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A chronic low dose of Δ^9-tetrahydrocannabinol (3 mg / kg / 21 d) reorganizes the disturbed wound healing process and accelerates wound closure in old female mice

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ABSTRACT

Wound healing in old mice is characterized by disturbed tissue homeostasis, manifested by delayed immune cell infiltration and reduced growth factor secretion, leading to a delayed onset and prolonged duration of the inflammatory phase. The endocannabinoid system (ECS) is an important regulator of tissue homeostasis and cell migration and is also considered to be subject to aging processes, which may contribute to observable aging phenomena. Therefore, stimulating the aged ECS could represent a therapeutic option to support tissue regeneration in aging.

Female old mice received a low-dose of medical THC daily for 3 weeks, before four excisional full skin wounds were created. At day 1, 3 and 7 post-surgery, the wound closure rate was analyzed and wound samples were examined immunohistochemically for the numbers of granulocytes, M1-macrophages and mesenchymal stem cells (MSCs). The concentrations of inflammatory cytokines and regenerative growth factors were determined by ELISA.

Administration of THC improved the wound healing rate of old mice between day 1 and 7, which was associated with an altered timing and quantity of infiltrating immune cells and decreased levels of inflammatory cytokines in wound tissue on days 1 and 3 post-injury. THC treatment significantly increased MSC infiltration but had no effect on the growth factor release.

The present study confirmed the anti-inflammatory activity of THC *in vivo*. The THC-treatment improved wound healing in old mice by coordinating the temporal sequence of immune cell infiltration and cytokine release. Thus, restoration of ECS signaling could be an effective strategy to support age-related skin regeneration.

1. Introduction

Physiological wound healing is a process coordinating three consecutive phases for tissue repair, *i.e.*, the inflammatory phase, the proliferative phase, and the remodeling phase (Zomer and Trentin, 2018). However, the progression of these phases maybe impaired or interrupted at elevated age (Gerstein et al., 1993). We recently observed that the inflammatory phase appears delayed by 3–4 days in old (18 months) compared to young (2 months) female mice (Plum et al., 2025). Postponed neutrophil and macrophage recruitment (Nishio et al., 2008), impeded neutrophil chemotaxis (Brubaker et al., 2013), and a delayed but lingered release of inflammatory cytokines in wounds of old animals reflect the disturbed tissue homeostasis, which in turn is crucial for the temporal organization of regular skin tissue repair (Shallo et al., 2003). Especially in old animals, the inflammatory response can persist and lead to a chronic inflammatory state that hinders the transition to the subsequent healing processes (Franceschi and Campisi, 2014). This is indicated by a reduced content of growth factors in wound tissue of old animals, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming growth factor (TGF)-β1 (Komi-

Abbreviations: ASCs, Adipose Stem Cells; BSA, Bovine Serum Albumin; CBD, Cannabidiol; CD90, Cluster of Differentiation 90; ECS, Endocannabinoid System; HGF, Hepatocyte Growth Factor; IGF-1, Insulin-like Growth Factor 1; IL-6, Interleukin-6; Ly-6G, Lymphocyte Antigen 6 Complex, Locus G; MAC-3, Macrophage Antigen Complex-3; MCP-1, Monocyte Chemoattractant Protein-1; MSCs, Mesenchymal Stem Cells; PFA, Paraformaldehayde; RT, Room Temperature; s.c., subcutaneous; TGF-ß, Transforming Growth Factor Beta; THC, tetrahydrocannabinol; TNF-α, Tumor Necrosis Factor Alpha; VEGF, Vascular Endothelial Growth Factor; Veh, Vehicle.

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Kuramochi et al., 2005; Plum et al., 2025; Swift et al., 1999).

The delicate balance between age-promoting influences and antagonizing homeostatic processes significantly determines the aging process. The endocannabinoid system (ECS) is a regulatory network, which is highly conserved during vertebrate evolution and essentially involved in maintenance of local tissue homeostasis (Rodriguez de Fonseca et al., 2005). In a simplified way, it consists of synthesizing and degrading enzymes of the endocannabinoids that interact with the cannabinoid receptors in various tissues (Correia-Sá et al., 2020; Toth et al., 2019). Thereby, the ECS contributes to normal development and function of the skin by regulating cell growth, differentiation, migration and survival (Tóth et al., 2011), as well as by modulating inflammation and oxidative stress (Dawidowicz et al., 2021; Ruhl et al., 2021a; Ruhl et al., 2021b). In addition, cannabinoids promote tissue regeneration by enhancing the secretion of growth factors by mesenchymal stem cells (MSCs) (Ruhl et al., 2020; Ruhl et al., 2021c). However, emerging evidences suggest that the ECS is subject to age-related changes (Lee et al., 2023). In the CNS, it is well established that endocannabinoid synthesis and their binding affinity as well as the cannabinoid receptor density decrease by age (Berrendero et al., 1998; Bilkei-Gorzo, 2012; Nidadavolu et al., 2022; Romero et al., 1998), while the concentration of degrading enzymes increases (Piyanova et al., 2015). Although it is unclear whether cutaneous cannabinoid receptor expression changes with age, it has been found that the genetic deletion of the cannabinoid receptor CB1 induces an aging-like phenotype in the skin of mice. At the age of one year, CB1-knockout mice display a reduction in the subdermal fat layer when compared to their age-matched wildtype counterparts (Bilkei-Gorzo et al., 2012). Furthermore, CB1 receptor deficiency promotes aging-like changes such as a decrease in collagen content that manifests in disorganized fibers with increased inter-fiber spaces, impaired skin antioxidant capacity and an increased proinflammatory environment (Leal et al., 2021). Olah et al. demonstrated that mitochondrial CB1 activation reduces mitochondrial activity in human keratinocytes, suggesting that stimulation of this receptor could be a potential target for reducing oxidative damage during aging (Oláh et al., 2020). Based on these findings, it has been suggested that the underlying processes associated with ECS aging may also contribute to skin aging and its related consequences.

Previous studies have exclusively investigated the therapeutic potential of systemic ECS stimulation on wound healing in young mice. Due to its higher expression in the skin, they tested highly specific synthetic cannabinoid ligands for the CB2 receptor without considering the CB1 receptor (Del Río et al., 2016; Du et al., 2018; Parikh et al., 2024). Activating CB2 reduces neutrophil and macrophage infiltration, it decreases the secretion of inflammatory cytokines, *i.e.*, monocyte chemotactic protein (MCP)-1, Interleukin (IL)-6 and tumor necrosis factor (TNF)-α (Wang et al., 2016), and modulates fibrogenesis (Li et al., 2016). On the other hand, phytocannabinoids with broader receptor affinity have so far only been investigated for topical application (Parikh et al., 2024). For instance, Cannabidiol (CBD), which does not bind to the classical cannabinoid receptors CB1 and CB2, increases levels of antioxidants and wound repair keratins in the intact skin of 6 months old mice (Casares et al., 2020), while it does not affect the wound healing rate in young horses (McIver et al., 2020).

