

Anti-inflammatory activity of topical THC in DNFB-mediated mouse allergic contact dermatitis independent of CB1 and CB2 receptors

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Abstract

Background: Δ^9 -Tetrahydrocannabinol (THC), the active constituent of *Cannabis sativa*, exerts its biological effects in part through the G-protein-coupled CB1 and CB2 receptors, which were initially discovered in brain and spleen tissue, respectively. However, THC also has CB1/2 receptor-independent effects. Because of its immune-inhibitory potential, THC and related cannabinoids are being considered for the treatment of inflammatory skin diseases. Here we investigated the mechanism of the anti-inflammatory activity of THC and the role of CB1 and CB2 receptors.

Methods: We evaluated the impact of topically applied THC on DNFB-mediated allergic contact dermatitis in wild-type and CB1/2 receptor-deficient mice. We performed immunohistochemical analyses for infiltrating immune cells and studied the influence of THC on the interaction between T cells, keratinocytes and myeloid immune cells *in vitro*.

Results: Topical THC application effectively decreased contact allergic ear swelling and myeloid immune cell infiltration not only in wild-type but also in CB1/2 receptor-deficient mice. We found that THC (1) inhibited the production of IFN γ by T cells, (2) decreased the production of CCL2 and of IFN γ -induced CCL8 and CXL10 by epidermal keratinocytes and (3) thereby limited the recruitment of myeloid immune cells *in vitro* in a CB1/2 receptor-independent manner.

Conclusions: Topically applied THC can effectively attenuate contact allergic inflammation by decreasing keratinocyte-derived pro-inflammatory mediators that orchestrate myeloid immune cell infiltration independent of CB1/2 receptors. This has important implications for the future development of strategies to harness cannabinoids for the treatment of inflammatory skin diseases.

Allergic contact dermatitis caused by small molecules (haptens) is one of the leading causes for occupational diseases. The pathophysiology has been extensively investigated in experimental mouse models of contact hypersensitivity (CHS) using obligate contact sensitizers such as DNFB (1, 2). We discovered that inflamed skin tissue in DNFB-mediated CHS contains elevated levels of endogenous cannabinoids (3, 4). Mice lacking CB1 and CB2 receptors (*Cnr1/2^{-/-}*) develop enhanced allergic ear swelling to the obligate contact sensitizer DNFB, likely through increased expression of immune cell-recruiting pro-inflammatory chemokines (3). These observations demonstrated that the endogenous cannabinoid system attenuates contact allergic dermatitis.

Skin inflammation has become an attractive target for cannabinoid-based therapies (5–7). Systemic and topical application of THC reduces DNFB-mediated CHS responses in mice (3). We initially hypothesized that THC exerts its anti-inflammatory potential through CB2 receptors, which are predominantly expressed by immune cells but also by epidermal keratinocytes (3, 7, 8). However, topical application of the CB2 agonist HU-308 increased contact allergic ear swelling, whereas the pharmacological blockade of CB2 receptors suppressed the allergic immune response in mice (3, 9). These observations indicated that CB2 receptor activation exerts pro-inflammatory effects in the skin. THC could also act through CB1 receptors, which were initially discovered

in the central nervous system but are also expressed in peripheral tissues including the skin (3, 7, 10). Alternative explanations for the anti-inflammatory effects of THC could be the activation of other G-protein-coupled receptors or receptor-independent effects (6). The objective of the present study was to further analyse the mechanism of the anti-inflammatory effect of THC and the role of CB1 and CB2 receptors in DNFB-mediated mouse allergic contact dermatitis.

Methods

Animals

Wild-type (WT) and CB1/CB2 receptor-deficient (*Cnr1*^{2^{-/-}}) C57Bl/6J mice were bred at our animal facility. All experiments were conducted according to the institutional and national guidelines for the care and use of laboratory animals and were approved by the local government authorities (LANUV NRW, Germany).

