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RESEARCH PAPER

Δ^9Tetrahydrocannabinol attenuates Staphylococcal enterotoxin B-induced inflammatory lung injury and prevents mortality in mice by modulation of miR-17-92 cluster and induction of T-regulatory cells

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BACKGROUND AND PURPOSE

Staphylococcal enterotoxin B (SEB) is a potent activator of Vβ8+T-cells resulting in the clonal expansion of ~30% of the T-cell pool. Consequently, this leads to the release of inflammatory cytokines, toxic shock, and eventually death. In the current study, we investigated if Δ^9tetrahydrocannabinol (THC), a cannabinoid known for its anti-inflammatory properties, could prevent SEB-induced mortality and alleviate symptoms of toxic shock.

EXPERIMENTAL APPROACH

We investigated the efficacy of THC against the dual administration (intranasal and i.p.) of SEB into C3H/HeJ mice based on the measurement of SEB-mediated clinical parameters, including cytokine production, cellular infiltration, vascular leak, and airway resistance. In addition, the molecular mechanism of action was elucidated *in vitro* by the activation of splenocytes with SEB.

KEY RESULTS

Exposure to SEB resulted in acute mortality, while THC treatment led to 100% survival of mice. SEB induced the miRNA-17-92 cluster, specifically miRNA-18a, which targeted Pten (phosphatase and tensin homologue), an inhibitor of the PI3K/Akt signalling pathway, thereby suppressing T-regulatory cells. In contrast, THC treatment inhibited the individual miRNAs in the cluster, reversing the effects of SEB.

CONCLUSIONS AND IMPLICATIONS

We report, for the first time a role for the miRNA 17–92 cluster in SEB-mediated inflammation. Furthermore, our results suggest that THC is a potent anti-inflammatory compound that may serve as a novel therapeutic to suppress SEB-induced pulmonary inflammation by modulating critical miRNA involved in SEB-induced toxicity and death.

Abbreviations

miRNA, microRNA; PTEN, phosphatase and tensin homologue; SEB, Staphylococcal enterotoxin B; T-regs, regulatory T-cells; THC, Δ^9tetrahydrocannabinol

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[d]
CB_1 receptor	Akt (PKB)
CB_2 receptor	CASP2 (caspase 2)
Nuclear hormone receptors[b]	CASP8 (caspase 8)
RORG (RAR-related orphan receptor-γ)	ERK
Catalytic receptors[c]	JAK
Death receptor	MAPK
IL1RL1	PI3K
IL6ST	PIK3R1
TGFBR2	PTEN
TGFBR3	
TNFRSF1B	

LIGANDS	
Cannabidiol (CBD)	MCP-1 (CCL2)
Dexamethasone	Rapamycin
IFN-γ	TGF-β
IL-2	THC
IL-6	TNF-α
IL-10	TNFSF11
IL-12	VEGF-A

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d]Alexander *et al.*, 2013a,b,c,d).

Introduction

Staphylococcal enterotoxin B (SEB) is a potent activator of the immune system resulting in the clonal expansion of 5–30% of the T-cell pool and massive release of cytokines (Choi *et al.*, 1989; Faulkner *et al.*, 2005). As a consequence, it is associated with a number of diseases ranging from food poisoning, multi-organ failure and lethal toxic shock (Dinges *et al.*, 2000; Alouf and Muller-Alouf, 2003; Larkin *et al.*, 2009). When SEB is inhaled, the combination of cellular infiltration and cytokine production, results in vascular leak, pulmonary oedema, tissue damage and eventually acute inflammatory lung injury (Saeed *et al.*, 2012; Rao *et al.*, 2014). Due to its ability to be easily aerosolized and for its possible role as a biological weapon, SEB is considered a Center for Disease Control and Prevention – Category B select agent (Ulrich *et al.*, 2001). While it is known that the interaction of SEB with the T-cell receptor (TCR) results in the activation of inflammatory pathways such as the PI3K, MAPK and NFκB (Krakauer, 2013), a recent study from our laboratory has suggested that microRNA (miRNA) may play an important role in SEB-mediated inflammation (Rao *et al.*, 2014).

miRNA are ~22 nucleotide, small, non-coding RNA that target mRNA, leading to its degradation and/or translational repression (Guo *et al.*, 2010). Consequently, they control the development and differentiation of various immune cells thereby leading to the regulation of immune responses (Lindsay, 2008). Upon receiving inflammatory signals, active changes occur within the transcriptional repertoire accompanied with altered expression of a number of miRNA resulting in the up- or down-regulation of several important genes (O'Connell *et al.*, 2012). One such modulator of gene expression is the miR-17-92 cluster. Originally recognized as an oncogenic miRNA, this cluster that comprises of six miRNA (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1) is known to be an important regulator of B and T-cell responses (Olive *et al.*, 2010). For example, miR-17-92 transgenic mice that express the cluster in both B and T-cells develop lymphoproliferative disease (Xiao *et al.*, 2008). In addition, miR-19b and miR-17 within the cluster are reported to regulate CD4 + T-cells and enhance Th1 responses (Liu *et al.*, 2014), thereby demonstrating an important role for the cluster during inflammation.

Although current therapeutic strategies against SEB include the use of non-specific human immunoglobulins (intravenous immunoglobulins or IVIg) as well as monoclonal antibodies against SEB-induced cytokines (Miethke *et al.*, 1992; Matthys *et al.*, 1995; Larkin *et al.*, 2009; 2010) its efficacy remains inefficient (Darenberg *et al.*, 2004). Additionally, corticosteroids have been shown to attenuate SEB-induced toxic shock and acute lung injury (Krakauer and Buckley, 2006; Huzella *et al.*, 2009), but the immunosuppressive property of corticosteroids is either accompanied by a number of side effects or have remained ineffective clinically (Bernard *et al.*, 1987; Meduri *et al.*, 1998; Wajanaponsan *et al.*, 2007). As a result, there is a need for alternative agents that mitigate SEB-triggered inflammation with the potential to modulate SEB-induced inflammatory miRNA.

Δ^9-Tetrahydrocannabinol (THC) is a marijuana plant-derived cannabinoid known for its robust anti-inflammatory and immunosuppressive properties (Klein, 2005; Nagarkatti *et al.*, 2009; 2010). It mediates its action by binding to two main cannabinoid receptors, CB_1 and CB_2, found primarily in the brain and on immune cells respectively (Felder and Glass, 1998). Previously, we have demonstrated that THC induces apoptosis in Jurkat leukemia T-cells and dendritic cells (Do *et al.*, 2004). THC suppresses the production of Th1 cytokines IFN-γ and TNF-α (Klein *et al.*, 1995; Srivastava *et al.*, 1998; Klein, 2005; Sun *et al.*, 2008), while increasing Th2 cytokines, IL-10 and TGF-β (Sun *et al.*, 2008). Additionally, while we have earlier reported that THC induces the production of myeloid derived suppressor cells (MDSCs; Hegde *et al.*,

2010),we have for the first time demonstrated that THC-mediated miRNA control the development of these MDSCs (Hegde *et al.*, 2013). Taken together, THC, by virtue of its anti-inflammatory and immunosuppressive properties and its recently discovered ability to regulate miRNA expression could serve as an effective therapeutic agent in the attenuation of SEB-mediated lung injury.

Thus, in the current study, we tested the hypothesis that THC treatment ameliorates SEB-induced toxicity through regulation of miRNA. Our data demonstrate that THC treatment down-regulates the members of the miR-17-92 cluster and ameliorates inflammatory symptoms associated with SEB exposure in the lungs.

