



Beneficial effects of cannabinoids (CB) in a murine model of allergen-induced airway inflammation: Role of CB₁/CB₂ receptors

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ABSTRACT

The endocannabinoid system (ECS) consists of two cannabinoid (CB) receptors, namely CB₁ and CB₂ receptor, and their endogenous (endocannabinoids) and exogenous (cannabinoids, e.g. delta-9-tetrahydrocannabinol (THC)) ligands which bind to these receptors. Based on studies suggesting a role of THC and the ECS in inflammation, the objective of this study was to examine their involvement in type I hypersensitivity using a murine model of allergic airway inflammation. THC treatment of C57BL/6 wildtype mice dramatically reduced airway inflammation as determined by significantly reduced total cell counts in bronchoalveolar lavage (BAL). These effects were greatest when mice were treated during both, the sensitization and the challenge phase. Furthermore, systemic immune responses were significantly suppressed in mice which received THC during sensitization phase. To investigate a role of CB_{1/2} receptors in this setting, we used pharmacological blockade of CB₁ and/or CB₂ receptors by the selective antagonists and moreover CB₁/CB₂ receptor double-knockout mice (CB₁^{-/-}/CB₂^{-/-}) and found neither significant changes in the cell patterns in BAL nor in immunoglobulin levels as compared to wildtype mice. Our results indicate that the activation of the ECS by applying the agonist THC is involved in the development of type I allergies. However, CB₁/CB₂ receptor-independent signalling seems likely in the observed results.

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Introduction

Allergic asthma belongs to the type I hypersensitivity reactions which are accompanied by chronic airway inflammation dominated by eosinophil and lymphocyte infiltration, increased cytokines, mucus hyperproduction, airway hyperresponsiveness,

and elevated serum IgE levels (Robinson et al. 1993). Within this concept of the predominance of a Th2-type response in type I allergies, Th2 cells and Th2-like cytokines, namely IL-4, -5, and -13, are pivotal in the pathology of this disease (Holgate and Polosa 2008). Although there are several therapeutic strategies for the prevention and treatment of asthma, the identification and development of promising new drug candidates which exert anti-asthmatic effects is highly appreciated.

The class of cannabinoids is known to act as immunomodulators (Cabral and Staab 2005; Klein et al. 2003; Tanasescu and Constantinescu 2010), and their potential use as therapeutic has been widely discussed (Baker et al. 2003; Croxford and Yamamura 2005; Klein 2005). Cannabinoids are discussed for their use in the treatment of T cell-mediated autoimmune diseases (e.g. multiple sclerosis) as they affect both cells of neuronal and immunocompetent origin (Baker et al. 2007; Baker and Pryce 2003; Kubajewska and Constantinescu 2010).

Cannabinoids as well as endocannabinoids bind specifically to G protein-coupled receptors, CB₁ and CB₂ receptors (Devane et al. 1988; Munro et al. 1993), which negatively regulate adenylyl

Abbreviations: ANOVA, analysis of variance; BAL, bronchoalveolar lavage; c, challenge phase; CB, cannabinoid; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell scanning; Ig, immunoglobulin; IL, interleukin; IFN γ , interferon-gamma; i.p., intraperitoneal; Ova, ovalbumin; PBS, phosphate-buffered saline; s, sensitization phase; SR1, SR141716A, N-(piperidine-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR2, SR144528, N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-ethylbenzyl)-pyrazole-3-carboxamide; Th, T helper cell; THC, delta-9-tetrahydrocannabinol; WT, wildtype.

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cyclase activity and/or stimulate mitogen activated protein kinase (MAPK) activity. CB₁ is thought to be mainly expressed in central and peripheral nerve terminals and on a wide range of tissues such as adipose tissue, liver, muscle, gastrointestinal tract, and pancreas (Bouaboula et al. 1993). CB₂ receptor is considered to be restricted to immune-related organs or tissues such as the tonsils, spleen, thymus, and bone marrow with particular high expression levels on B cells and natural killer cells (Bouaboula et al. 1993; Howlett 2002). However, the dogma that CB₂ receptors are immune cell-specific seems no longer valid as the CB₂ receptor is present in the brain and spinal cord, e.g. on microglial cells (Onaivi et al. 2008). Moreover, there is growing evidence that cannabinoids do not exert all of their effects via CB₁ or CB₂ receptors (Kaplan et al. 2003; Rockwell et al. 2006; Zygmunt et al. 2002). A line of studies demonstrated the existence of additional receptors which are shown to mediate the effects of cannabinoids, such as the orphan receptor GPR55, the transient receptor potential vanilloid type-1 receptor (TRPV1), or the peroxisome proliferator-activated receptors (PPARs) which is reviewed by Brown (2007).

While it has been shown that cannabinoids have immunomodulatory capacity, there still remain many questions about the molecular mechanism by which cannabinoids exert their mode of action which seems to be predominately immunosuppression (Croxford and Yamamura 2005; Klein and Cabral 2006; Klein et al. 1998, 2000; Roth et al. 2002).

There are studies demonstrating a beneficial impact of cannabinoids on several diseases including asthma (Baker et al. 2003). The effects of cannabinoids and further endocannabinoids on the immune system are broad and involve responses associated with innate, humoral, and cell-mediated immunity. The discussed pathways through which cannabinoids act are apoptosis, inhibition of proliferation, suppression of cytokine and chemokine production, and induction of T regulatory cells (Tregs). Numerous studies indicated that B cells and T cells are two major targets of cannabinoids such as THC (Klein et al. 1995). T cell-dependent cellular immune responses against infection, such as herpesviruses, were shown to be decreased after THC exposure *in vivo*. Furthermore, T and B cell proliferation was decreased dose-dependently after application of anandamide (Schwarz et al. 1994) and antibody production was inhibited in the presence of endocannabinoids as well as cannabinoids (Kaminski et al. 1994; Titishov et al. 1989). As during the establishment of a Th2 response the secretion of Th2 like cytokines and the induction of IgE antibodies play a key role in the development of asthma, the question arises whether and how cannabinoids can modulate the allergic inflammation.

Based on studies suggesting a role of cannabinoids and the ECS in allergic airway inflammation (Giannini et al. 2008; Jan et al. 2003) and allergic contact dermatitis (Karsak et al. 2007), the objective of this study was to further examine the involvement of cannabinoids and their receptors, in particular CB₁ and CB₂ receptors, in the development of type I hypersensitivity using a murine model of allergic airway inflammation. Several key immunologic features associated with the allergen-induced airway response, such as induction of airway inflammation and humoral immune response were investigated.

Methods

Materials

Chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany) unless otherwise noted. Cremophor was from Fluka (Deisenhofen, Germany). Cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany) unless otherwise noted. The

CB₁ receptor antagonist SR1 and the CB₂ receptor antagonist SR2 were kindly provided by Sanofi-Aventis Recherche (Montpellier, France). All chemicals for injection (THC, SR1, SR2) contained undetectable levels of endotoxin as determined by Limulus amoebocyte lysate analysis kit (Lonza, Wuppertal, Germany).

Stock solutions of THC (25 mg/ml) were in 100% ethanol and stock solutions of antagonists (10^{−2} M) in DMSO. Experimental concentrations were obtained by dilution of stock solutions in one volume cremophor and further in sterile PBS to yield the final concentration. Vehicle controls consisted of equal amounts of ethanol/DMSO and cremophor in PBS.

Animals

Female C57BL/6 wildtype mice (6–8 week old) were obtained from Charles River (Sulzfeld, Germany). CB₁/CB₂ receptor double-knockout mice (CB₁^{−/−}/CB₂^{−/−}) were generated as described previously (University of Bonn, Germany) (Jarai et al. 1999). Homozygous CB₁^{−/−}/CB₂^{−/−} mice were bred in our animal facility. All mice were housed in our animal facility at the Helmholtz Zentrum München in isolated ventilated cages (VentiRack; Biozone, Margate, UK) in a specific pathogen-free environment. They received a standard pellet diet and water *ad libitum*. The study was conducted under the guidelines of Helsinki convention for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Zentrum München.