Rodents are generally well suited for studying various aspects of wound healing, but they have shown limited utility for translation to humans (Elliot et al., 2018). The differences between the structure and physiology of rodent and human skin can distort the collected data on wound healing and make certain data difficult to extrapolate to humans (Chen et al., 2015). However, wound healing studies designed with these differences in mind can provide valuable translational information (Masson-Meyers et al., 2020), *i.e.*, when rodents are used to study specific therapeutics in the skin, they can serve as sufficient models due to their similar dermis and epidermis layers (Flynn et al., 2023).

Due to the close relationship of the cutaneous ECS and its putative role in skin aging and regeneration, the present study is the first to investigate whether prolonged exposure to a low dose of Δ^9-tetrahydrocannabinol (THC), which has an affinity for various cannabinergic binding sites, has lasting effects on skin wound healing in old mice. Therefore, female old mice (18 months) received a chronic low-dose of THC for a period of 3 weeks before performing four excisional wounds (diameter 5 mm) on the dorsum. The wound closure rate was monitored for 7 days. On the days 1, 3, and 7 post-surgery, the concentrations of growth factors (VEGF, HGF, and TGF-ß1) and inflammatory cytokines (MCP-1, IL-6 and TNF-α) were determined in wound-tissue homogenates by ELISA. For histological evaluation, the numbers of granulocytes, M1-macrophages and MSCs were determined by immuno-histochemistry.

2. Materials and methods

2.1. Animals

Female mice (strain = C57BL/6; age = 18 months; n = 30) were purchased from Janvier Laboratories (Saint-Berthevin, Cedex, France). The mice were randomly divided into two groups, the untreated control and the THC group. According to the experimental design, five mice per group and time point were required to address the stated questions in the animal experiments. This number is determined by the inter-animal variability and the number of experimental parameters, so that statistically differences can be obtained. This is also based on experience from previously completed studies on mouse wound healing (Plum et al., 2025; Ruhl et al., 2021b). We therefore expect biologically relevant differences between the untreated control group and the THC group of 25–50 %, which corresponds to a size effect of Cohen's d = 0.5–0.8.

This study examined only female mice to minimize sex-specific differences in wound healing (Kopcewicz et al., 2020), as wounds of female mice heal significantly faster than wounds of males (Rowland et al., 2023). Mice were housed in groups of 5 animals per cage in the facilities of the Institute of Laboratory Animal Science, University Hospital, RWTH Aachen. The mice were kept at a room temperature of 20–24 °C under a 12-h light/dark cycle, the humidity level was 45–65 %. The animals had access to food and water *ad libitum* throughout the study. The experimental procedures were performed according to the EU Directive 2010/63/EU for animal experiments, they followed the guidelines of the animal welfare laws and were approved by the Animal Care and Use Committee of the state of North Rhine-Westphalia, Germany (AZ-81-02.04.2021.A352).

2.2. Daily substance application

The test group received a daily injection (200 µL *s.c.* into the inguinal fat pads) of THC (3 mg/kg bodyweight) diluted in a vehicle (Veh) solution (Poloxamer P188, methanol, saline at a ratio of 1:1.5:17.5), whereas the control group received the Veh only (Ruhl et al., 2025). Injection sites alternated daily between the right and left inguinal region to minimize tissue irritation and skin burden. The inguinal body region was selected for substance injection to avoid interfering skin irritations as it is distant from the surgical site. After 21 days of pharmacological stimulation, excisional wounds were created on the dorsum of the animals (Fig. 1).

2.3. Anesthesia and surgery

The procedure of the animal experiments corresponded to the same procedure as described earlier (Ruhl et al., 2021b). The animals were isolated one day prior surgery to prevent unwanted manipulation on the individual's wound healing post-surgery. The animals received metamizole for peri-operative analgesia (p.o. 125 mg / 100 mL) and were anaesthetized using 2 % Isoflurane (Abbott, Germany). The animals received an *i.p.* injection of ketamine for intra-operative analgesia (100 mg/kg bodyweight). All surgeries were performed under sterile conditions. The dorsum was shaved and sterilized with Octeniderm (Schülke,

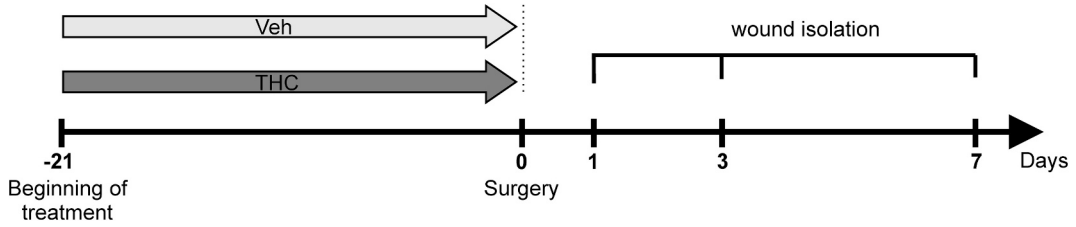

Fig. 1. Timeline of the experimental procedure: The pharmacological treatment was initiated 21 days prior to the surgery. The two groups were administered daily either the vehicle (Veh, light grey arrow) or THC solution (dark grey arrow). On day 0, the surgery was performed to create 4 excisional wounds. The animals' wound closure rate was monitored and wound tissue was collected at day 1, day 3, and day 7 post-surgery.

Germany). With a surgical punch device (diameter: 5 mm), each mouse received four excisional full wounds - proximal: 2 cm caudal to the ears; distal: 3 cm caudal to the proximal row. The *panniculus carnosus* was preserved to keep the function of skin contraction, thereby following the advice not to prevent the natural contraction, *e.g.* by mechanical fixation using splints, which could induce additional stress potentially affecting the natural skin repair (Chen et al., 2015).