Contact hypersensitivity and THC treatment

The DNFB (1-Fluoro-2,4 dinitrobenzene, Merck, Schwalbach, Germany) was diluted in acetone/olive oil (4 : 1) immediately before use. CHS experiments were performed as previously described (3). Briefly, 8- to 12-week-old mice were sensitized by painting 50 µl of 0.2% DNFB on the shaved abdomen on two consecutive days. Mice were challenged by painting both sides of one ear with 10 µl of 0.3% DNFB on days 5 and 12. 30 µg of THC (Δ^9 -tetrahydrocannabinol, Sigma-Aldrich, Munich, Germany) was applied topically in 20 µl acetone 30 min. before as well as 24 h and 48 h after challenge. Ear swelling was determined 24 h, 48 h and 72 h after challenge by measuring the difference between the hapten-exposed and the untreated ear with a spring-loaded calliper in millimetres.

Histology

Paraffin-embedded ear tissue was stained with primary rat anti-mouse antibodies for Gr-1 (RB6-8C5, BD Pharmingen, Heidelberg, Germany) and CD3 (MCA1477, Serotec, Düsseldorf, Germany). Slides were incubated with biotinylated rabbit anti-rat IgG, followed by streptavidin-AP and colour development with Fast Red (Dako, Hamburg, Germany). Counterstaining was performed with haematoxylin. The number of Gr-1+ and CD3+ cells was determined in 5 high-power fields (HPF, 200× magnification).

Myeloperoxidase assay

Ears were harvested 48 h after the second challenge and immediately frozen in liquid nitrogen. Tissues were homogenized in HTAB buffer (50 mM KH₂PO₄ + 0.5% hexadecyltrimethylammonium pH 6.0). Homogenates were centrifuged, and 50 µl supernatant was added to 200 µl MPO reagent (50 mM potassium phosphate buffer containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.005% H₂O₂). Absorbance (OD) was measured at 460 nm and normalized to the value obtained in untreated control groups.

Antigen-specific restimulation

Mice were sensitized with DNFB as described above. On day 5, lymphocytes from spleen and draining lymph nodes were isolated. Dendritic cells generated from bone marrow precursors with GM-CSF and IL-4 (Peprotech, Hamburg, Germany) were haptenized with 2.5 mM DNBS (MP Biomedicals, Eschwege, Germany), the water-soluble analogue of DNFB. Sensitized lymphocytes (1×10^5 /well) were restimulated with haptenized DCs (1×10^4 cells/well) for 24 h in 96-well plates without or with THC diluted in ethanol/cremophor/medium (1 : 1 : 18) and added at a final concentration of 0.1 or 1 µM.

Primary keratinocyte culture

Primary epidermal keratinocytes were isolated from the skin of newborn mice according to standard procedures, resuspended in keratinocyte growth medium 2 with 0.05 mM Ca²⁺ (PromoCell, Heidelberg, Germany) and 5×10^6 cells seeded in 75-cm² flasks. Confluent keratinocyte cultures were in part exposed to 0.1 or 1 µM THC as described above. Half of the cultures were stimulated with 1000 U/ml recombinant IFN γ (R&D Systems, Wiesbaden, Germany). Culture supernatants were collected after 24 h for chemokine measurements and migration experiments.

ELISA

Cytokine and chemokine levels in supernatants were determined using sandwich ELISAs for IFN γ , CCL2, CCL8 and CXCL10 (R&D Systems) according to standard protocols.

Transwell migration assays

Macrophages generated from bone marrow precursors with fibroblast supernatant were starved for 24 h, and 1×10^5 cells/well seeded on PEG-coated membrane inserts in 24-well plates. DMEM containing 1% FBS and 100 ng/ml CCL2, CCL8 or keratinocyte supernatants were added to the lower chamber of triplicate wells. 24 h later, cells in the upper chamber were removed. Cells that migrated through the membrane were stained with 5 µM CFSE, fixed with 4% paraformaldehyde and the mean of 5 HPF counted in each well using a fluorescence microscope.

