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ORIGINAL ARTICLE

Intranasal Administration of Perillyl Alcohol Activates Peripheral and Bronchus-Associated Immune System In Vivo

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Abstract Perillyl alcohol (POH) presents antitumoral activity but clinical application is hampered by adverse effects following oral administration. This work aimed to verify the cytotoxic effect of intranasal POH administration in the histology of lung, liver, brain; the cellularity and function of peripheral and bronchoalveolar-associated immune system. C57 adult mice received 1-min inhalation with POH, vehicle 70 % ethanol or saline buffer, once (84 μg/day) or twice (164 μg/day) during five consecutive days, and were killed 72 h after treatment. Spleen, cervical and mesenteric lymph nodes were removed for ^{3}H-thymidine proliferation assay, leukocyte cellularity and flow cytometry analysis. Peripheral blood and bronchoalveolar lavage cells were collected to assess cellularity and immunoglobulin (IgA, IgM) levels. Intranasal POH did not alter body weight or liver, brain and lung morphology, but increased splenocyte and cervical lymph node cell proliferation, and IgM production without altering peripheral lymphocyte subsets. Treatment also increased the percentage of alveolar macrophages (83 %) and IgA-producing lymphocytes (15 %), a pattern characteristic of activated bronchoalveolar innate immune system. Intranasal administration of POH activated peripheral immune system and innate immunity of bronchus-associated lymphoid tissue, thus suggesting a possible role for POH as a chemotherapeutic drug also in pathological processes affecting the lung.

Keywords Perillyl alcohol · Intranasal administration · Therapeutic strategy · Immune system · Bronchoalveolar fluid

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Introduction

Perillyl alcohol (POH), perillic acid and *cis/trans*-dihydroperillic acid are systemic metabolites of *d*-limonene and considered effective inhibitors of cellular proliferation in vitro. Studies using human HT-29 colon carcinoma cell line have suggested that POH, a naturally occurring monoterpene, is a potent inhibitor of small G protein isoprenylation (Crowell et al. 1994). POH exhibits potent cytotoxic activity to tumor cells but not normal cells, inducing activation of transforming growth factor β pathway and/or inhibition of p21 Ras signaling, leading to tumor cell differentiation or apoptosis (Haag et al. 1992; Hudes et al. 2000). Phase I/II clinical trials suggest that oral administration of POH could be useful in clinical practice. However, oral delivery of POH has the potential to cause unwanted side effects (Azzoli et al. 2003) due to hepatic first-pass

metabolism which requires high doses (4.8–11.2 g/m^2 per day), causing gastrointestinal distress, vomiting and diarrhea. The maximum-tolerated POH dose delivered orally (4 times/day 8.4 g/m^2) has shown only modest clinical responses as anticancer agent (Azzoli et al. 2003; Bailey et al. 2004; Belanger 1998). Standard chemotherapeutic regimens for the treatment of brain tumors are frequently inefficient due to the inability of drugs to reach and maintain effective concentrations within the brain tissue for an appropriate length of time (Caruso et al. 2011). Drug transport into the central nervous system (CNS) is dependent upon the blood–brain barrier (BBB) permeability and restricted according to the size of the molecules or presence of polar functional groups. Accordingly, the role of the olfactory epithelium as a gateway for substances entering the CNS have lead us to develop a novel and ground breaking methodology for POH delivery to treat patients with malignant brain tumors (da Fonseca et al. 2011a, b). Intranasal delivery is a non-invasive method that allows rapid access of drugs and hormones to the CNS, bypassing the BBB, minimizing systemic exposure and dramatically reducing adverse side effects (Dhuria et al. 2010). Our group has pioneered the use of intranasal POH for the treatment of patients with recurrent malignant gliomas and published extensive evidence to support that intranasal administration of POH substantially increased survival, with virtually no adverse side effects (da Fonseca et al. 2008b, 2011a, b). Since intranasal administration is emerging as the route of choice to improve drug delivery to the brain, it was important to evaluate the effects of intranasal administration of POH on cellularity and function of peripheral and bronchoalveolar-associated immune system; and histology of lung and brain tissues.

Materials and Methods

Animals

Male and female C57BL/6J mice at 6–8 weeks were obtained from the animal housing facilities at Institute of Biology from Fluminense Federal University. Mice were kept at constant temperature (20 °C) with a light cycle of 12:12 h, receiving acidified water and enriched diet ad libitum. All experiments were approved by the Committee for Ethics in Animal Research of the Fluminense Federal University and followed the guidelines of the Brazilian College for Animal Experimentation in agreement with international regulations.