Methods

Mice

Female C3H/HeJ mice (6–8 weeks) were purchased from The Jackson laboratory. All mice were housed at the Animal Resource Facility (ARF), University of South Carolina, under specific pathogen free conditions with a maximum of five animals per cage. Animals were kept under 12 light/12 dark cycle at a temperature of ~18–23 °C and 40–60% humidity. Food and water were available *ad libitum*. All experiments involving the use of vertebrate animals were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at USC and the US National Research Council's 'Guide for the Care and Use of Laboratory Animals'. A total of 80 animals were used in the experiments described here. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

SEB administration and THC treatment schedule

THC was provided by National Institute on Drug Abuse (Bethesda, MD, USA) and SEB was procured from Toxin Technologies (Sarasota, FL, USA). The treatment schedule comprised of injecting vehicle or THC i.p. at a concentration of 20 $mg \cdot kg^{-1}$ body weight. The rationale behind administering the current dose of THC is based on body surface area normalization (Reagan-Shaw *et al.*, 2008), whereby 20 $mg \cdot kg^{-1}$ THC dose converts to 60 $mg \cdot m^{-2}$, which is within the range of synthetic THC used in the clinic (90 $mg \cdot m^{-2} \cdot day^{-1}$). THC or vehicle was first administered in a 100 µL volume dissolved in ethanol (day 1). The following day (day 2), THC (20 $mg \cdot kg^{-1}$) was once again administered via the i.p. route. Thirty minutes later, SEB was delivered as a 'Dual Dose' as described previously (Huzella *et al.*, 2009). Briefly, SEB dissolved in sterile PBS (2 $mg \cdot mL^{-1}$) was administered first by the intranasal (i.n.) route at a concentration of 5 µg per mouse in a volume of 25 µL. Two hours later, a second dose of SEB was delivered i.p. at a concentration of 2 µg per mouse in a 100 µL volume. On day 3, mice were treated i.p. with THC (20 $mg \cdot kg^{-1}$). Survival of mice was monitored up to 5 days after SEB exposure and any moribund mice were immediately killed.

Assessment of lung damage and function

Vascular leak in the lungs was determined as described previously (Rieder *et al.*, 2011; 2012; Saeed *et al.*, 2012). Briefly, mice were injected with 1% Evans blue in sterile PBS i.v., 72 h after the second dose of SEB. Two hours later, mice were killed and lungs were perfused with heparin-containing PBS. Lungs were incubated in formamide at 37°C for 24 h to extract the dye. The OD of the supernatant was measured spectrophotometrically at 620 nm and % increase in vascular leak was calculated using the following formula – $(OD_{sample} - OD_{control}/OD_{control}) \times 100$. Airway resistance was measured using whole-body plethysmography (Buxco, Troy, NY, USA). Each mouse was restrained in a two-chamber plethysmographic tube and was first allowed to acclimatize, followed by exposure to saline for 2 min. This was followed by a 2 min exposure to increasing doses of methacholine. The specific airway resistance (sRaw) measurement at each methacholine dose was calculated and plotted as % airway resistance. To examine lung morphology and histology, lungs were fixed in 10% formalin, paraffin embedded and stained with haematoxylin and eosin. The slides were observed under a light microscope at 20× magnification.

Cell preparation and flow cytometry

Vehicle, SEB + vehicle and SEB + THC mice (five mice per group) were killed 72 h after dual exposure to SEB. The lungs were perfused with heparin-containing PBS, harvested and homogenized using Stomacher® 80 Biomaster blender from Seward (Davie, FL, USA) in 10 mL of sterile PBS. After being washed with sterile PBS, cells were layered carefully onto Ficoll-Histopaque ®-1077 from Sigma-Aldrich (St Louis, MO, USA) at a 1:1 ratio. Mononuclear cells were separated by density gradient centrifugation as a distinct layer as described previously (Rieder *et al.*, 2012; Rao *et al.*, 2014) and enumerated by Trypan blue exclusion. To determine the phenotypical characteristics of the infiltrating cells, mononuclear cells were stained with the following fluorescent conjugated antibodies – FITC-conjugated anti-CD8 (clone: 53-6.7), anti-CD3 (clone: 145.2 C11). Phycoerthyrin (PE)-conjugated anti-CD4 (clone: GK 1.5), anti NK1.1 (clone: PK136) from Biolegend (San Diego, CA, USA). FITC-conjugated anti-Vβ8 (clone: K516) from Ebioscience (San Diego, CA, USA). Intracellular staining of Foxp3 was carried out using Biolegend's Foxp3 Fix/Perm buffer set following manufacturer's instructions and using anti-foxp3 alexa flour 488 (clone MF-14) from Biolegend.

Cytokine analysis

To assess serum cytokines, mice were bled 3 h after dual exposure to SEB. Cytokines from the broncheoalveolar lavage fluid (BALF) were obtained at the time by binding the trachea with a suture and excising the lung along with the trachea, as described previously (Rieder *et al.*, 2012; Rao *et al.*, 2014). Sterile, ice-cold PBS was injected through the trachea to aspirate the fluid. The samples were centrifuged to obtain the supernatants. All cytokine levels were measured using Biolegend ELISA MAX™ standard kits.

miRNA target predictions and transfections

miRNA target candidate *Pten* was predicted using Ingenuity Pathway Analysis (IPA) software from Ingenuity Systems® (Mountain View, CA, USA). Briefly, highly predicted and experimentally observed targets of the individual miRNA in

the miR-17-92 cluster were selected. A core analysis was carried out and significant (Fisher's exact test) biological functions associated with the data set were generated. Additionally, a bar graph highlighting key canonical pathways associated with the data set was also generated. miRSVR score and alignment of miR-18a with *Pten* was obtained from www.microRNA.org, target prediction website. To validate *Pten* as a target of miR-18a, splenocytes from naïve C3H/HeJ mice were harvested and cultured in complete (10% FBS, 10 mM L-glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol and 100 μg·mL^{-1} penicillin) RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA). Cells were seeded at 2×10^5 cells per well in a 24-well plate and transfected for 24 h with 40 nM synthetic mmu-miR-18a (MSY0000528) or mock transfected with HiperFect transfection reagent from Qiagen (Valencia, CA, USA). For inhibition of miR-18a, SEB-activated cells were similarly transfected for 24 h with 100 nM synthetic mmu-miR-18a (MIN0000528) or mock transfected.

Total RNA extraction and qRT-PCR

Total RNA (including small RNA) was isolated from lung-infiltrating mononuclear cells or *in vitro* from splenocytes using miRNeasy kit from Qiagen following the manufacturer's instructions. The purity and concentration of the RNA was confirmed spectrophotometrically using Nanodrop 2000c from Thermo Scientific (Wilmington, DE, USA). For miRNA validation and quantification, we used SYBR Green PCR kit (Qiagen) and for mRNA validation, SSO Advanced™ SYBR green PCR kit from Biorad (Hercules, CA, USA). Fold change of miRNA was determined by normalization to Snord96_an internal control, whereas mRNA levels were normalized to β-actin. The following qRT-PCR primers were used: *β-actin* (F) 5′GGCTGTATTCCCCTCCAT G-3′ and (R) 5′-CCAGTT GGTAACAATGCCATGT-3′; *Foxp3* (F) 5′ AGCAGTCCACTTCACCAAGG 3′ and (R) 5′ GGATAACGCCAGAGGAGCTG 3′; *Pten* (F) 5′ TGGATTCGACTTAGACTTGACCT 3′ and (R) 5′ GCGGTGTCATAATGTCTCTCAG 3′.

In vitro *cell culture assays*

Splenocytes from naïve C3H/HeJ mice were harvested and cultured in complete RPMI. Cells were seeded at 1×10^6 cells per well of a 96-well plate and either left unstimulated or stimulated with SEB (1 μg·mL^{-1}). Cell were either treated with THC or with an allosteric Akt 1/2 kinase inhibitor (A6730), that is pleckstrin homology (PH) domain dependent and does not have an inhibitory effect against PH domain lacking Akts, or related kinases (Sigma-Aldrich) at the doses indicated. Twenty-four hours later, cells were harvested and centrifuged. The cell supernatants were collected for assessment of IFN-γ levels by ELISA and the cell pellets were used for total RNA extraction and qRT-PCR. To determine the effect of other immunosuppressive compounds on the miR-17-92 cluster, SEB-activated splenocytes were treated with cannabidiol (CBD) obtained from the National Institute on Drug Abuse (Bethesda, MD, USA), dexamethasone (Dexa) (#D4902) and rapamycin (Rapa) (#R8781) from Sigma. Cell proliferation was determined by incubating the cells as described above for 48 h. [^{3}H]-thymidine (2μCi) was added to the cell cultures in the last 12 h of incubation. Cultures were collected using a cell harvester and thymidine incorporation was measured using a scintillation counter (Perkin Elmer, Waltham, MA, USA).