Statistical evaluation

Statistically significant differences were calculated with the nonparametric Wilcoxon–Mann–Whitney *U*-test using SPSS 12 software (SPSS, IBM, Ehningen, Germany). *P*-values are given as follows: **P*<0.05; ***P*<0.01; ****P*<0.001.

Results

Topical THC application attenuates contact allergic ear swelling independent of CB1/2 receptors

To examine the role of CB receptors for the anti-inflammatory effect of topical THC on contact allergic inflammation,

we sensitized WT and $Cnr1/2^{-/-}$ C57BL/6J mice with DNFB. Half of the mice were treated with THC on the ears immediately before as well as 24 h and 48 h after DNFB challenge on days 5 and 12 of the experiments. The other half of the mice received vehicle only and served as controls (Fig. 1A).

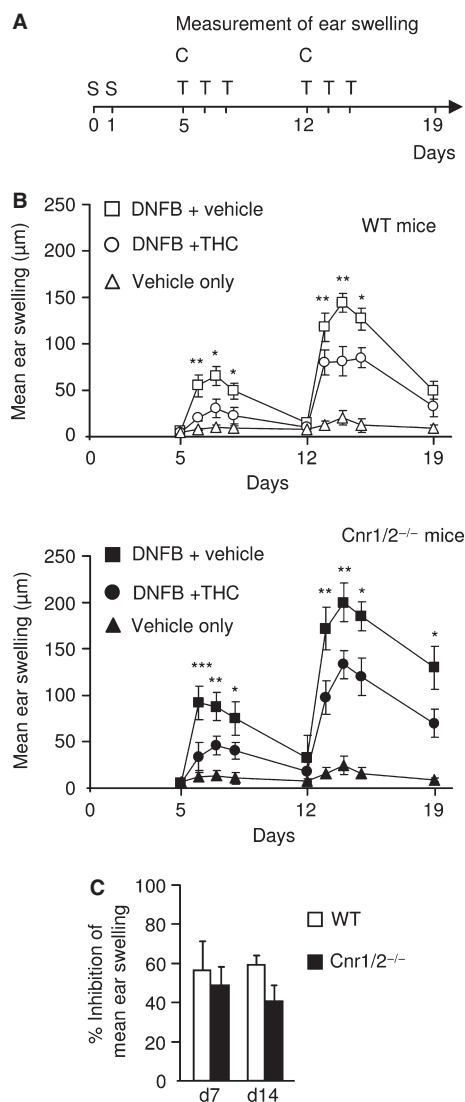


Figure 1 Topical THC application attenuates contact allergic ear swelling in WT and CB1/2 receptor-deficient mice. (A) Experimental protocol: Groups of 10 WT and $Cnr1/2^{-/-}$ C57BL/6 mice were sensitized (S) and challenged (C) with DNFB. Half of the mice were treated topically with THC (T) immediately before as well as 24 h and 48 h after challenge on days 5 and 12. The other half of the mice received vehicle only. Additional control groups of 5 WT and $Cnr1/2^{-/-}$ C57BL/6 mice were sensitized with DNFB and challenged with vehicle only. (B) Shown is the mean ear swelling in the indicated groups measured 24 h, 48 h and 72 h after challenge on days 5 and 12 ($\pm$ SEM). Similar results were obtained in three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) Shown is the relative inhibition of mean ear swelling in THC-treated compared with control WT and $Cnr1/2^{-/-}$ mice 48 h after the 1st and 2nd challenges ($\pm$ SEM).

As reported previously, contact allergic inflammation was markedly increased in $Cnr1/2^{-/-}$ compared with WT mice (3). Topical THC treatment decreased the magnitude of CHS responses to a similar extent in both WT and $Cnr1/2^{-/-}$ mice (Fig. 1B,C). These results demonstrated an anti-inflammatory effect of THC on DNFB-mediated allergic contact dermatitis independent of CB1/2 receptors.