Intranasal POH Treatment

For efficient intranasal delivery, commercially available POH (Sigma Aldrich, St. Louis, MO) formulated (v/v) as 10 % in 70 % ethanol (vehicle) was administered into nostrils using an ultrasonic inhalator (NS RespiraMax RPXFC, São Paulo, Brazil) with a mask adapted to the mouse snout. According to the manufacturer's instructions, the nebulization rate was adjustable to 0.3 ml/min, and each mouse received 84 µg POH/day. Dosing schedule was calculated based on the protocol used on phase I/II clinical trial for patients with recurrent glioma and adjusted considering mouse corporal area, brain and body weight (da Fonseca et al. 2008a, b, 2011a). Mice were subjected to 1-min inhalation once or twice a day (12/12 h) for five consecutive days using POH (Sigma, MO, USA), 70 % ethanol (vehicle) or saline PBS buffer (pH 7.2) as sham control group to assess the impact of stress ($N = 5$/group). Animals were killed 72 h following the last treatment. Experiments were repeated three times.

Collection and Analysis of the Bronchoalveolar Cells

Immediately after the mice were euthanized, the left pulmonary hilum was tied off and a 20-gauge catheter was inserted into the trachea. The bronchoalveolar lavage (BAL) fluid was collected by lavage in the right lung with four 0.5 ml aliquots of 0.9 % NaCl supplemented with 0.6 mM EDTA. After three consecutive lavages, the BAL fluid was centrifuged at 200×g during 10 min at 10 °C (Universal 320R, Hettich, Germany). The BAL cellularity was determined using a haemocytometer, and differential cellularity determined from examination of cytocentrifuge slides (Thermo Shandon, Pittsburgh, PA, USA) pre-coated with 1 % bovine serum albumin/PBS solution and stained with May-Grünwald Giemsa.

Analysis of Leukocyte Subsets by Flow Cytometry and Cell Proliferation Assay

Spleen, cervical and mesenteric lymph nodes were carefully removed, finely minced in ice-cold complete RPMI (Sigma Chem Co, USA) culture medium, filtered through 40 µm nylon mesh (BD Falcon, Bedford, MA), and centrifuged at 200×g during 10 min at 10 °C (Universal 320R, Hettich, Germany). The following antibodies were used: PE rat anti-mouse CD4, biotin mouse anti-mouse NK-1.1 (BD-Pharmingen, San Diego, CA), rat anti-mouse CD8, FITC rat anti-mouse CD45R/B220 (Southern Biotech, Birmingham, AL) and Streptavidin-Quantum Red (Sigma, St. Louis, MO). For proliferation assay, 10^6 cells were plated in 96-well plates, incubated with 1 µCi ^{3}H-thymidine for 3 h at 37 °C and radioactivity count determined (Packard, model 1600 TR).

Histological Examination

Following euthanasia, lungs, brain (cortex, cerebellum), liver and bone marrow were carefully removed, fixed

overnight in formalin-buffered Millonig solution (pH 7.2), and embedded in paraplast (Sigma, St Louis, MO). Five-μm thick tissue sections were stained with hematoxylin–eosin to assess morphological alterations. Sirius red staining was used for collagen deposition analysis.

Determination of IgG and IgA Levels by Enzyme Immunoassay

50 μL of BAL diluted 1:2 and serum diluted 1:500 in saline buffer were used for quantitative sandwich enzyme immunoassay (Southern Biotech, USA). Measurements were done at 492 nm in a Thermoplate *TP* Reader.

Statistical Analysis

Data are expressed as arithmetic mean ± SD. Statistical analysis was performed by one-way analysis of variance with Tukey's Multiple Comparison post test employed to compare the studied groups. All data were analyzed using GraphicPad Prism (GraphicPad Software Inc., CA, USA). Differences were considered significant at $p < 0.05$.

Results

Intranasal Administration of POH Is Not Toxic to C57 Mice

Mice which have received intranasal administration of POH once (84 μg) or twice (164 μg) a day did not present clinical signs of disease such as weight loss, seizures, diarrhea, ruffled fur or behavioral changes when compared to vehicle 70 % ethanol or sham control groups (data not shown). Likewise, no significant differences in the cellularity of peripheral blood leukocytes (PBL) were observed after 72 h treatment with POH once (control 392 ± 43.8/mm^3, vehicle 250 ± 24.3/mm^3, POH 385 ± 46.4/mm^3) or twice a day (control 411 ± 21.5/mm^3, vehicle 354 ± 21.8/mm^3, POH 365 ± 30/mm^3) when compared to the control groups (Fig. 1b, c). A transitory reduction of PBL cellularity after treatment with ethanol once a day was noticed.