Planimetric wound closure was assessed by measuring the larger (L) and minor (M) diameter of each wound (dermal border) and determined by applying the formula: $\left(\frac{L}{2} \times \frac{M}{2}\right) \times \pi$. The original wound size was measured immediately after the surgery and the wound closure rate was calculated as percentage of this value. After the surgery, the animals were carefully returned to their home cages and monitored individually until they had fully recovered from anesthesia. Five mice per group were euthanized by cervical dislocation on day 1, 3 and 7 post-surgery. Subsequently, the diameter of each wound was measured by a blinded researcher. Each wound was excized with adjacent intact tissue and frozen at −80 °C for later analysis. Briefly, three of the four wounds (approx. 0.1 g) were homogenized in 2 mL lysis buffer (pH = 7.5, 10 mM HEPES, 0.5 % Triton X-100, protease inhibitor) on ice using a tissue tearer (Thermo Fisher Scientific, Schwerte, Germany). Homogenates were centrifuged at 2000 ×*g* for 10 min at room temperature to remove large particles. The clear supernatant was further centrifuged at 14,000 ×*g* for 40 min at 4 °C. The aliquots were used for cytokine and growth factor determination by ELISA.

2.4. Immunohistochemistry and tissue analysis

One wound tissue sample of each mouse was fixed with 4 % PFA for 3 h, embedded in paraffin and cut into 5 µm transversal sections using a sliding microtome (pfmmedical, Cologne, Germany). Heat-induced antigen retrieval was performed with citrate buffer (pH = 6) using a steamer (SpectraLab, Markham, Canada) for 30 min. Blocking was performed in two steps with Bloxall (Vector, Newark, USA) for 10 min and with 4 % BSA for 30 min, followed by washing in PBS. Tissue sections were incubated with monoclonal antibodies *vs.* Lymphocyte antigen 6 complex, locus G (Ly-6G)/granulocytes (1:2000 µL, rabbit, abcam, Cambridge, United Kingdom), Macrophage Antigen Complex-3 (Mac-3)/M1-macrophages (1:2000 µL, rat, abcam, Cambridge, United Kingdom), and Cluster of Differentiation 90 (CD90)/MSCs (1:2000 µL, rat, abcam, Cambridge, United Kingdom) diluted in blocking solution. The ImmPress HRP Horse Anti-Rabbit/Anti-Rat IgG Polymer Detection Kit (Vector, Newark, USA) containing the secondary antibody was applied following the manufacturer's instructions. Afterwards, the sections were washed with PBS and labelled cells were visualized using the ImmPact Amec Red Peroxidase Substrate (Vector, Newark, USA). Following counterstaining with hematoxylin (Roth, Karlsruhe, Germany), the stained slices were photographed using an EVOS FL auto imaging system (Thermo Fisher Scientific, Waltham, USA). Marked cells were quantified in five randomly selected areas (50 × 50 µm^2) using the free software Image J (Wayne Radband, Institutes of Health, Bethesda, USA), and calculated as percentage of the total cell number.

2.5. Cytokine and growth factor determination by enzyme-linked immunosorbent assay (ELISA)

To determine the concentrations of soluble factors (TNF-α, MCP-1, IL-6, VEGF, HGF and TGF-β1), the wound homogenates were thawed and analyzed by ELISA Duo-Sets (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Extinction was measured in duplicates on a microplate reader (BMG Labtech, Ortenberg, Germany). Cytokine concentrations were expressed as the amount of each factor (pg) per milligram of total protein amount. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Rockford, Ill., USA) following the manufacturer's instructions.

2.6. Statistics

The measurements of all experiments were grouped to examine the observations for each type of experimental investigation on day 1, 3, and 7 post-wounding. The data were tested for normal distribution using the Kolmogorov-Smirnov test, and pairwise comparison between THC and Veh treatment was tested by the Student's *t*-test for independent samples (SPSS 24, SPSS Inc., Chicago, USA). The results were expressed as mean with standard error of the mean (± SEM). Differences associated with $p \leq 0.05$ were considered statistically significant. The effect size was measured using Cohen's d formula. The calculated d-values of 0.2–0.49 were considered small, 0.5–0.79 were considered medium, and d > 0.8 was interpreted as a large effect size. Figures were created using the graphics program Corel Draw X5 (Corel Corporation, Ottawa, Canada).

3. Results

3.1. The THC effect on the wound closure rate in old mice

During pharmacological treatment, two mice of the THC group had to be excluded from the wound healing experiments because of skin abnormalities (sore spots on the tail and the extremities, which were not related to pharmacological stimulation). The remaining mice ($n = 28$) survived the procedures during the full time course of the study. They tolerated the daily injection paradigm without showing physical or psychological irritations caused by the THC or Veh administration, and they exhibited regular healing responses upon skin wounding, which includes weight gain and maintaining their pre-surgery behavior. The wounds were clean and free of any signs of infection or necrosis (Fig. 2a). On day 1 post-surgery, the mean wound size of the Veh group (89 %) did not differ from that of the THC group (79 %; $t(25.27) = 0.9$, $p = 0.378$; d = 0.3; Fig. 2b). On day 3 post-surgery, wound closure in the THC group progressed to 36 %, whereas the control group was at 57 %. This difference was significant ($t(25.94) = 3.4$, $p = 0.002$; d = 0.9; Fig. 2b). For day 7 post-surgery, the wounds were not completely closed in both groups. The mean value of wound closure for the THC group was 10 %, which was not significantly different from the vehicle group (17 %), although it showed a strong tendency ($t(20.63) = 1.9$, $p = 0.071$; d = 0.7; Fig. 2b).

