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Marijuana-derived Δ^9 -tetrahydrocannabinol suppresses Th1/Th17 cell-mediated delayed-type hypersensitivity through microRNA regulation

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Abstract

Δ^9 -Tetrahydrocannabinol (THC) is one of the major bioactive cannabinoids derived from the *Cannabis sativa* plant and is known for its anti-inflammatory properties. Delayed-type hypersensitivity (DTH) is driven by proinflammatory T helper cells including the classic inflammatory Th1 lineage as well as the more recently discovered Th17 lineage. In the current study, we investigated whether THC can alter the induction of Th1/Th17 cells involved in mBSA-induced DTH response. THC treatment (20 mg/kg) of C57BL/6 mice with DTH caused decreased swelling and infiltration of immune cells at the site of antigen rechallenge. Additionally, THC treatment decreased lymphocyte activation as well as Th1/Th17 lineage commitment, including reduced lineage-specific transcription factors and cytokines. Interestingly, while DTH caused an overexpression of miR-21, which increases Th17 differentiation via SMAD7 inhibition, and downregulation of miR-29b, an IFN- γ inhibitor, THC treatment reversed this microRNA (miR) dysregulation. Furthermore, when we transfected primary cells from DTH mice with miR-21 inhibitor or miR-29b mimic, as seen with THC treatment, the expression of target gene message was directly impacted increasing SMAD7 and decreasing IFN- γ expression, respectively. In summary, the current study suggests that THC treatment during DTH response can simultaneously inhibit Th1/Th17 activation via regulation of microRNA (miRNA) expression.

Keywords

Hypersensitivity; microRNA; Cannabinoid; THC; Inflammation; Th1/Th17 regulation

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Compliance with ethical standards All experiments were conducted under an approved Institutional Animal Care and Use Committee animal protocol. This model of footpad induced DTH is well established and approved by IACUC (protocol# 2214-100895-090814: Mouse Models of Innate Immunity 1031 of the series Methods in Molecular Biology 101-107). The manuscript does not contain clinical studies or patient data.

Conflict of interest The authors declare that they have no competing interests.

Introduction

T helper cell differentiation has classically represented distinct T cell lineages impacted by cytokine milieu, Th1 (IL-12), Th2 (IL-4), Th17 (IL-6 and TGF β), and T regulatory cells (Tregs-TGF β), leading to terminally differentiated T cell subsets [1]. Over the years, it has been shown that interaction between Th cell subsets, such as Th1 and Th2, can regulate the differentiation of other T cell lineages [2, 3]. While the Th1, proinflammatory, versus Th2, anti-inflammatory, responses have been clearly defined (as reviewed by [4]), the discovery of additional proinflammatory T helper cell populations, such as Th17, has complicated our understanding of T cell interactions. Recent evidence has shown that Th1 secreted cytokines, IFN- γ and IL-2, can inhibit Th17 differentiation and that while IL-17a/f expression cannot directly inhibit the Th1 responses, elevated levels of TGF β associated with active Th17 cells can [5, 6]. Further muddling these proinflammatory cell interactions is the fact that Th17 and Th1 lineages may have some overlap due to immune cell plasticity and the emergence of proposed “Th17/Th1” and “non-classic” Th1 lymphocytes [7, 8].

Heritable genetic modifications, known as epigenetics, have arisen recently as the explanation for selective gene expression [9]. Epigenomics broadly encompasses DNA methylation, histone acetylation, and methylation, as well as microRNA (miRNA or miR) expression. Evidence now exists which highlights the influence of epigenetic modifications on immune cell differentiation and activation [10–12]. miRNA are short strands of RNA which most commonly inhibit gene expression through the binding of messenger RNA (mRNA) sequences [13]. Each miR can bind numerous targets and therefore regulate the expression of multiple genes [13]. Interestingly, lineage-defining transcription factors and the downstream targets associated with Th cell subsets have become a major focus for epigenetic modification such as miRNA binding [14]. Inflammation seen in delayed-type hypersensitivity (DTH) plays a critical protective role against intracellular pathogens. However, such inflammation is often seen against innocuous non-pathogenic antigens leading to severe damage to the tissue as seen during contact dermatitis against nickel or poison ivy. Classically, DTH was considered to be a Th1-driven inflammatory disease; however, treatment with anti-IFN- γ showed that such mice continue to exhibit signs of DTH [15]. More recently, the importance of Th17 cells in DTH disease progression was highlighted in IL-17 knockout mice which showed significantly reduced ear swelling in an allergenic contact dermatitis model [16]. Thus, depending on the antigen, it is likely that both Th1 and Th17 cells play an effector cell role.

