead to the identification of novel immunoregulatory pathways.

The efficacy of tapinarof in the treatment of both psoriasis and atopic dermatitis also suggests that it may be useful in the treatment of other inflammatory skin diseases, including lupus, vitiligo and contact dermatitis. If our in vitro data suggesting that tapinarof reduces T_{RM} generation and persistence are confirmed in larger studies, tapinarof may be also useful in the growing number of dermatologic conditions known to have T_{RM} contributions.

In addition to its AhR agonist activity, tapinarof is a potent antioxidant. Each molecule of tapinarof scavenges two molecules of ROS, and tapinarof induces the transcription of antioxidant genes via activation of the NRF2 pathway.[2] ROS-driven tissue injury and inflammation contribute to fibrosis in the skin and lung.[72,73] Tapinarof's unique combination of anti-inflammatory and antioxidant activity suggests that it may also have efficacy in the treatment of fibrotic skin diseases such as scleroderma, morphea and chronic radiation dermatitis.

Tapinarof upregulates the expression of proteins implicated in skin barrier function in keratinocytes.[2] Skin barrier gene expression is reduced in patients with atopic dermatitis and

leads to an increased risk for infection and allergen sensitization.[74] A deficiency in skin barrier proteins may also contribute to skin inflammation in psoriasis.[75] Tapinarof may improve atopic dermatitis and psoriasis in part by upregulation of the expression of filaggrin and improvement in skin barrier function. Other dermatologic diseases such as ichthyosis vulgaris are characterized by deficiencies in filaggrin and could potentially be improved by topical tapinarof.[76]

Tapinarof may act via multiple mechanisms of action to improve skin inflammation in atopic dermatitis and psoriasis. A combination of anti-inflammatory, immunoregulatory and antioxidant activities may be responsible for its observed anti-inflammatory effects. Its ability to target common pathways in inflammation and immunoregulation make it a promising candidate for testing in the treatment of other inflammatory skin diseases. A better understanding of its mechanism of action will also provide novel insights into the normal role of AhR signaling in healthy skin homeostasis.

In conclusion, our in vitro studies suggest that tapinarof may inhibit the generation, persistence, cytokine-driven proliferation, and cytokine production of $CD4^+$ T_{RM}. Because T_{RM} drive lesional recurrence in patients with psoriasis, this ability may explain the durable remissions observed by patients with psoriasis after treatment with topical tapinarof. Tapinarof also inhibited early events in $CD4^+$ and $CD8^+$ T cell activation and upregulated expression of CD39. Additional studies are needed to clarify the mechanisms of action of tapinarof and elucidate its effect on both immune and stromal cell types in skin.

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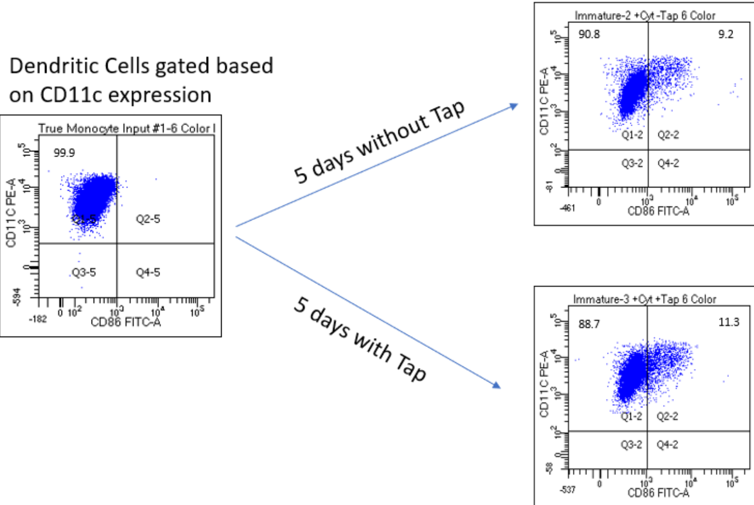
Supplementary Figures

Figure S1.

a.