Western blots

SEB-activated splenocytes were treated with THC (20 μM) for 18 h and protein extracts (~15 μg) were separated on a 10% SDS-PAGE by electrophoresis (60V for ~2 h). Separated protein was transferred onto a nitrocellulose membrane. The membrane was probed with antibodies against pan-Akt (#4685), phosphor-Akt-Ser^{473} (\$9271S), β-actin 13E5 (#4970) from Cell Signaling Technology® (Danvers, MA, USA) and phosphatase and tensin homologue (PTEN) (#SC 6817-R) from Santa Cruz Biotechnology®, Inc (Dallas, TX, USA).

Statistical analysis

All statistical analyses were carried out using GraphPad Prism Software (San Diego, CA, USA). In all experiments, the number of mice used was 4–5 per group, unless otherwise specified. Results are expressed as means ± SEM. Student's *t*-test was used to compare the two groups, whereas multiple comparisons were made using one-way ANOVA, followed by *post hoc* analysis using Tukey's method. A *P*-value of <0.5 was considered statistically significant. Individual experiments were performed in triplicate and each experiment was performed independently at least three times to test reproducibility of results. Survival analysis was carried out using a log-rank test.

Results

THC strongly attenuates SEB-mediated inflammation and prevents acute mortality

Dual SEB exposure has been previously used to study acute lung injury leading to 100% death in C3H/HeJ mice (Huzella *et al.*, 2009). In the current study, we found that 100% of the mice exposed to SEB died between 96 and 120 h. Remarkably, in the THC-treated groups, all mice survived (Figure 1A). SEB-exposed mice displayed signs of lethargy, hunching, ruffled fur and respiratory stress, whereas THC-treated mice were as active as vehicle-treated mice and did not display hunched posture, ruffling of fur or signs of respiratory distress. To gauge the extent of pulmonary damage, we measured airway resistance using whole-body plethysmography and found that SEB exposure resulted in a significant percent increase in airway resistance, while THC-treated mice recorded sRAW values similar to vehicle alone (Figure 1B). Further, we measured the percent increase in vascular leak by administration of Evans blue dye. Evans blue binds to serum albumin and is a measure of vascular permeability, as shown previously (Rieder *et al.*, 2012). Our results demonstrated that while SEB exposure had a profound increase in vascular leak when compared with vehicle only, THC treatment caused a significant decrease in vascular leak (Figure 1C). Acute inflammatory lung injury is characterized by massive immune cell infiltration into the lung. Accordingly, we found an increase in the total number of mononuclear cells after SEB exposure and a subsequent decrease with THC treatment (Figure 1D). This was confirmed by histopathological examination of the lungs, whereby SEB-exposed mice displayed an increase in infiltrating immune cells around the bronchioles and air vessels while the THC-treated mice, yielded significantly fewer layers (Figure 1E). To identify the immune subsets

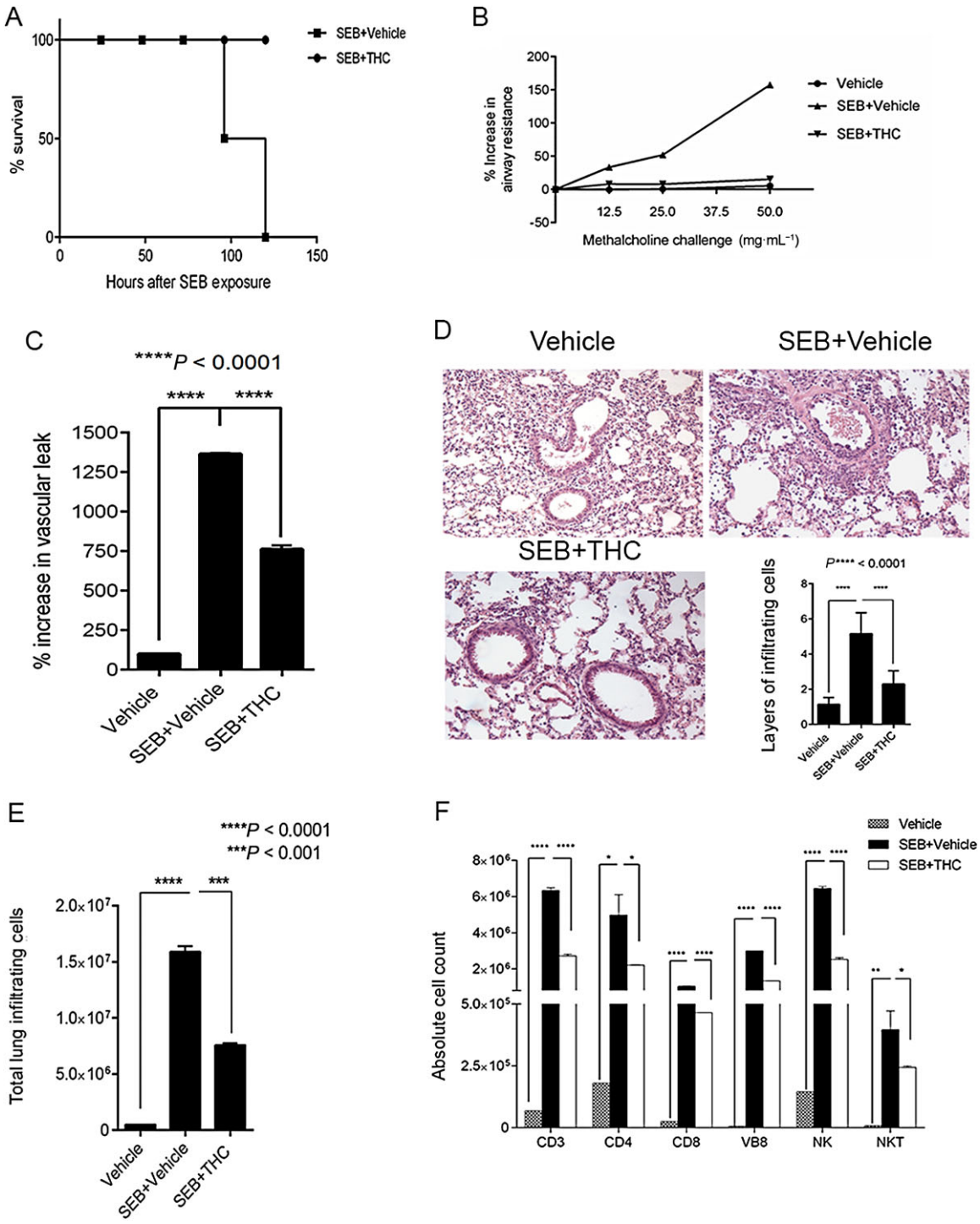

Figure 1

THC prevents mortality and alleviates SEB-induced inflammation in the lung. (A) Survival curve of mice receiving SEB+ vehicle when compared with mice treated with SEB+THC. (B) Measurement of airway hyperreactivity (sRAW) using whole-body plethysmography. (C) Assessment of vascular leak in the lungs; % vascular leak was calculated by measuring absorbance at 620 nm. (D) Histopathological examination of lungs as determined by haemotoxylin and eosin staining. Total layers of infiltrating cells were counted around 10 different capillaries and enumerated in the bar graph. (E) Total number of infiltrating mononuclear cells obtained from the lung was enumerated by trypan blue exclusion method. (F) Flow cytometric analysis to identify immune subsets was carried out. Mononuclear cells were stained with antibodies against T-cells (CD3), T-helper cells (CD4), cytotoxic T-cells (CD8), Vβ8-region of the T-cell receptor (Vβ8), NK cells and natural killer T-cells (NKT). Absolute cell numbers were calculated using the formula: total number of cells isolated from the lungs × % of specific cells/100, and plotted as a bar graph. Bar graphs summarize the means ±SEM from 3–5 independent experiments. All experiments above were carried out 72 h after exposure to SEB. Statistical significance is indicated as follows: *$P < 0.005$; **$P < 0.01$.

