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Differential role of cannabinoids in the pathogenesis of skin cancer

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ABSTRACT

Aim: Cannabinoids (CB) like Δ^9-tetrahydrocannabinol (THC) can induce cancer cell apoptosis and inhibit angiogenesis. However, the use of cannabinoids for the treatment of malignant diseases is discussed controversially because of their immunomodulatory effects which can suppress anti-tumor immunity. Here we investigated the role of exogenous and endogenous cannabinoids in mouse skin cancer.
Main methods: First we examined the effect of THC, which binds to CB receptors (CB1, CB2), on the growth of the mouse melanoma cell lines B16 and HCmel12 *in vitro* and *in vivo* in wild type (WT) and CB1/CB2-receptor deficient mice (Cnr1/2$^{-/-}$). Next we evaluated the role of the endogenous cannabinoid system by studying the growth of chemically induced melanomas, fibrosarcoma and papillomas in WT and Cnr1/2$^{-/-}$ mice.
Key findings: THC significantly inhibited tumor growth of transplanted HCmel12 melanomas in a CB receptor-dependent manner *in vivo* through antagonistic effects on its characteristic pro-inflammatory microenvironment. Chemically induced skin tumors developed in a similar manner in Cnr1/2$^{-/-}$ mice when compared to WT mice.
Significance: Our results confirm the value of exogenous cannabinoids for the treatment of melanoma but do not support a role for the endogenous cannabinoid system in the pathogenesis of skin cancer.

1. Introduction

The endogenous cannabinoid system (ECS) consists of specific G-protein coupled receptors (CB1, CB2), their lipid ligands (endocannabinoids) and the enzymes for their synthesis and degradation. The ECS has a protective physiologic role in the central nervous system (CNS) by adjusting synaptic inputs and limiting excessive neuronal activity [21]. It has also been shown to participate in the downregulation of inflammatory immune responses using *in vivo* in models for atherosclerosis, inflammatory bowel disease (chemically induced colitis) and contact allergic inflammation [17,22,29].

In the last years, many studies have explored the therapeutic use of exogenous cannabinoids (CB) like Δ^9-tetrahydrocannabinol (THC) or the pharmacological modulation of the endocannabinoid system for the treatment of malignant tumors. Using different *in vitro* and *in vivo* models for glioblastoma multiforme [13], thyroid carcinoma [2] and breast cancer [14] it has been demonstrated that cannabinoids are able to inhibit tumor growth. They exert their anti-tumor effects in part by directly acting on cancer cells, thereby affecting cell proliferation or programmed cell death [6]. Additionally, cannabinoids are able to modulate tumor progression through their effects on neo-angiogenesis [4], cell migration and the immune system [27]. Nevertheless, due to their immunosuppressive potential, a tumor promoting effect of cannabinoids has also been described. In an experimental mouse model of lung cancer the chronic application of the CB1/CB2 receptor agonist THC leads to an increased tumor growth *in vivo* [36]. Similar results were found in a model for breast cancer [25].

Cannabinoids and the endogenous cannabinoid system also regulate immune responses and tumor growth in the skin. For example, the ECS attenuates cutaneous allergic inflammation [17] and promotes epidermal barrier functions [12]. Using synthetic CB receptor ligands like WIN-55,212-2 it has been shown that cannabinoid receptors are involved in the growth regulation of subcutaneously inoculated melanoma and basal cell carcinoma cell lines in wild type and nude mice [3,7]. To further study the role of cannabinoids and the ECS in the skin, we examined the effect of systemically applied THC on the growth of transplantable melanoma cell lines in wild type and CB1/CB2-receptor deficient animals (Cnr1/2$^{-/-}$). Additionally, we investigated the pathogenesis of chemically induced fibrosarcomas, papillomas and melanomas in Cnr1/2$^{-/-}$ mice.