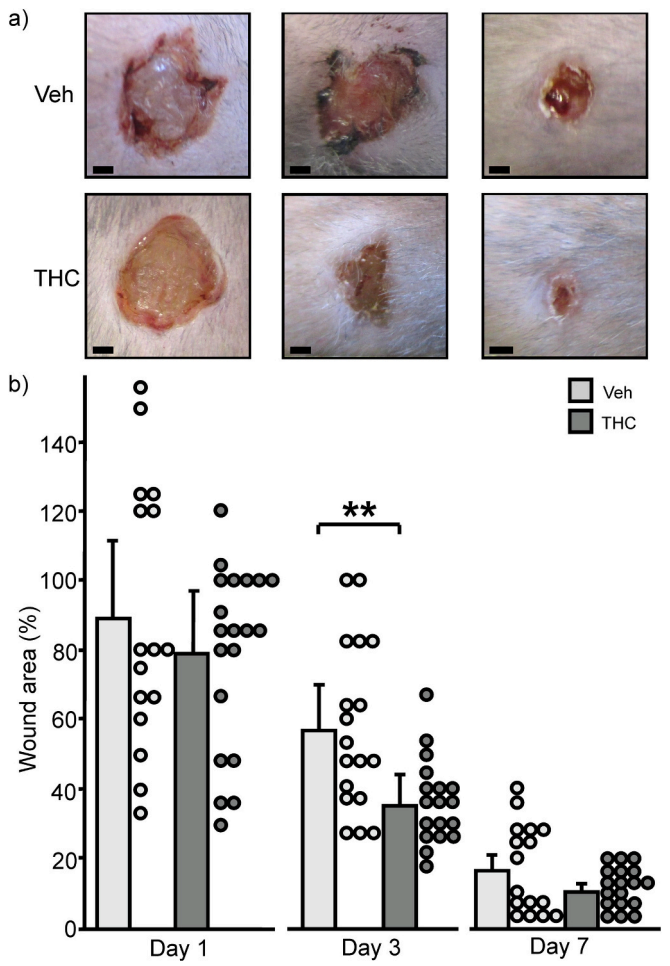

Fig. 2. The effect of a chronic low dose of THC (3 mg/kg bodyweight) on the wound closure in old mice. Representative images of wounds (a) from vehicle- (Veh) and THC-treated mice on days 1, 3 and 7 post-surgery (bars = 1 mm). The difference between the application of Veh (light grey) and THC (dark grey) on the wound closure rate (calculated in % relative to the original wound size) for the days 1, 3 and 7 post-surgery (b). Data are expressed as mean values (+ SEM) with the data points in a scatter plot. The number of animals was $n = 4$–5 mice for each group and time-point. Pairwise comparison was performed by Student's *t*-test; **$p \leq 0.01$.

3.2. The THC effect on the inflammatory phase (day 1 post-surgery)

The infiltration of immune cells and MSCs into the wound tissue was evaluated by calculating the percentage of neutrophils (positive for Ly-6G expression), M1-macrophages (positive for Mac-3 expression) and MSCs (positive for CD90 expression) relative to the total cell number. The maximum of Ly-6G cells was detected for both groups on day 1 post-surgery, with significantly higher number for the Veh than for the THC group ($t(17) = 3.6$, $p = 0.002$; $d = 1.8$; Fig. 3a). At the same timepoint, the wounds of the THC group also contained significantly less Mac-3 positive cells compared to the Veh group ($t(18) = 4.7$, $p < 0.001$; $d = 2.2$; Fig. 3b). Approximately 6 % of the total cell count in wounds of the THC treated mice was stained positive for CD90, which was about three times higher than in the Veh group. This difference was significant ($t(14) = 2.4$, $p = 0.031$; $d = 0.8$; Fig. 3c).

The TNF-α levels were significantly lower in the THC group (mean = 37.1 pg/mg) compared to the vehicle group (mean = 80.7 pg/mg) on day 1 post surgery ($t(6) = 2.9$, $p = 0.029$; $d = 2.3$; Fig. 4a). The same applies for the MCP-1 concentration, which was significantly lower in the wound tissue of the THC (mean = 308.2 pg/mg) than in the Veh treated animals (mean = 637.1 pg/mg; $t(7) = 2.3$, $p = 0.05$; $d = 1.8$; Fig. 4b). Wound tissue of THC treated mice had lower levels of IL-6 compared to the Veh group, however, this difference was not significant ($t(7) = 0.8$, $p = 0.433$; $d = 0.7$; Fig. 4c). In contrast to the inflammatory cytokines, the application of THC had no effect on the release of the investigated growth factors (VEGF, HGF, TGF-ß1; Fig. 4d–f).

3.3. The THC effect on the transition from the inflammatory to the proliferative phase (day 3 post-surgery)

On day 3 post-surgery, the number of Ly-6G cells was not affected by the THC treatment ($t(13.89) = 0.9$; $p = 0.395$; $d = 0.4$; Fig. 5a), whereas the number of Mac-3 cells was significantly higher in the THC than in the Veh group ($t(15) = 4.0$; $p = 0.001$; $d = 2.1$; Fig. 5b). The difference in CD90 labelled cells was not significant between both experimental groups ($t(19) = 0.7$; $p = 0.242$; $d = 0.3$; Fig. 5c).

The THC effect on the release of inflammatory cytokines was comparable to that observed on day 1 post-surgery. Although the maximum concentration of TNF-α was recorded for both groups on day 3 post-surgery, the level remained significantly lower in wounds of the THC (mean = 78 pg/mg) than in the Veh group (mean = 116 pg/mg; $t(8) = 2.4$; $p = 0.047$; $d = 1.6$; Fig. 5d). The same applies for MCP-1 ($t(6) = 0.4$; $p = 0.029$; $d = 2.3$), which decreased in both groups compared to day 1 post-surgery (Fig. 5e). The THC treatment did not affect the release of IL-6 ($t(8) = 0.6$; $p = 0.570$, $d = 0.8$; Fig. 5f). The concentrations of the investigated growth factors were also not affected by THC (Fig. 5g–i).

3.4. The THC effect on the transition from the proliferative to the remodeling phase (day 7 post-surgery)

At day 7 post-surgery, the numbers of cells stained positive for Ly-6G ($t(9.89) = 4.0$; $p = 0.003$; $d = 1.9$) and Mac-3 ($t(18) = 4.7$; $p = 0.037$; $d = 1.1$) were significantly higher in wounds of the Veh than of the THC group (Fig. 6a&b). THC did not affect the number of CD90 positive cells ($t(22) = 0.4$; $p = 0.347$; $d = 0.2$; Fig. 6c). The levels of TNF-α, MCP-1, and of IL-6 decreased from day 3 to day 7. There were no differences in the concentrations of inflammatory cytokines in the wound tissues of both the Veh and the THC group (Fig. 6d–f). The same applies for the concentrations of VEGF, HGF and TGF-β1 (Fig. 6g–i).

4. Discussion

In the present study, a chronic low dose of THC improved the wound healing process of old female mice. Accelerated wound closure was associated with earlier invagination of immune cells and decreased concentrations of inflammatory cytokines, which resulted in a shortened inflammatory phase that enabled a faster transition to the proliferative phase upon THC treatment.

The effectiveness of the immune system diminishes and the body's regenerative capacities decline by age. This is substantiated by reduced migration and secretory activity of the immune cells leading to impaired tissue repair capacity (Brubaker et al., 2013). Wound healing in old mice is in particular associated with a disturbed tissue homeostasis that is expressed in an impaired progress of the wound healing stages, *i.e.*, a delayed and prolonged inflammatory phase (Plum et al., 2025). Increasing evidence suggests that the activity and effectiveness of the ECS declines with age. Given the important role of the ECS in modulating immune responses and improving cellular functions, stimulating the ECS represents a promising option to support tissue regeneration in aging.