Experimental protocols

Allergen sensitization/challenge protocol

The general protocol for allergen sensitization and challenge is shown in Fig. 1A. Mice (C57BL/6 wildtype or CB₁^{−/−}/CB₂^{−/−} mice) were sensitized by repetitive i.p. injections of 1 or 10 µg ovalbumin (Ova; chicken egg albumin; grade V) adsorbed to 2 mg alum (ImjectAlum; Pierce Chemical Co., Rockford, IL) in PBS on days 0, 7, 14, and 28. Non-sensitized control mice received PBS. All mice were challenged with 1% Ova-aerosol (in PBS) on days 42, 44, and 46 for 20 min delivered by Pari-Boy nebulizer (Pari, Starnberg, Germany). BAL analysis was performed 24 h after the last Ova challenge.

Treatment protocols

A schematic representation of the treatment protocols is shown in Fig. 1B and C.

THC treatment (Fig. 1B): mice were sensitized and challenged with Ova as described in Fig. 1A. THC (50 mg/kg bodyweight) was administered by i.p. injection for 3 consecutive days before each Ova sensitization (Ova/THC(s)), before each Ova challenge (Ova/THC(c)), or before each sensitization and challenge (Ova/THC(s+c)) as previously reported (Jan et al. 2003). THC was administered for 3 days before each Ova-treatment to ensure an effective dose of THC in serum and further to allow an immune reaction to take place. Control mice were given vehicle (5% ethanol and 5% cremophor in PBS).

Treatment with CB receptor antagonists SR1 and SR2 (Fig. 1C): mice were sensitized and challenged with Ova as described above. CB₁ and CB₂ receptor antagonists SR1 or/and SR2 (each 1 µg/kg bodyweight) were administered by i.p. injection 30 min before each Ova sensitization and challenge (Ova/SR1(s+c), Ova/SR2(s+c), Ova/SR1+SR2(s+c)). Doses were selected according to previous records (McMahon and Koek 2007). Antagonists were injected 30 min prior to Ova-treatment to ensure receptor-binding at the time point of Ova injection. Control mice were given vehicle (5% DMSO and 5% cremophor in PBS).

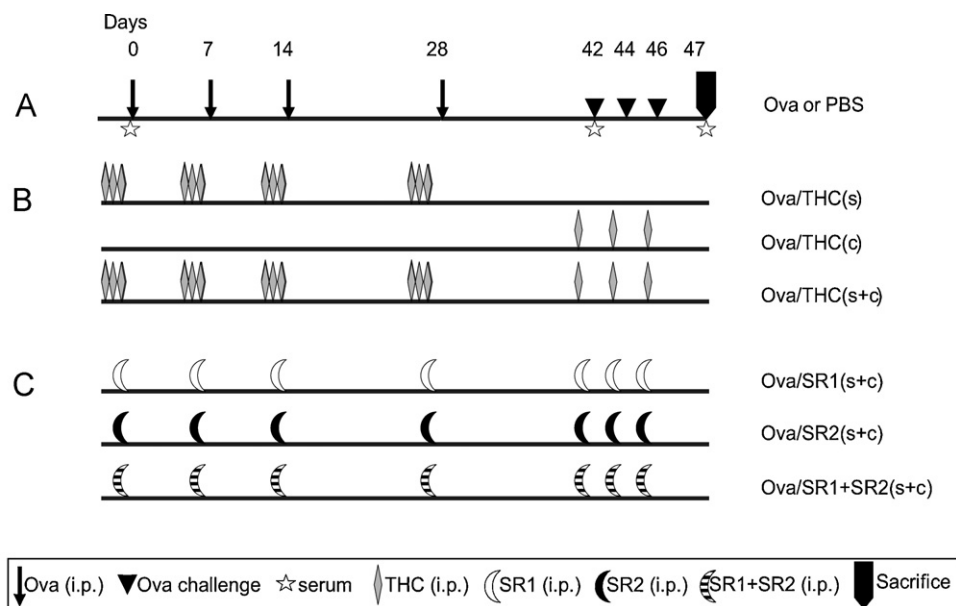


Fig. 1. Experimental design for systemic sensitization and pharmacological treatment. (A) General sensitization protocol: mice were sensitized by repetitive i.p. injections of 1 or 10 μ g Ova/alum on days 0, 7, 14, and 28. Non-sensitized control mice received PBS. All mice were challenged with 1% Ova-aerosol (in PBS) on days 42, 44, and 46 for 20 min. Serum samples were obtained on days 0, 42, and 47. (B) THC-treatment: mice were sensitized and challenged with Ova as described above. THC (50 mg/kg bodyweight) was administered by i.p. injection for 3 consecutive days before each Ova sensitization (Ova/THC(s)), before each Ova challenge (Ova/THC(c)), or before each sensitization and challenge (Ova/THC(s+c)). Control mice were given vehicle. (C) Treatment with CB₁ and CB₂ receptor antagonists SR1 or/and SR2 (each 1 μ g/kg bodyweight) were administered by i.p. injection 30 min before each Ova sensitization and before each OVA challenge (Ova/SR1(s+c), Ova/SR2(s+c), Ova/SR1+SR2(s+c)). Control mice were given vehicle.

Sample preparation

Collection and analysis of bronchoalveolar lavage (BAL)

Twenty-four hours after the last Ova challenge, BAL was performed as described previously (Alessandrini et al. 2006). Briefly; the airways were rinsed 5 times with 0.8 ml PBS. The supernatant of the first lavage was used to measure cytokine levels. Total cell viability and yield were quantified by trypan blue exclusion. Cytospin preparations were prepared and stained with Diff Quick Staining Set (Medion Diagnostics GmbH, Düringen, Germany). Determination of differential BAL cells was done by counting a minimum of 400–500 cells per preparation. Percentages of macrophages, eosinophils, neutrophils, and lymphocytes were calculated from absolute cell number and total cell counts.

Serum samples

Blood samples were taken by puncturing the retroorbital plexus under ether anesthesia on days 0, 42, and 47. Serum samples were obtained by centrifugation and stored at -20°C until use.

Splenocyte isolation and culture

Spleens were removed aseptically and single cell suspensions were generated. Splenocytes were cultured in complete media (RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M β -mercaptoethanol, 1 mM minimum essential media (MEM) sodium pyruvate, 0.1 mM MEM nonessential amino acids, 20 mM HEPES, and 10% FCS (PAA, Coelbe, Germany) in quadruplicates during polyclonal stimulation with 2.5 μ g/ml anti-CD3 mAb (clone 17A2; eBioscience, San Diego, CA) or 2 μ g/ml anti-CD28 (clone 37.51; eBioscience). After 4 days of culture at 37°C in 5% CO_2 , the supernatants were harvested and stored at -80°C until determination of IL-4, IL-5, and IFN γ cytokine levels by ELISA.

Analysis

Cytokine ELISA

Levels of IL-4, IL-5, IFN γ in BAL fluids or cell culture supernatants were determined by ELISA according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany). A commercially available ELISA kit was used to assess levels of IL-13 in BAL fluid and cell culture supernatants (Biosource Deutschland GmbH, Solingen, Germany).

Serum immunoglobulin ELISA

Ova-specific IgE and IgG1 were measured in dilutions of serum samples by ELISA as described previously (Herz et al. 1998). Total IgE in serum was analyzed using an isotype-specific sandwich ELISA for murine IgE with a lower detection limit of 1 ng/ml as described (Alessandrini et al. 2001).

Determination of lymphocyte subpopulations by flow cytometry

For determination of Foxp3-positive CD4/CD25 cells, a commercial available kit from eBioscience was used.