2. Materials and methods

2.1. Animals

CB1 receptor-deficient (Cnr1$^{-/-}$) and CB2 receptor-deficient (Cnr2$^{-/-}$) animals have been previously described [5,37]. CB1/CB2-receptor-deficient mice (Cnr1/2$^{-/-}$) and their wild type (WT) controls were bred at our animal facility. Hgf-Cdk4^{R24C} mice were bred as described previously [30]. All mice were maintained on the C57BL/6

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background. Cnr1/2$^{-/-}$ mice were crossed into the Hgf-Cdk4^{R24C} melanoma mouse model to generate mice with a dark skin phenotype which develop CB1 and CB2 receptor-deficient melanomas. 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 (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany).

2.2. Reagents

Δ9-Tetrahydrocannabinol ethanol solution (THC), 7,12-dimethylbenz(a)anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA) and 3-methylcholanthrene (MCA) were purchased from Sigma-Aldrich.

2.3. Cell culture

The melanoma cell line HCmel12 was established from a primary DMBA-induced HGF-CDK4^{R24C} melanoma [1]. HCmel12 and B16 melanoma cells were routinely cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS (Biochrome), 2 mM L-glutamine, 10 mM non-essential amino acids, 1 mM Hepes (all from Life Technologies), 20 μM 2-mercaptoethanol (Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

2.4. In vitro effects of THC on melanoma cell growth

B16 and HCmel12 cells were cultured as described and seeded in 6 well plates (1×10^4 cells/well). THC was diluted in ethanol/chremophor/medium (1:1:18) and added in various concentrations (5 μM, 10 μM). Control cells were treated with vehicle only. Cell growth was documented by counting cells after 24 h, 48 h and 72 h.

2.5. RT-PCR

Cells were harvested and immediately snap-frozen in liquid nitrogen. Total RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel) and was reverse-transcribed using Superscript III (Invitrogen). Quantitative PCR was performed using 3 μg cDNA and Fast SYBR Green Master Mix (ABI). Relative gene expression was calculated using the 2-dCt method. Sequences of primers from 5′ to 3′: **CB1** *TCCTCTACGTGGGCTCAAATGACA* (forward), *GTGTCTCCTGCTGGAACCAACGG* (reverse), ***CB2*** *TGGTGCTGGCTGTGCTG* (forward), TAACAAGGCACAGCATGGAA (reverse), ***UbiC*** *AGGCAAGACCATCACCTTGGACG* (forward), and *CCATCACACCCAAGAACAAGCACA* (reverse).

2.6. Transplantable melanoma model

1×10^5 B16 or HCmel12 melanoma cells were injected intracutaneously (i.c.) into the flanks of WT and Cnr1/2$^{-/-}$ animals. THC was diluted as described and mice received daily subcutaneous (s.c.) injections (5 mg/kg body weight). Control mice received the appropriate vehicle solution only. Tumor development was monitored by inspection and palpation. Tumor sizes were measured and recorded as $0.5 \times$ length $\times$ width $\times$ $0.5 \times$ (length + width). Mice with tumors exceeding 500 mm^3 were sacrificed. All experiments were performed in groups of five mice and repeated independently at least twice.

2.7. Methylcholanthrene-induced skin carcinogenesis

WT and Cnr1/2$^{-/-}$ mice were inoculated s.c. in the hind flank with 100 μg of 3-methylcholanthrene (MCA) in 0.1 ml of olive oil. Development of fibrosarcomas was monitored periodically over the course of 100–200 days. Tumors >2 mm in diameter and demonstrating progressive growth were recorded as positive.

2.8. DMBA/TPA-induced papillomas

8–10 week old WT and Cnr1/2$^{-/-}$ mice were treated once with 100 nmol DMBA in 200 μl acetone on the shaved back skin. Seven days later treatment with 10 nmol TPA in 200 μl acetone was initiated and TPA was applied topically twice per week. Incidence was calculated and numbers of papillomas per mouse were counted.

2.9. DMBA-induced primary melanomas

8–10 week old Hgf-Cdk4^{R24C} or Hgf-Cdk4^{R24C} $\times$ Cnr1/2$^{-/-}$ mice were shaved on the back and treated locally with 100 nmol DMBA solved in 200 μl acetone to accelerate and synchronize melanomagenesis. Tumor development was monitored by inspection and palpation. When progressively growing tumors exceeded 2 mm in diameter, they were considered as melanomas. Incidence was calculated and numbers of melanomas per mouse were counted.