Immune cells are well established to express cannabinoid receptors; however, their precise role in the regulation of immune response remains unclear [17]. Δ^9 -Tetrahydrocannabinol (THC), one of the most well-studied bioactive cannabinoids derived from the *Cannabis sativa* plant, has long been shown to reduce T cell driven inflammation via apoptosis, induction of immuneoregulatory cells, regulation of Th cell cytokines, and even epigenetic modifications [18, 19, 10, 20–22]. Majority of THC research, in regard to inflammation and autoimmunity, has centered on the Th1/Th2 axis. Specifically, we and others have found that THC treatment increases anti-inflammatory cytokines causing a subsequent drop in proinflammatory cytokines such as IL-12, IFN- γ , and TNF- α as well as reduced T cell activation [18, 19]. THC treatment has also been found to induce Tregs which are known to

inhibit both Th1/Th17 lineage differentiation [21, 23]. Furthermore, THC has been found to significantly reduce cytokines associated with Th17 differentiation such as IL-6 and IL-17a [24]. Recently, our lab has found that the ability of THC to shift the immune response from proinflammatory to antiinflammatory involves, in part, miRNA regulation [21].

While cannabinoid receptor agonists can attenuate DTH, the role of THC regulation of epigenetic modifications in this model has not been assessed [25–27]. Furthermore, the ability of THC treatment to inhibit both Th1/Th17 activation within a hypersensitivity model has not been reported. With this in mind, we set out to determine if THC treatment could inhibit both Th1 and Th17 T cell lineages, in a methylated BSA (mBSA) sensitization/rechallenge model of DTH, via miRNA regulation. Our results suggest that THC can attenuate DTH response to mBSA by inhibiting both Th1/Th17 cells through reversal of the miRNA alterations brought about during the DTH response.

Methods

Mice

Female C57BL/6 (BL6) mice, aged 6–8 weeks and at an average weight of 20 g, were obtained from Harlan Laboratories. All mice were housed in pathogen-free conditions and allowed ad libitum access to filtered water and Teklad rodent diet 8604 (normal chow) at the Animal Research Facility located at the University of South Carolina School of Medicine. All experiments were conducted under an approved Institutional Animal Care and Use Committee animal protocol.

DTH and THC treatment

DTH was induced in BL6 mice using a sensitization/rechallenge method [27]. Briefly, mice ($n = 5$ per experimental group) were sensitized with a subcutaneous injection (100 μ L/hind flank) of 1.5 mg/mL methylated BSA (mBSA—Sigma Aldrich) emulsified in complete Freund's adjuvant (CFA—Sigma Aldrich). Six days later, the mice were rechallenged with a footpad injection (20 μ L/footpad) of 10 mg/mL mBSA in 1 $\times$ PBS. ⁹-Tetrahydrocannabinol (THC—NIDA-NIH) in ethanol (20 mg/mL) was diluted in 1 $\times$ PBS to a concentration of 20 mg/kg. THC or vehicle (ethanol diluted in 1 $\times$ PBS) was administered intraperitoneally (i.p.) at a volume of 0.1 mL/mouse into DTH mice daily starting on day 3. Percent swelling was measured as [(thickness(mBSA rechallenged footpad)-thickness(1 $\times$ PBS rechallenged footpad))/thickness(1 $\times$ PBS rechallenged footpad)]*100.

Monoclonal antibodies, reagents, and flow cytometer

Antibodies used for flow cytometric analysis (BioLegend) included Fc block (93), PE Cy7-conjugated anti-CD3 (145-2C11), PE-conjugated anti-CD19 (6 days), FITC-conjugated anti-CD69 (H1.2F3), FITC-conjugated anti-IL17a (TC11-18H10.1), and PE-conjugated anti-CD4 (GK1.5). In short, popliteal lymph node cells (PopLN—10⁶ cells in 25 μ L) from rechallenged BL6 mice were incubated with FC receptor antibodies (5–10 min) and incubated with conjugated antibodies (20–30 min at 4 °C). After incubation with conjugated antibodies, cells were washed twice with 1 $\times$ PBS/2% fetal bovine serum buffer. The stained cells were then assessed by flow cytometer (FC500; Beckman Coulter) and the resulting

data analyzed by Cytomics CXP software (Beckman Coulter). Intracellular Cytokine Staining Kit (BD biosciences) was used for IL-17a staining per manufacturer's specifications. Briefly, cells were first stained for cell surface CD4 receptor and then fixed/permeabilized. Fixed/permeabilized cells were then stained for IL-17a. Three-color flow cytometric analysis was used to profile the lymphocyte activation. Two-color flow cytometric analysis was used to look for Th17 cell induction.

