INTRODUCTION

Alveolar echinococcosis (AE) is a very severe zoonotic helminthic disease, which is fatal if patients are not appropriately diagnosed and subsequently treated. AE is characterized by a chronically progressing hepatic damage caused by the continuous proliferation of the larval stage (metacestode) of Echinococcus multilocularis (E. multilocularis), that behaves like a slowly growing and metastasizing liver cancer, thus progressively also invading other host tissues and organs such as lungs and brain, among others. For the treatment of AE, surgery is, so far, the only potentially curative option. Inoperable AE cases must undergo long-term chemotherapy, often lifelong. Extensive animal experimentations and observations in human patients suffering from AE have demonstrated that ABZ and MBZ exhibit a parasitostatic rather than a parasiticidal effect. Therefore, recurrence rates after interruption of therapy are high. Beside the inconvenience of a daily and long-term drug administration, some patients experience side effects.

Summary

The growth potential of the tumour-like Echinococcus multilocularis metacestode (causing alveolar echinococcosis, AE) is directly dependent upon the nature/function of the periparasitic adaptive host immune-mediated processes. PD-1/PD-L1 pathway (programmed cell death 1), which inhibits lymphocytic proliferation in tumour development, is over-expressed at the chronic stage of AE. We tested the impact of a PD-1/PD-L1 pathway blockade on the outcome of both chronic AE (intraperitoneal metacestode inoculation, secondary AE and SAE) and acute AE (peroral egg infection, primary AE and PAE). To assess the parasite proliferation potential, we measured parasite mass weight for SAE and liver lesion number for PAE. In both models, the parasite load was significantly decreased in response to anti-PD-L1 antibody treatment. In SAE, anti-PDL1 administration was associated with increased Th1 response parameters and decreased Treg responses, while in PAE anti-PDL1 administration was associated with fewer lesions in the liver and decreased Treg/Th2 responses. Our findings highly suggested that a PD-1/PD-L1 pathway blockade triggered the host immune responses in favour of an immune-mediated control of E. multilocularis proliferation. Based on this, future studies that combine PD-1/PD-L1 blockade with a parasitostatic albendazole medication may yield in a putatively curative therapeutic approach to control alveolar echinococcosis.

KEYWORDS

anti-PD-L1, Echinococcus multilocularis, immunotherapy, PD-1, PD-L1
such as hepatotoxicity\(^6,7\) and drug interactions,\(^8\) thus alternative treatment options are required, especially when this could offer a curative prognosis.

Mechanistically, *E. multilocularis* metacestode infection is critically modulated by adaptive immune response of the host. In particular, an initial acute inflammatory Th1 response (putatively immune protective) is gradually converting into a mixed Th1/Th2 response during the chronic phase of AE\(^9,10\) thus allowing parasite survival upon regulation via CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T (Treg) cells and Th17 cells,\(^10\) and thus finally leading to a lethal outcome of disease due to continuous long-term parasite proliferation and maturation.

In recent years, specific immunotherapies such as checkpoint blockade has become of great interest to researchers and clinicians, particularly in its promise to treat various forms of cancer,\(^11\) but also infectious diseases increasingly gained respective interest.\(^12\) With regard to helminth infection, it was shown that cestode *Taenia crassiceps* infections in mice induce macrophages alternatively activated with strong suppressive activity involving PD-1/PD-L1 blockade against secondary AE.\(^13\) Blockade of the PD-1/PD-L1 pathway during infections with certain pathogens such as *Toxoplasma* restored exhausted CD8\(^+\) T cell response,\(^14\) and promoted brain leukocyte infiltration and diminishes cyst burden in another mouse infection model.\(^15\) It was also shown that blocking PD-L1 signaling in *Leishmania donovani*-infected mice resulted in restoration of protective type 1 responses by both CD4\(^+\) and CD8\(^+\) cells, which resulted in a significant decrease in the parasitic burden.\(^16\) With regard to echinococcosis, significantly higher levels of sPD-L1 in patients with cystic echinococcosis were observed compared with healthy controls, and elevated levels of Th2 cytokines in the sera of patients with CE.\(^17\) A recent study showed that upregulation of PD-1 on CD4\(^+\)CD25\(^+\) cells is associated with immunosuppression in liver of mice infected with *E. multilocularis*.\(^18\) *Echinococcus multilocularis* proliferation and some malignant tumours are both sharing similar features such as local immune evasion, induction of tolerance and disruption of T cell signalling\(^9,10,19\) and T cell exhaustion at late stage of infection.\(^20\) Monoclonal antibodies targeting PD-1 or PD-L1 are in clinical use demonstrating high efficacy in lung, colon, head and neck gastric cancers, in addition to renal cell carcinoma and melanoma.\(^21-23\)

