Immunosuppressive activity of non-psychoactive *Cannabis sativa* L. extract on the function of human T lymphocytes

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Abstract

Background: *Cannabis sativa* L. extracts (CSE) are used for treating inflammatory conditions, but little is known about their immunomodulatory effects. We investigated a novel CSE with high (14%) CBD and low (0.2%) THC concentration in comparison with pure CBD on primary human lymphocytes.

Methods: Proliferation, cell cycle distribution, apoptosis/necrosis and viability were analysed with standard methods. Genotoxicity was evaluated with the comet-assay. The effect on T lymphocyte activation was evaluated via CD25/CD69 marker expression, degranulation assays and the production of cytokines. The influence on the transcription factors was analysed using Jurkat reporter cell lines. Specific CB2 receptor antagonist SR144528 and TRPV1 receptor antagonist A78416B were used to study the involvement of CB2 or TRPV1 receptors.

Results: CSE inhibited the proliferation of activated T lymphocytes in a dose-dependent manner without inducing apoptosis, necrosis, or affecting cell viability and DNA integrity. The inhibitory effect was mediated via the suppression of T lymphocytes activation, particularly by the suppression of CD25 surface marker expression. Furthermore, CSE interferes with the functionality of the T lymphocytes, as indicated by inhibition of degranulation, IL-2, and IFN-γ production. AP-1-and-NFAT-reporter activation was reduced implicating an AP-1-and-NFAT-mediated mode of action. The effects were in part reversed by SR144528 and A78416B, showing that the effects were mainly mediated by CB2 and TRPV1 receptors.

Conclusion: CSE and CBD have immunomodulatory effects and interfere with the activation and functionality of T lymphocytes. A comparison between CSE and CBD suggests that the immunosuppressive effect of CSE is mostly due to the effect of CBD.

Keywords: *Cannabis sativa* L., cannabidiol, lymphocytes, inflammation, immunomodulation

1 Introduction

The immune system protects the human body from diseases and has an important role in maintaining tissue homeostasis. Inflammation is part of the body's defence mechanism that is crucial to health. The acute inflammatory response is a result of such a process [1]. However, acute inflammatory responses that fail to regulate can become chronic and cause a variety of inflammatory diseases, including cardiovascular diseases, inflammatory bowel diseases, diabetes, arthritis, joint diseases, respiratory diseases, mental disorders, cancer, and autoimmune disorders [1,2]. In the case of autoimmune diseases, such as type 1 diabetes mellitus, rheumatoid arthritis (RA), or multiple sclerosis (MS), the body attacks and damages its own tissues and causes inflammation [3,4]. Worldwide, there is an increasing number of people suffering from autoimmune diseases, including a significant cause of death [5,6]. The loss of immune cells' ability to distinguish between non-self and self-antigens leads to an aberrant immune reaction against healthy cells and tissues, which ultimately leads to the over-production of autoreactive immune cells and the development of these diseases [7,8].

Autoimmunity is initiated by the activation of antigen-specific T cells, which respond to self-antigens. The activation of T cells is characterised by robust production and release of pro-inflammatory mediators, including cytokines, chemokines, and perforin or granzymes, killing target cells [8]. The inhibition of T lymphocyte functions has great potential to improve the quality of life for patients suffering from autoimmune diseases. The treatment of autoimmune diseases generally focuses on immunosuppressive and anti-inflammatory drugs, such as corticosteroids, azathioprine, and cyclophosphamide, to inhibit the activity of the overreacting

immune system [9,10]. However, these drugs have numerous side effects [11]. Consequently, there is great interest in studying the mode of action of natural medicine products.

Cannabis sativa L. (*C. sativa*) belongs to the Cannabaceae family, an annual herbaceous species popularly and widely used for recreational and medicinal purposes. Cannabis and cannabinoids have been shown to have benefits in diseases such as MS, nausea, cancer, epilepsy, and neurodegenerative disorders [12–14]. Over the last few years, its importance as a botanical medicine has increased dramatically. In 2018, it was the top-selling herbal supplement, and its sales increased by an astounding 332.8% compared with the previous year [15]. Apart from the psychoactive compound Δ^9-tetrahydrocannabinol (THC), it contains other non-psychoactive cannabinoids, e.g. cannabidiol (CBD) and cannabigerol (CBG) in various amounts. CBD possesses anti-inflammatory and immunomodulatory effects, suppresses proliferation and cytokine production of mouse splenocytes and might therefore be beneficial for the treatment of autoimmune and inflammatory diseases [16–18]. Cannabis is prohibited in many countries due to its mind-altering properties. Therefore, new varieties of cannabis containing little or no THC, and rich in other non-psychoactive cannabinoids, have been developed. Cannabis and its cannabinoids might have the ability to improve different inflammatory conditions; therefore, this study aimed to investigate the immunomodulatory effects of a new standardised CBD-rich (14%) *C. sativa* extract with a low THC (0.2%) content, compared with pure CBD, using human primary lymphocytes.

2 Material and methods

2.1 Ethics statement

The blood donors gave written informed consent for the use of their blood for research purposes prior to the start of the experiments. All experiments conducted on human material were approved by the ethics committee of the University of Freiburg (55/14).

2.2 Plant material and cannabidiol

CSE and pure CBD were kindly provided by BaFa neu GmbH (Malsch, Germany). CSE is prepared from leaves of the *C. sativa* Fedora through a standardised procedure. The plant material is harvested when the seeds mature, are dried, and then processed. The extract is prepared with a CO_2 extraction method followed by dewatering and decarboxylation. The main components and their percentage present in the CSE were analysed by ÖHMI Analytic GmbH (Magdeburg, Germany) and are listed in Table 1. For biological assays, the extract and CBD were solved in dimethyl sulphoxide (DMSO).

Table 1: Quantification of compounds in the CSE.

Compounds	**mg/kg**	**%**
Total, Δ^9-Tetrahydrocannabinol (THC)	2018	0.2018
Δ^9-Tetrahydrocannabinol (THC)	2012	0,2012
Δ^9-Tetrahydrocannabinol acid (THCA)	6.33	0.0006
Total, Cannabidiol (CBD)	144432	14.4432
Cannabidiol (CBD)	144415	14.4415
Cannabidiol acid (CBDA)	18.55	0.0019

2.3 Preparation and cultivation of human peripheral blood mononuclear cells (PBMC)

PBMC were isolated from the blood of healthy donors obtained from the blood transfusion centre (University Medical Centre Freiburg, Freiburg, Germany). Venous blood was layered and centrifuged on a LymphoPrep gradient (density: 1.077 g/cm^3, 20 min., 500 g, 20 °C; Progen, Heidelberg, Germany). The PBMC were collected and washed twice with phosphate buffer saline (PBS; GE Healthcare, München). The cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FBS) (Life Technologies), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin, and 100 U/ml streptomycin (Life Technologies). Cells were cultured at 37 °C in a humidified incubator with a 5% CO_2/ 95% air atmosphere.

2.4 Cell lines

Jurkat reporter cell lines AP-1::eGFP, NFAT::eGFP and NF-κB::eGFP were described earlier [19] and were used in this study. All cell lines were obtained from Prof. Dr. Peter Steinberger (Center for Pathophysiology, Infectiology and Immunology, Institute of Immunology, Medical University of Vienna, Vienna Austria) and cultured in an RPMI 1640 medium, supplemented with 10% heat-inactivated FBS, 2 mM L Glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, and 1% HEPES. All the cells were cultured at 37 °C in a humidified incubator with a 5% CO_2/ 95% air atmosphere in a standard cell culture flask (75 cm^3).