POH Treatment Modulates Cellular Proliferation of Lymphocyte Subsets

Since the nasal cavity is highly vascularized and contains a rich supply of lymphatic vessels, it was important to determine the effects of intranasal administration of POH on lymphocyte subpopulations within peripheral lymph nodes. Mice treated once or twice a day with POH, vehicle or sham controls showed similar percentage of T lymphocyte subsets CD4$^+$, CD8$^+$; B lymphocytes and natural killer cells (data not shown) in the cervical (Fig. 2a), mesenteric lymph nodes (Fig. 2b) and spleen (Fig. 2c).

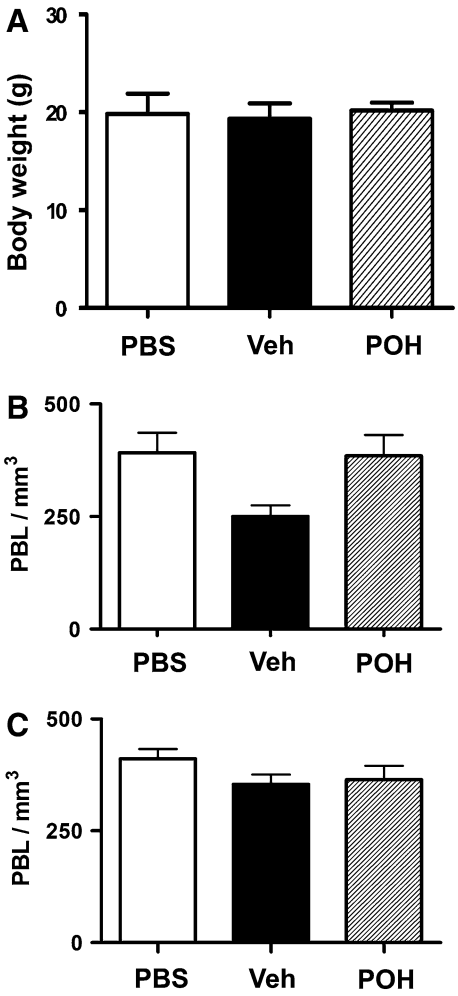

Fig. 1 Cellularity and body weight. Intranasal administration was performed once (**a**, **b**) or twice (**c**) a day with POH, vehicle (70 % ethanol) or sham control (PBS). $N = 5$ per treatment group. Data represent the average of three independent experiments. Results are expressed as mean ± SD

We further used a short-term radioactive assay with ^{3}H-thymidine uptake to assess the effect of POH treatment upon cell proliferation by measuring the amount of labeled thymidine incorporated during DNA synthesis. POH inhalation increased the proliferative capacity of cervical lymphocytes and splenocytes but not mesenteric lymphocytes (Fig. 3). Such effect was more evident ($p < 0.006$) in cervical lymphocytes of mice treated with POH twice a day (698.6 ± 78.7 cpm) when compared with vehicle 70 % ethanol (468 ± 25.6) or PBS sham control groups (410.6 ± 24.7 cpm). In comparison with control groups, intranasal administration of POH once (A, B, C: left panel) or twice (A, B, C: right panel) a day induced significant increase of splenocyte proliferation. Such effect was evident after POH treatment once ($p < 0.0012$, C left panel) in