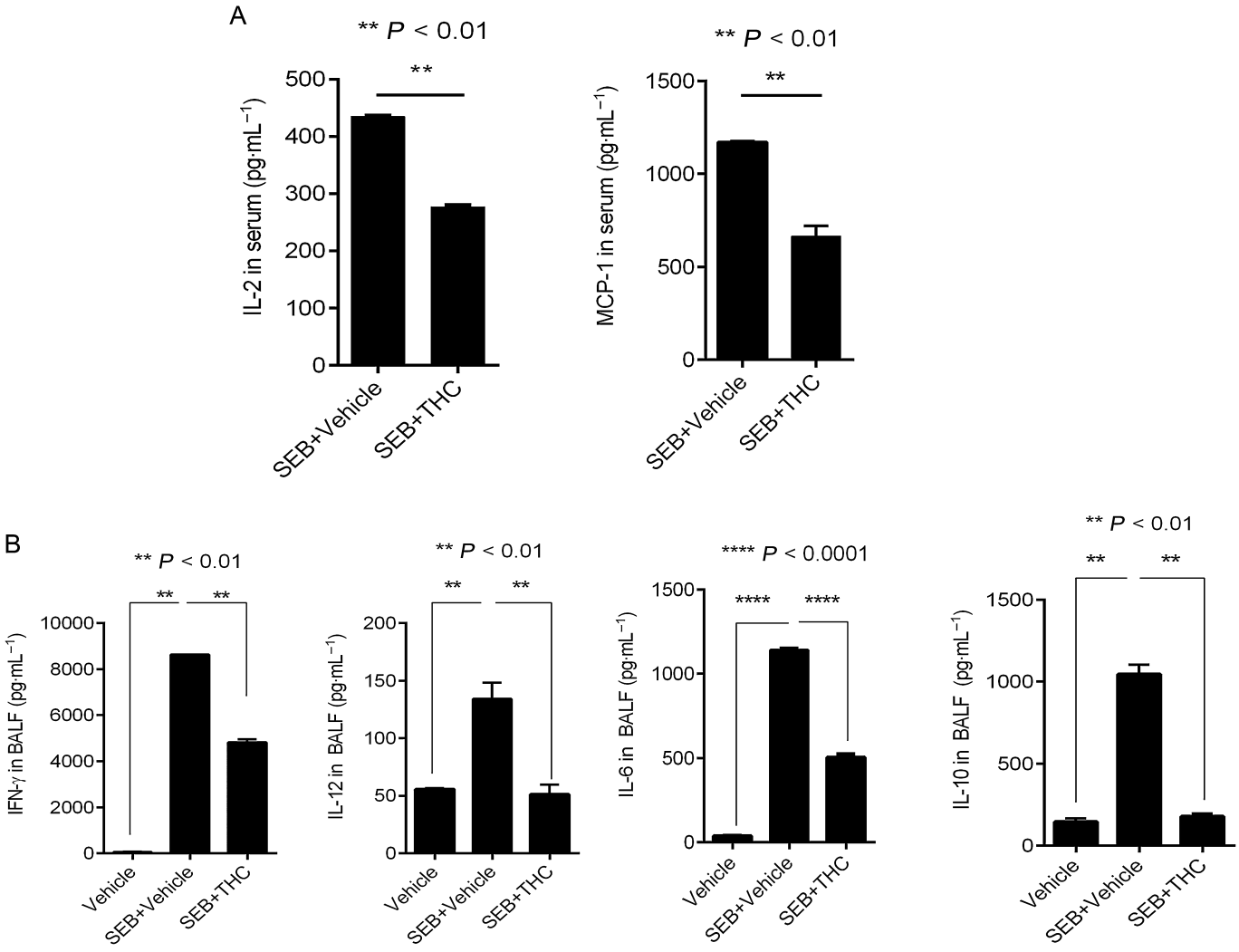

Figure 2

THC decreases SEB-induced cytokine secretion. (A) Measurement of early cytokines, IL-2 and MCP-1 in serum 3 h after SEB exposure. (B) Measurement of IFN-γ, IL-12, IL-10 and IL-6 in the BALF. All cytokine concentrations were determined using ELISA. Bar graphs summarize the means ±SEM from 3–5 independent experiments. For cytokines from the serum, unpaired, two-tailed *t*-test was used to determine significance from SEB. For cytokines from the BALF, one-way ANOVA, followed by *post hoc* analysis using Tukey's method was used.

among the infiltrating mononuclear cells, we stained the cells with various fluorescein-conjugated anti-mouse antibodies. We found that exposure to SEB resulted in increased CD3+ (T-cells), CD4+ (T-helper cells), CD8+ (cytotoxic T-cells), Vβ8+ NK+ (NK cells) and NK1.1 + CD3+ (NK T-cells), THC treatment caused an overall decrease in the absolute cell numbers (Figure 1F).

A hallmark of SEB-mediated inflammation is the abundant release of cytokines. To determine if THC was able to blunt cytokine secretion, we first analysed the concentration of early cytokines IL-2 and MCP-1 in the serum. Mice were bled at 3 h, 6 h and 24 h after SEB exposure. While IL-2 and MCP-1 peaked at 3 h (data not shown), we found that the THC-treated group showed diminished secretion of both IL-2 and MCP-1 as early as 3 h after SEB exposure (Figure 2A), supporting the potent anti-inflammatory role of THC in this model. Moreover, an examination of cytokines in the BALF revealed that THC treatment led to the substantial decrease in IFN-γ, IL-6, IL-12 and IL-10 (Figure 2B). Overall, these data suggest that THC attenuates SEB-induced immune cell infiltration, decreases early and late cytokine secretion, and prevents mortality of the mice.

THC modulates the expression of the miR-17-92 cluster

Antigenic stimulation and the activation of the TCR is known to result in the induction of miR-17-92 cluster (Wu *et al.*, 2012). Consequently, we reasoned that SEB exposure would lead to the expression of this prominent miRNA cluster. Seventy-two hours after exposure to SEB, we measured miRNA expression in mononuclear cells isolated from the lung by qRT-PCR. Interestingly, we observed high levels (up to 600-fold) of the miRNA cluster, although individual miRNA were induced to different levels. More interestingly,

THC treatment was able to down-regulate the individual members of the cluster significantly (Figure 3), strongly suggesting that THC may exhibit its powerful anti-inflammatory activity through the modulation of inflammatory miRNA. In order to establish that the ability to modulate the cluster was unique to THC alone, we also compared the effect of other known immunomodulatory compounds dexamethasone, rapamycin and CBD *in vitro* on the cluster. We observed that while CBD only significantly down-regulated miR-19a and miR-20a, dexamethasone only affected miR-19a and miR-19b-1 and rapamycin, decreased levels of miR-19b-1, miR-92a-1 and miR-17 suggesting that while these compounds may affect a few miRNA derived from the cluster via a mechanism distinct from THC, they are unable to modulate the entire cluster (Supporting information Figure S1).

miR-17-92 cluster is linked to the activation of the PI3K/Akt/pathway

Because SEB exposure resulted in the strong induction of the miR-17-92 cluster, we sought to explore the significance of this particular cluster in our study. Therefore, we carried out computational analysis on the highly predicted and experimentally observed targets of the cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1) using IPA. IPA results suggested that the members of the cluster were involved in a number of biological functions relevant to the expansion of T-helper lymphocytes, the development of regulatory T-cells (T-regs), the proliferation of cells and apoptotic processes (Figure 4A). In addition, analysis of the canonical pathways associated with the cluster indicated the involvement of the MAPK, NFκB and PI3K/Akt signalling pathways,