2.5 Activation and treatment of primary lymphocytes

The PBMC were stimulated with 100 ng/ml anti-human CD3 (clone OKT3) and 100 ng/ml anti-human CD28 (clone 28.6) mAbs (eBioscience, Frankfurt, Germany). The cells were left untreated or were treated with 5 µg/ml ciclosporin A (CsA; Sandimmum; Novartis Pharma, Basel, Switzerland), 300 µM camptothecin (CPT; Tocris, Bristol, UK), 0.5% Triton-X 100 (Sigma-Aldrich, Taufkirchen, Germany), and 2.5 µM ethyl methanesulfonate (EMS; Sigma-Aldrich Chemie, Steinheim, Germany) as controls, or with different concentrations of CSE and CBD, respectively. After cultivation, the biological activities of the cells were assessed as described below.

2.6 Analysis of cell division using CFSE staining

To determine cell proliferation, PBMC were harvested, washed twice with cold PBS, and resuspended in PBS. The cells (5×10^6 cells/ml) were stained with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma-Aldrich), diluted 1/1000, and incubated for 10 min at 37 °C. The staining reaction was stopped by washing twice with supplemented RPMI medium. Next, the stained cells were treated for 72 h. The proliferation was analysed by flow cytometry, using a FACS Calibur analyser (BD Bioscience, Becton Dickinson, Franklin Lakes, New Jersey (NJ)).

2.7 Cell cycle analysis

The T lymphocytes were treated for 72 h. The DNA content was measured after permeabilisation and fixing the cells with 70% ethanol for at least 2 h at 4 °C. Afterwards, the staining of the DNA was carried out using PI master mix, which contains 40 µg/ml PI and 100 µg/ml RNase (both from Sigma, Taufkirchen, Germany) in PBS, and incubated at room temperature for 30 min in the dark. The fluorescence

detection was performed by flow cytometry (FACS Calibur, BD Bioscience). FlowJo software was utilised to analyse the DNA frequency histograms and to analyse the proportion of cells in the particular phases of the cell cycle.

2.8 Determination of apoptosis and necrosis

The cells were treated for 72 h, washed with PBS, and stained with the annexin V-FITC apoptosis detection kit (eBioscience, Frankfurt, Germany) according to the manufacturer's instructions. In brief, after annexin V staining for 15 min, PI (eBioscience, Frankfurt, Germany) was added, and the cells were incubated for an additional 15 min at room temperature in the dark, followed by flow cytometric analysis (FACS Calibur, BD Bioscience).

2.9 Measurement of cell viability

The cell viability was analysed using a WST-1 assay, which is based on the cleavage of the tetrazolium salt WST-1 by cellular mitochondrial dehydrogenases to formazan. The cells were treated for 72 h, washed twice with PBS, and then resuspended in 100 µl of freshly prepared cell proliferation reagent WST-1 (Roche, Mannheim, Germany). The incubation was carried out at 37 °C for 1.5 h. The viability of the cells was measured using the Tecan Infinite M 200 microplate-reader at 450 nm wavelength.

2.10 Assessment of genotoxicity

To detect the genotoxic properties of CSE and CBD, an alkaline comet assay was performed according to Steinborn et al. (2017) with a few modifications. After 24 h of treatment, the cells were harvested and washed twice with PBS and prepared for a comet assay analysis. The DNA damage analysis was done using a Leica

fluorescence microscope (Leica DMLS; Wetzlar Germany). At least 100 nucleoids per slide were randomly scored using image analysis software (Pylon Viewer 5.0.10). The genotoxic effects were quantified with ImageJ software, and the indicator of DNA damage was percent tail DNA.

2.11 Electron paramagnetic resonance (EPR) spectroscopy

To detect the level of intracellular oxidative stress in activated T cells, electron spin resonance spectroscopy (e-scan EPR, model NOX-E.11-ESR), equipped with a temperature and gas controller Bio III (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany), was used. The cells (5×10^5 cells/ml) were treated for 24 h and thereafter exposed to 200 µM menadione for 1 h. Afterwards, cells were washed with Krebs-HEPES buffer (KHB; Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). The cells were then incubated for 20 min at 37 °C with KHB buffer supplemented with 25 µM deferoxamine methanesulfonate (DFO), 100 µM high cell-permeable spin probe 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrolidine hydrochloride (CMH), and 5 µM diethyldithiocarbamic acid sodium (DETC) (all from Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). The samples were then kept on ice, and EPR spectra were measured in 50 µl glass capillaries.

2.12 Determination of CD25 and CD69 activation markers

The T lymphocytes activation was assessed by analysing the expression of the surface markers CD25 and CD69 after treatment for 24 h. The cultured cells were washed with PBS and stained with APC-labelled anti-human CD4, PE-labelled anti-human CD25, and FITC-labelled anti-human CD69 mAbs (eBioscience, Frankfurt,

Germany) for 20 min at 4 °C. The expression of CD25 and CD69 was measured for $CD4^+$ and $CD8^+$ T cells by flow cytometry (FACS Calibur analyzer, BD Bioscience).

2.13 Determination of degranulation

To analyse the T cell granulation, the cells were treated for 20 h and restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for another 4 h. Afterwards, PE-conjugated anti-CD107a mAb (eBioscience, Frankfurt, Germany) was added, and they were incubated for 1 h at 37 °C before GolgiStop (BD Biosciences, Heidelberg) treatment for another 3 h. The samples were analysed by flow cytometry (FACS Calibur, BD Biosciences).

2.14 Determination of cytokine production

The cells were treated for 48 h and restimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Taufkirchen, Germany), 500 ng/ml ionomycine (Sigma-Aldrich, Taufkirchen, Germany), and BD GolgiPlug (BD Biosciences, Heidelberg) for an additional 4 h at 37 °C. Afterward, the cells were fixed and permeabilised, using 4% paraformaldehyde (Sigma-Aldrich) and Prem/Wash solution (Becton Dickinson, Franklin Lakes, NJ), followed by staining with either PE-conjugated anti-human interferon-gamma (IFN-γ) mAb, PE-conjugated anti-human interleukin 2 (IL-2) mAb (eBioscience, Frankfurt, Germany), or PE-conjugated anti-human tumor necrosis factor-alpha (TNF-α) mAb (all from eBioscience, Frankfurt, Germany). The samples were analysed with FACS LSR Fortessa analyzer (BD Biosciences, Becton Dickinson).

2.15 Influence on AP-1, NFAT, and NF-κB activity, using Jurkat reporter cell lines

The different Jurkat reporter cells were harvested, washed twice with PBS, and adjusted to a concentration of 5 × 10^5 cells/ml in a 5% FCS RPMI medium. In a 96-well F-bottom plate, 200 µl cells/well were seeded and stimulated with an ImmunoCult human CD3/CD28 T cell activator (25 µg/ml; StemCell™ Technologies, Cologne, Germany). The cells were treated with different inhibitor controls for AP-1 (1 µM; SP100030; Tocris Bioscience, R&D Systems, Abingdon, UK), NFAT (5 µg/ml; ciclosporin A), and NF-κB (10 µM; parthenolide; Sigma-Aldrich, Taufkirchen, Germany), as well as CSE (3, 11, 33, and 100 µg/ml) or CBD (0.5, 1.6, 4.8, and 14.4 µg/ml) and incubated at 37 °C for 24 h. Thereafter, the cells were washed twice with PBS, and the eGFP was measured by flow cytometry (FACS Calibur, BD Biosciences).