Topical THC limits myeloid immune cell infiltration in contact allergic ear tissue independent of CB1/2 receptors

To understand how THC treatment can attenuate the CHS response to DNFB, we determined the infiltration of myeloid immune cells, which are recruited in large numbers to the site of allergen challenge during the effector phase. Inflamed ear tissue was harvested 48 h after the second challenge and stained for Gr-1, a marker expressed by granulocytes and monocytes. Consistent with our previous observations, we found increased numbers of Gr-1-positive cells in allergic ears of $Cnr1/2^{-/-}$ compared with WT mice. Topical THC treatment diminished the recruitment of Gr-1+ cells in both strains of mice to a similar extent (Fig. 2A,B). The impact of THC on myeloid immune cell infiltration independent of CB1/2 receptors was confirmed by measurements of myeloperoxidase (MPO) enzyme activity, an index for neutrophil accumulation (Fig. 2C). These results strongly confirmed the anti-inflammatory effect of THC on DNFB-mediated allergic contact dermatitis independent of CB1/2 receptors.

THC inhibits the hapten-specific IFN γ production by sensitized T lymphocytes independent of CB1/2 receptors

Next, we studied the impact of THC on T lymphocytes, the principal effector cells of contact allergic inflammation. Immunohistochemical investigations revealed similar low numbers of CD3+ T lymphocytes in inflamed ears irrespective of genotype and THC treatment (Fig. 3A,B). Because cannabinoids have been shown to impair T-lymphocyte functions including proliferation and cytokine secretion *in vitro*, we also investigated the effect of THC on the antigen-specific release of IFN γ by T lymphocytes isolated from DNFB-sensitized mice upon restimulation with haptenized dendritic cells. Sensitized $Cnr1/2^{-/-}$ T lymphocytes secreted higher levels of IFN γ compared with sensitized WT T lymphocytes (Fig. 3C). Importantly, THC inhibited the secretion of IFN γ in a dose-dependent manner in both strains of mice. These results suggest that topical THC does not impair the recruitment of T lymphocytes to the site of allergen challenge, but limits their antigen-specific effector functions.

THC decreases the production of immune cell-recruiting pro-inflammatory chemokines by keratinocytes independent of CB1/2 receptors

Epidermal keratinocytes are important regulators of skin inflammation (11). Through the production of pro-inflammatory