Specific binding of mAbs was analyzed by direct immunofluorescence using a FACSCalibur (BD Biosciences). Briefly, 2×10^5 cells in PBS containing 2% FCS and 0.1% sodium azide were incubated in the presence of saturating concentrations of anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), and anti-CD45R/B220 (clone RA3-6B2) (BD Bioscience) in the dark on ice for 30 min. Specific binding of mAbs was controlled by incubation with isotype-matched control Abs.

Data analysis

For statistical evaluation, one-way analysis of variance (ANOVA) was used to compare more than two groups. Significant ANOVAs were further analyzed by the Bonferroni/Dunn's post hoc test. Stu-

dent's *t*-Tests or Mann-Whitney *U*-tests were used to compare two groups. A *p*-value ≤ 0.05 was considered to be significant; **p* ≤ 0.05 , ***p* ≤ 0.01 , ****p* ≤ 0.001 .

Results

THC treatment of C57BL/6 wildtype mice ameliorates Th2-like airway inflammation with differential effects during sensitization and challenge phase

C57BL/6 wildtype mice which were sensitized and challenged with Ova as described in Fig. 1 developed the immunological abnormalities characteristic of an allergic response. Thus, the total number of BAL cells in the Ova-sensitized (Ova) group raised 6.7-fold ($p \leq 0.001$) when compared with non-sensitized (PBS) control animals which showed very low total cell numbers, indicating that no inflammation occurred (Fig. 2A). This rise in total cell number was mainly due to a rise in absolute cell numbers of eosinophils ($p \leq 0.001$) and also macrophages ($p \leq 0.001$) as indicated in Table 1. Thus, Ova mice showed a very high percentage of eosinophils and a lower percentage of macrophages when compared with PBS mice (Fig. 2B).

We examined whether the treatment with THC had an influence on the development of allergic airway inflammation and whether the timing of THC administration is important with respect to sensitization or challenge phase. We treated Ova-sensitized mice with THC either during sensitization (Ova/THC(s)), or during challenge phase (Ova/THC(c)), or during both phases (Ova/THC(s+c)). Administration of THC in either phase reduced total BAL cell numbers (Fig. 2A) when compared with the Ova-sensitized vehicle treated group (Ova). However, this reduction was most prominent for mice treated during sensitization and challenge phase (Ova/THC(s+c)) ($p \leq 0.001$). However, total BAL cell numbers was also reduced when mice received THC in sensitization (Ova/THC(s)) or challenge phase (Ova/THC(c)) alone, although this failed statistical significance.

Further, THC treatment resulted in an altered distribution of absolute cell numbers when compared to Ova control mice as shown in Table 1. Administration of THC during sensitization and challenge (Ova/THC(s+c)) showed a reduction in absolute BAL macrophage numbers ($p \leq 0.001$) and eosinophils ($p \leq 0.001$) when compared with Ova control group. The relative numbers of BAL cells in this group shifted towards an increase of the macrophage fraction ($p \leq 0.001$) and a decrease of the eosinophil fraction ($p \leq 0.001$) (Fig. 2B). THC treatment during sensitization phase showed a less prominent phenotype with an increased macrophage fraction and decreased eosinophil fraction. THC treatment only during challenge phase did not induce these effects.

BAL fluid cytokine production after THC treatment during sensitization and challenge phase

To further investigate allergen-induced airway inflammation, BAL fluid was assessed for the presence of the cytokines (Fig. 2C) IL-4, IL-5, and IL-13. Levels of IL-4 remained the same in all study groups. When analysing levels of IL-5 and IL-13, we found decreased levels of these cytokines in groups which also showed lower numbers of total BAL cells. These differences, however, did not reach statistical significance.

THC treatment of C57BL/6 wildtype mice during sensitization phase ameliorates Th2-like serum immunoglobulin levels

We further investigated the efficacy of THC to influence the levels of serum immunoglobulins. We quantified total IgE as well as Ova-specific IgE and IgG1. Non-sensitized (PBS) animals

showed no increase in immunoglobulin levels, however, levels of all immunoglobulins increased after Ova-sensitization and again after Ova-aerosol challenge (Fig. 3).

The administration of THC during Ova-sensitization significantly reduced total IgE as well as Ova-specific IgE and IgG1 levels in the Ova/THC(s) group compared to the Ova group at pre-challenge levels on day 42 as well as after challenge on day 47.

Mice treated with THC additionally during challenge phase (Ova/THC(s+c)) displayed even lower levels for Ova-specific IgE ($p \leq 0.01$) and a reduction of total IgE and Ova-specific IgG1 on day 47.

THC treatment only during challenge phase (Ova/THC(c)) significantly reduced IgG1 levels ($p \leq 0.05$) on day 47, whereas total IgE was only partially down-regulated and Ova-specific IgE levels were not affected.

These results indicate a Th2-ameliorating effect of THC administration at the time of sensitization.

Leukocyte phenotypes in spleen after THC treatment

Furthermore, splenocyte preparations were assessed for their expression of CD4, CD8, intracellular expression of Foxp3 within the CD4/CD25-positive population, and functional cytokine production after *ex vivo* restimulation. The percentage of CD4-positive and CD8-positive cells in non-sensitized and Ova-sensitized mice was similar (Fig. 4A). However, Ova-sensitized mice treated with THC during both phases (Ova/THC(s+c)) showed a reduced amount of CD4-expressing cells ($p \leq 0.001$), but a constant number of CD8-positive cells.

The percentage of Foxp3 positive cells in non-sensitized and Ova-sensitized mice was similar (Fig. 4B). Moreover, THC treatment of mice during both phases did not alter the expression of Foxp3 in these mice.

Further, cell culture supernatants of splenocytes from Ova and Ova/THC(s+c) mice which were stimulated with anti-CD3 and anti-CD28 for 4 days did not show any differences in their potency to produce cytokines (Fig. 4C).

Pharmacological blockade of CB receptors by selective antagonists does not influence allergic outcome

To investigate the role of the CB₁ and CB₂ receptors in the induction of allergic airway inflammation, we examined C57BL/6 wildtype mice after pharmacological blockade of the CB receptors by administration of the CB₁ receptor antagonist SR141716A (SR1) or/and the CB₂ receptor antagonist SR144528 (SR2), respectively. We administered SR1 and/or SR2 by i.p. injection 30 min before each sensitization and challenge to block either the CB₁ receptor, the CB₂ receptor, or both receptors.

We could not detect any significant changes neither in total numbers of BAL cells (Fig. 5A) nor in differential cell counts (Fig. 5B) between the different study groups. Moreover, cytokine levels in BAL fluid (Fig. 5C) were similar.

Furthermore, serum immunoglobulin levels for total IgE, Ova-specific IgE, and Ova-specific IgG1 were not significantly changed in the presence of the antagonists (Fig. 6).

The percentages of CD4-positive, CD8-positive, or Foxp3-expressing CD4/CD25-positive cells within the splenocytes of mice treated with both antagonists were not altered when compared with the Ova-sensitized vehicle-treated group (Fig. 7B).