2.16 Influence on cannabinoid receptor 2 (CB2) and transient receptor potential vanilloid subtype 1 (TRPV1)

To evaluate the involvement of CB2 and TRPV1 receptors, isolated PBMC were activated as described in 2.6 and pre-incubated for 2 h with the selective CB2 receptor antagonist SR144528 (5 µM; Sigma-Aldrich) or TRPV1 receptor antagonist A78416B (100 nM; Tocris, UK) followed by a further treatment with CSE (33 µg/ml) and CBD (5 µg/ml). After cultivation for 48 h the cells were assessed for IL-2 cytokine production (see 2.15) and after 72 h treatment for proliferation (see 2.7).

2.17 Data analysis

For statistical analysis, the data were processed using Microsoft Excel (Version 2016, Microsoft Cooperation, Redmond, USA) and SPSS software (IBM, Version 22.0, Armonk, USA). The values are presented as the mean ± standard deviation (SD) for the indicated number of independent experiments. The statistical significance was determined using the student t-tests or one-way ANOVA followed by Dunnett's post hoc pairwise comparisons. The asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) represent the significant differences from the controls. The dose-response inhibition was analysed by non-linear regression, using GraphPad Prism software (Prism, version 7.1.0 La Jolla, CA), and the half-maximum inhibitory concentration (IC_{50}) value was finally calculated.

3 Results

3.1 Effect of CSE on T lymphocyte proliferation

To analyse the impact of CSE in relation to CBD on proliferation, different concentrations (1–100 µg/ml) were tested on human T lymphocytes. The proliferation of activated T cells was inhibited by ciclosporin A (CsA) compared with the stimulated control cells (Fig.1 A). CSE and pure CBD dose-dependently decreased the proliferation of activated T cells, with a calculated half-maximum inhibitory concentration (IC_{50}) of 26.2 µg/ml (CSE) and 4.7 µg/ml (CBD) (Fig. 1 A, B). The observed inhibition of the T cell proliferation may be due to changes in the cell cycle.

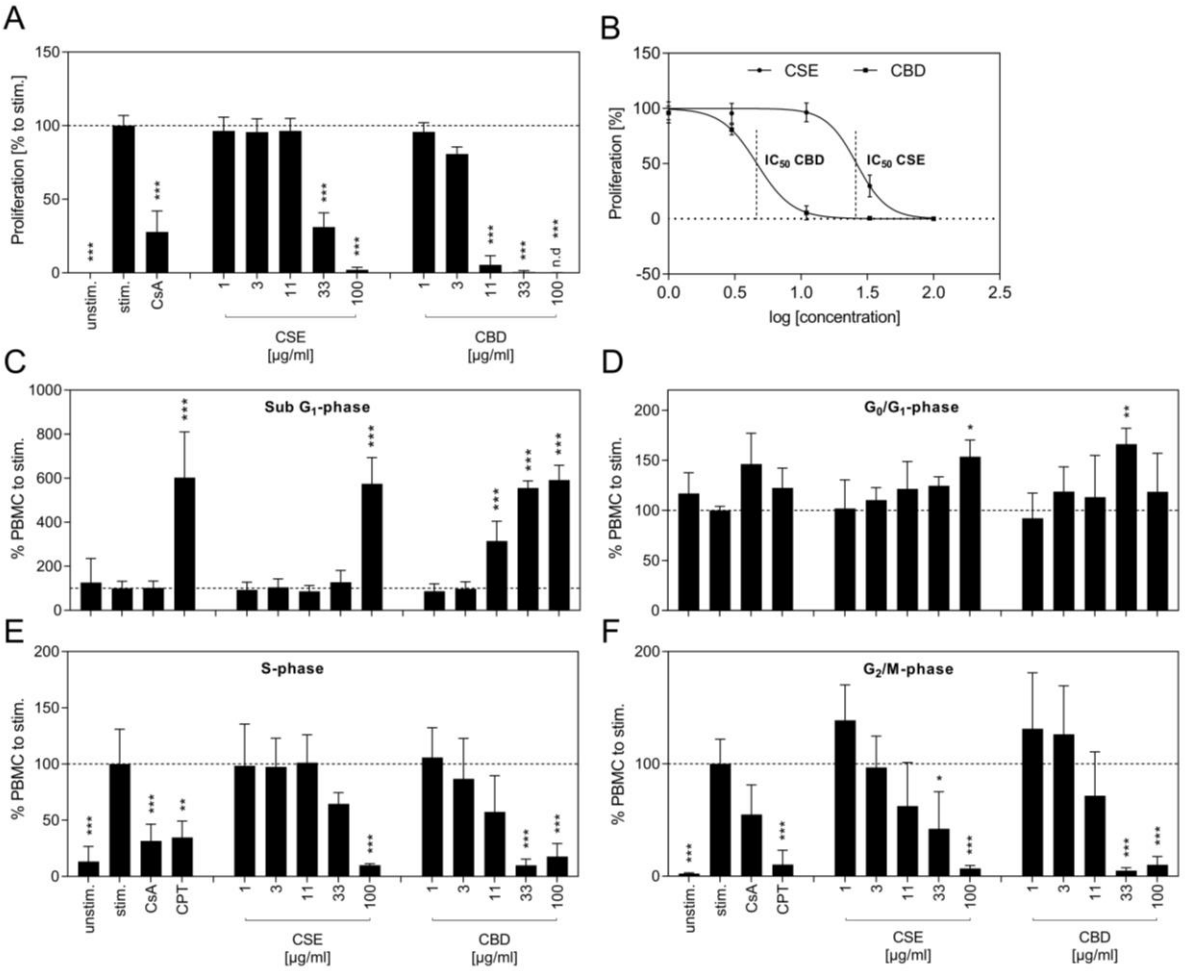

Fig. 1: Effect of CSE and CBD on proliferation and cell cycle of activated T lymphocytes. (**A**) The CFSE-labelled primary human lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs (except the unstimulated (unstim.)) and ciclosporin A (CsA; 5 µg/ml) or different concentrations (1-100 µg/ml) of *C. sativa* extract (CSE) and cannabidiol (CBD) were added. After 72 h of culturing, the cell division analysis was determined by flow cytometry. (**B**) The half-maximum inhibitory concentration (IC_{50}) was calculated based on the proliferation data. (**C-F**) Human primary lymphocytes were stimulated with anti-human 100 ng/m CD3/CD28 mAbs (except unstim.) and CsA (5 µg/ml), camptothecin (CPT; 300 µM), or different concentrations (1-100 µg/ml) of CSE and CBD were added. After 72 h of incubation, the percent distribution of the cell cycle for Sub G_1-phase (**C**), G_0/G_1-phase (**D**), S-phase (**E**), and G_2/M-phase (**F**) were determined by flow cytometry. The data are presented as the mean ± standard deviation of three (**A**, **B**) or five (**C**-**F**) independent experiments in relation to the untreated stimulated control. *p < 0.05, **p < 0.01, ***p < 0.001. n.d = not detectable.