Mice with melanomas larger than 10 mm in diameter were sacrificed.

2.10. Flow cytometry

Hcmel12 melanomas were dissociated mechanically before incubation in 1 mg/ml collagenase D + 0.02 mg/ml DNAseI (Roche, Germany) in PBS containing 5% FBS (Biochrom, Germany) for 30 min at 37 °C. Staining was performed with the fluorochrome-conjugated antibodies against CD45, CD11b and Gr-1 (BD Biosciences). Gr1- and CD11b-positive cells were analyzed in the CD45+ gate. Fluorescence was measured with a FACSCanto flow cytometer system and data analyzed with FlowJo software.

2.11. Statistical analyses

Statistically significant differences were calculated with Student's t test using SPSS 12 software and two-tailed p values are given as follows: $^*p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Effect of THC on melanoma cell growth in vitro

Previous studies described that cannabinoids are able to inhibit or promote the growth of various melanoma cell lines *in vitro* [15,16]. Based on these contradictory findings we evaluated the effect of the plant-derived cannabinoid THC, which binds to both known CB receptors, on the growth of the murine melanoma cell lines HCmel12 and B16. As shown in Fig. 1A, CB1 and CB2 receptors can be detected on these cell lines, even though their expression levels are relatively low. Melanoma cells were cultured in the presence of 5 μM or 10 μM THC. Viable cells were counted after 24 h, 48 h and 72 h using the trypan blue dye exclusion assay. The treatment with THC had no effect on cell proliferation of HCmel12 or B16 cells *in vitro* (Fig. 1B)

3.2. Effect of THC on melanoma cell growth in the transplantable tumor model

In a next set of experiments we evaluated the effect of THC on the growth of HCmel12 or B16 cells *in vivo*. HCmel12 or B16 melanoma cells were injected subcutaneously into the flanks of wild type animals. Additionally, mice received daily injections of THC or were treated with vehicle only (Fig. 2A). Independent of the treatment with THC, HCmel12 melanoma bearing mice developed palpable tumors after 11 days. After an average of 25 days vehicle-treated mice were sacrificed since melanomas reached a volume of 500 mm³. In contrast, THC treatment significantly reduced the growth of HCmel12 melanomas *in vivo* with tumors only reaching 250 mm³ after 25 days (Fig. 2B, top). The growth

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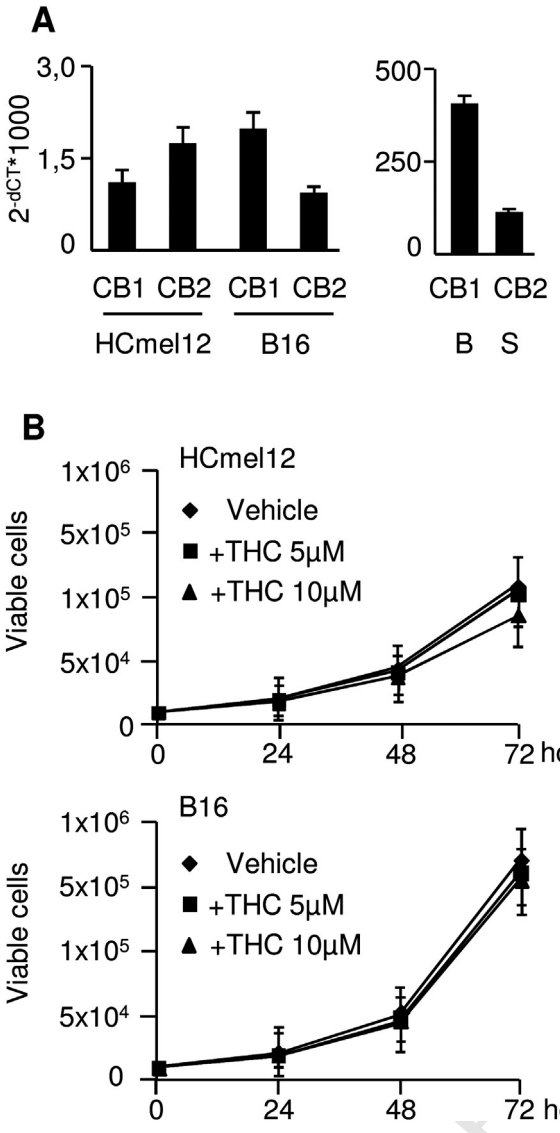