Previous studies have investigated the effect of systemic ECS stimulation on wound healing in young mice using several specific receptor agonists and antagonists targeting the CB2 receptor (Niyangoda et al., 2024; Parikh et al., 2024). For instance, beta-caryophyllene improved reepithelization (Koyama et al., 2019), and GP1a decreased inflammation during skin wound healing in young mice (Du et al., 2018; Zhao

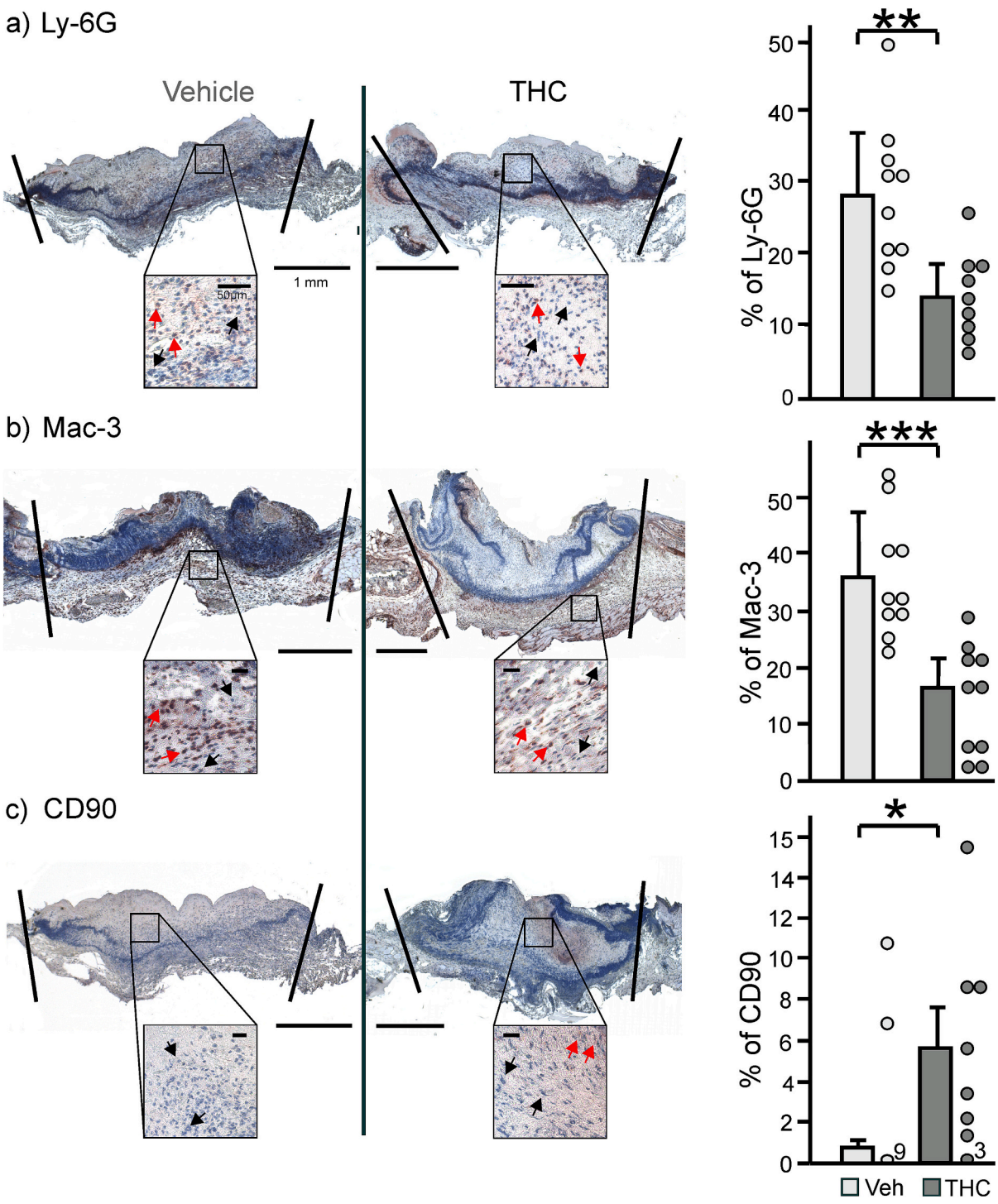

Fig. 3. Cell invagination during the inflammatory phase on day 1 post surgery. Numbers of cells expressing Ly-6G (granulocytes), Mac-3 (M1-macrophages), and CD90/Thy1 (MSCs) were determined in wound-tissue sections of old mice. Representative microphotographs (bars = 1 mm) of the sections from Vehicle (Veh, light grey) and THC (dark grey) treated mice stained for cells expressing Ly-6G (a), Mac-3 (b), and CD90/Thy1 (c). Black lines represent the dermal border. Detection of labelled cells (red arrows) *vs.* non-labelled cells (black arrows) in the high magnification box (bar = 50 µm; see also Supplementary Fig. 1). The numbers of marked cells (+ SEM) are shown in bar charts, with data points presented as dots. For clarity: numbers next to the dots indicate the exact number of data points at that value. Pairwise comparison was performed by the Student's t-test; *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2021). By contrast, phytocannabinoids with unspecific receptor affinity have so far only been tested topically. The treatment of burn wounds with hemp oil led to faster wound healing in young mice, as evidenced by increased wound contraction, a shorter epithelialization time and reduced inflammation (Mehrabani et al., 2016). Further murine and equine models have been used to test the wound healing properties of ECS stimulation, specifically in acute wounds such as oral ulcers and skin incisions (for review see Niyangoda et al., 2024). The major effects on wounds are enhanced re-epithelialization without any report on significant side effects. In human subjects, the use of cannabis