To further investigate the inhibitory effect of CSE and CBD on proliferation, cell cycle progression parameters were analysed after 72 h of treatment. The percent distribution of the cells in the four phases of the cell cycle is commonly referred to as the “sub-G_1”, “G_0/G_1”, “S”, and G_2/M phases. In the G_0/G_1 phase, the cell grows and contains a complete set of chromosomes. In the S or synthesis phase, DNA replication takes place and results in a double set of chromosomes. The cells with the double set of chromosomes are now in the G_2 phase and preparing for mitotic division, which starts the cell cycle again [21]. The sub-G_1 phase (Fig. 1 C) contains apoptotic cells with fractional DNA content or cell debris [22]. The flow cytometry analysis revealed that CSE and CBD dose-dependently arrested the cells in the G_0/G_1-phase (Fig. 1 D) and concomitant inhibition in the number of cells in the S-phase (Fig.1 E) and the G_2/M-phase (Fig.1 F) of the cell cycle compared with untreated stimulated cells.

3.2 Effect of CSE on T lymphocyte viability

To quantify whether the inhibitory effect of CSE on proliferation is due to a toxic effect we analysed the induction of apoptosis and necrosis with annexin V/PI staining (Fig. 2 A, B). Only the highest CSE concentration (100 µg/ml) induced apoptosis, whereas CBD showed a dose-dependent induction of apoptosis (Fig. 2 A). CSE and CBD showed no significant effects on necrosis induction (Fig. 2 B). The analysis of the mitochondrial dehydrogenase activity of the cells was in line with the results of apoptosis/necrosis and proliferation experiments, where only 100 µg/ml CSE and 11-100 µg/ml CBD could reduce the viability (Fig. 2 C). Interestingly, 33 µg/ml CSE could effectively inhibit T cell proliferation without inducing apoptosis or necrosis in the cells.

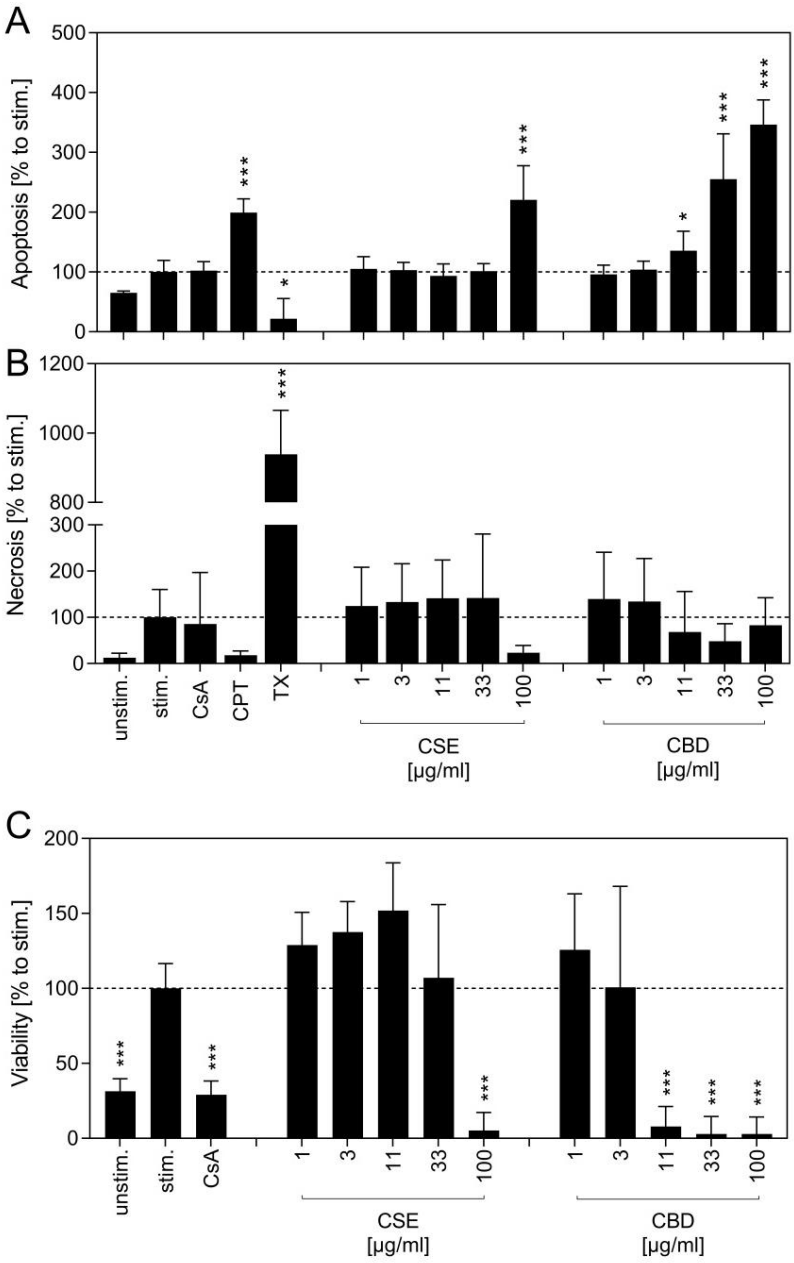

Fig. 2: Effect of CSE and CBD on apoptosis, necrosis and viability of activated T lymphocytes. (**A, B**) Human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs (except unstimulated (unstim.)) and ciclosporin A (CsA; 5 µg/ml), camptothecin (CPT; 300 µM), and triton-X 100 (TX; 0.5%), or different concentrations (1-100 µg/ml) of *C. sativa* extract (CSE) and cannabidiol (CBD), were added. After culturing for 72 h, the cells were stained with annexin V-FITC and propidium iodide (PI) to determine the percentage of apoptotic (**A**) and necrotic (**B**) cells. (**C**) Human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs (except unstim.) and CsA (5 µg/ml), or different concentrations (1-100 µg/ml) of CSE and CBD were added.

After 72 h of incubation, the viability of the cells was determined by mitochondrial dehydrogenase activity using a WST-1 assay. The data were presented as the mean ± standard deviation of three (**A**, **B,** and **C**) independent experiments in relation to the untreated stimulated (stim.) control. ***p < 0.001.

3.3 Effect of CSE on genotoxicity and ROS formation

We then used a comet assay, which detects DNA strand breaks to assess genotoxic stress after treating T lymphocytes with CSE or CBD. For a better understanding, the effect was evaluated using different concentrations of CSE (3-100 µg/ml) and the corresponding concentrations of pure CBD (0.5-14.4 µg/ml). In the presence of EMS (positive control for DNA damage), the untreated stimulated cells (positive control) showed intact DNA and only the highest concentration of CSE (100 µg/ml) induced DNA damage in the cells.