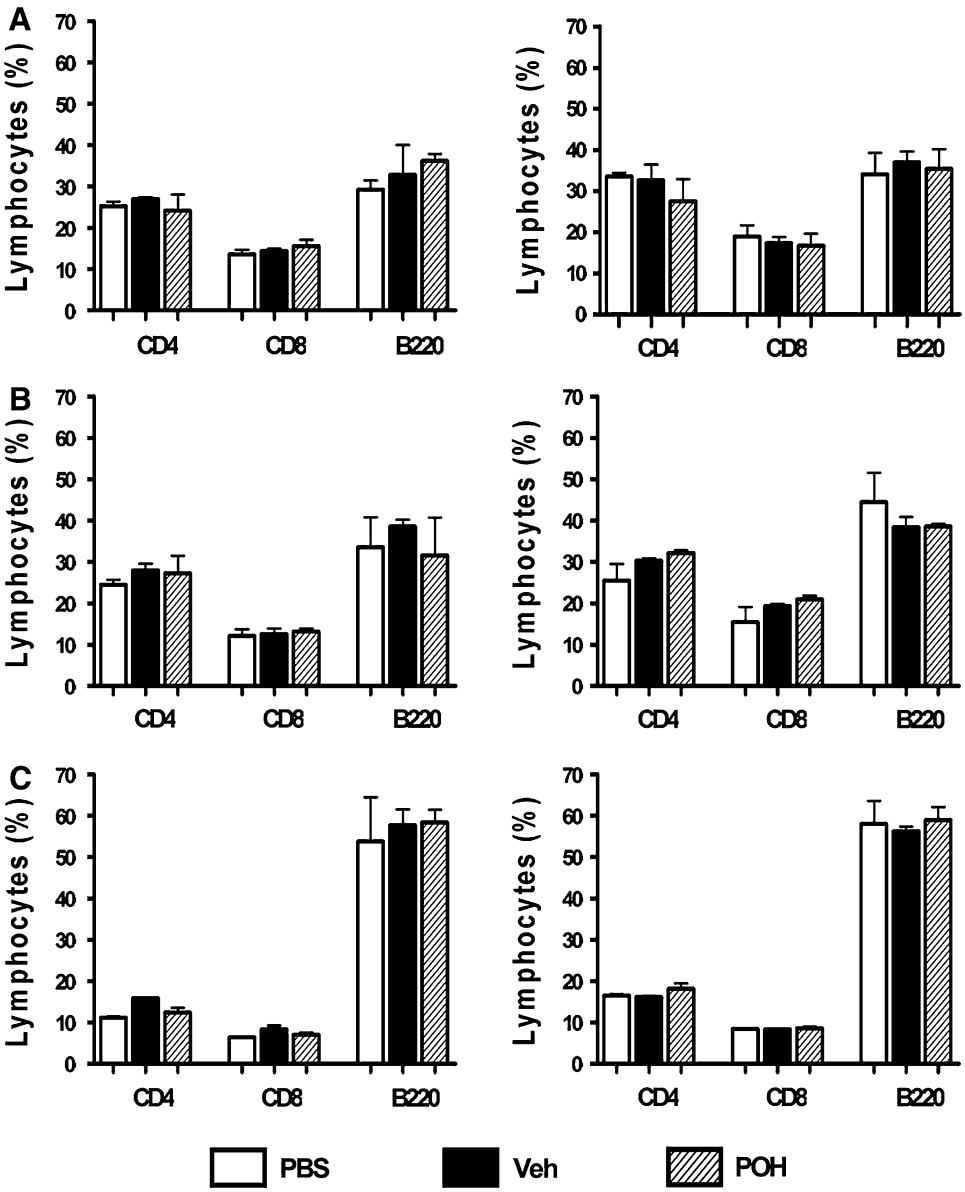

Fig. 2 Mononuclear cell phenotype. Intranasal administration was performed once (*left panel*) or twice (*right panel*) a day with POH, vehicle (70 % ethanol) or sham control (PBS). Results are expressed as percentage of lymphocyte subsets in the cervical lymph node (**a**), mesenteric lymph node (**b**) and spleen (**c**). $N = 5$ per treatment group. Data represent the average of three independent experiments. Results are expressed as mean ± SD

relation to vehicle, and treatment twice a day ($p < 0.0002$, C left panel) in relation to PBS, and $p < 0.0012$ (C right panel) in relation to vehicle 70 % ethanol.

Intranasal POH Treatment Increases Immunoglobulin Production

Given the impact of intranasal delivery of POH on lymphocyte cellularity in the cervical lymph nodes and spleen, it was of interest to analyze the levels of circulating immunoglobulin following POH treatment (Fig. 4). Mice treated with POH twice a day showed significant ($p < 0.0011$) increase in IgM serum levels (755.6 ± 41.3) as compared to control groups (vehicle 464 ± 24.8, PBS 522 ± 18.7; Fig. 4). However, no differences in IgM or IgG serum levels were observed following intranasal treatment with POH once a day. Serum levels of IgA were not altered upon treatment with POH either once or twice a day. In contrast, increased IgA content was consistently observed in the BAL fluid of mice treated with POH once ($p < 0.007$; Fig. 5a) or twice a day ($p < 0.028$; Fig. 5b).