Allergen-induced airway inflammation is similar in C57BL/6 wildtype mice and CB₁^{-/-}/CB₂^{-/-} mice

To examine the role of the CB₁ and CB₂ receptor on airway eosinophilia C57BL/6 wildtype or CB₁^{-/-}/CB₂^{-/-} double-knockout

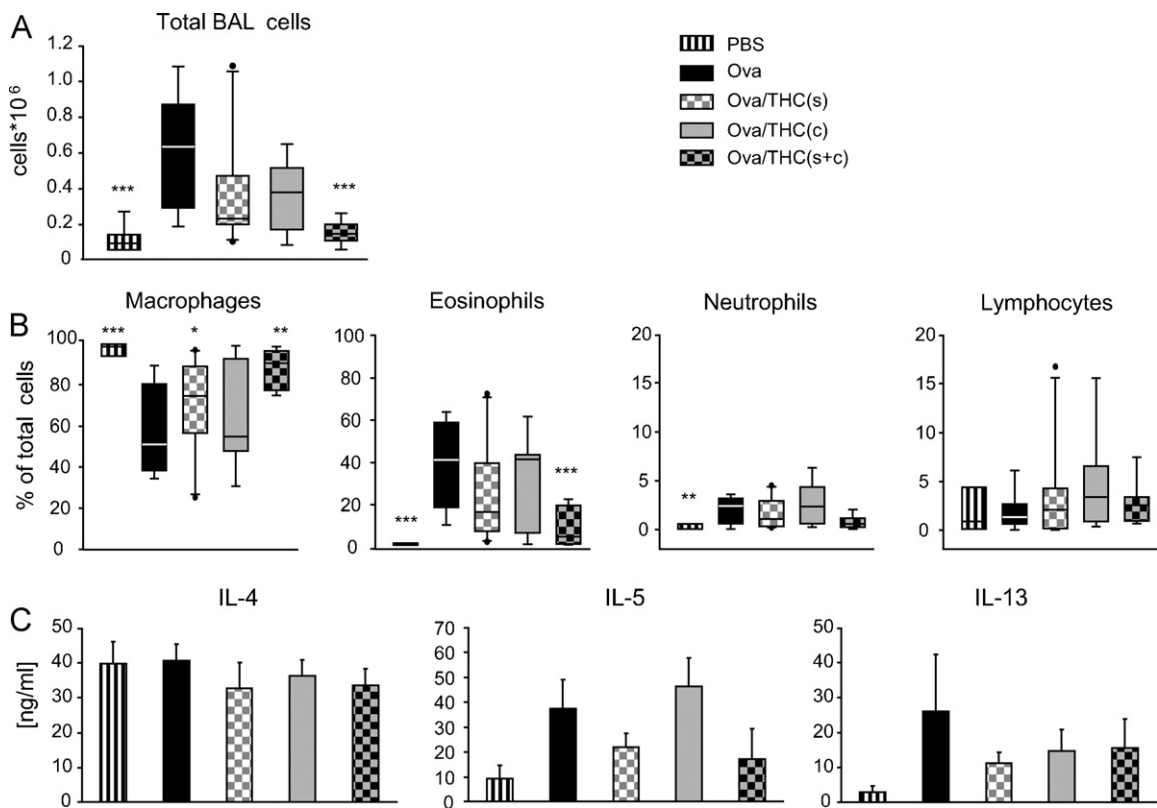


Fig. 2. Reduced allergic phenotype in BAL of C57BL/6 wildtype mice after THC treatment. C57BL/6 wildtype mice were either non-sensitized (PBS) or sensitized (Ova) with 10 µg Ova and were Ova challenged as shown in Fig. 1A. THC (50 mg/kg bodyweight) or vehicle was administered via i.p. injection for 3 consecutive days before each Ova sensitization (THC(s)), before each Ova challenge (THC(c)), or during both phases (THC(s+c)) as described in Fig. 1B. (A) THC treatment reduced absolute numbers of total cell count in BAL. (B) THC treatment converted the percentage distribution of differential cell counts in BAL towards a non-inflamed phenotype. Data for BAL cell analyses are displayed as boxplots. (C) BAL fluid was analyzed for cytokine levels by ELISA. Data are shown as mean + S.E.M. All results are calculated from one experiment with at least 9 mice per group. Significance: **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001 compared to the Ova group.

Table 1
Absolute numbers in BAL in C57BL/6 wildtype mice treated with THC.

	Macrophages	Eosinophils	Neutrophils	Lymphocytes
PBS	81,109 ± 12,418***	331 ± 1717***	u.d.**	594 ± 1424
Ova	293,209 ± 30,517	235,467 ± 78,450	6,917 ± 4017	9027 ± 2772
Ova/THC(s)	195,988 ± 18,991*	35,770 ± 80,152	2,526 ± 4363	5653 ± 3578
Ova/THC(c)	212,450 ± 30,059	218,267 ± 41,293	14,868 ± 3421	21,911 ± 6443
Ova/THC(s+c)	109,815 ± 17,211**	4,570 ± 6460***	914 ± 510	3252 ± 1886

C57BL/6 wildtype mice were either non-sensitized (PBS) or sensitized (Ova) with 10 µg Ova and were challenged three times with aerosolized Ova as shown in Fig. 1A. THC (50 mg/kg) was administered dvia i.p. injection for 3 consecutive days before each Ova sensitization (Ova/THC(s)), before each Ova challenge (Ova/THC(c)), or during both phases (Ova/THC(s+c)) as described in Fig. 1B. All other mice received vehicle as control. BAL cells were analyzed for their absolute numbers of differential cell count after morphological characterization. Results are expressed as median ± S.E.M.

* *p* ≤ 0.05.
** *p* ≤ 0.01.
*** *p* ≤ 0.001 compared to the Ova group.

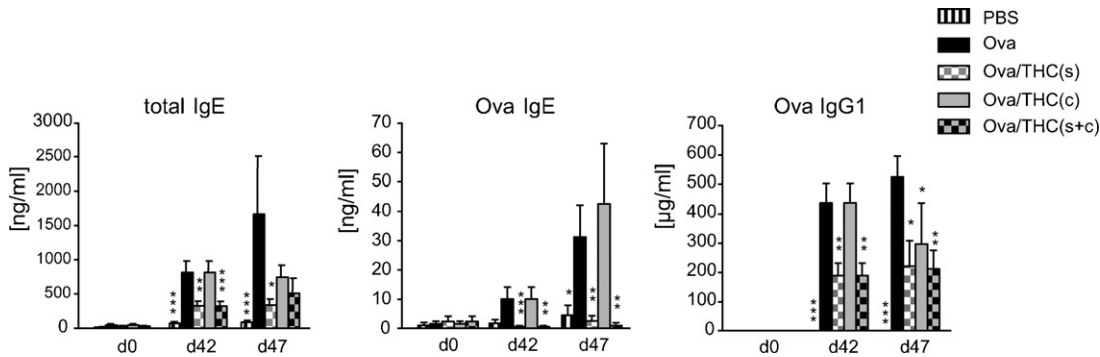


Fig. 3. Reduced humoral immune response in serum of C57BL/6 wildtype mice after THC treatment. C57BL/6 wildtype mice were treated as described in Fig. 2. Serum was obtained at indicated days (d0, d42, d47) and analyzed for levels of immunoglobulins by ELISA. Levels of total IgE, Ova-specific IgE, and Ova-specific IgG1 were significantly reduced in mice treated with THC during sensitization or sensitization and challenge phase. Data are expressed as mean + S.E.M. All results are calculated from one experiment with at least 9 mice per group. Significance: **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001 compared to the Ova group.