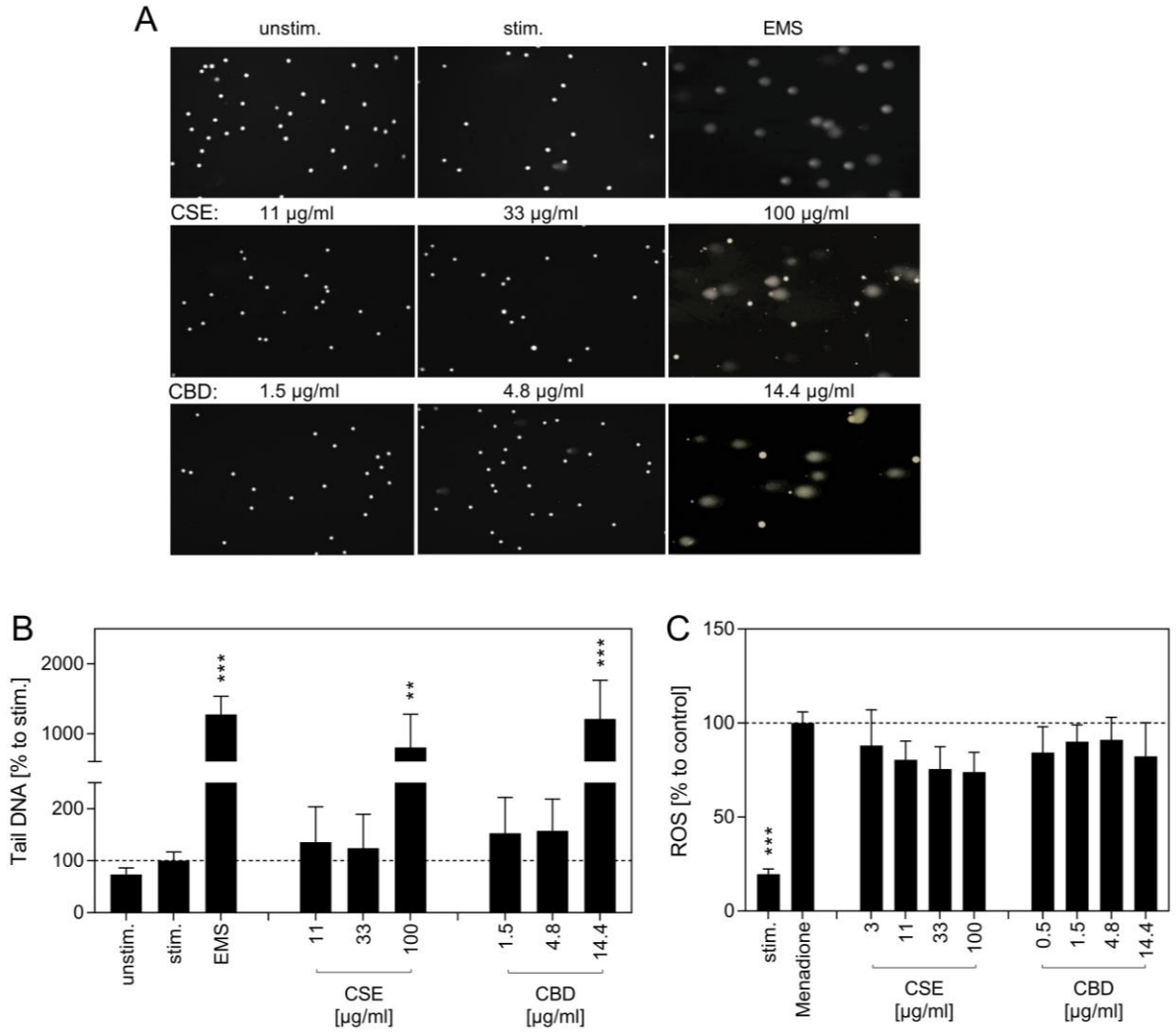

Fig. 3: Effect of CSE and CBD on the induction of DNA damage and ROS formation. (**A,B**) The human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs and ethyl methanesulfonate (EMS; 5 µM) or *C. sativa* extract (CSE; 11-100 µg/ml), and cannabidiol (CBD; 1.5-14.4 µg/ml) was added. After 24 h of culturing, a comet assay was performed for the detection of a genotoxic effect. The electrophoresed and fixed cells were stained with ethidium bromide (5 µg/ml) and analysed with a fluorescence microscope. (**A**) The illustration represents the stained nucleus of the cells. (**B**) The data were presented as the mean ± standard deviation of three independent experiments in relation to the untreated stimulated (stim.) control. (**C**) The human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs and CSE (3-100 µg/ml) and CBD (0.5-14.4 µg/ml) was added. To induce ROS, the cells were re-stimulated with 200 µM menadione (except stim.) after 24 h of culturing, and ROS production was evaluated using the EPR method. The data

were presented as the mean ± standard deviation of three independent experiments in relation to menadione control. **p < 0.01, ***p < 0.001.

This damage seems to be induced by CBD, since pure CBD used at the concentration that is contained in the CSE extract (14.4 ug/ml) also induced DNA-damage (Fig. 3 A, B). One of the major causes of DNA damage is ROS [23]. Therefore, we examined the formation of ROS in primary T cells after CSE and CBD treatment. Electron paramagnetic resonance spectroscopy showed that there was a slight dose-dependent inhibition of ROS formation in cells treated with CSE, but the effect was not statistically significant (Fig. 3 C). However, the CBD showed no effect.

3.4 Effect of CSE on activation markers of T lymphocyte

Further investigation on the effects of CSE and CBD on the effector function of the T cells was performed. The stimulation of T cells via the T cell receptor (TCR) induces an upregulation of activation markers CD25 and CD69 on the cell surface [24]. To activate TCR signalling, the cells were stimulated with CD3 and CD28, which significantly induced the upregulation of CD25 and CD69 in $CD4^+$ and $CD8^+$ T cells (Fig. 4 A, B, C). Treatment with CSE and CBD lowered CD25 expression dose-dependently in both $CD4^+$ and $CD8^+$ T cells. However, this was not the case with CD69 (Fig. 4 A, B, C).

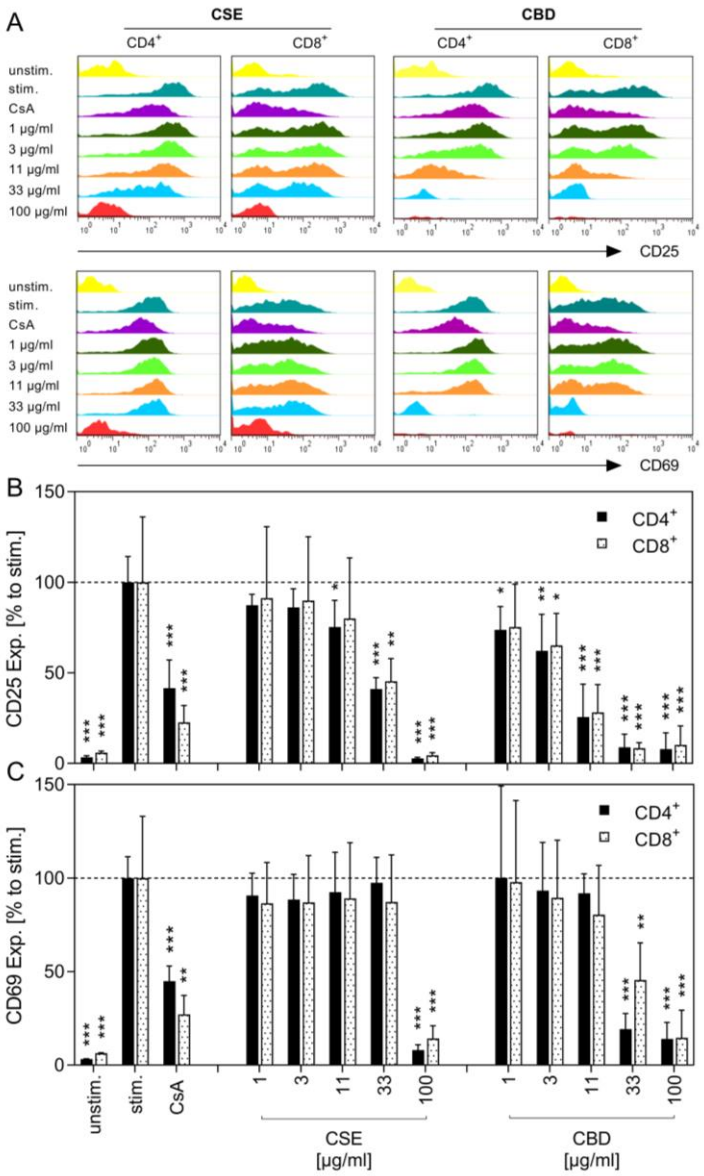

Fig. 4: Effect of CSE and CBD on the expression of activation marker of activated T lymphocytes. Human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs (except unstimulated (unstim.)) and ciclosporin A (CsA; 5 µg/ml), or different concentrations (1-100 µg/ml) of *C. sativa* extract (CSE) and cannabidiol (CBD) were added. After 24 h of culturing, the cells were stained with anti-CD25-PE or anti-CD69-FITC and anti-CD4-APC mAbs and analysed by flow cytometry. (**A**) Histogram plots show the expression of CD25 and CD69 surface markers in activated $CD4^+$ and $CD8^+$ T lymphocytes. (**B**, **C**) The data were presented as the mean ± standard deviation of three independent experiments in relation to the untreated stimulated control. *p < 0.05, **p < 0.01, ***p < 0.001.