Intranasal Treatment with POH Activates the Bronchoalveolar-Associated Immune System

Analysis of the leukocyte populations in the BAL fluid showed that in contrast to control PBS (Fig. 5c) and vehicle 70 % ethanol (Fig. 5d) which showed normal distribution of alveolar macrophages (93 %), lymphocytes (4 %) and neutrophils (3 %), mice treated once a day with POH showed increased number of lymphocytes (9 %) (Fig. 5e). Such effect was even more evident after

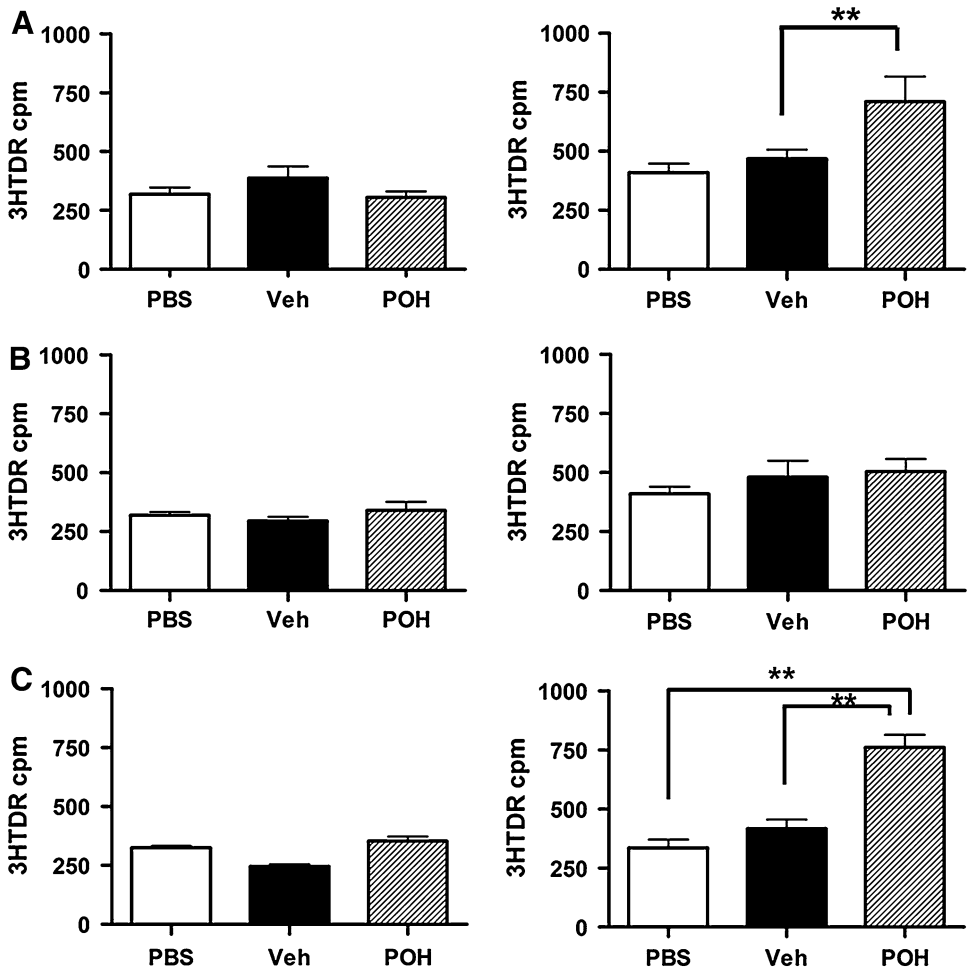

Fig. 3 Intranasal POH increased cellular proliferation of cervical lymph node cells and splenocytes. 10^6 mononuclear cells from cervical lymph node (**a**), mesenteric lymph node (**b**) and spleen (**c**) of C57 mice were incubated with 1 μCi ^{3}H-thymidine for 3 h at 37 °C. Intranasal administration was performed once (*left panel*) or twice (*right panel*) a day with POH, (70 % ethanol) or sham control (PBS). $N = 5$ per treatment group. Data represent the average of three independent experiments. Results are expressed as mean cpm ± SD. Increased proliferation of cervical lymph node cells after POH treatment twice ($p < 0.006$, **a** *right panel*); and also splenocytes after POH treatment once ($p < 0.0012$, **c** *left panel*) in relation to vehicle, and treatment twice a day ($p < 0.0002$, **c** *left panel*) in relation to PBS, and $p < 0.0012$ (**c** *right panel*) in relation to vehicle 70 % ethanol