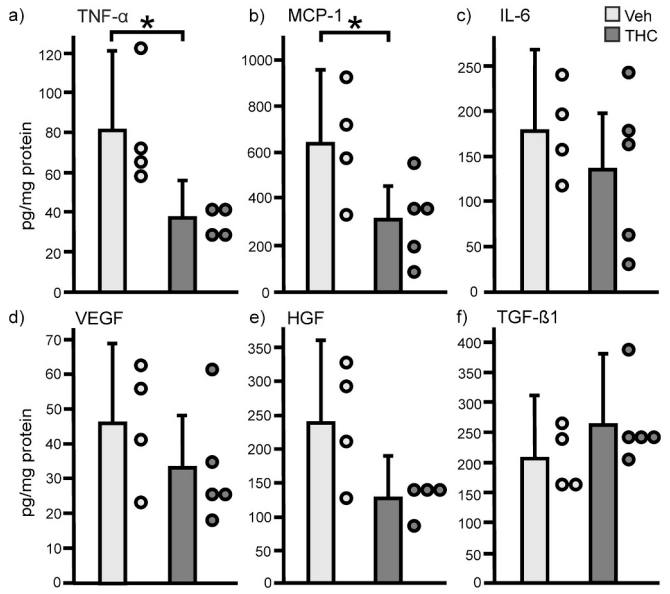

Fig. 4. Content of soluble factors in wound tissue on day 1 post surgery. Concentrations of inflammatory cytokines (a–c) and growth factors (d–f) were measured in the wound homogenates of Veh (light grey) and THC (dark grey) treated animals by ELISA. The amount of each factor (pg) was normalized to the total protein content (mg). All data are presented as mean values (+ SEM) with the data points in a scatter plot. Pairwise comparison was performed by the Student's t-test; *p ≤ 0.05.

oils led to reduced blistering, shortened healing times, and alleviated symptoms, thus, improving quality of life through topical, oral, and sublingual routes.

All previous studies exclusively investigated acute cannabinergic modulation of wound healing in young rodents with an efficiently functioning ECS. By contrast, the present study is the first to explore if the chronic stimulation of the aged ECS prior injury has lasting effects on disturbed tissue regeneration in old mice. Although the influence of aging on the cutaneous ECS has not yet been investigated, one could speculate that the expression of cannabinoid receptors in the skin decreases with age, as has already been observed for the central nervous system (Piyanova et al., 2015). In this context, it has been found that chronic low-dose THC administration adapts the gene expression profile of cells from mature mice to that of young control animals in a CB1 receptor-dependent manner (Bilkei-Gorzo et al., 2017). This includes the upregulation of anti-aging transcripts such as Klotho (Kl) and the downregulation of genes with potential pro-aging effects, *e.g.*, caspase-1 (Casp1) and connective tissue growth factor (Ctgf). Furthermore, it is also possible that long-term exposure to THC increases the expression of cannabinoid receptors (Zhuang et al., 1998), which could, in turn, improve the ECS tone achieved by low-dose THC treatment and normalize the weak cannabinoid signaling in aged mice in the long term.

Furthermore, earlier studies mainly tested synthetic cannabinoids allowing to focus on a single receptor, *i.e.*, CB2, while the use of THC enables the activation of further cannabinoid binding sites. Treatment with THC also offers the advantage that after administration by continuous injection, the concentration in adipose tissue reaches its peak after 30 to 120 min and does not decrease thereafter (Torrens et al., 2022). Thus, fat tissue acts as a reservoir for the lipophilic THC, allowing slow and continuous release into local tissues upon stimulation (Gunasekaran et al., 2009).

The initial inflammatory phase of physiological wound healing begins in the first hours after injury and lasts for approximately 2–3 days (Almadani et al., 2021). On the other hand, wound healing in old mice is characterized by a delayed and prolonged inflammatory response (Plum et al., 2025). Neutrophils are drawn to the wound site and secrete

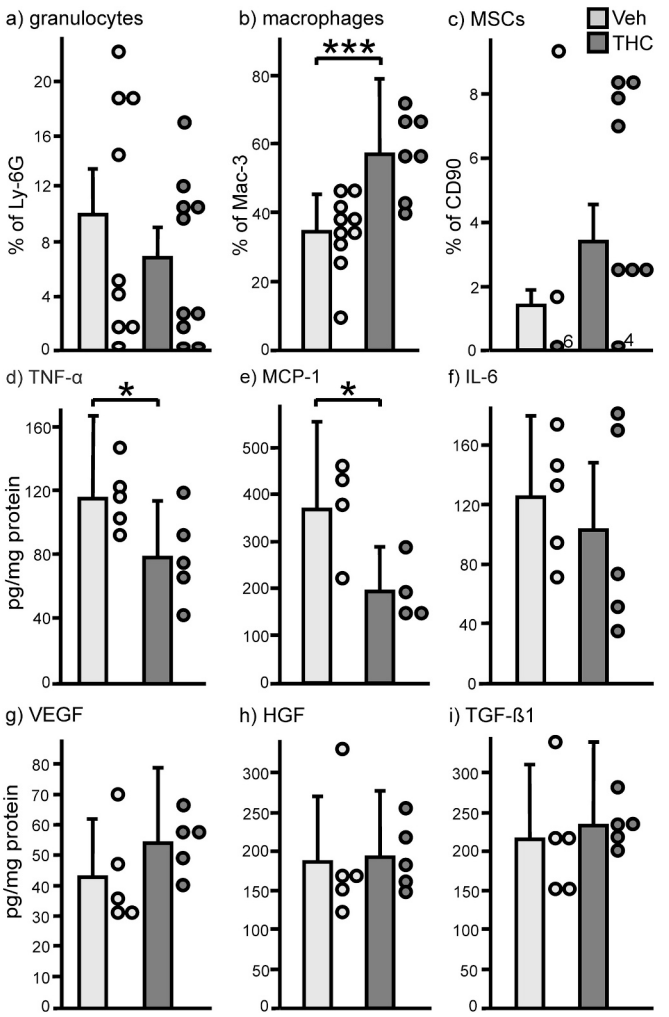

Fig. 5. Cell distribution and cytokine concentrations in wound tissue on day 3 post surgery. The percentual average (+SEM) of cells positive for Ly-6G (granulocytes, a), Mac-3 (M1-macrophages, b) and CD90/Thy1 (MSCs, c). Small numbers next to the dots indicate the exact number of data points at that value. The inflammatory cytokines TNF-α (d), MCP-1 (e), IL-6 (f), and the growth factors VEGF (g), HGF (h) and TGF-ß (i) were measured in the wound homogenates of Veh (light grey) and THC (dark grey) treated animals and normalized to the total protein content (pg/mg). Pairwise comparison was performed using the Student's t-test; *p ≤ 0.05 and ***p ≤ 0.001.