Fig. 1. THC does not inhibit the growth of melanoma cell lines *in vitro*. (A) Expression of CB1 and CB2 receptors in HCmel12 (HC) and B16 melanoma cell lines as measured by quantitative PCR. Spleen (S) and brain (B) tissue were used as a positive control. (B) HCmel12 (top) and B16 (bottom) melanoma cell lines were cultured in 1×10^4 cells/well. THC was added in various concentrations (5 μM, 10 μM), and control cells were treated with vehicle only. Viable cells were counted at the indicated time points.

of B16 melanomas was not affected through the systemic application of THC (Fig. 2B, bottom).

It has been reported previously that the *in vivo* effects of THC are in part independent of CB1- or CB2 receptors [11,28,32]. To evaluate CB receptor independent effects of THC, experiments with HCmel12 cells were repeated in mice lacking CB1- and CB2 receptors (Cnr1/2$^{-/-}$) in comparison to wild type animals. As shown in Fig. 2C there was no significant difference in the growth kinetics of HCmel12 melanomas in wild type or Cnr1/2$^{-/-}$ animals treated with THC pointing to a CB receptor dependent effect.

Since THC did not influence the growth of melanoma cells *in vitro* we hypothesized that the inhibitory effect on the transplantable tumor model may be due to effects on the interaction with immune cells and/or effects on tumor angiogenesis. Flow cytometric analyses of HCmel12 melanomas from THC-treated animals revealed a reduced infiltration of melanomas with CD45+ immune cells. CD45+ cells largely consist of myeloid derived macrophages and neutrophils. Both populations were significantly reduced in tumors of THC treated