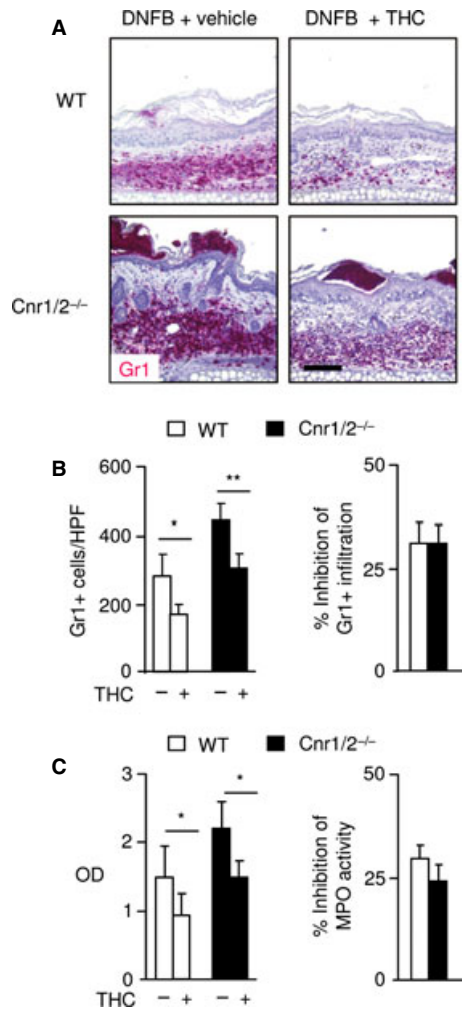


Figure 2 Topical THC application decreases myeloid immune cell infiltration in contact allergic ear tissue in WT and CB1/2 receptor-deficient mice. (A) Ear tissue of mice treated as indicated was harvested 48 h after the second challenge with DNFB. Shown are representative immunohistochemical stains for the myeloid immune cell marker Gr-1 in inflamed ear tissue of WT and Cnr1/2^{-/-} mice treated as indicated (bar = 100 μ m). (B) Left: Mean numbers of Gr-1+ cells per HPF in contact allergic ear tissue of five mice ($\pm$ SEM) in the indicated groups. Right: Relative inhibition of Gr-1+ immune cell infiltration in THC-treated compared with control WT and Cnr1/2^{-/-} mice ($\pm$ SEM). (C) Left: Mean MPO activity in contact allergic ear tissue of five mice ($\pm$ SEM) in the indicated groups. Right: Relative inhibition of MPO activity in THC-treated compared with control WT and Cnr1/2^{-/-} mice ($\pm$ SEM). * P < 0.05; ** P < 0.01.

chemokines, they recruit myeloid immune cells to the site of allergen challenge and thereby orchestrate the allergic inflammatory reaction. Cultured primary keratinocytes show an inflammatory phenotype and constitutively produce CCL2. THC inhibited the CCL2 production in a dose-dependent manner in both WT and CB1/2 receptor-deficient keratinocytes (Fig. 4A). In response to T-cell-derived IFN γ , keratinocytes

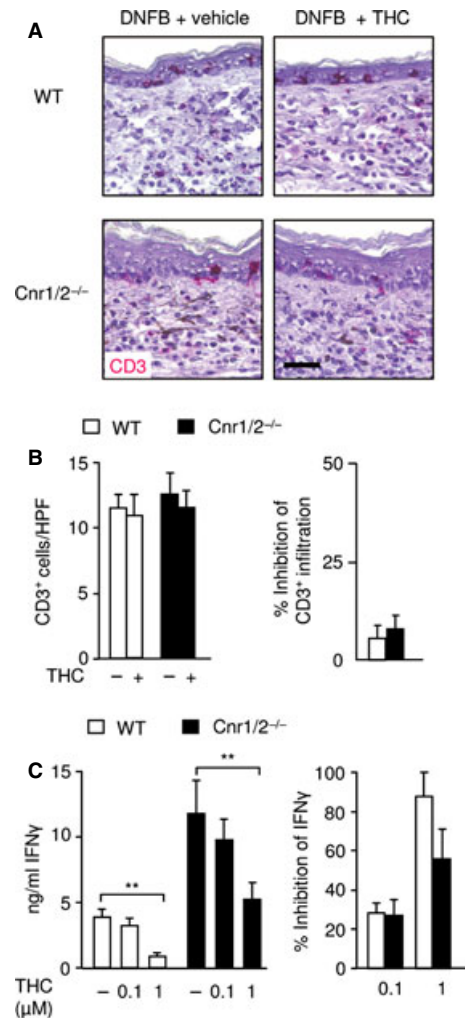


Figure 3 Δ^9 -Tetrahydrocannabinol inhibits the hapten-specific IFN γ production by T lymphocytes isolated from sensitized WT and CB1/2 receptor-deficient mice. (A) Groups of 5 WT and Cnr1/2^{-/-} C57BL/6 mice were sensitized and challenged with DNFB with or without THC treatment as described. Ear tissue was harvested 48 h after the second challenge. Representative immunohistochemical stains for the T-lymphocyte marker CD3 are shown (bar = 50 μ m). (B) Left: Mean numbers of CD3+ cells per HPF in contact allergic ear tissue of five mice ($\pm$ SEM) in the indicated groups. Right: Relative inhibition of CD3+ T-lymphocyte infiltration in THC-treated compared with control WT and Cnr1/2^{-/-} mice ($\pm$ SEM). (C) Left: Mean IFN γ levels measured by ELISA in the supernatant of T lymphocytes isolated from sensitized WT and Cnr1/2^{-/-} mice following restimulation with haptenized WT and Cnr1/2^{-/-} dendritic cells and exposure to THC as indicated ($\pm$ SEM of three independent cultures). Right: Relative inhibition of IFN γ production by THC-exposed T lymphocytes compared with controls ($\pm$ SEM). ** P < 0.01.