Thymidine analysis of lymph node proliferation

Draining lymph node cells from mBSA-sensitized mice (1.5 mg/mL in vivo) were co-cultured in triplicate (0.2 mL/well in a round bottom 96-well plate) with mBSA (40 mg/mL) for 48 h. Sixteen hours before collection and analysis [³H]thymidine (2 µCi/well) was added to the cell cultures. The radioactivity was measured using a liquid scintillation counter (MicroBeta Trilux).

Histological assessment of rechallenged footpad

Footpads from THC- or vehicle-treated DTH mice were isolated for histological examination. Rechallenged feet were cut distal to the ankle and decalcified/fixed in Cal-Rite (Fisher scientific). The footpad was then embedded in paraffin and sectioned into 6-µm sections and stained for hematoxylin and eosin (H&E) for assessment of swelling, or edema, and immune cell infiltration [27].

Cytokine analysis in cell culture supernatants

ELISA MAX sandwich enzyme-linked immunosorbent assay (ELISA) kits (BioLegend) were used to assess the level of IL-17a/f, IFN-γ, and TNF-α cytokines. Cells were isolated from spleen of DTH mice, plated at 1×10⁶ cells/well, cultured overnight (18–20 hours) for spontaneous cytokine secretion in complete RPMI media (1 % v/v penicillin/streptomycin, 1 % v/v HEPES buffer, 10 % v/v heat inactivated FBS, and 0.0002 % v/v 2-mercaptoethanol). Cytokine production was quantified from cell supernatants (stored at –20 °C). Absorbance was measured at 450 nm using a Victor2 1420 Multilable counter (Wallac).

qPCR analysis of gene expression

Total RNA was isolated from PopLN and purified using miRNeasy kit (Qiagen), following manufacturer's procedure. iScript complementary DNA (cDNA) synthesis kit (BioRad) was used according to manufacturer's specifications to reverse transcribe cDNA. qPCR was performed using Sso Advanced SYBR Green (BioRad) on a CFX Connect (BioRad). Samples were assessed for expression of β actin, Tbx21 (Tbet), RORγT, IRF4, Pten, NFκB, IFN-γ, and SMAD7. Primers, as found on PrimerBank (Harvard Medical School), were synthesized from IDT DNA technologies with annealing temperatures of 60 °C (Table 1).

Western blot analysis

Protein was isolated from PopLN lymphocytes using radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz), as described [22]. Five micrograms of protein was loaded and ran on a polyacrylamide gel (12 %) at 60 V for 2.5 h. Protein was then transferred to nitrocellulose at 100 V for 1 h. Protein transfer was confirmed using 3 mL of Ponceau S staining solution.

Nitrocellulose was then blocked in 5 % nonfat dry milk in TBS. Nitrocellulose was then probed with Cell Signaling antibodies to Akt (pan [C67E7] 1:100), phospho-Akt ([S473] 1:100). HRP conjugated secondary antibodies were used at 1:1000 dilution. For control protein bands β -actin (Poly6221) was used at a dilution of 1:1000. ECL substrate (Thermo) was added for 1 min; exposure time varied.

microRNA (miRNA or miR) array and array analysis

Affymetrix miRNA array version 4, which integrates all mature miRNA sequences in miRBase Release 20 (miRBase v20), was used to profile miR expression in the PopLN 24 h after mBSA rechallenge in DTH mice. The array identified over 3000 unique mouse miRs as identified in miRBase v20 using the FlashTag biotin HSR hybridization technique, as previously described [28]. In brief, log transformation of fluorescent intensities obtained from the hybridization was used to generate heat maps. Hierarchical clustering, using Ward's method, and similarities were measured using half square Euclidean distance. Fold changes in miR expression were obtained from the raw array data, and only those miRs with greater than twofold change were considered for further analysis. miR predicted targets, alignments, and miR SVR scores were determined using online databases (TargetScan, microRNA.org, and miRWalk). Determination of miR interaction and pathway analysis was completed using Ingenuity Pathway Analysis (IPA— Ingenuity Systems). Venn diagram construction was done using LucidChart Venn diagram maker (https://www.lucidchart.com/pages/examples/venn_diagram_maker).

miR validation in PopLN cells

miR levels were validated using miScript II RT kit (Qiagen), following manufacturer's specifications for 5 $\times$ miScript HiSpec Buffer. qPCR was performed using miScript SYBR Green (Qiagen) on CFX Connect (BioRad). Samples were assessed for expression of SNORD96A, miR-21a-5p, miR-223-3p, miR-29b-3p, miR-125a-5p, and miR-142-5p (Qiagen).