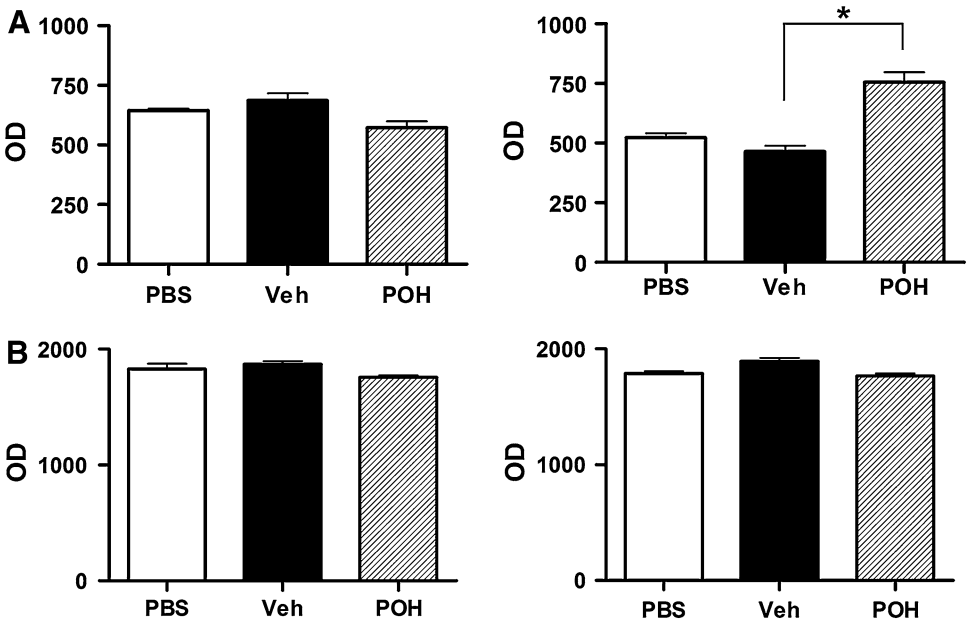

Fig. 4 Effect of intranasal POH treatment in serum immunoglobulins. Determination of IgM (**a**) and IgA (**b**) serum levels following intranasal administration performed once (*left panel*) or twice (*right panel*) a day with POH (70 % ethanol) or sham control (PBS). $N = 5$ per treatment group. Data represent the average of three independent experiments. Results are expressed as mean ± SD. *Significant increase ($p < 0.0011$) of IgM after POH treatment twice a day

treatment with POH twice a day (Fig. 5b). Indeed, intranasal administration of POH twice a day led to a further increase in the percentage of plasmocytoid lymphocytes (17 %) and alveolar macrophages (82 %) with activated characteristics (Fig. 5e) in contrast to 70 % ethanol (vehicle) or sham control groups (PBS). Altogether, the

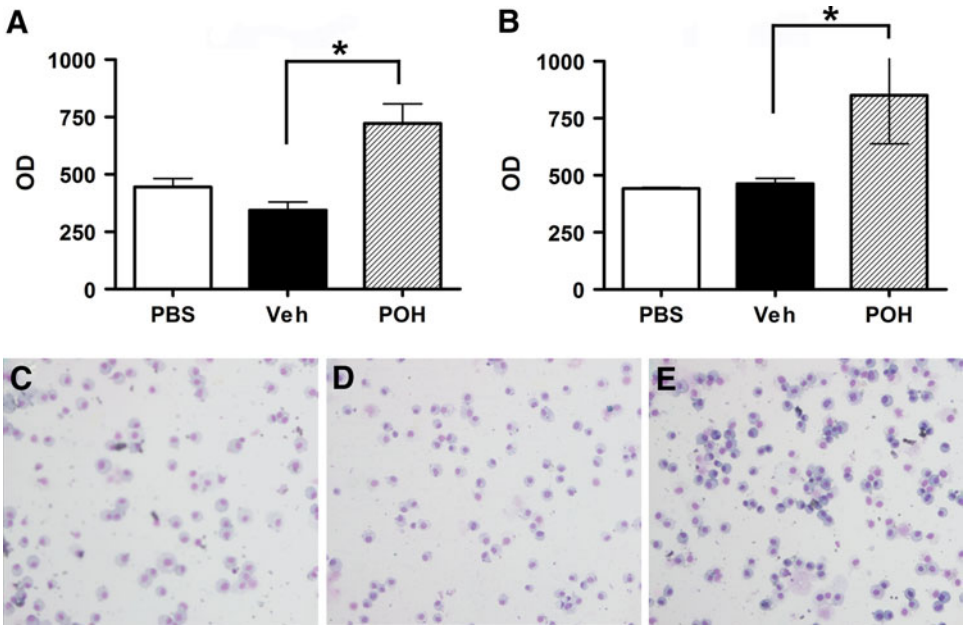

Fig. 5 Intranasal POH treatment activates bronchoalveolar system. IgA levels in the BAL fluid were determined by enzyme immunoassay after intranasal POH treatment once (**a** *right panel*, $p < 0.007$), and twice a day (**b** *left panel*, $p < 0.028$). Representative figure depicts cytospin preparation stained with Wright-Giemsa PBS (**c**), 70 % ethanol (**d**) or POH (**e**). Intranasal POH treatment increased numbers of plasmocytoid lymphocytes and alveolar macrophages with activated characteristics (Fig. 5e) in contrast to 70 % ethanol (vehicle) or sham control (PBS) groups. $N = 5$ per treatment group. Data shown are from a representative experiment. Experiments were repeated three times. Magnification ×40

results indicate that POH treatment activated the bronchoalveolar-associated lymphoid tissue within the pulmonary microenvironment.