3.5 Effects of the *CSE* on the function of T lymphocytes

Next, we analysed whether CSE or CBD affected the release of perforin and granzymes in T cells. The activation of T lymphocytes initiates cytolytic activities to kill the target cells via the release of granule content, such as perforin/granzymes [25]. The lytic granule membrane contains lysosomal-associated membrane proteins (LAMPs), such as LAMP-1 (CD107a) and fuses with the membrane of activated T cells. Therefore, analysis of the LAMP-1 surface expression can be directly related to the identification of degranulating cells. Flow cytometric analysis on the LAMP-1 was performed using anti-CD107a mAb, and the results showed that the degranulation of the activated T cells was reduced by ciclosporin A (CsA) compared with the stimulated control cells. The presence of CSE and CBD significantly reduced degranulation in a concentration-dependent manner (Fig. 5 A). To further investigate the effects of CSE and CBD treatment on T lymphocyte function, we analysed the secretion of the cytokines interleukin-2 (IL-2), tumor necrosis factor-α (TNFα) and interferon-γ (IFN-γ) upon TCR stimulation with CD3 and CD28. The production of all the cytokines was inhibited by the inhibition control CsA, compared with the untreated stimulated control. The treatment with CSE in a nontoxic concentration (33 µg/ml) significantly lowered the production of IL-2 and IFN-γ (Fig 5 B, C, D). In addition, the non-toxic concentration of CBD (3 µg/ml) also significantly lowered the production of IL-2 and IFN-γ (Fig 5 B, C, D). No effect of CSE and CBD on TNFα could be observed (Fig. 5 D).

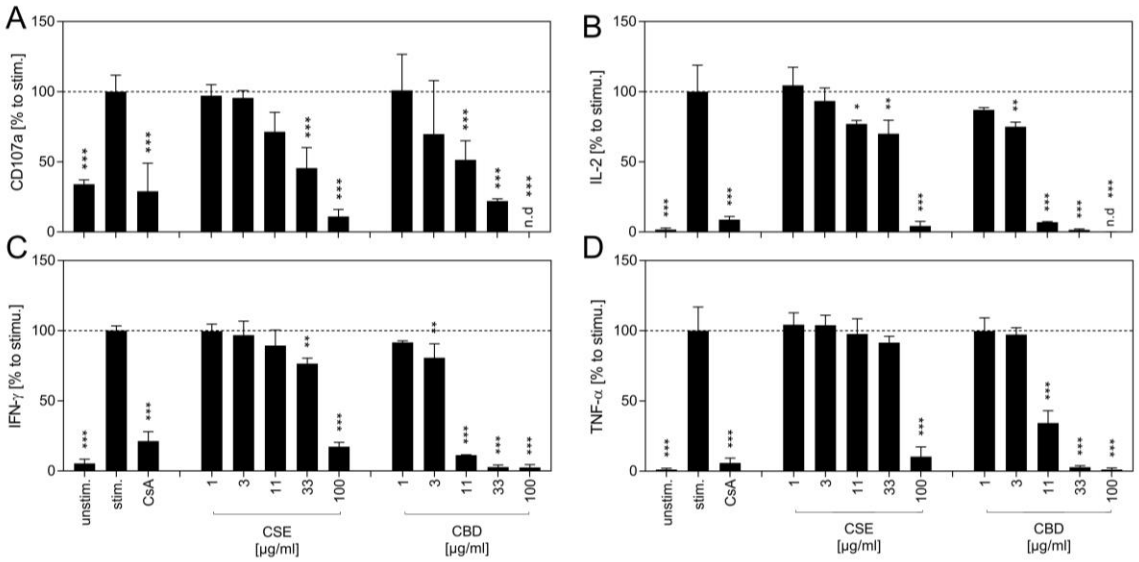

Fig. 5: Effect of CSE and CBD on the degranulation and cytokines production of activated T lymphocytes. Human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs (except unstimulated (unstim.)) and incubated in the presence of ciclosporin A (CsA; 5 µg/ml), or different concentrations (1-100 µg/ml) of *C. sativa* extract (CSE) and cannabidiol (CBD) for 24 (**A**) or 48 h (**B**-**D**). Subsequently, the cells were re-stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for an additional 4 h. The cells were stained with either PE-coupled anti-CD107a mAb, (**A**) or anti-IL-2 (**B**), or anti-IFN-γ (**C**), or anti-TNF-α (**D**) mAbs, and analysed using flow cytometry. The data were presented as the mean ± standard deviation of three independent experiments in relation to the untreated stimulated control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.6 Effect of CSE on the AP-1, NFAT and NFκB

For a better understanding of how CSE induces the immunosuppressive effect on human T lymphocytes, the transcription factors activator protein 1 (AP-1), nuclear factor of activated T-cells (NFAT), and nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), which are known to play a major role in T cell activation processes, were analysed. One important function of these transcription factors (TFs) is to bind to the IL-2 gene and induce the expression of IL-2, which is important for T cell proliferation [26]. To investigate the effect on these TFs, we used reporter cell

lines generated from Jurkat cells [19]. The cells were treated with CSE (100-3 µg/ml) and with corresponding concentrations of CBD (14.4-0.5 µg/ml) or with specific inhibitors. In the presence of inhibitory controls SP100030, CsA, and parthenolide, the activity of AP-1, NFAT, and NF-κB, respectively, was completely inhibited. CSE and CBD inhibited AP-1 and NFAT reporter activation, whereas NF-κB activity was not affected (Fig. 6 A, B, C).