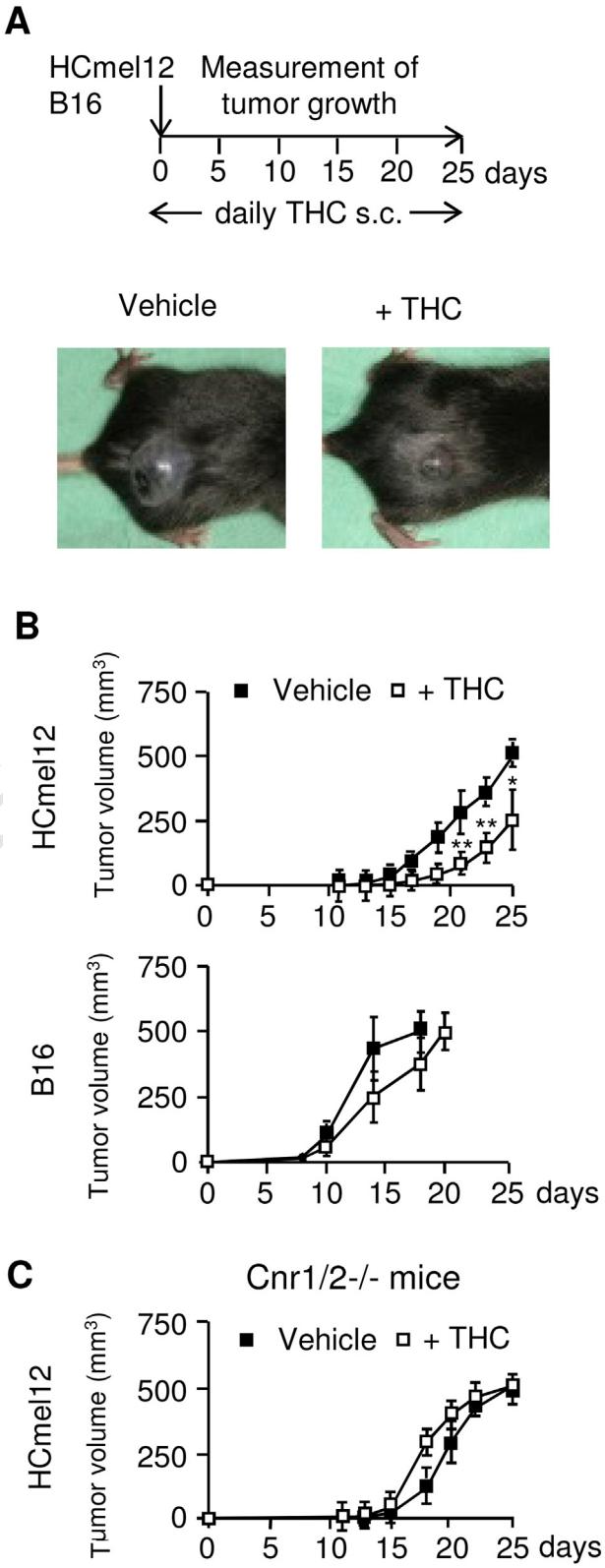

Fig. 2. THC inhibits growth of HCmel12 melanomas and decreases inflammatory immune cell infiltrates in the tumor microenvironment *in vivo*. (A) Experimental protocol: HCmel12 or B16 melanoma cells were injected into mice. THC was applied daily, and controls received vehicle only. Tumor growth was monitored over time. (B) Growth of HCmel12 (top) and B16 (bottom) melanoma cell lines in wild type (WT) animals. Representative pictures of HCmel12 tumor growth ± THC are shown. (C) Growth of HCmel12 in CB1 and CB2 receptor-deficient animals (Cnr1/2$^{-/-}$). Shown is the tumor volume in the indicated groups measured over time (±SEM). Similar results were obtained in three independent experiments. *$p < 0.05$; **$p < 0.01$.

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animals in comparison to controls (Fig. 3A). In contrast, the density of blood vessels was not significantly affected (Fig. 3B).

3.3. Role of the endogenous cannabinoid system on the growth of chemically induced skin tumors

To evaluate if the endogenous cannabinoid system inhibits or promotes the development of skin tumors we used three different mouse chemical carcinogenesis models. For the induction of fibrosarcomas, wild type and Cnr1/2$^{-/-}$ were inoculated once with 3-methylcholanthrene subcutaneously. Then tumor growth at the site of injection was monitored. As shown in Fig. 4A we did not find a significant difference in the development of fibrosarcomas in WT and CB receptor-deficient animals. In a next set of experiments we used the two-stage DMBA-TPA model for the initiation and promotion of skin papillomas. WT and Cnr1/2$^{-/-}$ animals were treated once with DMBA on the shaved back skin followed by TPA application twice a week. Both strains developed papillomas and there was no difference in the number of papillomas per mouse between WT and Cnr1/2$^{-/-}$ animals (Fig. 4B). To evaluate the impact of the ECS on the pathogenesis of melanomas Cnr1/2$^{-/-}$ animals were crossed with melanoma-prone Hgf-Cdk4^{R24C} mice. Here, the development of melanomas can be induced through a single epicutaneous application of DMBA [20,31]. 8–10 week old Hgf-Cdk4^{R24} and Hgf-Cdk4^{R24}-Cnr1/2$^{-/-}$ were treated on the shaved back skin and tumor development was monitored over time. We found no difference in melanoma incidence or the number of melanomas per mouse between the two strains (Fig. 4C). These results indicate that the endogenous cannabinoid system does not influence the development of chemically induced skin tumors.