miR transfection of primary cells

Transfection of primary cells was completed using the HiPerFect Transfection kit (Qiagen) following the manufacturer's protocol. Briefly, DTH draining lymph node cells were plated (4×10^5 cells/well) in complete RPMI media. Transfection, 6 h at 37 °C, with mimic (40 ng/well), inhibitor (100 ng/well), and negative mimic (negative control siRNA—40 ng/well) was done in serum free RPMI media (1 % v/v penicillin/streptomycin, 1% v/v HEPES buffer, and 0.0002 % v/v 2-mercaptoethanol) with 6 μ L HiPerFect transfection reagent. Transfection complex was added one drop at a time into cell culture. Transfection mimic, inhibitor, and negative mimic were purchased from Qiagen. Twentyfour hours from initial incubation cells were collected, total RNA isolated (miRNeasy kit- Qiagen), and cDNA reverse transcribed (miScript II RT kit- Qiagen) for miR validation and target gene expression analysis.

Animal welfare and ethics statement

Experimental procedures used in these studies comply with ARRIVE recommendations and were carried out as humanely as possible. Additionally, all experiments were conducted

under an approved Institutional Animal Care and Use Committee animal protocol at the University of South Carolina.

Statistical analysis

Data is shown as mean $\pm$ S.E.M. Student's *t* test was used to compare data between two groups. One-way ANOVA with Tukey post hoc test was used to compare three or more groups. Experimental groups were compared to controls, $p < 0.05$ was considered significant. All experiments were repeated in duplicate or triplicate to ensure reproducible findings.

Results

Hypersensitivity is decreased in THC-treated DTH mice

Delayed-type hypersensitivity (DTH) is a T cell driven response to antigens, mediated primarily by Th1 and Th17 cells. In the current study, we used methylated BSA (mBSA) as an antigen to induce DTH in mouse footpads as described previously [27]. Briefly, mice were sensitized by injecting mBSA (1.5 mg/mL in CFA) into the footpads on day 0 followed by rechallenge with mBSA on day 6 (10 mg/mL in 1xPBS) (Fig. 1a). These mice received 20 mg/kg THC or vehicle (ethanol diluted in 1xPBS) on days 4, 5, and 6 after initial sensitization (Fig. 1a). We used relative percent swelling of footpad as the primary indicator of DTH response 24 h after rechallenge with mBSA. THC treatment of DTH mice significantly reduced swelling in the hind footpad compared to vehicle treatment (Fig. 1b) as well as draining lymph node (popliteal lymph node, PopLN) cellularity (Fig. 1c). Additionally, we looked at lymphocyte proliferation, due to ex vivo rechallenge (40 mg/mL mBSA) of sensitized draining lymph nodes, via [³H]thymidine incorporation. We found that THC treatment was able to significantly decrease mBSA-induced lymphocyte proliferation compared to vehicle-treated DTH mice (Fig. 1d). Moreover, histological analysis showed reduced swelling in THC-treated mice correlated with a significant decrease in lymphocyte infiltration in the rechallenged footpad (Fig. 1e – h).

THC treatment reduced lymphocyte activation

Furthermore, assessment of T cell activation, using the early lymphocyte activation marker CD69, in the PopLN showed significant decrease in THC-treated DTH mice compared to vehicle (Fig. 2a). As CD69 can be used as an early activation marker for both T and B cells, we next looked at both CD3+CD69+ T cell and CD19+ CD69+ B cell populations. THC-treated DTH mice showed a decrease in both proportion (Supp. Fig. 1a–b) and absolute cell counts (Fig. 2b). With recent findings of cannabinoid receptor agonists negatively regulating IL-17 [29, 30], we next studied Th17 cells. The gating of such cells, on CD4, revealed that IL-17a producing T cells also showed significant decrease in proportion between DTH+ THC-treated mice and DTH + Veh mice (Supp. Fig. 1c) suggesting that THC was affecting T helper cell response.

THC treatment reduces expression of proinflammatory transcription factors

To show that THC treatment was inhibiting Th cell driven inflammation, we next looked at proinflammatory cytokines which have been linked to DTH including IL-17a/f, IFN- γ , and