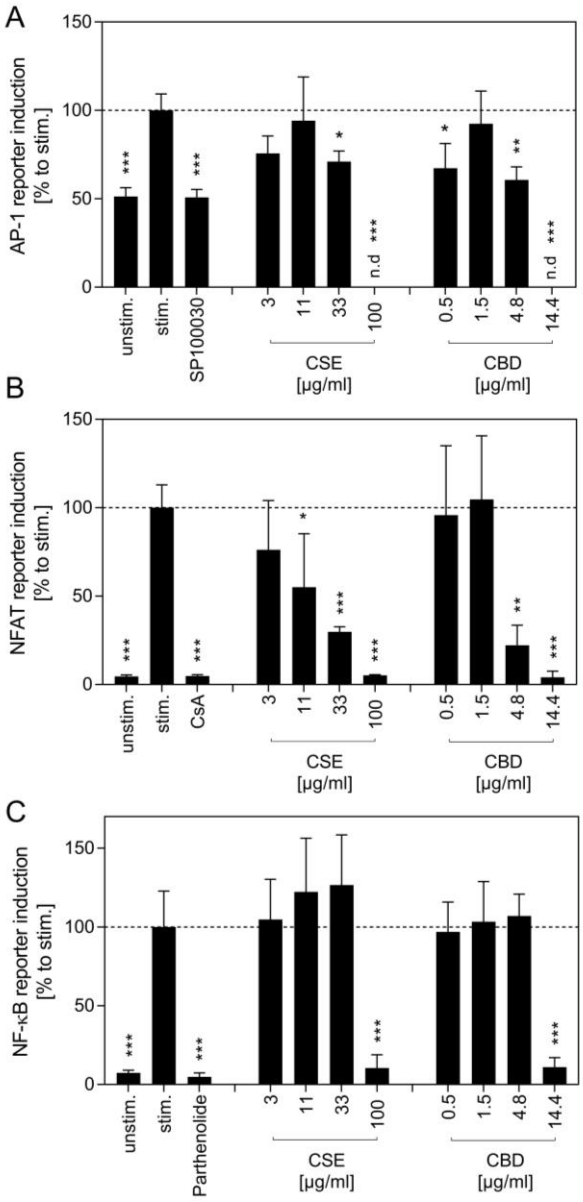

Fig. 6: Influence of the CSE on the activity of transcription factors AP-1 (A), NFAT (B), and NF-κB (C) in Jurkat cells. The AP-1-, NFAT-, and NF-κB-eGFP-Jurkat reporter cells were left unstimulated (unstim) or were stimulated with 25 µl/ml ImmunoCult Human CD3/CD28 T Cell Activator and incubated in the presence of specific inhibitors (1 µM SP100030 for AP-1; 5 µg/ml ciclosporin A for NFAT; and 10 µM parthenolide for NF-κB) or *C. sativa* extract (CSE; 3-100 µg/ml) and cannabidiol (CBD; 0.5-14.4 µg/ml). Following 24 h of stimulation, the eGFP expression in the AP1- (A), NFAT- (B), and NF-κB-reporter cells (C) was examined using flow cytometry. The data were presented as the mean ± standard deviation of three independent experiments in relation to the untreated stimulated (stim.) control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n.d. = not detectable

3.7 Effects on the proliferation and IL-2 production after blocking CB2 and TRPV1 receptor

We hypothesized that the anti-proliferative and immunosuppressive effect of CSE and CBD is mediated by CB2 and TRPV1. The observed inhibitory effect of CSE and CBD would be reversed by a receptor specific antagonist, when CB2 or TRPV1 are involved. As specific CB2 receptor antagonist SR144528 and as TRPV1 receptor antagonist A78416B was used, respectively. Pre-incubation with the different antagonists alone showed no effect on the proliferation and IL-2 compared to untreated stimulated cells (= 100%) (Fig. 7 A and B). The anti-proliferative effect of CSE was significantly inhibited by SR144528 and A78416B (Fig. 7 A). The CSE-induced inhibition of IL-2 could be prevented by co-treatment with SR144528 but not with A78416B (Fig. 7 B). Similar to CSE, the corresponding concentration of pure CBD mediated anti-proliferative effect could be reversed with SR144528 and A78416B (Fig. 7 A). Both antagonists were not able to reverse the effect of CBD in terms of IL-2 production (Fig. 7 B).

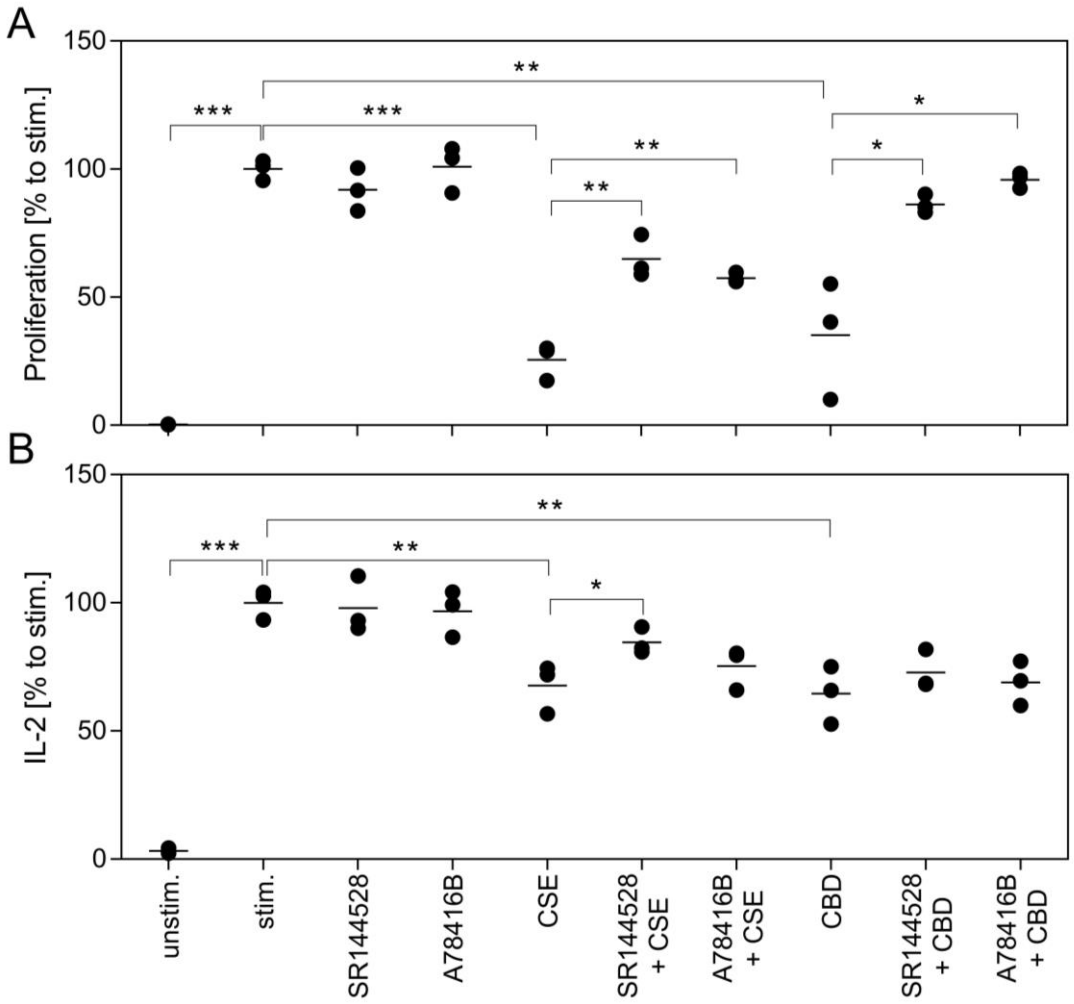

Fig. 7: Effect of CSE and CBD on T lymphocyte proliferation and IL-2 after blocking CB2 and TRPV1 receptor. Human primary lymphocytes were stimulated with 100 ng/ml anti-human CD3/CD28 mAbs (except unstimulated (unstim.)), and incubated in the presence of specific CB2 antagonist SR144528 (5 µM) and specific TRPV1 antagonist A78416B (100 nM) for 2 h. The cells were further treated with *C. sativa* extract (CSE; 33 µg/mL) and corresponding concentration of cannabidiol (CBD; 5 µg/mL). IL-2 production was determined after 48 h whereas the proliferation was assessed after 72 h. Data and mean of three independent experiments are presented for proliferation (**A**) and IL-2 production (**B**) in relation to untreated stimulated (stim.) cells. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

4 Discussion

For centuries, cannabis has been used in the treatment of different inflammatory diseases [27,28]. Based on the assessment of the last 50 years, its psychoactive properties due to THC outweigh its potential therapeutic benefits. Therefore, there is an increasing interest in using CSE with no or low THC content and in evaluating its potential health-improving effect. In this study, we investigated the effect of a cannabis extract with a high CBD (14%) and a low THC (0.2%) content in comparison with pure CBD on human T-lymphocytes. The activation, differentiation, and development of T cells are accompanied by extensive changes in the expression pattern of inflammatory cytokines and transcription factors, and correlate with their survival and function [8]. However, the over-activation of these cells causes hyper-inflammation and tissue damage. Therefore, the inhibition of T lymphocyte activation is a viable treatment option for autoimmune diseases or other conditions associated with aberrant immune responses.