upregulate the production of CCL8 and CXCL10, which further drives myeloid immune cell recruitment. In line with our previous observations, we found increased CCL8 levels in

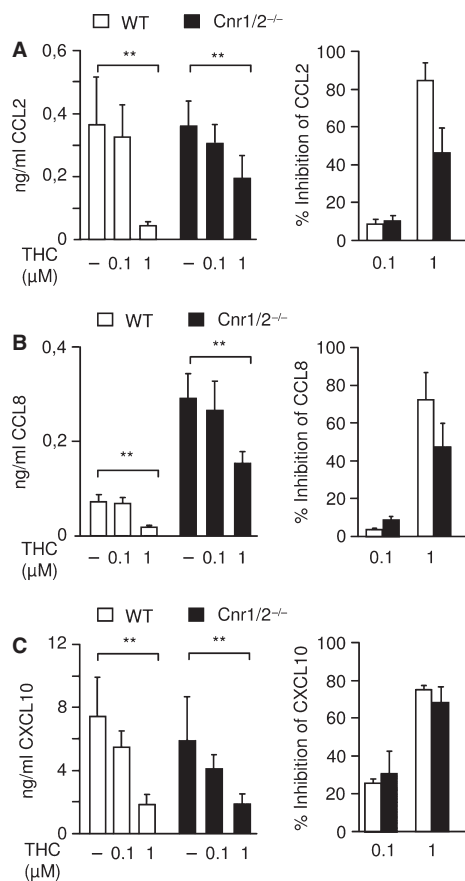


Figure 4 Δ^9 -Tetrahydrocannabinol decreases the production of immune cell-recruiting pro-inflammatory chemokines by cultured primary keratinocytes derived from WT and CB1/2 receptor-deficient mice. (A) Left: Mean CCL2 levels measured by ELISA in the supernatants of primary WT and Cnr1/2^{-/-} keratinocytes exposed to THC as indicated ($\pm$ SEM of 10 independent cultures). Right: Relative inhibition of CCL2 production by THC-exposed keratinocytes compared with controls ($\pm$ SEM). (B) Left: Mean CCL8 levels measured by ELISA in the supernatant of IFN γ -stimulated primary WT and Cnr1/2^{-/-} keratinocytes exposed to THC as indicated ($\pm$ SEM of 10 independent cultures). Right: Relative inhibition of CCL8 production by THC-exposed keratinocytes compared with controls ($\pm$ SEM). (C) Left: Mean CXCL10 levels measured by ELISA in the supernatant of IFN γ -stimulated primary WT and Cnr1/2^{-/-} keratinocytes exposed to THC as indicated ($\pm$ SEM of 10 independent cultures). Right: Relative inhibition of CXCL10 production by THC-exposed keratinocytes compared with controls ($\pm$ SEM). ** $P < 0.01$.

IFN γ -exposed CB1/2 receptor-deficient keratinocytes (Fig. 4B). Importantly, THC also inhibited the IFN γ -induced production of CCL8 and CXCL10 in a dose-dependent fashion in both WT and CB1/2 receptor-deficient keratinocytes (Fig. 4B,C). These results indicate that THC limits the production of immune cell-recruiting pro-inflammatory chemokines by keratinocytes in a CB1/2 receptor-independent manner.