Tissues Morphology were not Altered Following POH Treatment

Morphological analysis of hematoxylin–eosin stained tissues harvested from animals treated with intranasal POH once or twice a day revealed no histological alterations (Fig. 6). In addition, there was no evidence of treatment-associated fibrosis in tissue sections stained with sirius red to determine changes in collagen deposition.

Discussion

The brain tissue is one of the least accessible organs for delivery of active pharmacological compounds (Caruso et al. 2011). POH is a relatively nontoxic naturally occurring hydroxylated monocyclic monoterpene with demonstrated preclinical activity against various tumors, such as breast and liver. POH is an effective radio and chemosensitizer (Rajesh et al. 2003) that arrests G2/M phase of glioma cells cycle and presents anti-angiogenic properties (Loutrari et al. 2004). In addition, POH is known to suppress several signaling pathways, including post-translational isoprenylation of Ras small GTPase superfamily of proteins, inducing apoptosis through activation Bax in different glioma cell lineages (Chaudhary et al. 2009; Cho et al. 2012; da Fonseca et al. 2008a; Hohl and Lewis 1995; Holstein and Hohl 2003; Loutrari et al. 2004; Rajesh et al. 2003). Importantly, the lipophilic characteristics of POH allow the molecule to bypass the BBB, indicating that POH may be an effective agent for treatment of patients with brain tumors (da Fonseca et al. 2008a, 2011a). In the phase I/II clinical trials conducted by our group in adult glioma patients, the POH dosage 6.3 mg/kg administered by intranasal route on a long-term basis was safe, reduced tumor growth and increased the overall survival of patients with recurrent malignant glioma (da Fonseca et al. 2008a, 2011a). The in vivo dosages used in our experiments were within the pharmacological range that is achievable in humans and set based on average mouse body (∼20 g) and brain (∼490 mg) weights which corresponded to 84 μg POH per day. Using a mouse model, we could demonstrate that a short-term intranasal treatment with POH did not cause any toxic effect in the lung, liver, brain and bone marrow but was capable to activate peripheral lymphoid and bronchoalveolar lymphoid-associated tissues without causing lipoid-lung inflammation or lipofuscin aggregate deposits within alveolar macrophages.

Malignant brain tumors present high rates of morbidity and mortality, as clinically management is difficult due to