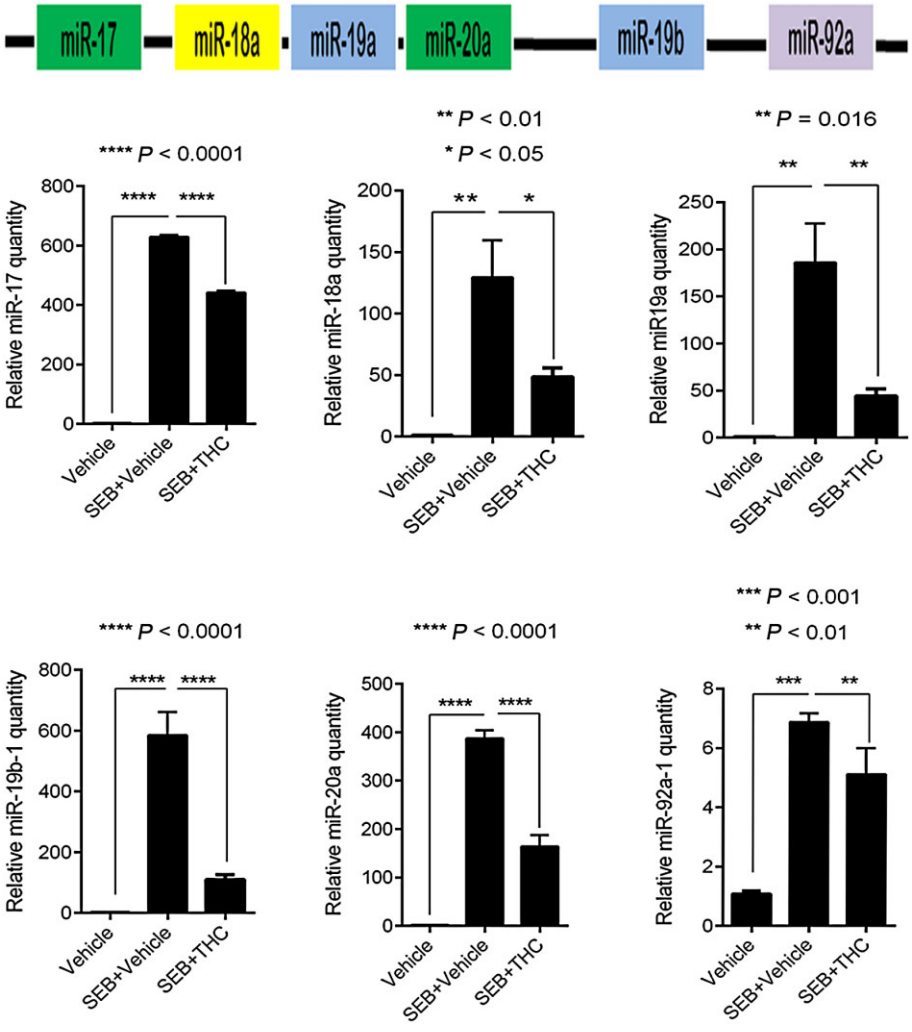

Figure 3

THC significantly down-regulates SEB-induced expression of the miR-17-92 cluster. Real-time (RT) PCR validation of the individual miRNA (miR-17, miR-18a, miR-19a, miR-19b-1, miR20a and miR-92a-1) of the miR-17-92 cluster obtained from lung-infiltrating mononuclear cells. Data are normalized to internal control Snord_96a. Statistical significance was assessed using ANOVA, Tukey's multiple comparison test.

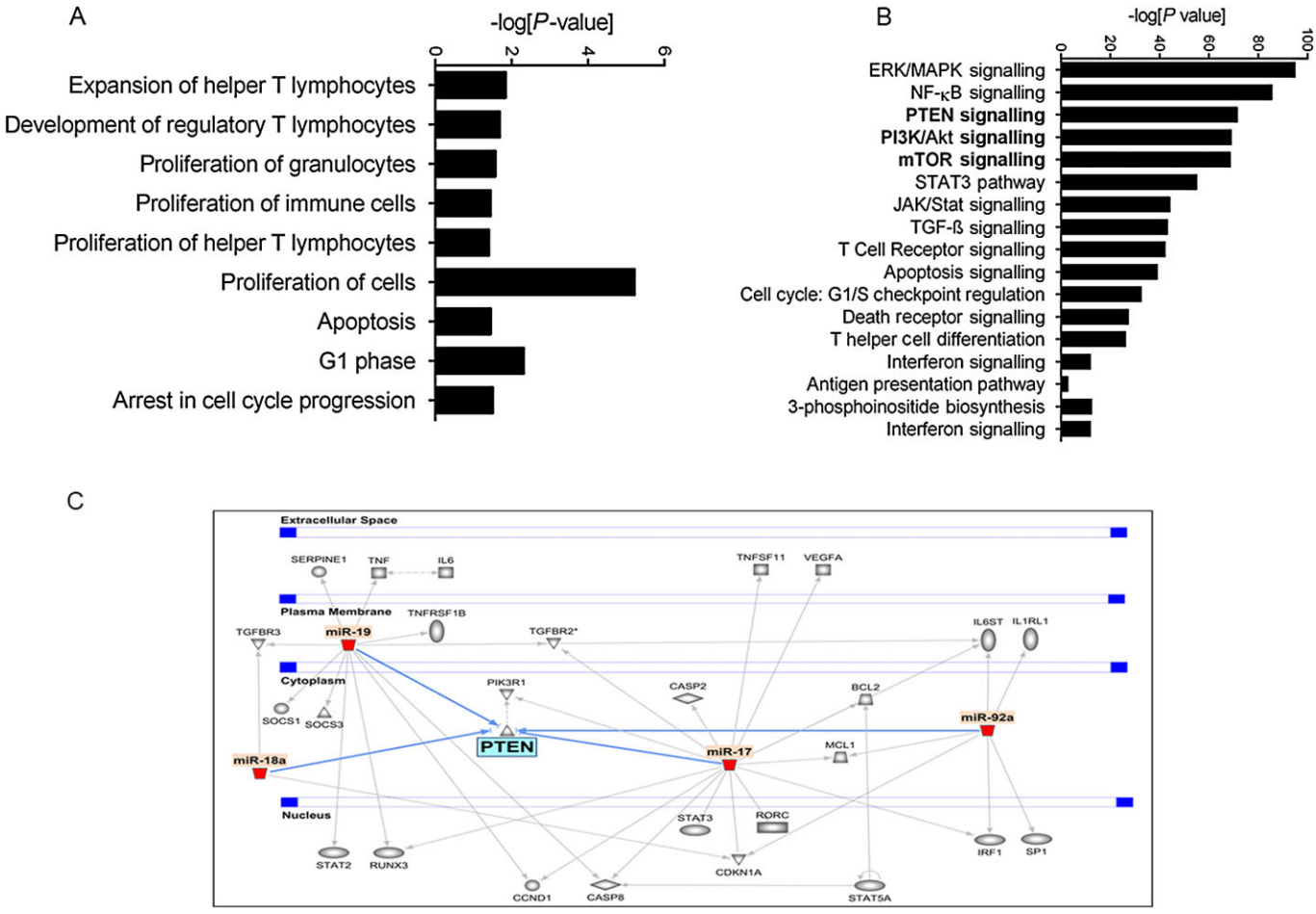

Figure 4

The involvement of the miR-17-92 cluster in key biological pathways. (A) Graphical representation of the biological functions associated with significantly up-regulated SEB-induced miRNA as determined by IPA. (B) Canonical pathways associated with miRNA target genes. IPA was used to filter highly predicted and experimentally observed targets of only the significantly up-regulated miRNA in response to SEB. A graphical representation of the significant (Fisher's exact test) pathways of these particular target genes was generated. (C) IPA pathway demonstrating the convergence of the members of the miR-17-92 cluster on *Pten*.

(processes that are all reported to be SEB-triggered). Interestingly, while the PTEN signalling and mTOR pathways were highlighted by IPA as significant (Figure 4B), pathway analysis also indicated that the miRNA in the cluster are highly predicted or experimentally observed to converge on *Pten* (Figure 4C). As a result, we reasoned that the SEB-induced miRNA cluster is involved in the activation of the PI3K/Akt signalling pathway while THC-mediated down-regulation of this cluster inhibits the aforementioned activation. To test if the cluster was indeed involved in the activation of the PI3K/Akt pathway, we activated splenocytes with SEB and treated them with an Akt1/2 inhibitor. Among the individual members of the cluster that were up-regulated by SEB activation, miR-18a in particular was significantly down-regulated upon Akt1/2 inhibition, while the other members of the cluster either remained unaltered, were insignificantly down-regulated or found to be further up-regulated after Akt1/2 inhibition (Figure 5A). This indicated the direct involvement of miR-18a in SEB-mediated activation of the PI3K/Akt pathway.