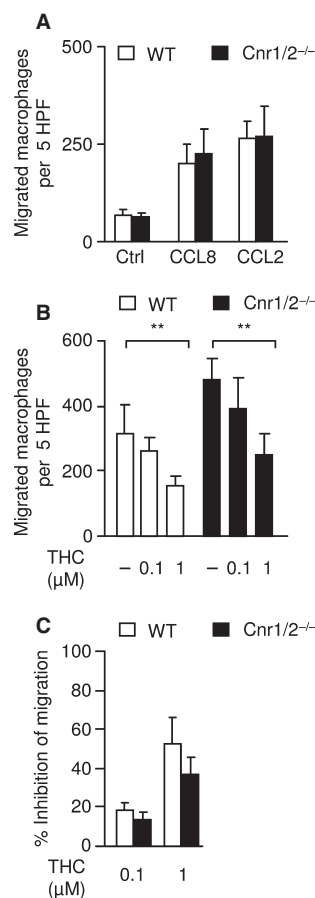


Figure 5 Macrophage migration in response to supernatants of THC-exposed, IFN γ -activated keratinocytes is reduced compared with controls in both WT and CB1/2 receptor-deficient experimental systems. (A) Mean number of migrated WT and Cnr1/2^{-/-} macrophages in response to medium containing 1% FBS alone, supplemented with CCL2 or with CCL8 ($\pm$ SEM). (B) Mean number of migrated macrophages in response to culture supernatants derived from IFN γ -activated keratinocytes exposed to THC as indicated in WT and CB1/2 receptor-deficient experimental systems ($\pm$ SEM). ** $P < 0.05$. (C) Relative inhibition of macrophage migration of THC-exposed cultures compared with controls ($\pm$ SEM).

Macrophage migration in response to supernatants of THC-exposed, IFN γ -activated keratinocytes is decreased compared with controls independent of CB1/2 receptors

We hypothesized that the decreased production of pro-inflammatory chemokines by THC-treated IFN γ -activated keratinocytes *in vitro* contributes to the decreased myeloid immune cell infiltration upon topical THC application *in vivo*. To support this hypothesis, we performed macrophage transwell migration experiments. The chemokines CCL2 and CCL8, which are produced by IFN γ -activated keratinocytes, effectively promoted migration of CCR2-expressing WT and Cnr1/2^{-/-} macrophages generated from bone marrow precursors (Fig. 5A). Macrophage migration was reduced in

response to supernatants of THC-exposed IFN γ -activated keratinocytes compared with controls in both WT and CB1/2 receptor-deficient experimental systems (Fig. 5B,C).

Discussion

In our work, we demonstrated that topical application of the plant-derived cannabinoid THC has an anti-inflammatory effect on DNFB-mediated mouse allergic contact dermatitis independent of CB1 and CB2 receptors. Histopathological analyses of inflamed ear tissue revealed that THC diminished the number of infiltrating Gr-1⁺ myeloid immune cells not only in WT but also in CB1 and CB2 receptor-deficient mice. This finding was confirmed by measuring MPO enzyme activity, an index for neutrophil accumulation. Our observations are consistent with a report that the chronic systemic application of THC attenuates OVA-induced allergic airway inflammation in C57Bl/6 mice as indicated by a reduced number of lung-infiltrating immune cells and of the serum IgE and IgG levels. In this model, the suppressive effect of THC on serum immunoglobulins was also observed in Cnr1/2^{-/-} mice (12).

We further investigated the mechanism of the CB1/2 receptor-independent anti-inflammatory effects of THC on contact allergic skin inflammation. Memory T lymphocytes are the key effector cells in the elicitation phase of CHS responses to DNFB. *In vitro* experiments revealed an inhibitory effect of THC on hapten-specific IFN γ production by T lymphocytes isolated from sensitized WT and Cnr1/2^{-/-} mice after restimulation with haptenized WT and Cnr1/2^{-/-} dendritic cells, respectively. This result suggested that topically applied THC impairs effector functions of allergen-specific T lymphocytes in the skin of both mouse strains. Our results agree with several publications reporting effects of THC on immune cell functions. Plant-derived cannabinoids have been shown to modulate cytokine secretion, cell proliferation, migration and apoptosis of mouse and human B and T lymphocytes as well as macrophages (13). For example, THC inhibited the proliferation of mouse lymphocytes and decreased the production of IL-2 and IFN γ (14, 15). THC also reduced the secretion of pro-inflammatory cytokines like IL-1 α , IL-1 β and TNF α in microglial cells. This effect was not inhibited by the CB1 receptor antagonist SR141716 or the CB2 receptor antagonist SR144528, suggesting a CB1/2 receptor-independent mechanism (16).