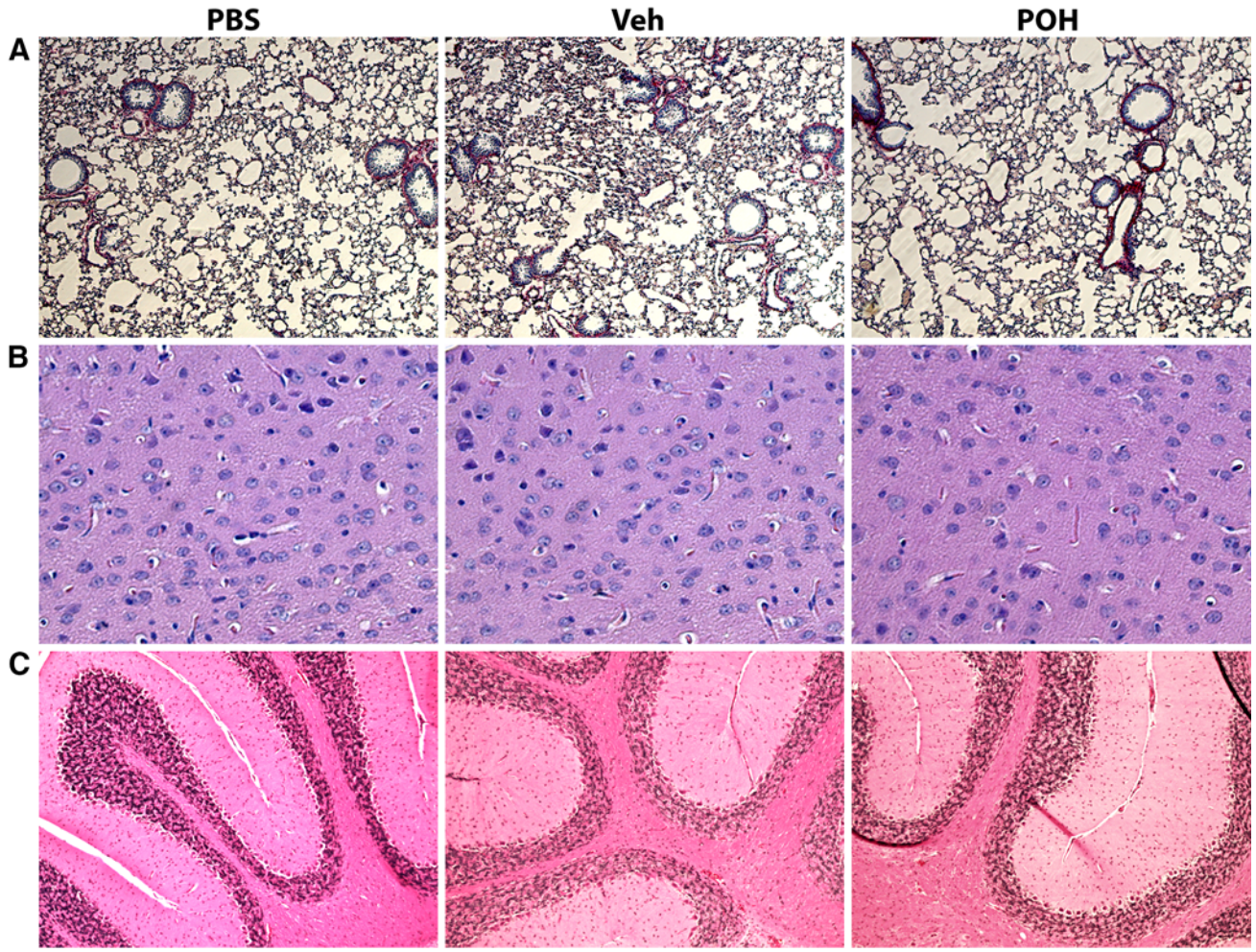

Fig. 6 Effect of intranasal POH in the histology of brain and lung. Intranasal administration was performed twice with POH, vehicle or PBS. Representative photomicrography of 5 μm tissue sections of lung (**a**), brain cortex (**b**) and cerebellum (**c**) stained with hematoxylin–eosin, and with sirius red to observe collagen deposition in the pulmonary microenvironment (**a**)

limitations of conventional therapies and inefficient delivery of active pharmacological compounds. This is partly due to specific physical interfaces that tightly regulate the exchange between the peripheral blood circulation and the brain tissue (Caruso et al. 2011). In addition, brain tumor cells present high degree of altered molecular signaling pathways, marked angiogenesis, down regulation of apoptotic genes, and multiple mutations in proto-oncogenes and tumor-suppressor genes encoding proteins that play an important role in signal transduction. Therapies targeting specific molecules may result in killing tumor cells effectively while keeping normal cells intact (Cho et al. 2012; da Fonseca et al. 2008a). Intranasal delivery of POH may be a non-invasive therapeutic strategy with less systemic side effects due to rapid drug absorption through the cribriform plate directly into the brain tissue (Banks et al. 2009). Pathways of intranasal route involve the olfactory and trigeminal nerves, the vasculature, and reaching the cerebrospinal fluid where can be rapidly distributed throughout the CNS, via bulk flow mechanisms and mix brain interstitial fluid (Dhuria et al. 2010). Clearance products and drug metabolites can also exit these spaces in the reverse direction from CNS to the periphery (Dhuria et al. 2010). The intensity of therapeutic response by nasal absorption is high due to profuse vascularization of the nasal mucosa with extensive surface, about 150 cm^2, which results in rapid absorption and action. In this context, we can envisage as a promising antitumoral therapeutic strategy, the synthesis of biological active hybrid molecules containing POH as a carrier conjugated to drugs specifically targeting critical regulators of cell proliferation (Cho et al. 2012; Das et al. 2010). Such multifunctional drug delivered by intranasal route may successfully be employed to treat brain tumors to overcome apoptosis resistance, angiogenesis and neuroinflammation—hallmarks of malignant gliomas, and also pathological processes affecting lung tissue.

In conclusion, intranasal administration of POH activated peripheral immune system and innate immunity of

bronchus-associated lymphoid tissue, thus indicating that such strategy may successfully be employed to treat brain tumors and also pathological processes affecting lung tissue.

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Conflict of interest The authors declare that they have no conflict of interest.

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