TNF- α [31]. We observed significant decreases in spontaneous secretion of IFN- γ , TNF- α , and IL-17 in DTH+ THC-treated mice when compared to DTH+ Veh treated mice (Fig. 2c). Having seen a decrease in Th1/Th17 proinflammatory cytokines, IFN- γ and IL-17a/f respectively, we next looked at lineage-dependent transcription factors Tbx21 (Tbet for Th1) and ROR γ T and IRF4 (for Th17) in the PopLN cells [32–34]. THC treatment significantly decreased the expression of both Th1/Th17 lineage-specific transcription factors, compared to vehicle-treated DTH mice (Fig. 2d). Having shown that markers specific to Th1/Th17 driven inflammation, IFN- γ and IL-17a/f, respectively, had been decreased with THC treatment, we next looked at more generalized markers of cellular proliferation and inflammation. As such, we used expression of Pten, the PIK $_3$ /Akt inhibitor, and Akt phosphorylation as indicators of immune cell proliferation [35] and NF κ B expression as a marker of inflammation [36]. Interestingly, THC treatment of DTH mice caused a significant increase in Pten levels (Fig. 2e) and a correlative drop in Akt phosphorylation (Fig. 2f) suggesting that THC was decreasing proliferation. Furthermore, NF κ B expression (Fig. 2g) was decreased by half in DTH+ THC compared to DTH+ Veh mice, indicative of a reduced inflammatory response.

Induction of DTH in mice causes miRNA dysregulation

Immune cell differentiation and activation are believed to be regulated, at least in part, by epigenetic modifications including miRNA [11, 12]. As such, dysregulation of miRNA seen in disease models especially those considered inflammatory and/or autoimmune has become a topic of major interest for possible therapeutic targets [37]. The importance of miRNA in hypersensitivity was recently highlighted by the observance of similarly elevated miRNA expression in both human and mouse allergic contact dermatitis skin samples [38]. Using Affymetrix miRNA array version 4 (JHMI deep sequencing and microarray core), we assessed the expression of over 3000 miRNA in the PopLN from naïve and DTH mice. In order to visualize the miRNA dysregulation caused by DTH, a heat map was constructed using log transformation of fluorescent intensities obtained from the FlashTag biotin HSR hybridization (Fig. 3a). One thousand five hundred twenty-nine miRs were up/downregulated in DTH mice compared to naïve; however, the expression of only 70 miRNA were altered at greater than twofold change, 51 upregulated, and 19 downregulated (Fig. 3b). miR-21 and miR-223 were selected from the top upregulated miRNA (Fig. 4b) as potential inflammatory markers associated with DTH response and validated by qPCR (Fig. 3c). Also, of interest, for possible therapeutic targets, were the top downregulated miRs (Fig. 4c) of which miR-29b, miR-125a, and miR-142 were selected and validated by qPCR (Fig. 3e).

THC treatment reverses DTH-mediated miRNA dysregulation

Having validated two upregulated miRs, and three downregulated miRs as markers potentially associated with DTH response, we next set out to determine what impact, if any, THC treatment in DTH mice would have on the expression of these miRNAs. Interestingly, DTH+THC mice showed a significant reversal of DTH dysregulated miRNA (Fig. 4a, b). THC treatment caused downregulation of miR-21 and –223, reduced by half, and upregulation of miR-29b and miR-142, increased greater than fivefold, while having no significant effect on miR-125a. Next, we selected 20 miRs, evenly distributed, from the top up- and downregulated miRs and using ingenuity pathway analysis (IPA) tried to connect

these miRs of interest to Th1/Th17 differentiation. In this analysis, we were able to identify only two miRs, miR-21 which was decreased by half, and miR-29b which was increased nearly sevenfold in DTH+THC mice that were directly connected to the proinflammatory Th cell differentiation pathway (Fig. 4c).

THC-mediated regulation of miR-21 and miR-29b inhibits Th1 and Th17 stable lineage commitment

As implicated by the pathway analysis, IFN- γ is a target of miR-29b, supported here by the miR SVR score (miRWalk—Fig. 5a) and alignment (miRNA.org—Fig. 5b). We then inferred that THC-mediated upregulation of miR-29b contributed to decreased IFN- γ induction. Additionally, SMAD7, a negative regulator of TGF β signaling and subsequent upregulation of IL-17 [39], is a strong target of miR-21, indicated here by the miR SVR score (miRWalk—Fig. 5a) and alignment (miRNA.org—Fig. 5d). Thus, THC-mediated downregulation of miR-21 may have contributed to increased SMAD7 and decreased IL-17. Next, we tried to confirm the expression of these molecules in the PopLN cells using qPCR. Our findings showed that THC treatment of DTH mice was in fact able to decrease IFN- γ (Fig. 5c) and increase SMAD7 expression in PopLN cells (Fig. 5e) when compared to DTH + Veh mice.