Our results demonstrate that CSE inhibited the proliferation of activated human T lymphocytes (Fig. 1 A, B) with an IC_{50} value of 26.2 µg/ml. For pure CBD, the inhibitory effect on proliferation was stronger with an IC_{50} value of 4.7 µg/ml. The anti-proliferative effect has also been observed in mouse splenocytes [29]. The authors demonstrated that CBD has a stronger anti-proliferative effect compared with a high (20-30%) CBD/THC cannabis extract. A concentration of 33 µg/ml CSE was sufficient to inhibit T cell proliferation without affecting apoptosis/necrosis or cell viability (Fig. 2 A, B, D). Our data showed a cell cycle arrest in the G_1 phase and concomitant dose-dependent decrease in the S and G_2/M phases of the cell cycle (Fig. 1 C, D, E, F). These results suggest that the inhibitory effects of CSE and CBD on the proliferation

of activated T cells are due to the inhibition of DNA replication (S phase), growth (G2 phase), and mitosis (M phase), in which genetic information is distributed between two different daughter cells. Studies have also shown that CBD induces dose-dependent apoptosis in immune cells, such as murine splenocytes [30], thymocytes [31], HL-60 [32], and primary human monocytic cells [33]. The cytotoxic effect of CBD has already been determined for many cell lines, including human HT-29 colorectal carcinoma [34] NIH3T3 fibroblast, B16 melanoma cells, A549 lung cancer cells, SNU-C4 colon cancer cells, and MDA-MB-231 breast cancer cells [35]. With regard to viability, the *in vitro* inhibitory effects of CBD were reported in differentiated human neuronal SY-SH5Y cells with IC_{50} = 5 µg/ml [36].

The activation and proliferation of T lymphocytes is initiated by the ligation of the T-cell receptor (TCR) to cognate antigens, which results in the upregulation of several cell surface markers, each at a distinct phase of the activation process. CD69 (C-type lectin protein) is an inducible cell surface glycoprotein expressed at the early phase of activation via TCR [24]. On the other hand, CD25 (the alpha chain of the trimeric IL-2 receptor) is a late activation marker, upregulating its expression on the surface, and is considered to be the most prominent cellular activation marker [37]. The expression of CD25 dose-dependently decreased for CSE and CBD in $CD4^+$ and $CD8^+$ T lymphocytes, while the expression of CD69 was not affected. After the stimulation and activation of T lymphocytes, several cytokines are released, which trigger specific immune responses including IL-2, IFN-γ, and TNF-α. IL-2 causes clonal expansion of T cells [38] and promotes the production of pro-inflammatory cytokines, such as IFN-γ and TNF-α.

Our data further demonstrate that CSE and CBD treatment reduced the capacity of degranulation of activated T cells, as well as IL-2 and IFN-γ production. The suppression of IL-2 and IFN-γ production, CD25, and CD107a surface markers appear to be potential mediators of the proliferation inhibition observed in this study. Therefore, we conclude that CSE and CBD interfere with the functionality of the T lymphocytes, which results in the inhibition of proliferation. The transcription factors AP-1, NFAT, and NF-κB, are responsible for the transcription of the IL-2 gene. The suppression of IL-2, which leads in the end to inhibition of T cell proliferation, is linked to AP-1 and NFAT transcription factor suppression (Fig. 6).

Previous studies showed that CBD may mediate the anti-inflammatory effect on immune cells via the CB2 receptor [39,40]. This receptor is mainly expressed on immune cells, and it was shown that immune cell function was modulated by CB2 in inflammatory diseases [41]. In addition, the novel non-cannabinoid TRPV1 receptor has been proposed to bind phytocannabinoids as well [42]. TRPV1 is expressed in neuronal cells [43] and immune cells [44]. By utilizing selective antagonists of CB2 (SR144528) and TRPV1 (A78416B) receptors, we tried to analyse the role of these receptors in CSE and CBD related immunosuppression. Pre-treatment with SR144528 or A78416B partly reversed the inhibitory effects of CSE and CBD on activated T lymphocytes proliferation and IL2-production. Inhibiting effects of CBD on IL-2 production were, however, in contrast to CSE, not reversed; hence other principles of action might be responsible. These findings highlight CB2 and TRPV1 as a molecular target for CSE as well as for CBD related immunosuppression.

CBD is the main compound known in this extract of cannabis, and its structure and biological activity have been well studied. It has been reported that CBD suppresses proliferation, mRNA expression of IL-2 and IFN-γ, and surface expression of CD25 in mouse splenocytes [45]. Other studies have provided further evidence that CBD inhibits IFN-γ production from splenocytes isolated from NOD mice [46] and from lymph node cells isolated from arthritic mice [47]. Moreover, various cannabinoid-based extracts suppress proliferation and cytokine secretion in mouse splenocytes [29]. There is no doubt that CBD has an immunosuppressive effect and could be an ideal candidate for the treatment of autoimmune diseases, but *in vivo* studies are still required. It is also important to know whether CBD on its own, or as part of a cannabis complex mixture, is more effective.

In the present study, the effects of CSE were similar to those of corresponding concentrations of pure CBD, indicating that most of the observed immunosuppressive effects of CSE can be explained by its CBD content. However, CSE is slightly more effective for the inhibition of proliferation (IC_{50}: CSE = 3.8; CBD = 4.7), degranulation, and NFAT activity, indicating that other vegetal complex matrices present in the extract possibly contribute to the effects. Consequently, CSE at non-toxic concentrations affects human T lymphocytes' function and could be used as an anti-inflammatory herbal drug.

5 Conclusion

This study demonstrates that a *C. sativa* extract rich in CBD has immunosuppressive effects, and this can be linked to an NFAT related signalling pathway and mediation via CB2 and TRPV1 receptors. The results support the use of CBD rich *C. sativa*

extract and CBD in the treatment of immune disorders associated with aberrant T cell activation. However, further investigation is required to corroborate these effects.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding this work.

CRediT authorship contribution statement

Seema Devi: Conceptualization, Investigation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing**. Amy M. Zimmermann-Klemd:** Conceptualization, Supervision, Writing – review & editing**. Bernd L. Fiebich:** Conceptualization, Writing – review & editing**. Michael Heinrich:** Conceptualization, Writing – review & editing, Validation**. Carsten Gründemann:** Formal analysis, Methodology, Conceptualization, Writing – review & editing, Validation**. Peter Steinberger:** Writing – review & editing, Validation**. Stefanie Kowarschik:** Conceptualization, Visualization, Supervision, Writing – review &

editing, Validation**. Roman Huber:** Formal analysis, Methodology, Conceptualization, Visualization, Supervision, Writing – review & editing, Funding acquisition**.**

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