miR-18a targets PTEN, a negative regulator of the PI3K/Akt pathway

Previous reports have established that a principal target of the miR-17-92 cluster is PTEN, an antagonist of the PI3K/Akt pathway (Xiao *et al.*, 2008; Liu *et al.*, 2014). Using the www.miRNA.org alignment tool, we found that miR18a is predicted to target the 3′ UTR of *Pten* with a good miRSVR score of –0.1453 (Figure 5B). To assess if miR-18a indeed targets *Pten*, we first transfected splenocytes with synthetic miR-18a mimic. Interestingly, we found that miR-18a mimic repressed *Pten* (Figure 5C). Further, the inhibition of SEB-activated cells with a miR-18a synthetic inhibitor led to the derepression of *Pten* (Figure 5D), suggesting that miR-18a, belonging to the cluster, plays a prominent role in the repression of *Pten* and consequently results in the activation of SEB-triggered PI3K/Akt pathway. Earlier, we observed that THC was able to dramatically decrease the expression of the miRNA cluster (Figure 3). Therefore, we wondered if THC treatment would be able to subsequently restore the

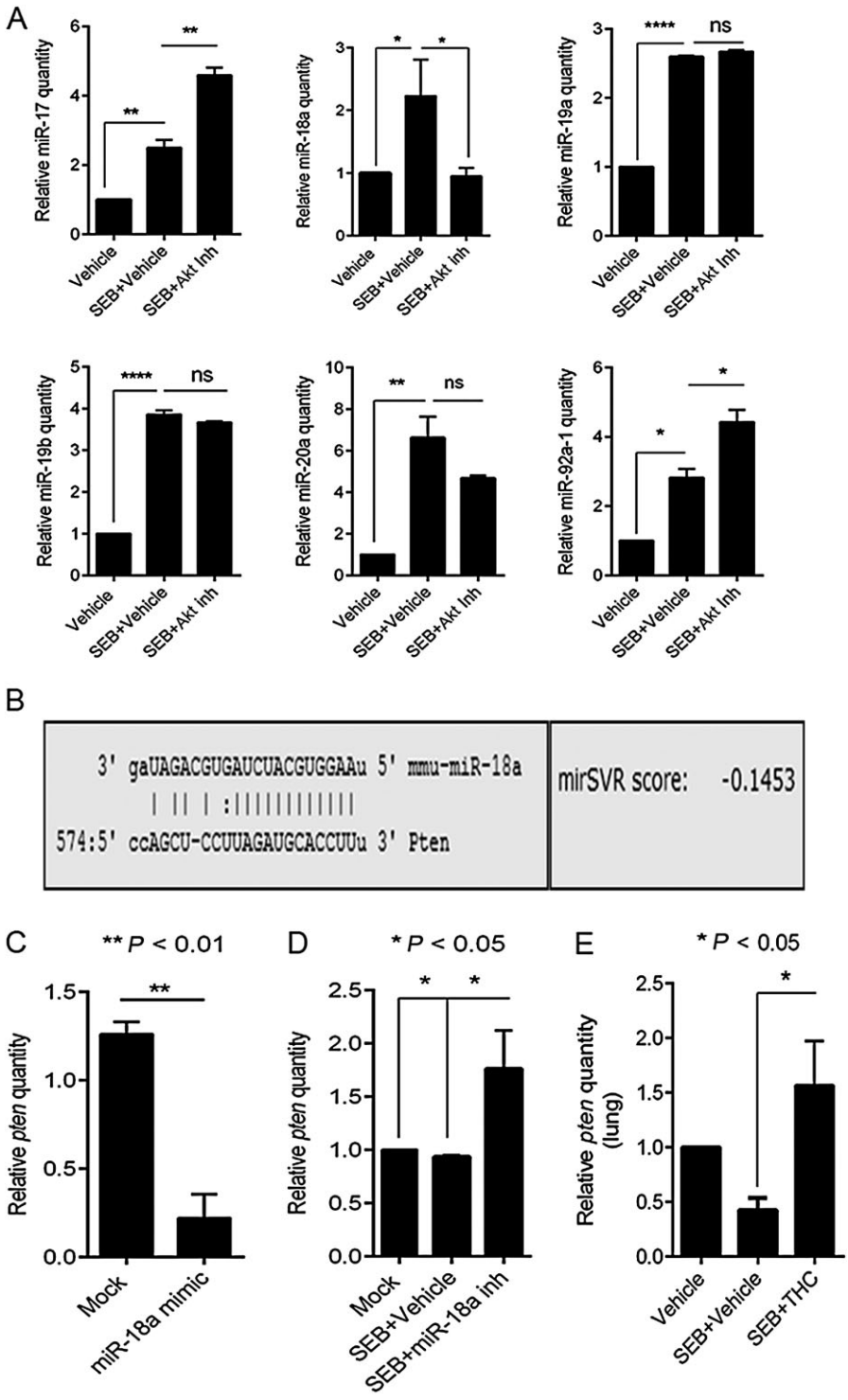

Figure 5

miR-18a targets *Pten*, an inhibitor of the PI3K/Akt pathway. (A) RT-PCR of members of the miR-17-92 cluster in splenocytes that were activated with SEB and treated with AKT inhibitor (20 μM). (B) Target prediction of miR-18a using miRanda (www.microrna.org), showing the alignment of the mature miRNA to the 3′ UTR of *Pten* mRNA. The miRSVR score represents the probability of mRNA target down-regulation and the cut-off for a good score was set at ≤ –0.01. (C) RT-PCR quantification of miR-18a and its target *Pten*. Splenocytes were transfected with 40 nmol of a miR-18a mimic for 24 h. (D) RT-PCR quantification of miR-18a and *Pten* after transfection with a synthetic miR-18a inhibitor. Splenocytes that were activated with SEB (1 $\mu g \cdot mL^{-1}$) were also transfected with miR-18a inhibitor for 24 h. (E) Real-time PCR quantification of *Pten* levels in lung-infiltrating mononuclear cells. All miRNA levels were measured relative to internal control Snord_96a and mRNA levels were normalized to β-actin. Statistical significance was assessed using ANOVA, Tukey's multiple comparison test.