THC treatment alters microRNA-specific target gene expression

In order to confirm that the reduced proinflammatory Th cell commitment was due to miRNA regulation, we next used primary cell transfections with miR-21 and miR-29b. Draining lymph node cells from DTH mice were transfected (HiPerFect- Qiagen) with either miRNA mimics or inhibitors (Qiagen) for 6 h with target gene expression assessed 24 h after transfection. The transfection was performed as detailed in our previous publications [11, 40]. We found that miR-29b inhibitor significantly increased IFN- γ levels compared to miR-29b overexpression (Fig. 5f). Additionally, SMAD7 levels were significantly elevated with miR-21 inhibition compared to miR-21 overexpression (Fig. 5g).

Discussion

While cannabinoid receptor agonists have been shown to reduce type IV hypersensitivity associated with DTH, the role of THC as an epigenetic regulator of proinflammatory T helper cell differentiation has not yet been fully elucidated in this model [25–27]. To this end, we investigated the effect of THC on mBSA-induced DTH response specifically addressing how THC may downregulate the transcription factors and cytokines that promote proinflammatory T cell activation. Additionally, we investigated how THC can induce miR expression to alter the expression of key regulatory pathways which directly impact Th17/Th1 stable lineage commitment.

The natural compound THC found in marijuana is well known for its immunoregulatory properties. Specifically, in regard to T cell activation, THC treatment has been shown to reduce secretion of proinflammatory cytokines, including IFN- γ and TNF- α , and suppress proliferation of T cells via apoptosis and/or induction of regulatory immune cells [18, 19, 21]. As such, THC has been shown to be highly effective therapeutically in numerous

models of T cell driven inflammatory disease including inflammatory lung injury, autoimmune hepatitis, and allogenic hematopoietic cell transplantation [21, 41]. With this in mind, we set out to determine the effects of THC on type IV hypersensitivity, as seen in contact dermatitis from nickel or poison ivy, in mice.

DTH response, however, is complicated by the fact that both Th1/Th17 cell subsets have been shown to contribute to the overall pathogenesis [42]. The ability of THC to act as an inhibitor of Th1-driven inflammation, which we saw in the current study, has been well documented in other models such as cancer and autoimmunity [18, 43]. However, current literature on the role of THC as a Th17 inhibitor has been more indirect focusing instead on THC-mediated Treg induction [21]. Nonetheless, in the current study, we show a direct inhibition of Th17 activation via a derepression of SMAD7, a known inhibitor of the Th17 lineage [39], which occurs upon THC treatment of DTH mice.

Although there is no direct evidence to show that THC can inhibit Th1/Th17 differentiation simultaneously, recently, cannabinoid receptor agonists were shown to reduce concurrent Th1/Th17 cell activation [44]. This corroborates our findings that THC treatment in mBSA-induced DTH mice reduces the secretion of Th1/Th17 activating cytokines, IFN- γ and IL-17a/ f, and lineage-specific transcription factors, Tbet and ROR γ T. Additionally, recent findings from our lab showed that treatment with the endogenous cannabinoid receptor ligand anandamide (AEA) reduced elevated levels of IL-10 production and inflammation associated with mBSA-induced DTH [27]. These findings support our current research as Th2 cell secretion of IL-10 has been connected to the suppression of hallmark cytokines from both Th1 and Th17 cells [45, 46]. Furthermore, the work of Jackson et al. showed that mBSA-sensitized T cells treated with IL-10 increased miR-125a which the current study found to be dysregulated upon induction of DTH [26]. Thus, THC and other cannabinoid receptor ligands work to correct cytokine profiles and Th cell lineage commitment associated with inflammation via regulation of miRNA.

Epigenetic modifications, including miRNA expression, have been linked to fundamental biological processes including, but not limited to, cellular differentiation and proliferation [47]. As such, dysregulation of miRNA seen in disease models, including psoriasis, multiple sclerosis, and rheumatoid arthritis, has become a topic of major interest for possible therapeutic targets [37]. In the current study, we identified two miRNA and two target genes as possible therapeutic modalities: miR-21 with its target SMAD7 and miR-29b with its target IFN- γ . miR-21 has been identified to play a critical role in multiple inflammatory models, including allergic contact dermatitis, rheumatoid arthritis, and experimental autoimmune encephalomyelitis, for the regulatory role it plays in Th17 differentiation [38, 39, 48]. As suggested by Vennegaard et al. who saw elevated levels of miR-21 in the skin of mice with allergic contact dermatitis, we observed greater than twofold increase in miR-21 expression in mice with DTH response when compared to naïve controls. Additionally, miR-29b has been shown to be necessary for regulation of Th1 differentiation in mice and is also implicated in various inflammation models [49, 50]. We observed a downregulation of miR-29b greater than twofold in mice with DTH when compared to naïve controls, indicating a loss of restriction in the Th1 cell lineage and therefore allowing differentiation.