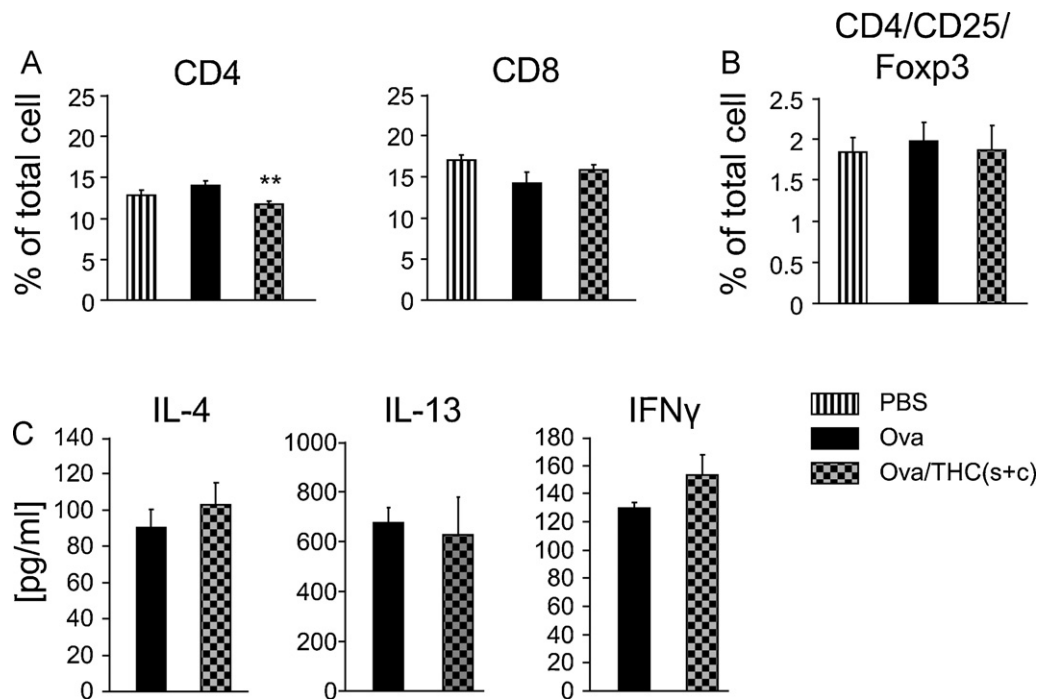


Fig. 4. Analysis of splenocyte populations of C57BL/6 wildtype mice after THC treatment. C57BL/6 wildtype mice were treated as described in Fig. 2. Splenocytes were analyzed for their cellular composition by flow cytometry. Viable splenic leukocyte populations were determined for the percentage of (A) CD4-expressing or CD8-expressing cells or (B) intracellular Foxp3-expressing CD4/CD25-positive cells. (C) Cytokine levels of splenocytes on day 4 after *ex vivo* restimulation were determined by ELISA. Values are expressed as mean $\pm$ S.E.M. Results are calculated from one experiment with at least 9 mice per group. Significance: * $p \leq 0.05$, ** $p \leq 0.01$ compared to the Ova group.

mice were sensitized to Ova and challenged with Ova-aerosol as shown in Fig. 1. As previously, non-sensitized wildtype or $CB_1^{-/-}/CB_2^{-/-}$ mice (PBS) showed a low number of inflammatory cells in the BAL, and Ova-sensitized animals (Ova) showed

increased total cell numbers in BAL after Ova-aerosol challenge (Fig. 8A). We studied two protocols in which mice received either 1 μ g or 10 μ g Ova and found that the number of total cells in BAL was markedly higher in both wildtype and double-knockout mice

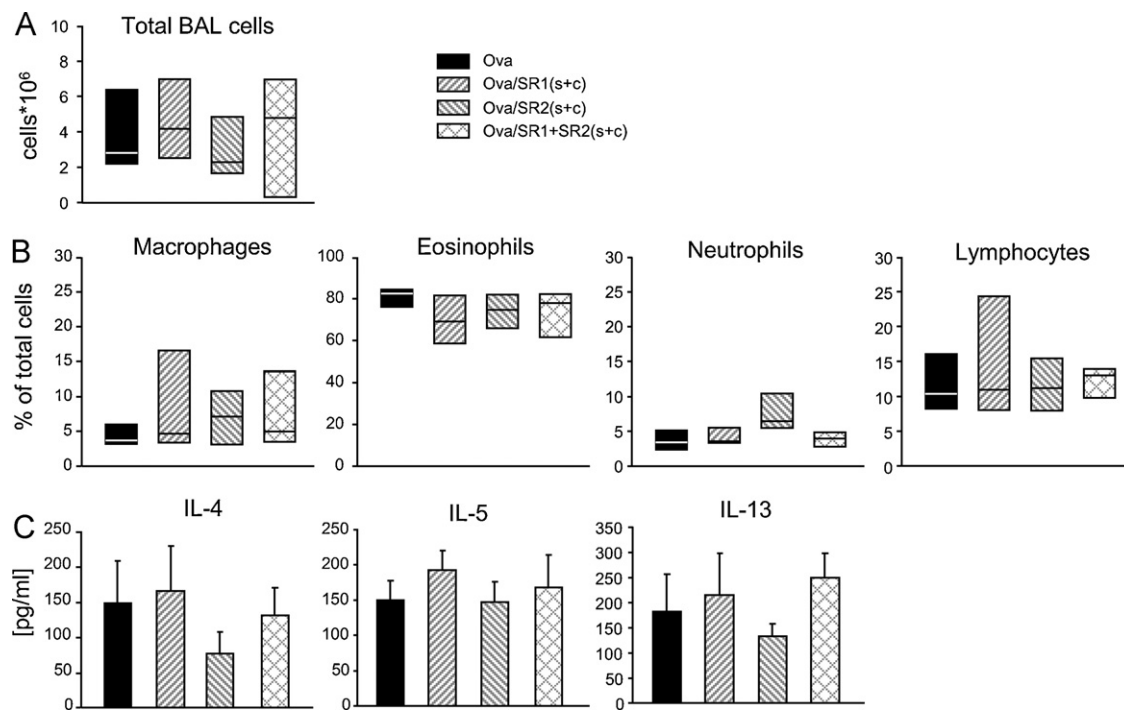


Fig. 5. Pharmacological blockade of CB receptors does not influence allergic airway inflammation in C57BL/6 wildtype mice. C57BL/6 wildtype mice were sensitized with 10 μ g Ova (Ova) and challenged with aerosolized Ova as shown in Fig. 1A. CB_1 and CB_2 receptors antagonists SR1 or/and SR2 (1 μ g/kg bodyweight) or vehicle were administered by i.p. injection before each Ova sensitization and Ova challenge (s+c) as indicated in Fig. 1C. BAL cells were analyzed for their (A) absolute numbers of total cell count and (B) percentage distribution of differential cell count after morphological characterization. Data are displayed as boxplots. (C) BAL fluid was analyzed for cytokine levels by ELISA. Data are shown as mean $\pm$ S.E.M. All results are calculated from one experiment with at least 7 mice per group.

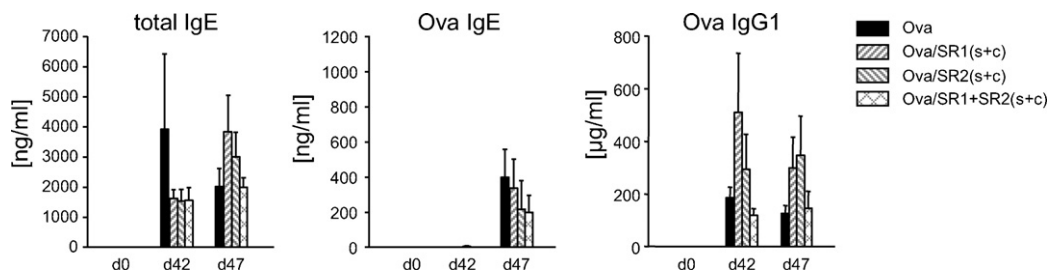


Fig. 6. Pharmacological blockade of CB receptors does not alter humoral immune response in C57BL/6 wildtype mice. C57BL/6 wildtype mice were treated as described in Fig. 5. Serum was obtained at indicated days (d0, d42, d47) and analyzed for levels of immunoglobulins by ELISA. Levels of total IgE, Ova-specific IgE, and Ova-specific IgG1 are expressed as mean + S.E.M. All results are calculated from one experiment with at least 7 mice per group.

given 1 μg than in those given 10 μg Ova. When comparing the two strains, $CB_1^{-/-}/CB_2^{-/-}$ mice elicited slightly lower but almost comparable total BAL cell numbers within the 1 μg and 10 μg Ova-sensitization groups.