soluble factors forming the foundation for tissue regeneration (Peña and Martin, 2024). Chemokines, including MCP-1, polarize monocytes into classically activated M1-macrophages (Park and Barbul, 2004). These traditionally called inflammatory M1 cells contribute to the inflammatory phase by releasing inflammatory cytokines, *e.g.* TNF-α and IL-6 (Shapouri-Moghaddam et al., 2018). A delay and prolongation of immune cell infiltration and inflammatory cytokine release may promote a chronic inflammatory status and disrupt the progress of subsequent healing stages (Franceschi and Campisi, 2014; Nishio et al., 2008; Shallo et al., 2003). Stimulating the ECS with THC appeared to primarily act by immunosuppression, as numbers of invading granulocytes and M1-macrophages were decreased on the first day post-wounding. However, it is also possible that THC did not reduce the granulocyte concentration but it rather shifted the granulocyte peak in the THC-treated group before day 1, which would correspond to the progression of wound healing in young mice (2 months), when neutrophil count peaks at 12 h after surgery (Wang et al., 2016). Furthermore, cannabinoids, including THC, modulate the cytokine release of immune cells (Blevins et al., 2022; Yekhtin et al., 2022). As these cells are the main source of

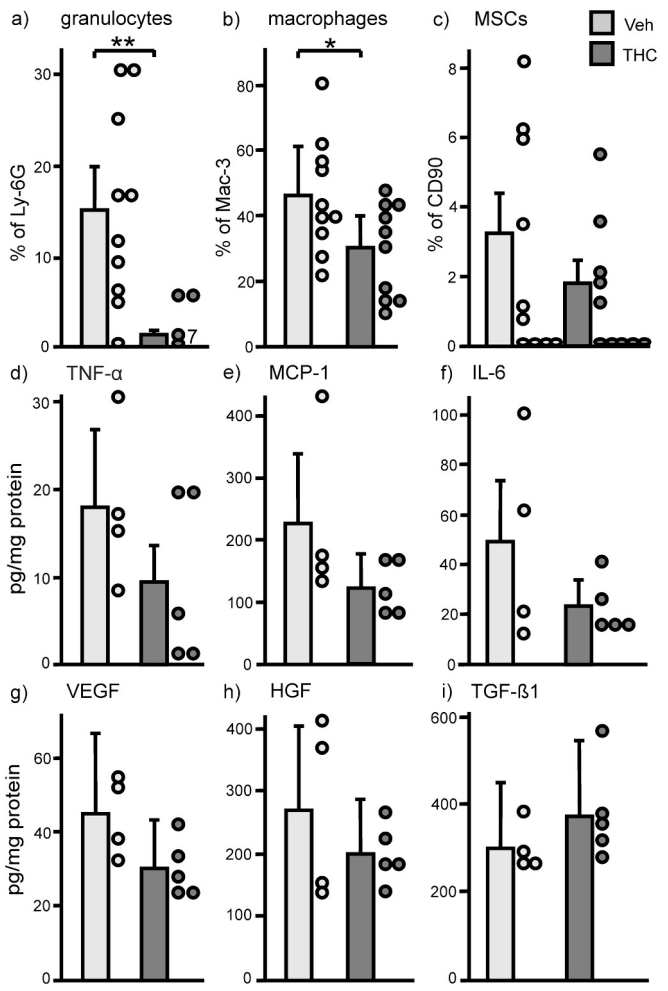

Fig. 6. Cell numbers and cytokine concentrations in wound tissue on day 7 post surgery. The percentual average (+SEM) of cells positive for Ly-6G (granulocytes, a), Mac-3 (M1-macrophages, b) and CD90/Thy1 (MSCs, c). Small number (a) next to the dots indicate the exact number of data points at that value. The inflammatory cytokines TNF-α (d), MCP-1 (e) and IL-6 (f), and the growth factors VEGF (g), HGF (h) and TGF-ß1 (i) were measured in the wound homogenates of Veh (light grey) and THC (dark grey) treated animals normalized to the total protein content (pg/mg). Statistical analysis was performed by pairwise comparison using the Student's t-test; *p ≤ 0.05 and **p ≤ 0.01 between Veh and THC.

inflammatory cytokines, both the reduced numbers of granulocytes and M1-cells and the suppression of their secretory activity may have contributed to the decreased TNF-α and MCP-1 levels in the wound tissue of THC treated mice. This effect depended in part on the interaction of THC with the CB2 receptor, since this finding corresponds well to a previous investigation on wound healing in young mice using a specific CB2 agonist (GP1a) (Wang et al., 2016). However, in contrast to the activation of CB2 alone, we were able to show *in vitro* that endocannabinoid binding to CB1 and CB2 downregulate TNF-α release by M1 macrophages even more effectively (Ruhl et al., 2021a). In addition, cannabinoids also have affinity to peroxisome proliferator-activated receptors (PPAR) (Pertwee et al., 2010), and especially their interaction with PPARγ has anti-inflammatory effects (O'Sullivan, 2016). Thus, it is likely that the observed THC effect on the concentrations of inflammatory cytokines was additionally driven by activation of CB1 and PPARγ.

The increase of MSC number in wound tissue of the THC group can also be explained by THC binding to CB2, since *in vitro* studies found enhanced MSC migration upon CB2 receptor activation (Miller et al., 2021; Schmuhl et al., 2014). Given that MSC deficiency and decelerated migration is a common characteristic of wound healing in old mice (Amini-Nik et al., 2022; Chen et al., 2015), the THC induced increased infiltration of MSCs might have contributed to an accelerated wound healing progress. MSCs have been described as an important factor contributing to tissue repair due to their multilineage differentiation potential (Guillamat-Prats, 2021). Moreover, MSCs exert beneficial paracrine effects. They are able to modulate inflammation, by switching macrophage polarization into the alternatively activated M2 phenotype (Andreeva and Buravkova, 2018; Zhang et al., 2010).

On day 3 post-surgery, the inflammatory phase gradually shifts into the proliferative phase. This transition represents a key step during wound healing. The number of M1-macrophages peaks before increased concentrations of anti-inflammatory cytokines promote the development of monocytes into activated M2a macrophages (Landen et al., 2016). The M2a, together with connective tissue cells, *i.e.* MSCs and fibroblasts, secrete regenerative growth factors such as VEGF, HGF, and TGF-β1 to stimulate angiogenesis and cell migration (Ahmad and Nawaz, 2022; Almadani et al., 2021). The duration of the inflammatory phase appeared reduced upon THC treatment, which probably led to accelerated wound closure by day 3. Comparably, other studies on topical and systemic application of CB2 receptor agonists have reported improved wound healing in 6–8 weeks-old mice between day 3 and day 10 (Mehrabani et al., 2016; Wang et al., 2016; Zhao et al., 2021). According to Wang et al., this effect was associated with a reduction in immune cell infiltration and lower concentrations of MCP-1 and TNF-α during the inflammatory phase (Wang et al., 2016). In our study, THC application induced the highest M1-macrophage count on day 3 post-surgery, which resembled the situation found for young mice (2 months) (Plum et al., 2025). The difference in cell infiltration is accompanied by reduced MCP-1 and TNF-α contents in wounds of the THC treated mice, as has been found for day 1 post-wounding.