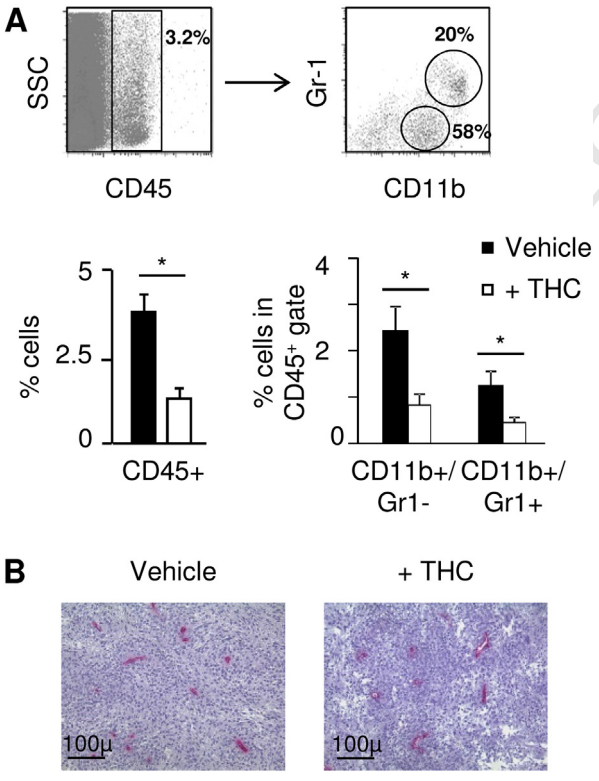

Fig. 3. THC decreases inflammatory immune cells in the microenvironment of HCmel12 melanomas. (A) HCmel12 tumors were taken on day 25, digested to prepare single-cell suspensions of the tissue, and stained with fluorescent Abs to identify infiltrating immune cells. Top: Flow cytometric dot plots for Gr1 and CD11b on CD45+ immune cells in tumors. Bottom: Analysis of infiltrating CD45+ immune cells and of tumor infiltrating Gr1+/CD11b+ immune cells in the CD45+ gate (n = 10/group, ±SEM). *p < 0.05. (B) Representative immunohistochemical stains for the blood vessel marker MECA (red) in HCmel12 tumors treated as indicated are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this chapter.)

4. Discussion

In this study, we first investigated the impact of the plant-derived cannabinoid tetrahydrocannabinol (THC) on the growth of the mouse melanoma cell lines HCmel12 and B16. We found that THC did not affect the growth of both melanoma cell lines *in vitro*. This is in contrast to publications of other groups who showed that the treatment with CB receptor agonists or antagonists influences the growth of tumor cells. Q4 Blázquez et al. demonstrated that CB receptors are expressed on human melanomas and melanoma cell lines and that THC as well as the synthetic agonist WIN 55,212-2 reduced the number of viable mouse and human melanoma cells *in vitro* in a CB receptor-dependent manner [3]. WIN 55,212-2 also inhibited the growth of mouse tumorigenic epidermal cell lines including PDV.C57 and HaCa4 cells [7]. One explanation for the lack of THC effects in our melanoma cell lines may be the very low expression levels of CB1 and CB2 receptors. McKallip and colleagues reported that human and mouse breast cancer cell lines, which do not express CB receptors, also did not respond to THC treatment [25]. Here, transfection of CB receptors into our cell lines might help to elucidate the role of direct cannabinoid receptor-dependent effects of THC on melanoma cells.

In our transplantable mouse tumor model the systemic application of THC significantly reduced the growth of HCmel12 melanomas when compared to vehicle-treated controls. This effect was not observed in mice lacking CB1 and CB2 receptors (Cnr1/2$^{-/-}$). Since THC had no direct effect on HCmel12 cell growth *in vitro* we hypothesized that it might modulate melanoma growth *in vivo* indirectly through effects on the tumor microenvironment. HCmel12 melanomas are characterized by the infiltration with pro-tumorigenic myeloid immune cells in their microenvironment [1]. The immunomodulatory properties of cannabinoids are well established. Depending on the cell type or the experimental set-up they have been shown to exert inhibitory or stimulatory effects on the immune system. THC affects the co-stimulatory activity of macrophages [5] and inhibits the cytolytic potential of natural killer cells [23]. Additionally, it suppresses the proliferation, signal transduction and IL-2 production of T-cells *in vitro* [10,26] and inhibits the development of Th1-cells [19]. Using the experimental mouse model of contact hypersensitivity we recently demonstrated that the systemic and topical application of THC attenuates contact allergic ear swelling and limits the local infiltration of immune cells [11]. Oral administration of THC also significantly reduced the recruitment of macrophages in an established model of atherosclerosis [29]. In a similar manner THC decreased the number of macrophages and neutrophils in HCmel12 melanoma tissues in our experiments. Taken together, we conclude from our results that THC antagonizes the infiltration of pro-tumorigenic myeloid immune cells in the microenvironment of HCmel12 melanomas that are known to drive their growth. The reduced recruitment of inflammatory immune cells into Hcmel12 tumors might result from a modified cytokine and chemokine expression pattern in THC-treated animals. We showed that the administration of THC diminished the number of infiltrating myeloid immune cells during contact allergic inflammation. This was due to the decreased production of immune cell-recruiting pro-inflammatory chemokines including CCL2 and CCL8 [11]. In atherosclerosis THC inhibited the migration of monocytes and macrophages through modulation of the CCL2 receptor, CCR2 [29]. Furthermore, exogenous cannabinoids including THC are also known to suppress immune responses *in vivo* and *in vitro* through their ability to induce apoptosis in T- and B-lymphocytes or dendritic cells [9,24].