Irrespective of the background (wildtype or $CB_1^{-/-}/CB_2^{-/-}$), non-sensitized animals (PBS) showed a high percentage of macrophages and a low percentage of eosinophils (Fig. 8B). However, non-sensitized $CB_1^{-/-}/CB_2^{-/-}$ mice had elevated percentages of eosinophils ($p \leq 0.01$) and lymphocytes ($p \leq 0.01$) when compared with C57BL/6 wildtype mice. As expected, the Ova-sensitized animals showed a low percentage of macrophages and a high percentage of eosinophils (Fig. 8B) which was similar for both Ova doses. Percentage of neutrophils ($p \leq 0.01$) was lower and percentage of lymphocytes ($p \leq 0.05$) was higher in $CB_1^{-/-}/CB_2^{-/-}$ mice received 10 μg Ova. No overall difference was found in differential cell count between C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice received 1 μg Ova.

BAL fluid cytokine production in C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice

Analysis of BAL fluid showed that non-sensitized (PBS) C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice showed lower levels of IL-4, IL-5, and IL-13 than Ova-sensitized (Ova) mice (Fig. 8C). Further, C57BL/6 wildtype mice that received 1 μg Ova showed higher cytokine levels than mice that received 10 μg Ova which is in line with the slightly elevated total BAL cell numbers in this group (Fig. 8A). $CB_1^{-/-}/CB_2^{-/-}$ mice showed decreased IL-4 levels in the 1 μg Ova group ($p \leq 0.05$) and decreased amounts of IL-5 in the 10 μg Ova group ($p \leq 0.05$) when compared with wildtype.

Serum immunoglobulin levels in C57BL/6 wildtype mice and $CB_1^{-/-}/CB_2^{-/-}$ mice

We examined serum from wildtype and double-knockout mice for their levels of total IgE, Ova-specific IgE, and Ova-specific IgG1 (Fig. 9). All mice that received Ova had elevated levels of all immunoglobulin isotypes compared to non-sensitized control mice (PBS) that had low levels of immunoglobulins near baseline.

When comparing the different Ova-doses in general, mice that received 10 μg Ova showed slightly higher Ova-specific IgG1 at day 42 than mice that received 1 μg Ova which was significant for C57BL/6 wildtype ($p \leq 0.01$) as well as $CB_1^{-/-}/CB_2^{-/-}$ mice ($p \leq 0.001$). There was still an increase on day 47 after Ova-aerosol challenge, although not significant. Levels of total IgE were also slightly elevated on day 42 in the high dose group, however not significant. Ova-specific IgE levels which were very low at the pre-challenge phase were similar in all groups.

No significant alterations were detectable by comparing corresponding C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice, although $CB_1^{-/-}/CB_2^{-/-}$ mice showed slightly reduced immunoglobulin levels, which was not statistically significant.

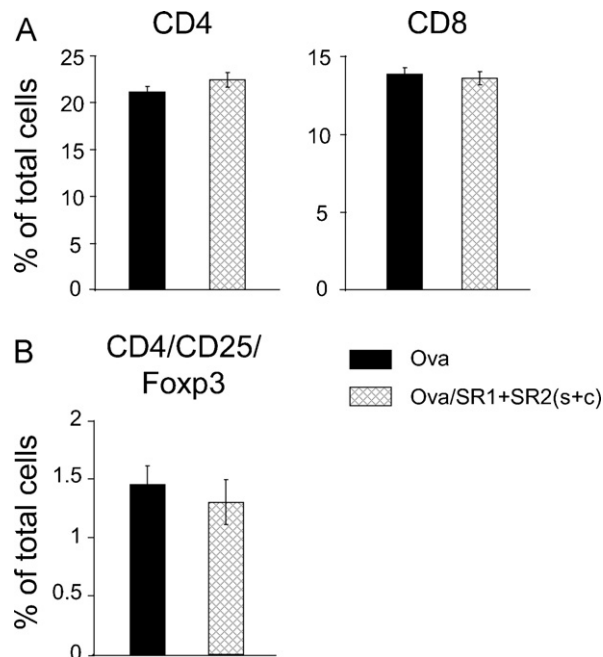


Fig. 7. Splenocyte population of C57BL/6 wildtype mice after pharmacological blockade of CB receptors. C57BL/6 wildtype mice were treated as described in Fig. 5. Viable splenic leukocyte populations were determined for the percentage of (A) CD4-expressing or CD8-expressing cells or (B) intracellular Foxp3-expressing CD4/CD25-positive cells. Data are shown as percentage of total viable cells. All results are expressed as mean + S.E.M and are calculated from one experiment with at least 9 mice per group.

Phenotypic analysis of leukocyte populations in spleens of C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice

Flow cytometric analysis of splenocytes of non-sensitized (PBS) or Ova-sensitized (Ova; 1 μg and 10 μg) C57BL/6 mice and $CB_1^{-/-}/CB_2^{-/-}$ mice was performed to determine whether there is a difference in the cellular composition of leukocytes. Significant differences were observed in the percentage of B cells (B220-positive), T cells (CD4-positive or CD8-positive), and regulatory T cells (Tregs; CD4/CD25/Foxp3-positive) between wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice irrespective of sensitization protocol (Fig. 10). $CB_1^{-/-}/CB_2^{-/-}$ mice that were either non-sensitized (PBS) or sensitized with 10 μg Ova had lower numbers of CD8-positive cells ($p \leq 0.01$ and $p \leq 0.001$) and more B cells ($p \leq 0.05$ and $p \leq 0.01$), while there were less Th cells ($p \leq 0.01$) and Tregs ($p \leq 0.01$) in $CB_1^{-/-}/CB_2^{-/-}$ mice which received 1 μg Ova.

THC treatment of $CB_1^{-/-}/CB_2^{-/-}$ mice reduces Th2-like serum immunoglobulin levels

We further investigated whether THC administration influences systemic immune response during sensitization and challenge