We suggest in this study that the ability of THC to impact Th cell lineage commitment stems from its ability to act as an epigenetic regulator. While only limited research exists on the role that THC plays in the regulation of epigenetic modifications, this research base is growing. THC treatment has been found to alter DNA methylation patterns in diseases which elicit inflammatory response such as, simian immunodeficiency virus (SIV) and even in regulatory immune cells including myeloid-derived suppressor cells (MDSC) [22, 51]. The role of THC in histone mark regulation, both acetylation and methylation, has only recently been studied by Yang et al. in T helper cell subsets [10]. Additionally, research centered on THC-mediated alterations of miRNA profiles is severely limited [21, 52]. Thus, to more fully understand what role THC treatment is playing in Th cell stable lineage commitment, we looked at the ability of THC to correct miRNA expression changes due to DTH. Interestingly, we saw a reversal of DTH-induced upregulation of miR-21 and miR-223, decreased by half, as well as downregulation of miR-29b and miR-142, increased by greater than fivefold. Having shown that THC treatment reduced Th1/Th17 specific cytokines and transcription factors, we focused in on miR-21 and miR-29b both of which are known to be directly involved in the Th1/ Th17 differentiation pathways. Using transfection of primary cells, we were able to prove direct targeting of SMAD7 and IFN- γ messages, by miR-21 and miR-29b, respectively.

In conclusion, the current study suggests that the elevated levels of IFN- γ seen in mice with DTH is caused by a loss of miR-29b expression (Fig. 6). Simultaneously, increased miR-21 expression and subsequent decrease in SMAD7 allowed for increased Th17 differentiation (Fig. 6). However, with THC treatment miR-29b, an inhibitor of IFN- γ , was overexpressed inhibiting IFN- γ expression and indirectly downregulating miR-21 thereby derepressing SMAD7 (Fig. 6). In short, THC treatment was able to effectively inhibit both Th1/Th17 lineages in the DTH model of hypersensitivity. Together, these findings suggest that THC can be used therapeutically in type IV hypersensitivity as well as autoimmune models where Th1/Th17 cells have been implicated in disease progression. Furthermore, our findings that THC regulates proinflammatory Th cell activation via altered miRNA expression are vital to fully elucidating the role cannabinoid receptor agonists play in adaptive immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key messages

- THC treatment inhibits simultaneous Th1/Th17 driven inflammation.
- THC treatment corrects DTH-mediated microRNA dysregulation.
- THC treatment regulates proinflammatory cytokines and transcription factors.

Fig. 1.

THC treatment reduces lymphocyte driven inflammation associated with DTH. Mice ($n = 5$ per experimental group) were sensitized, subcutaneously 1.5 mg/mL of mBSA in CFA. Sensitized mice were treated with THC (20 mg/kg) or vehicle, on days 4, 5, and 6 after sensitization. Mice were rechallenged (10 mg/mL mBSA) via a foot pad injection on day 6. Swelling was measured as

$$\frac{\text{thickness of mBSA footpad} - \text{thickness of vehicle footpad}}{\text{thickness of vehicle footpad}} \times 100\%.$$

a Schematic of

model and treatment regimen. **b** Relative percent swelling compared to naïve mouse footpad (for each hind paw foot pad). **c** Absolute cell count of draining lymph nodes (per mouse). Lymphocytes from mBSA-sensitized mice were rechallenged with mBSA, 40 mg/mL, and then cultured for 48 h. In the last 16 h of culture, the cells were pulsed with 2 μ Ci of [3 H]Thymidine. **d** The cells were harvested and analyzed for Thymidine uptake using a β -Scintillation counter. Histopathological analysis of mBSA rechallenged (DTH) footpad. The footpad was then embedded in paraffin and sectioned into 6- μ m sections and stained for hematoxylin and eosin. Mice were sensitized with mBSA, treated with either vehicle (**e**, **g**) or THC (**f**, **h**), and rechallenged with either vehicle (**e**, **f**) or mBSA (**g**, **h**). The footpad was isolated and fixed/decalcified in Cal-Rite. Representative data from replicate experiments. *Vertical bars* represent mean $\pm$ SEM. ANOVA/Tukey * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p > 0.0001$

Fig. 2.