Besides its impact on the immune system, THC may also affect tumor angiogenesis. Casanova et al. demonstrated that the systemic application of the synthetic CB agonists WIN 55,212-2 and JWH-133 significantly inhibited the growth of subcutaneously inoculated melanoma and basal cell carcinoma cell lines in wild type and nude mice. This effect was due to a reduced expression of pro-angiogenic factors and a decrease in blood vessel size in tumor tissue. Similar results were

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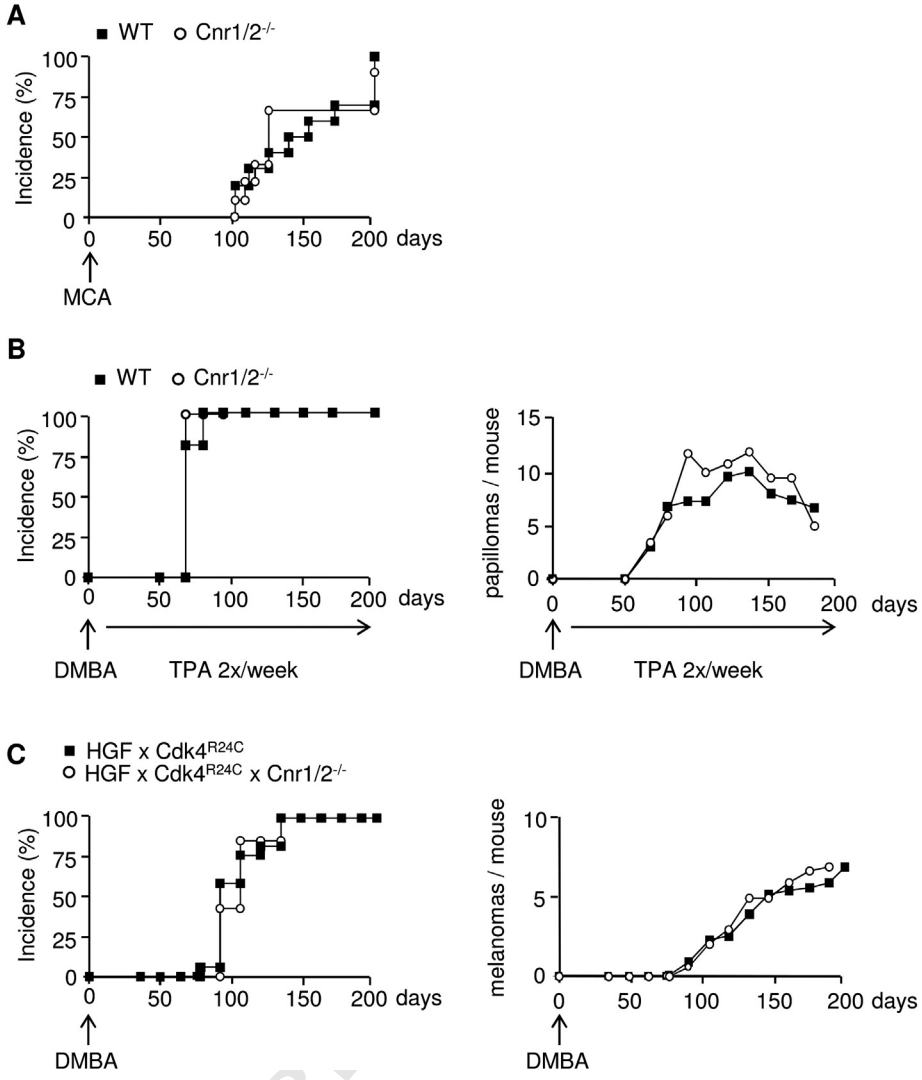