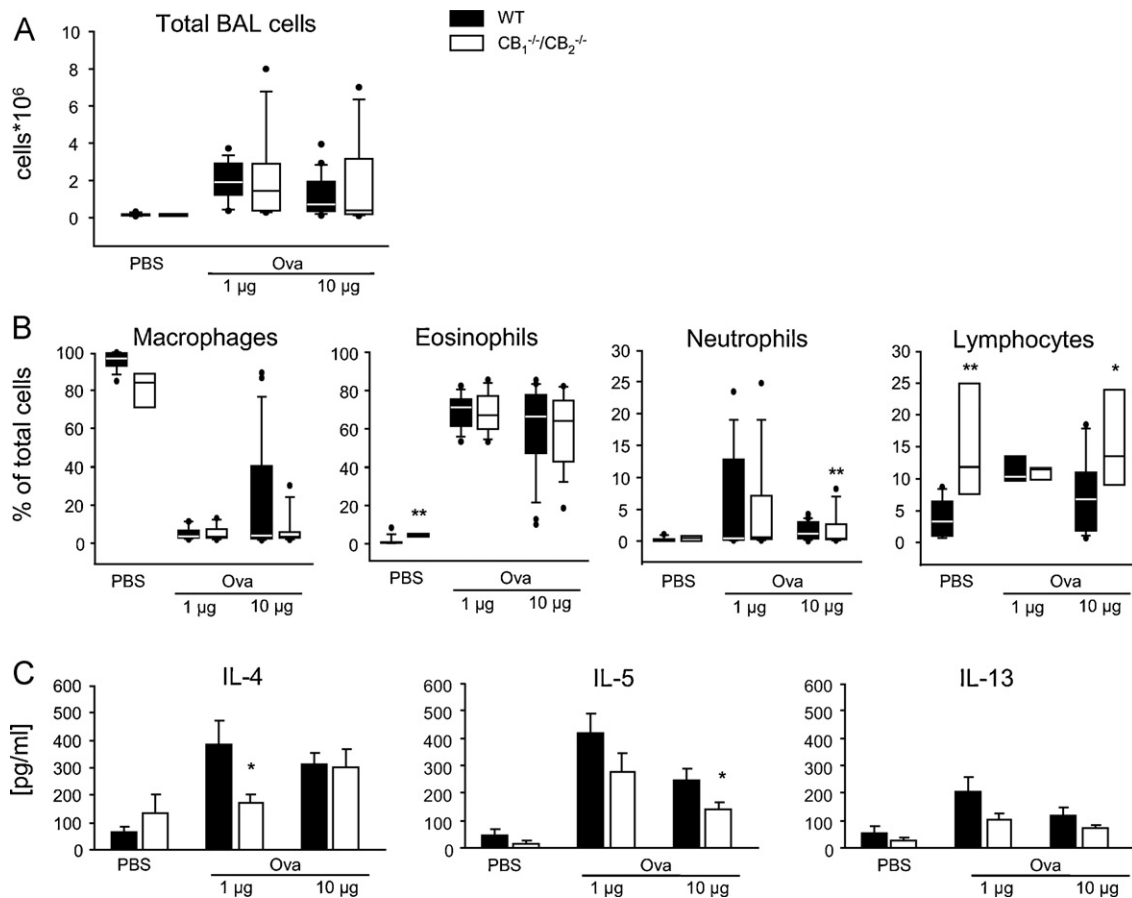


Fig. 8. Systemic sensitization to Ova results in a similar pattern of allergic airway inflammation in C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice. C57BL/6-wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice were either non-sensitized (PBS) or Ova-sensitized (Ova) with 1 μ g or 10 μ g as described in Fig. 1A. BAL cells were analyzed for their (A) absolute numbers of total cell count and (B) percentage distribution of differential cell count after morphological characterization. Values are displayed as boxplots. (C) BAL fluid was analyzed for cytokine levels by ELISA. Data are shown as mean $\pm$ S.E.M. All results are calculated from three independent experiments with at least 9 mice per group. Significance: * $p \leq 0.05$ compared to the corresponding dose group.

in $CB_1^{-/-}/CB_2^{-/-}$ mice by measuring serum immunoglobulins (Fig. 11). We quantified total IgE as well as Ova-specific IgE and IgG1.

The administration of THC during Ova-sensitization significantly reduced total IgE ($p \leq 0.05$) as well as Ova-specific IgE ($p \leq 0.01$) and IgG1 ($p \leq 0.05$) levels in the Ova/THC(s+c) group compared to the Ova group at pre-challenge levels on day 42 as well as after challenge on day 47.

These results indicate that THC administration during the preceding sensitization has a Th2-ameliorating effect.

Discussion

In this study, we showed that cannabinoids can influence the induction of an IgE-mediated allergic sensitization and allergen-induced airway inflammation in a murine model. We assessed the activation of the endocannabinoid system through the treatment with THC in wildtype and CB receptor deficient mice as well as the inactivation of the endocannabinoid system by both, the pharmacological blockade of the CB_1 and/or CB_2 receptors and by using CB receptor deficient mice. We conclude that cannabinoids modulate

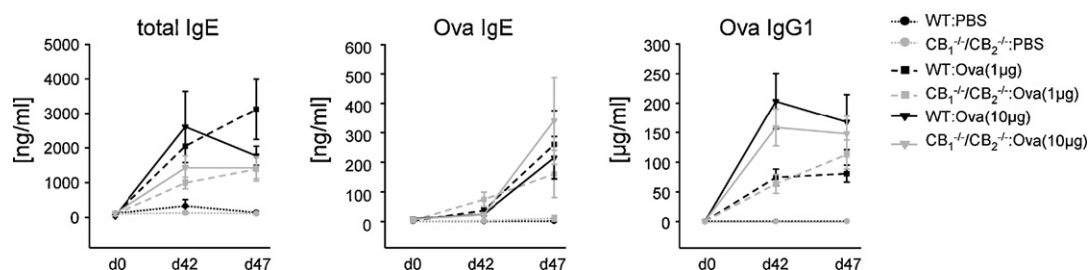


Fig. 9. Systemic sensitization of C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice to Ova leads to a similar humoral immune response in both strains. C57BL/6 wildtype and $CB_1^{-/-}/CB_2^{-/-}$ mice were treated as described in Fig. 8. Serum was obtained at indicated days (d0, d42, d47) and analyzed for levels of immunoglobulins by ELISA. Levels of total IgE, Ova-specific IgE, and Ova-specific IgG1 are expressed as mean $\pm$ S.E.M. All results are calculated from three independent experiments with at least 9 mice per group.

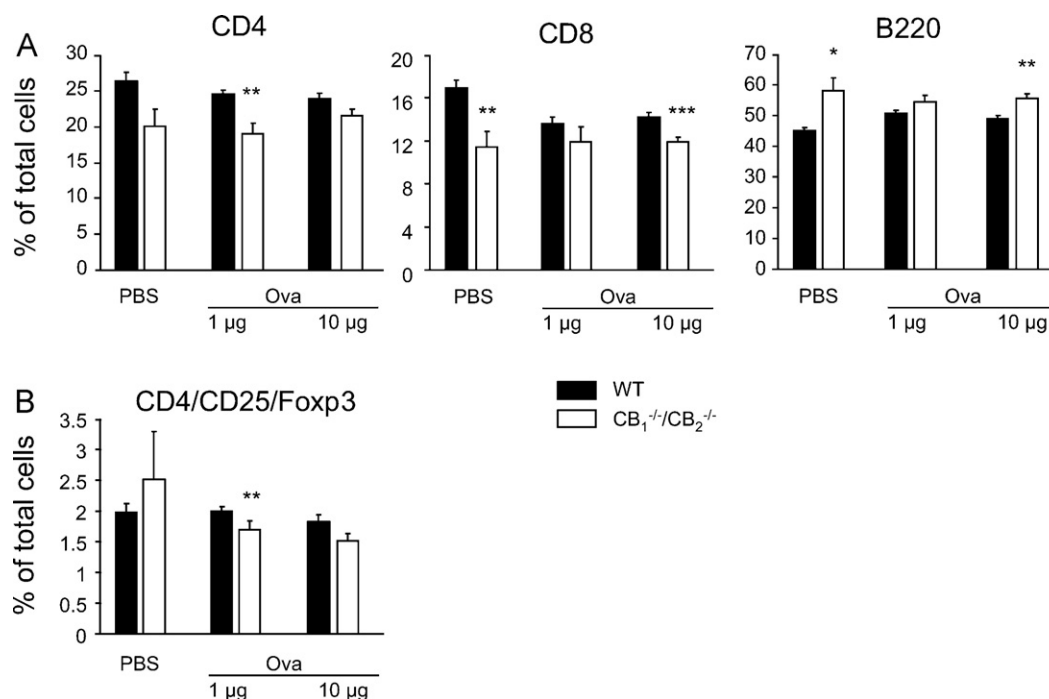


Fig. 10. Splenocyte populations of C57BL/6 wildtype and CB1^{-/-}/CB2^{-/-} mice. C57BL/6 wildtype and CB1^{-/-}/CB2^{-/-} mice were treated as described in Fig. 8. Viable splenic leukocyte populations were determined for the percentage of (A) CD4-expressing, CD8-expressing, or B220-expressing cells or (B) intracellular Foxp3-expressing CD4/CD25-positive cells. All results are expressed as mean + S.E.M. and are calculated from three independent experiments with at least 9 mice per group.

the induction of allergic airway inflammation at least in part by a non-CB₁/CB₂ receptor-mediated mechanism.