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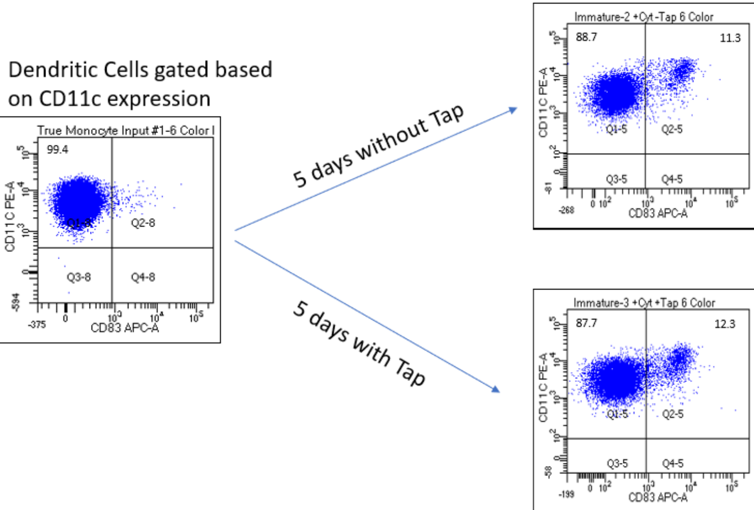
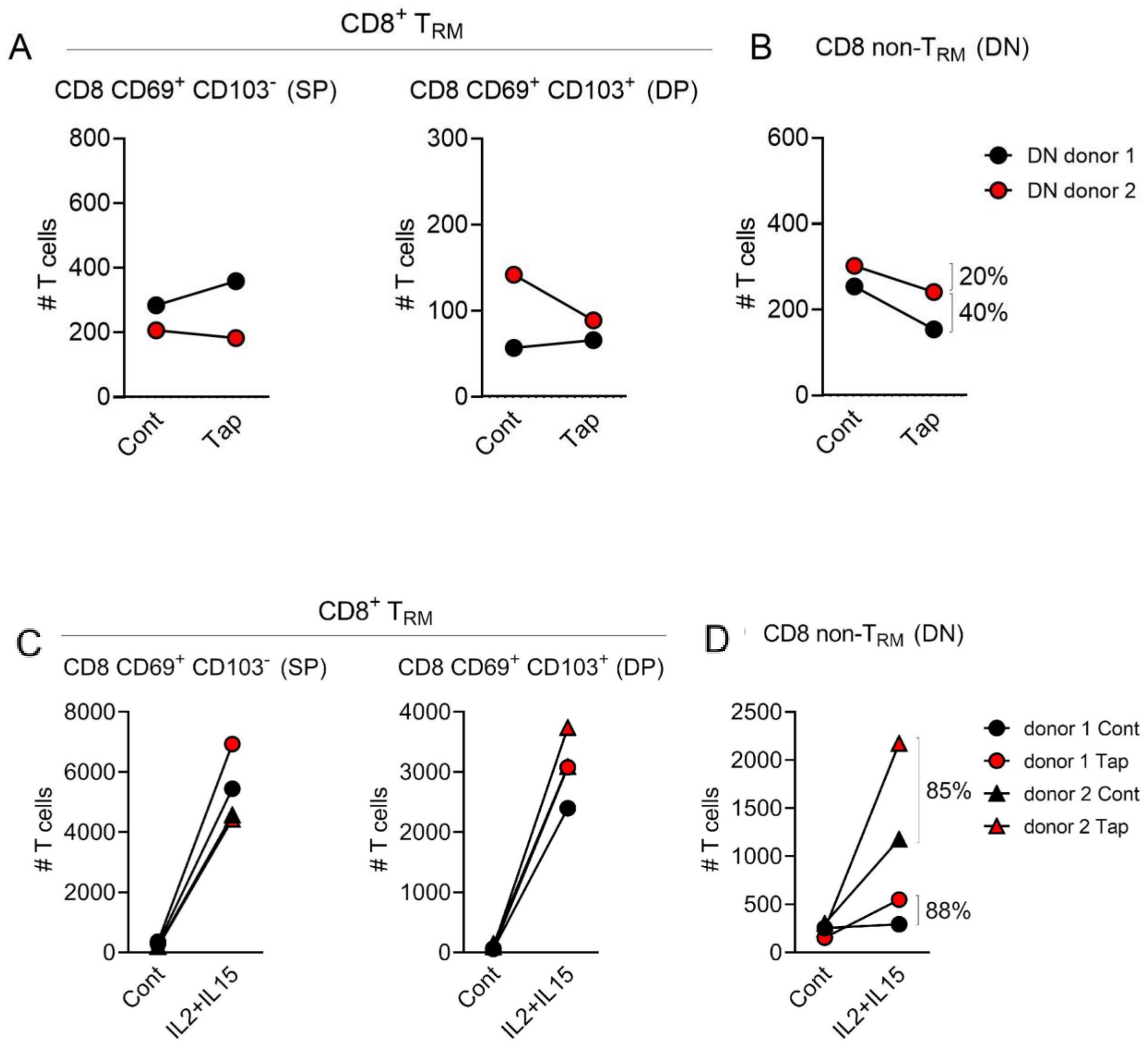


Figure S1: Successful Generation of Immature Dendritic Cells

a,b) Flow plots of $CD11c^+$ monocytes (left) given IL-4 and GM-CSF for 5 days to generate immature dendritic cells (right). Maturation is characterized by CD83 and CD86 expression

Figure S2.



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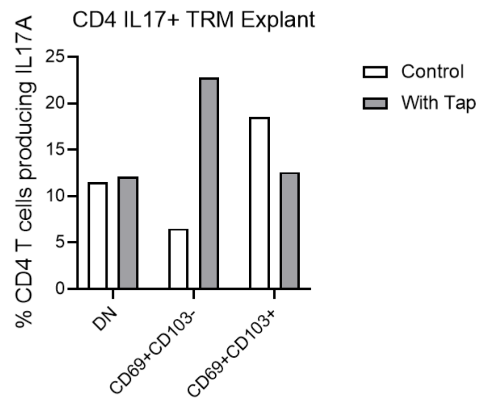

Figure S2. Tapinarof Did Not Affect CD8 Survival or Expansion in Human Explant Cultures and Increased IL-17A Production in SP CD4 T Cells

a) Absolute number of surviving SP, and DP CD8 T_{RM} cells in human explants without cytokines **b)** Absolute number of surviving non-T_{RM} cells in human explants without cytokines. **c)** Absolute number of expanded SP, and DP CD8 T_{RM} cells in human explants with IL-2 and IL-15. **d)** Absolute number of expanded non-T_{RM} CD8 cells in human explants with IL-2 and IL-15. **e)** Percentage of IL-17A-producing CD4 T cells separated by T_{RM} expression.