We also found that THC influenced the function of epidermal keratinocytes. These cells are the major target for topically applied THC in the skin. In the elicitation phase of the CHS response to DNFB, IFN γ secreted by allergen-specific effector T lymphocytes activates epidermal keratinocytes and induces the production of pro-inflammatory chemokines including CCL8 and CXCL10 (11). These chemokines help to orchestrate the allergic inflammatory reaction. THC suppressed the secretion of these pro-inflammatory chemokines by IFN γ -activated WT and Cnr1/2^{-/-} keratinocyte cultures. Macrophage migration experiments with supernatants of THC-exposed, IFN γ -activated keratinocytes *in vitro* supported our hypothesis that the inhibitory effect of THC on the production chemokines in the epidermis contributes to

the reduced recruitment of myeloid immune cells to the site of allergen challenge *in vivo*. Our results agree with several studies in the human system. For example, it was reported that the endogenous cannabinoid anandamide also inhibited the secretion of pro-inflammatory chemokines by human keratinocytes *in vitro*, independent of CB1 receptors as shown by blocking experiments with the CB1 receptor antagonist SR141716 (17). In another study, phytocannabinoids like cannabidiol, cannabidiol and THC were shown to exert a suppressive effect on human keratinocyte proliferation. Here, the synthetic CB receptor agonists HU-210, a structural analogue of THC, displayed an inhibitory effect on cell proliferation, which could not be blocked pharmacologically by CB1 and CB2 receptor inhibitors (18).

The molecular mechanism of how THC exerts its anti-inflammatory potential in Cnr1/2^{-/-} mice on the subcellular level remains to be determined. Due to their lipophilic nature, plant-derived cannabinoids are able to interact directly with cell membranes and change membrane lipid order and diffusion (19). These membrane perturbations may depend on higher concentrations of exogenously applied cannabinoids beyond physiological concentrations of endogenously released ligands, which may be reached in the epidermis following topical application of THC (20, 21). As an alternative possibility, THC could also act on other receptors. The transient receptor potential channel ankyrin 1 (TRPA1) expressed on sensory nerve fibres and epidermal keratinocytes (22, 23) is one example of a potential target for THC. TRPA1 activation induces the secretion of pro-inflammatory mediators in keratinocytes (24). As THC can bind to TRPA1, it may interfere with TRPA1 functions in keratinocytes. Another potential target for THC is the G-protein-coupled receptor GPR55, which has just recently been shown to be expressed in the skin where it supports keratinocyte proliferation (25). Plant-derived exogenous cannabinoids including THC and endocannabinoids like AEA have been shown to bind to GPR55 *in vitro* (25–27). The biological activity of THC at this receptor is not yet known. Finally, THC may exert its anti-inflammatory activity through the stimulation of nuclear peroxisome proliferator-activated receptors (PPAR) (28).

In conclusion, the data presented here clearly demonstrate the anti-inflammatory activity of the plant-derived cannabinoid THC beyond its interaction with specific CB1 and CB2 receptors. This is reminiscent of palmitoylethanolamide (PEA), an endogenous fatty acid with cannabimimetic properties that attenuates skin inflammation, although it does not bind to CB1 or CB2 receptor. Both THC and PEA can inhibit the secretion of pro-inflammatory chemokines in human keratinocytes and reduce acute skin inflammation in mice (23). Topical application of palmitoylethanolamide was also shown to have a profound antipruritic effect in patients in a clinical study (29). Taken together, these data warrant further exploration of the CB1/CB2 receptor-independent effects of THC, PEA and similar cannabinoids on the skin. These studies may open up new avenues for the topical treatment of inflammatory skin diseases with anti-inflammatory lipids.

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Author contributions

E.G. performed all mouse contact hypersensitivity experiments *in vivo* including histopathological analyses and MPO

assays. N.G. and M.C. performed all experiments with cultured keratinocytes *in vitro*. E.G. and T.T. conceived and supervised all aspects of the project, designed experiments, interpreted the data and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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