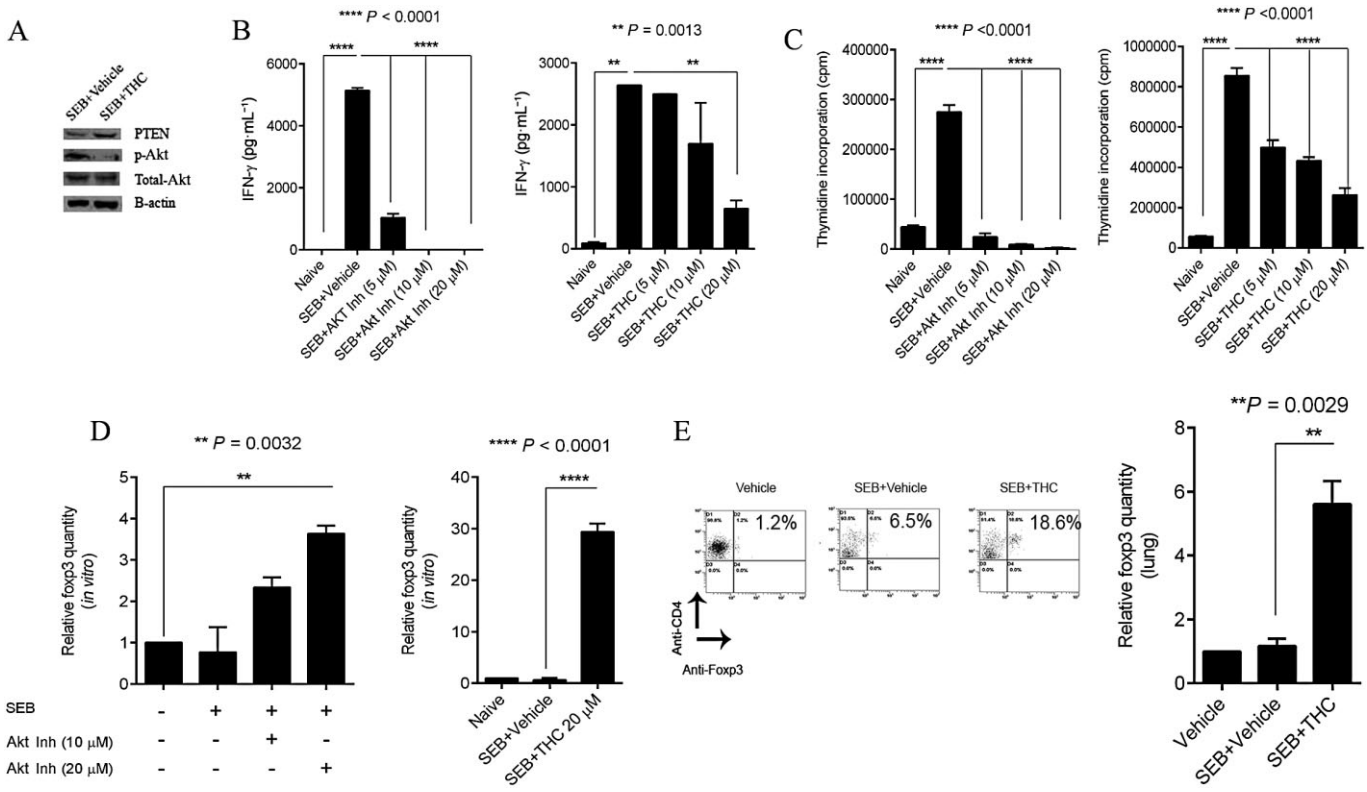

Figure 6

THC is an inhibitor of the Akt pathway and leads to the induction of CD4+Foxp3+ T-regulatory cells. (A) IFN-γ levels in supernatants of splenocytes that were activated with SEB and treated with varying doses of THC (5 μM,10 μM and 20 μM) or Akt inhibitor (5 μM,10 μM or 20 μM) as indicated. (B) Thymidine incorporation to measure proliferation of splenocytes activated with SEB or treated with the indicated doses of THC (5 μM, 10 μM and 20 μM) or Akt inhibitor (5 μM,10 μM or 20 μM). (C) RT-PCR of Foxp3 levels in splenocytes that were treated either with THC (20 μM)or Akt inhibitor (20 μM). mRNA levels measured relative to β-actin. (D) Flow cytometric analysis of CD4+Foxp3+ T-regulatory cells in lung-infiltrating mononuclear cells for the groups indicated. The bar graph represents the RT-PCR expression of Foxp3 in lung-infiltrating mononuclear cells. In all *in vitro* experiments, splenocytes were activated with 1 μg·mL^{-1} SEB. Statistical significance was assessed using ANOVA, Tukey's multiple comparison test.

SEB-induced suppression of *Pten*. Upon assessing *Pten* expression in lung-infiltrating mononuclear cells by qRT-PCR, we observed that while SEB exposure indeed resulted in the repression of *Pten*, THC treatment led to its increase (Figures 5E and 6A). Taken together, these data indicated that THC, via down-regulation of miR-18a, leads to the release of *Pten* and in doing so, may act as an Akt inhibitor.

THC functions as an Akt inhibitor

The activation of the PI3K/Akt pathway leads to cellular proliferation, the release of IFN-γ and inhibition of T-regulatory cells. Our results suggested that by antagonizing the PI3K/Akt axis via the down-regulation of miR-18a and the subsequent release of *Pten*, THC may mimic the properties of an Akt inhibitor. To confirm this notion, we activated splenocytes for 18 h with SEB or treated cells with THC and conducted a Western blot. We found a reduction in phosphorylated Akt in THC-treated cells when compared with SEB activation (Figure 6A). Next, we compared the properties of THC with an Akt inhibitor on IFN-γ production *in vitro*. Splenocytes were activated with SEB and treated with THC or Akt inhibitor. The resulting concentration of IFN-γ was measured by ELISA. As expected, Akt inhibition led to the dose-dependent decrease of IFN-γ (Figure 6B). Interestingly, THC treatment also demonstrated a trend in the dose-dependent decrease of IFN-γ, with a significant blunting of this cytokine at the highest dose (Figure 6C). Further, when cellular proliferation of cells was assayed by thymidine incorporation, we observed a similar dose-dependent decrease in cellular proliferation (Figure 6D) with THC and the Akt inhibitor. Moreover, the interruption of Akt signalling results in the induction of CD4+Foxp3+ T-regulatory cells (Sauer *et al.*, 2008; Merkenschlager and von Boehmer, 2010). Upon activating splenocytes with SEB *in vitro* and treating with either the Akt inhibitor or THC, we found significant induction of *Foxp3* (Figure 6E) compared with SEB alone. The induction was further confirmed by flow cytometric analysis and qRT-PCR of lung-infiltrating mononuclear cells (Figure 6E).Taken together, these data indicate that THC inhibits Akt signalling by modulating miR-18a and allowing for the release of *Pten*. The resulting decrease in cellular proliferation, IFN-γ production and induction T-regulatory cells, prevent SEB-mediated acute inflammatory lung injury and death.

Discussion

In the current study, we investigated the role of THC in preventing SEB-induced inflammatory lung injury and subsequent mortality via the modulation of miRNA. Through the use of qRT-PCR and computational tools, we found that the SEB-induced miRNA 17–92 cluster, which was overexpressed in the lungs, is down-regulated with THC treatment. Specifically, by performing gain-and loss-of function analysis using synthetic mimic and inhibitor, we confirmed the predominant role of miR-18a in the SEB-mediated activation of the PI3K/Akt pathway. Our studies also suggested that THC may function as an antagonist of the Akt pathway, at least in part, by its ability to significantly decrease the expression of miR-18a. Our results highlight the role of miRNA in facilitating severe inflammation and the ability of cannabinoids to suppress its expression. Importantly, we show that the previously reported potent anti-inflammatory role of THC can be explained, at least in part, by its ability to act upon SEB-mediated miRNA.

SEB specifically expands a large number of T-cells, by virtue of engaging the Vβ8 region of the TCR following binding to the MHC II on antigen presenting cells (Choi *et al.*, 1989; Hurley *et al.*, 1995). Consequently, SEB exposure leads to the massive release of inflammatory cytokines, proliferation of T-cells, tissue damage and SEB-mediated shock (Miethke *et al.*, 1992; DeVries *et al.*, 2011; Kissner *et al.*, 2011). Most models developed to study the effects of SEB exposure in mice, have employed the use of transgenic mice or external agents such as LPS or D-galactosamine to potentiate SEB-mediated inflammatory response (DaSilva *et al.*, 2002; Savransky *et al.*, 2003). In the dual administration of SEB as used in this study, microgram quantities of SEB were sufficient to cause inflammatory symptoms and toxicity reminiscent of SEB exposure in humans (Huzella *et al.*, 2009). In this model, mice succumb to SEB-mediated shock and severe respiratory damage (Huzella *et al.*, 2009). Consistent with these reports, we observed an overall increase in mononuclear cells in the lung, particularly T-cells. We also found SEB-mediated release of early monocyte and T-cell recruiting cytokines IL-2 and MCP-1 in the serum. Additionally, we noted the abundant release of late cytokines, especially IFN-γ in the BALF of the lungs resulting in compromised vascular permeability and the ultimate death of mice.

Earlier studies have reported that the molecular mechanism of action behind SEB-mediated inflammation and toxicity begins soon after the activation of the TCR (Goldbach-Mansky *et al.*, 1992; Linsley and Ledbetter, 1993). Following the increase in co-stimulatory molecules, a number of signalling pathways such as the MAPK, ERK, JNK and PI3K/Akt are simultaneously activated (Krakauer, 2013). These pathways culminate in the stimulation of various transcription factors such as NFκB and NFAT (Trede *et al.*, 1995; Tsytsykova and Goldfeld, 2000). However, with the recent discovery of miRNA, our understanding of the molecular mechanisms that govern gene regulation has been revolutionized. It is now evident that these small single-stranded RNA molecules are capable of targeting the 3′ UTR of mRNA thereby regulating biological processes such as cellular proliferation, differentiation and development (Davidson-Moncada *et al.*, 2010). miRNA are also induced upon a number of inflammatory cues and subsequently influence immune responses and immune cell development (Dai and Ahmed, 2011).