At elevated age, tissue repair is impaired due to reduced secretion of regenerative growth factors (Komi-Kuramochi et al., 2005; Plum et al., 2025; Swift et al., 1999). Although CB1 and CB2 cannabinoid receptor stimulation enhances the release of VEGF, HGF, and TGF-β1 by subcutaneous adipose stem cells (ASC) *in vitro* (Ruhl et al., 2020), and we found increased growth factor concentrations in adipose tissue of old mice after chronic THC exposure (Ruhl et al., 2025), this treatment paradigm did not affect growth factor concentrations in wound tissue. Skin wound healing involves the communication of multiple cell types to coordinate dermal repair. The ASCs are activated by cytokines from damaged tissue, they proliferate and infiltrate the wound tissue in parallel with fibroblasts. Due to their stem cell characteristics, *i.e.*, their capacity for self-renewal and multipotent differentiation, their contribution to tissue repair was previously thought to be limited to cell replacement (Ayala-Cuellar et al., 2019). However, it is now generally agreed that they support tissue regeneration through their paracrine activity (Caplan, 2017). In addition, mature adipocytes repopulate skin wounds following inflammation (Hu et al., 2018; Rodrigues et al., 2019). Corresponding functional analyses in lipoatrophic mice show that the activity of a functional subcutaneous adipose tissue is necessary for successful skin repair (Schmidt and Horsley, 2013). Since the subdermal fat layer decreases with increasing age (Baumann, 2007; Kim et al., 2014), we assume that THC application may have reached too few of the relevant target cells, while it predominantly acted on the existing immune cells and thus influenced the concentration of pro-inflammatory cytokines over the growth factor release. Therefore, a fat grafting beneath or aside the wounded skin combined with THC-stimulation could offer an additional regenerative effect.

After the decline of the inflammatory response, the wound enters the final stage of tissue repair around day 7 when tissue integrity is progressively restored in the remodeling phase (Rodrigues et al., 2019). Wound closure began to accelerate in the Veh group and reduced the difference to the THC-treated mice. Numbers of M1-macrophages and fibroblasts exhibited a delayed increase in the Veh compared to the THC

group, whereas the count of granulocytes was still elevated. Only towards the end of the observation period, the cellular profile in the Veh group resembled that of an active healing response, as indicated by high numbers of fibroblasts, granulocytes and macrophages. Cell infiltration occurred delayed and persisted longer, resulting in a gradual reduction in the difference in open wound area between the THC- and Veh-treated groups on day 7, with the Veh group partially 'catching up' in the final phase.

In summary, we found that chronic THC application prior wounding altered the timing and quantity of infiltrating granulocytes and M1-macrophages into the wound tissue of old female mice. The THC treatment resulted in an accelerated inflammatory response and caused both an earlier increase and a rapid decrease in granulocyte count. At the same time, M1-cell density remained constantly elevated in the Veh group, while their numbers were resolved at day 7 in THC treated mice. This type of temporal cell migration is very similar to the one observed in young mice, where the number of M1 macrophages peaks at day 3 and declines by day 7 (Plum et al., 2025). Furthermore, the THC application modulated the sequential release of inflammatory cytokines creating a wound environment comparable to that observed in young animals. The timely infiltration of immune and connective tissue cells into the wound tissue is essential for orchestrating the regular healing process (Rodrigues et al., 2019). Effective tissue repair requires both a balanced inflammatory response and its punctual resolution. The elevated levels of granulocytes and M1-macrophages in the Veh group indicated a prolonged inflammatory response, while the THC treatment reorganized the temporal disturbance in the progression of the wound healing process. This effect enables the restoration of tissue repair comparable to that of younger conspecifics. Admittedly, the use of only female mice poses some limitations to the generalizability of our results. As already mentioned, wound healing processes are subject to gender-specific peculiarities, which is why wound closure rate differs between male and female mice. Therefore, we cannot rule out that stimulation of the cutaneous ECS could also lead to different effects in old male and female animals.

Although human and murine wound healing differ significantly from each other, as a large portion of wound closure in mice occurs through contraction mediated by the *panniculus carnosus* muscle (Zomer and Trentin, 2018), mouse models still remain valuable for studying the underlying molecular processes during tissue repair including the investigation of the aging effect (Choudhary et al., 2024). Similar to mice, delayed wound healing is also observed in elderly humans, which is characterized by a prolonged inflammatory phase, impaired macrophage and reduced fibroblast migration (Ashcroft et al., 2002; Thomas, 2001). Existing therapeutic approaches to improve age-related wound healing disorders have notable limitations. Antiseptics and antibiotics can promote antibiotic resistance and should only be used in the presence of infection (Han and Ceilley, 2017). Hyperbaric oxygen therapy is costly, resource-intensive, and unsuitable for old patients with pre-existing conditions (Klakeel and Kowalske, 2022). Growth factor therapies have shown limited efficacy, as wound repair in aging is impaired by complex disruptions in the interactions among cytokines, extracellular matrix elements, and immune cells (Thomas, 2001). Given these limitations, alternative approaches are to be explored as potential regeneration stimulating strategies. In addition to systemic subcutaneous injection, THC can also be administered through other methods, such as Marinol® capsules or Sativex® oral spray, which are already medically used for treatment of pain, weight loss and spasticity (Legare et al., 2022). The present study indicated that stimulation of the ECS by systemic THC administration can affect age-related impairments and deterioration in the skin. The THC's rejuvenating ability may provide a foundation for the development of cannabinoid-based anti-aging therapies.

5. Conclusions

The present study suggests a novel role of chronic pre-injury ECS stimulation in counteracting age-related deficits for cutaneous tissue repair. Systemic THC-application altered the timing and quantity of infiltrating immune cells and MSCs into the wounds of old mice. Moreover, it coordinated the release of inflammatory cytokines creating a wound environment comparable to that observed in young animals. This research highlights the chronical stimulation of the ECS as a promising approach to improve physiological tissue repair in old individuals.

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CRediT authorship contribution statement

Melissa Plum: Writing – original draft, Methodology, Investigation, Formal analysis. **Justus P. Beier:** Writing – review & editing. **Tim Ruhl:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization.

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Declaration of competing interest

None.

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Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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