It is widely accepted that endocannabinoids and cannabinoids have immunomodulatory properties. The class of cannabinoids has been studied in many disease models including multiple sclerosis, diabetes, and rheumatoid arthritis (Baker and Pryce 2003; Croxford and Yamamura 2005; Klein 2005; Klein and Cabral 2006). However, it still remains elusive by which mechanisms cannabinoids mediate their activity. Four major pathways are characterized: induction of apoptosis, inhibition of cell proliferation, inhibition of cytokine and chemokine production, and induction of Tregs.

Our study confirms the immunosuppressive mode of action of THC, as we demonstrated that THC is able to counteract the allergen-induced airway inflammation in wildtype mice. Interestingly, not only the degree of airway inflammation was ameliorated. In addition, cellular distribution of recruited cell population was significantly shifted towards a reduced percentage of infiltrated eosinophils, which is central in the pathogenesis of allergic asthma. Furthermore, decreased levels of cytokines such as IL-4, -5, and -13 which are released by activated Th2 cells and are strongly correlated to the degree of eosinophil infiltration and thus are

closely linked an allergic response (Bradley et al. 1991) revealed an inhibitory effect of THC in our mouse model.

To date, it is unclear which are the main target cells for cannabinoid action. It is known that cannabinoids act on T as well as B lymphocytes by modulating proliferation and apoptosis (Klein et al. 1995; Schwarz et al. 1994). In addition, cannabinoids influence macrophage function, inflammatory cytokine production, chemokine secretion, and cell migration (Klein 2005; Klein et al. 2003).

The present study demonstrated that both, the induction of a humoral IgE-mediated allergic sensitization and the resulting allergic airway inflammation was reduced when THC treatment was applied during the whole time period of the experiment. As T cell activation and recruitment is a prerequisite for allergic airway inflammation, impaired T cell activity might account for the reduced airway inflammation in. However, THC treatment of already Ova-sensitized mice during challenge phase yielded in a less beneficial effect in both, the levels of immunoglobulin and airway inflammation. This might indicate that THC exerts its role rather during sensitization and establishment of an allergen-specific IgE response than during T cell activation and recruitment

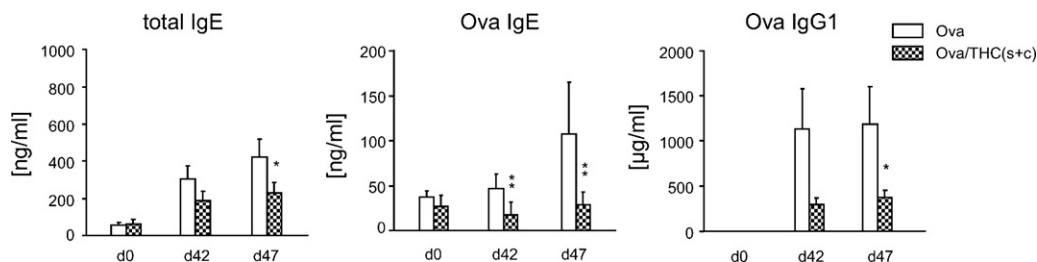


Fig. 11. Reduced systemic immune response after sensitization to Ova of CB1^{-/-}/CB2^{-/-} mice. CB1^{-/-}/CB2^{-/-} mice were Ova-sensitized (Ova) with 1 µg as described in Fig. 1A. THC (50 mg/kg bodyweight) or vehicle was administered via i.p. injection for 3 consecutive days before each Ova sensitization and each Ova challenge THC(s+c) as described in Fig. 1B. Serum was obtained at indicated days (d0, d42, d47) and analyzed for levels of immunoglobulins by ELISA. Levels of total IgE, Ova-specific IgE, and Ova-specific IgG1 were significantly reduced in mice treated with THC during sensitization and challenge phase. Data are expressed as mean + S.E.M. All results are calculated from one experiment with at least 9 mice per group. Significance: **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001 compared to the Ova group.

in an ongoing airway inflammation. In line, no difference in lymphocyte distribution in THC treated mice was detected. Several studies showed that endocannabinoids and cannabinoids can also inhibit antibody formation (Kaminski et al. 1994; Titishov et al. 1989) or proliferative response to LPS (Lee et al. 1995). In general, the effects of cannabinoids on immune cells are thought to be mediated by binding and activating the CB₂ receptor which is the predominant CB receptor on immune cells. CB₂ receptor stimulation by cannabinoids might then result in an anti-inflammatory reaction. It is known that B cells have the highest density of CB₂ receptor expression (Bouaboula et al. 1993; Howlett 2002). However, some studies suggest that cannabinoids target accessory cells such as T cells rather than directly acting on B cells (Schatz et al. 1993).

This, together with our data demonstrating a dramatic improve of the allergen-induced airway diseases after activation of the endocannabinoid system through THC treatment in wildtype mice, led us to the presumption that the inactivation of the endocannabinoid system and therefore a lack of endocannabinoid signalling through CB₁ and CB₂ receptor shows the converse outcome, an exacerbated allergic inflammation. Therefore, we blocked endocannabinoid signalling through the CB receptors by applying the antagonists SR1 and SR2 in C57BL/6 wildtype mice. To obtain independent evidence for the involvement of the single CB₁ or CB₂ receptors we applied the single antagonists SR1 or SR2, respectively. However, no altered outcome of systemic immune response or allergen-induced airway response was detected. Because knockout mice provide a more crucial model for investigation of the role of CB₁ and CB₂ receptor, we further evaluated allergic sensitization and allergen-induced airway inflammation in CB receptor-deficient mice. However, in our model of allergen-induced airway inflammation, these mice did not show an exacerbated immune response. Of note, airway inflammation and immunoglobulin levels were even slightly lower in CB₁^{-/-}/CB₂^{-/-} mice. To date we cannot clarify this controversy. It is noteworthy that in CB receptor deficient mice, endocannabinoids might be overproduced as a consequence of the lack of communication between receptors and ligands (Di Marzo et al. 2000) and as a consequence activate other pathways by binding to receptors which are distinct from CB receptors or even exert receptor-independent mechanisms. Anandamide, a well-known endocannabinoid, was shown to exert dose-dependent immunosuppressive effects on human T and B cell proliferation which is mainly regulated by apoptosis (Schwarz et al. 1994). This might explain, at least in part, how endocannabinoids might function as local modulators in the allergic immune response in mice which lack signalling through CB receptors.

In our study, a further and even more eminent evidence for a CB receptor-independent mechanism of cannabinoid-stimulation was achieved by THC treatment of CB₁^{-/-}/CB₂^{-/-} mice. Serum immunoglobulin levels in THC treated CB₁^{-/-}/CB₂^{-/-} mice were significantly reduced, which indeed suggested a mechanism independent from CB receptors. There is a growing number of studies which demonstrated that cannabinoids such as THC do not exert all their effects via CB₁ or CB₂ receptors (Kaplan et al. 2003; Zygmunt et al. 2002). A line of studies performed with CB receptor deficient mice further indicated that some effects of cannabinoids are mediated by neither CB₁ nor CB₂ receptors (Begg et al. 2005; Mackie and Stella 2006). Potential targets might be ion channels or intracellular lipid receptors such as the PPAR family. Suppression of IL-2 production by anandamide in activated T cells seems to occur via intracellular PPARγ (Rockwell et al. 2006). Two orphan G protein-coupled receptors of the non-CB₁/CB₂ receptor class have been suggested as novel cannabinoid receptors, namely GPR119 and GPR55. However, Brown et al. suggests that further, yet-to-be-identified novel cannabinoid receptors mediate these non-CB₁/CB₂ receptor effects (Brown 2007).

Together, our studies demonstrated that allergen-induced airway inflammation is regulated by cannabinoids. The mode of action of these substances still remains unclear; however, our data suggest at least in part a non-CB₁/CB₂ receptor mediated mechanism. As a consequence, the attenuation of allergic airway disease through cannabinoids seems to be a promising strategy for a therapeutic approach, especially when they do not signal through the CB₁ receptor cascade and thus do not induce psychoactive effects.

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