THC treatment reduces lymphocyte activation. DTH-induced mice were treated with THC or vehicle ($n = 5$ per experimental group) as described in Fig. 1 legend. **a** Absolute cell counts CD69+ lymphocytes (per popliteal lymph node PopLN). **b** Absolute cell counts (per PopLN) of CD3 + CD69+ T cells. Splenocytes, mBSA-sensitized in vivo, were cultured overnight without mBSA to analyze spontaneous cytokine secretion. **c** Culture supernatants were analyzed for proinflammatory cytokines IFN- γ , TNF- α , and IL-17 via ELISA MAX kit (Biolegend). **(d, e, g)** cDNA was isolated from the PopLN and analyzed using qPCR. **d** Analysis of proinflammatory T cell transcription factors Tbet, ROR γ T, and IRF4. **e** Analysis

for PIK₃/Akt regulator Pten and **f** Western blot of Akt and phospho-Akt (P-Akt) in PopLN of vehicle and THC-treated DTH mice (relative intensity based on β -actin normalized P-Akt/Akt). **g** Analysis of transcription factor NF κ B. Representative data from replicate experiments. *Vertical bars* represent mean $\pm$ SEM. Student's *t* test * p <0.05; ** p <0.01; *** p <0.001; **** p >0.0001

Fig. 3.

DTH reaction causes changes to microRNA expression in immune cells. Lymphocytes from PopLN of naïve and DTH mice were assessed for miR expression. **a** Heat map highlighting the microRNA dysregulation between naïve and DTH samples ($n = 5$ per experimental group). **b** Venn diagram depicting the up- and downregulated microRNA (greater than twofold). **c** IPA ingenuity analysis of top up- and downregulated microRNA. **d, e** qPCR validation of microRNAs of interest. **d** miR-21-5p and miR-223-3p. **e** miR-29b-3p, miR-125a-5p, and miR-142-5p. Representative data from replicate experiments. *Vertical*

bars represent mean $\pm$ SEM. Student's t test $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p > 0.0001$

Fig. 4.

THC treatment reverses DTH-mediated alterations in microRNA expression. DTH-induced mice were treated with THC or vehicle as described in Fig. 1 legend. **a, b** cDNA was isolated from DTH + Veh and DTH + THC PopLN lymphocytes for validation of microRNA array using qPCR. **a** Analysis of miR-21-5p and miR-223-3p. **b** Analysis of miR-29b-3p, miR-125a-5p, and miR-142-5p. **c** IPA ingenuity software was used to determine interactions between miR-21-5p and miR29b-3p and the proinflammatory T cell pathway. Representative

data from replicate experiments. *Vertical bars* represent mean $\pm$ SEM. Student's *t* test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p > 0.0001$

nodes, from DTH mice, were transfected for 6 h and then cultured overnight (18 h). **f, g**
Validation of proinflammatory T cell gene targets (**f**) IFN- γ and **g** SMAD7. Representative
data from replicate experiments. *Vertical bars* represent mean $\pm$ SEM. Student's *t* test or
ANOVA/Tukey * $p<0.05$; ** $p<0.01$; *** $p<0.001$

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Fig. 6.

Schematic illustration of the role microRNA play in mBSA-induced DTH. mBSA-induced DTH is classically associated with elevated Th1 and Th17 response, and increased expression of miR-21. Th17 differentiation is increased via miR-21 inhibition of SMAD7. THC treatment of DTH mice causes a decrease in the proinflammatory response through microRNA regulation, increasing expression of miR-29b (inhibitory of IFN- γ) and decreasing miR-21 expression. Thus, THC treatment reduces IFN- γ production and inhibition of IL-17 regulation via SMAD7 derepression (schematic built in PhotoShop)

Table 1

Primer sequences for qPCR analysis of gene expression in popliteal lymph node cells

Gene	Primer	Sequence (5'–3')	Annealing temp
β actin	Forward	GGC TGT ATT CCC CTC CAT CG	60 °C
	Reverse	CCA GTT GGT AAC AAT GCC ATG T	
Tbet	Forward	AAC CGC TTA TAT GTC CAC CCA	
	Reverse	CTT GTT GTT GGT GAG CTT TAG C	
ROR γ T	Forward	CAA GTT TGG CCG AAT GTC C	
	Reverse	CTA TAG ATG CTG TCT CTG C	
IRF4	Forward	GTC TCA CGG GTG CTT TCT GT	
	Reverse	GGC TGG GCA TTC TCA CGA TA	
Pten	Forward	CCT TTT GAA GAC CAT AAC CCA CC	
	Reverse	GAA TTG CTG CAA CAT GAT TGT CA	
NF κ B	Forward	ATG GCA GAC GAT GAT CCC TAC	
	Reverse	TGT TGA CAG TGG TAT TTC TGG TG	
IFN- γ	Forward	GCG TCA TTG AAT CAC ACC TG	
	Reverse	GAG CTC ATT GAA TGC TTG GC	
SMAD7	Forward	GCA TTC CTC GGA AGT CAA GAG	
	Reverse	CCA GGG GCC AGA TAA TTC GT	