The miR-17-92 cluster found on mouse chromosome 14 is among the many miRNA that are found to be overexpressed under inflammatory conditions (Sonkoly and Pivarcsi, 2009). It is transcribed as a single polycistronic unit and comprises of six miRNA – miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a and miR-92-1 (Wu *et al.*, 2012). Previously, it has been shown that naïve CD4+ T-cells polarized under Th1 inducing conditions show a significant increase in the miR-17-92 cluster. (Sasaki *et al.*, 2010) Further, a recent report demonstrated the critical role of the cluster in the expansion of T-cells upon antigenic stimulation, although they observed that individual miRNA within the cluster have differential roles in promoting Th1 responses. (Liu *et al.*, 2014). Prompted by these reports, we first investigated the role of miRNA in facilitating SEB-mediated lethality in mice and found that SEB exposure led to the overexpression of the miR-17-92 cluster in lung-infiltrating mononuclear cells. Similar to previous studies, we also found that the members of the cluster, were expressed to different levels, suggesting that one or more miRNA within the cluster may play a more prominent role.

It was reported that the overexpression of miR-17-92 cluster causes lymphoproliferation, autoimmunity and premature death of mice by targeting *Pten*, a well-established antagonist of the PI3K/Akt pathway (Xiao *et al.*, 2008). In the present study, we demonstrate that *Pten* is suppressed after SEB exposure. Whereas previous studies have shown a primary role for miR-19b in the targeting of *Pten* (Olive *et al.*, 2009; Liu *et al.*, 2014), we attribute the SEB-mediated suppression of *Pten* to the predominant role of miR-18a. These data are surprising because while we demonstrate a clear reversal in the suppression of *Pten* using a miR-18a inhibitor, a few reports have demonstrated its role in inhibiting cellular proliferation (Tsang and Kwok, 2009). It is possible that the functional targets of miRNA may differ based on the type of antigenic stimulation. Alternatively, the critical timing of SEB dual administration could contribute to the specific regulation of *Pten* by miR-18a. Moreover, it is also possible that because the cluster comprises of six miRNA with several predicted target genes, *Pten* is simply one of many genes simultaneously targeted during the course of inflammation. Nevertheless, our results show a clear induction of the miR-17-92 cluster and specifically the suppression of *Pten* by miR-18a.

The anti-inflammatory and immunosuppressive effects of THC are diverse and function effectively to abrogate a number of inflammatory processes. For example, THC has previously been reported to prevent the development of a murine model of multiple sclerosis and colitis (Lyman *et al.*, 1989; Jamontt *et al.*, 2010). In a mouse model of Con-A induced hepatitis, we have demonstrated its ability to act on acute inflammation, where it not only decreased the production of inflammatory cytokines, but also reduced cellular proliferation (Hegde *et al.*, 2008). Consistent with these reports, our data demonstrate a significant reduction in infiltrating immune cells in the lung, blunting of cytokines as early as 3 h after SEB exposure and more astonishingly, the 100% survival of mice.

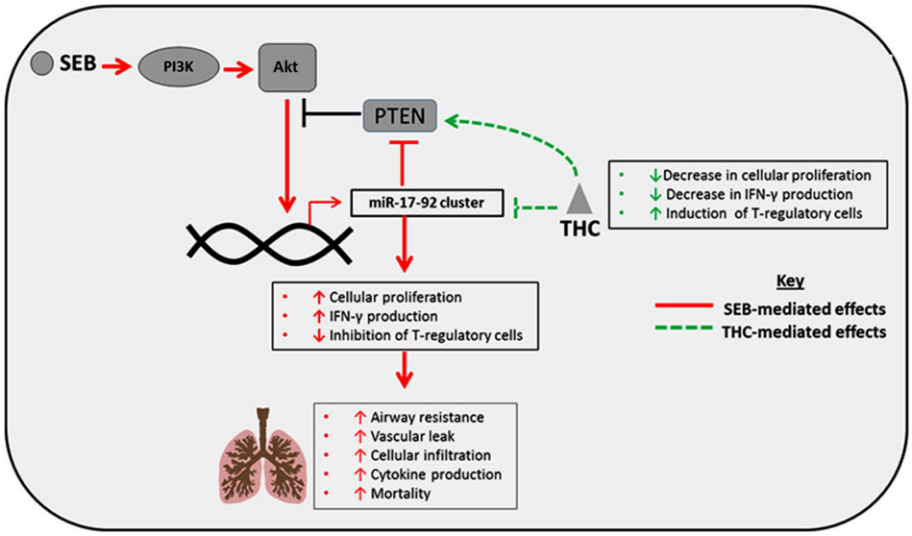

Figure 7

Schematic of the proposed working model. SEB administration leads to activation of the PI3K/Akt pathway via the induction of the miR-17-92 cluster and the subsequent down-regulation of *Pten*. As a result, SEB exposure leads to increased cellular proliferation, cytokine production, pulmonary damage and acute mortality. The down-regulation of the cluster by THC restores *Pten* levels and allows for the inhibition of the PI3K/Akt axis. This leads to attenuation of acute inflammatory lung injury and induction of T-regulatory cells, which together prevent SEB-induced mortality.

We have previously demonstrated that THC's anti-inflammatory properties can be credited, in part, to the induction of immunosuppressive cells such as T-regs and MDSC (Hegde *et al.*, 2008; 2010). Recently, we have identified a novel role for THC in modulating miRNA involved in the development of MDSCs (Hegde *et al.*, 2013), a finding that could shed mechanistic light on THC's mode of action. Thus, we rationalized that THC could potentially exert its strong anti-inflammatory activities by modulating SEB-induced miRNA. Our present findings validate our hypothesis and we observed a potent down-regulation of the miR-17-92 cluster. Interestingly, CBD, a cannabinoid also derived from the marijuana plant did not decrease all members of the cluster. Similarly immunosuppressive agents dexamethasone and rapamycin, previously shown to rescue C3H/HeJ mice from SEB induced toxicity (Krakauer and Buckley, 2006; 2012), also failed to effectively target the cluster, highlighting the unique property of THC to act specifically on the miR-17-92 cluster. Our results demonstrate the derepression of *Pten* suggesting that THC, via the down-regulation of the cluster might be an inhibitor of the PI3K/Akt pathway. Interestingly, earlier studies in cancer models have demonstrated that THC disrupts the PI3K/Akt signalling pathway (Greenhough *et al.*, 2007; Leelawat *et al.*, 2010). However, in light of our current observation that it modulates key miRNA, its anti-inflammatory properties via its role as a PI3K/Akt inhibitor are made more evident.

The disruption of the PI3K/Akt/mTOR axis using Akt inhibitors has been previously reported to decrease cellular proliferation, induce apoptosis and decrease IFN-γ production (Shin *et al.*, 2002; Mandal *et al.*, 2005). Further, rapamycin, an inhibitor of mTORC1, specifically diminishes SEB-induced IL-2, IFN-γ and T-cell proliferation (Krakauer *et al.*, 2010). In line with these reports, our results also demonstrated decreased cellular proliferation and IFN-γ production with THC treatment. Additionally, the PI3K/Akt activation of PTEN-deficient T-cells led to the suppression of CD4+Foxp3+ T-cells, which was reversed with PI3K/Akt inhibition (Sauer *et al.*, 2008). Similarly, we observed the induction of T-regs in the lung upon THC treatment, further confirming that THC is an inhibitor of the PI3K/Akt signalling pathway, and that part of its mechanism involves down-regulation of the miR-17-92 cluster, particularly miR-18a.

Taken together, our data demonstrate that THC is a strong anti-inflammatory agent capable of rescuing mice from SEB-mediated toxicity and death. By affecting the SEB-induced miR-17-92 cluster, it restores *Pten*, and enables the proper regulation of the PI3K/Akt signalling pathway. Consequently, it reduces cellular proliferation, diminishes the production of pro-inflammatory cytokine and induces T-regs (Figure 7).

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Authorship contribution

Statement of author contributions: R. R., M. N., P. S. N. contributed to the design of the experiments. R. R. performed the experiments. R. R., M. N. and P. S. N. analysed the data. R. R., M. N. and P. S. N. contributed to the writing of the manuscript. M. N. and P. S. N. directed the research.

Conflict of interest

The authors have no conflicts to report.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13026

Figure S1 Regulation of miR-17-92 cluster by THC, dexamethasone (Dexa), rapamycin (Rapa) and cannabidiol (CBD) *in vitro*. Splenocytes were activated with SEB and treated with THC (20 μM), CBD (20 μM), dexamethasone (200 nM) and rapamycin (100 nM). Total RNA was extracted and RT-PCR was conducted to measure miRNA levels relative to Snord_96a internal control. Statistical significance was assessed using ANOVA, Tukey's multiple comparison test.