Fig. 4. The endogenous cannabinoid system has no effect on the pathogenesis of chemically induced skin tumors *in vivo*. (A) Methylcholanthrene was inoculated s.c. into wild type (WT) and CB1/CB2 receptor deficient mice ($Cnr1/2^{-/-}$). Shown is the percentage of mice with fibrosarcomas over time (n = 20 mice/group). (B) Cohorts of 8–10 weeks old WT and $Cnr1/2^{-/-}$ mice were treated once with 100 nmol DMBA followed by treatment with 10 nmol TPA twice a week. Left: Shown is the percentage of papilloma-bearing mice over time. Right: Shown is the average number of papillomas developing in the different cohorts of mice over time. Scoring was performed on a weekly basis (n = 20 mice/group). (C) Cohorts of 8–10 weeks old $HgfxCdk4^{R24C}$ and $HgfxCdk4^{R24C}$ $Cnr1/2^{-/-}$ mice were treated once with 100 nM DMBA. Left: Shown is the percentage of melanoma-bearing mice over time. Right: Shown is the average number of melanomas developing in the different cohorts of mice over time. Scoring was performed on a weekly basis (n = 20 mice/group).

obtained using JWH-133 for the treatment of s.c. inoculated rat glioma cells [4,7]. In our model we could not observe a clear anti-angiogenic effect of THC.

We also showed for the first time that the absence of CB1 and CB2 receptors did not affect the development of chemically induced skin tumors, including fibrosarcomas, papillomas and melanomas. To our knowledge there is only one publication working with $Cnr1/2^{-/-}$ mice to evaluate the role of the endogenous cannabinoid system for the pathogenesis of epithelial skin tumors. Here, a two-stage carcinogenesis model using DMBA and repeated UVB irradiation was established to create an inflammatory milieu in the skin which promotes the growth of papillomas. Interestingly, CB1/2 receptor deficient animals had reduced signs of UV-induced inflammation and developed less papillomas in comparison to wild type mice [35]. In another autochthonous mouse model, the role of the endogenous cannabinoid system for the pathogenesis of colorectal cancer has been studied. Here the genetic deletion of CB1 receptors accelerated the growth of intestinal adenomas in $Apc^{Min/+}$ mice whereas the pharmacological activation of CB1 receptors attenuated tumor growth [34]. In humans the development of adenomas and colorectal cancer is often associated with chronic intestinal inflammation [33]. After treatment with pro-inflammatory agents $Cnr1^{-/-}$ mice show increased signs of colonic inflammation suggesting a protective role of CB1 receptors against colonic inflammation [22,8]. Surprisingly, we did not find a difference between CB receptor deficient mice and wild type animals in our tumor model. It is possible that both the nature of the stimulus and the stimulated cell types in different tissues are crucial in determining the effects of the endogenous cannabinoid system on tumorigenesis.

5. Conclusion

In conclusion, our studies suggest that the plant-derived CB receptor agonist THC inhibits the growth of transplanted melanoma cells through antagonistic effects on its characteristic pro-inflammatory microenvironment. Using different *in vivo* models we provide evidence that the endogenous cannabinoid system does not influence the growth of chemically induced skin tumors. Our results provide new insights into the potential role of natural or synthetic CB receptor agonists in the treatment of cancer types characterized by a protumorigenic inflammatory microenvironment.

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Conflict of interest statement

The authors declare no conflict of interests.

Uncited reference

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