Based on these observations, the basic hypothesis of the present study was PD-1/PD-L1 activation couple may represent a potential target to treat the tumour-like lesion development in AE. The major aims of the present study were as follows: (a) to determine the efficacy of PD-1/PD-L1 pathway blockade in the control of AE; and (b) to understand how it is acting by observing what happens in normal mice and in treated mice, and it is related adaptive (CD4\(^+\) T cell) and innate immune responses (DC, NK and NK T cell). To address these questions, we made use of two different mouse infection models, namely (a) intraperitoneal (i.p.) metacestode inoculation (secondary AE, SAE), representing a chronic and rather advanced, but not final stage of infection; and (b) peroral infection with parasite eggs (primary AE, PAE), representing the natural human infection mode (early or acute stage of infection at 2 weeks post infection (p.i.)).

## 2 MATERIALS AND METHODS

### 2.1 Ethics statement

The animal studies were performed in strict accordance with the recommendations of the Swiss Guidelines for the Care and Use of Laboratory Animals. The protocol was approved by the governmental Commission for Animal Experimentation of the Canton of Bern (approval no. BE112/14 and BE112/17).

### 2.2 Mice

Female 8-week-old wild-type C57/BL6 mice were purchased from Charles River GmbH (Sulzfeld, Germany). All animals were housed under specific pathogen-free (SPF) conditions according to recommendations of the Federation of European Laboratory Animal Science Association (FELASA), and additionally monitored by daily inspection, including the assessment of the appearance of health status, putative weight loss or gain during the whole course of the experiment. All experiments with animals were performed within a laminar flow safety enclosure.

### 2.3 Experimental design, infection and PD-L1 blocking

#### 2.3.1 Experiment 1. PD-1/PD-L1 pathway blockade against secondary AE

**Parasite and intraperitoneal infection of mice**

Intraperitoneal infection with *E. multilocularis* metacestodes was performed as previously described.\(^24\) Briefly, *E. multilocularis* (H95) was isolated and maintained by serial passages (vegetative transfer) in C57BL/6 mice as previously described.\(^24\) In order to prepare the infection material for mice, metacestode tissue was obtained from previously infected mice by aseptic removal from the peritoneal cavity. After grinding the tissue through a sterile 50 μm sieve, approximately 100 freshly prepared vesicular cysts were suspended in 100 μL sterile PBS (Gibco, Basel, Switzerland) and intraperitoneally injected. Each experimental group included six animals unless otherwise stated. Control mice received 100 μL of sterile PBS only. Upon end of experiments, mice were sacrificed by CO\(_2\)-euthanasia at 4 weeks post infection (corresponding to middle stage of chronic infection). Parasite tissues were dissected and, if present, fat and connective tissues were carefully removed for subsequent determination of the parasite mass.

**PD-L1 blocking**

All mice belonging to the PD-L1 blocking group (AE PD-L1) received 200 μg of anti-PD-L1 MAb i.p. (BioXcell, clone 10F.9G2, West Lebanon, NH, USA) dissolved in 100 μL PBS at 1 day before infection and maintained for 4 weeks, with a subsequent frequency of one injection/mouse every 4 days.\(^25\) All mice were daily monitored for survival and morbidity.
2.3.2 | Experiment 2. PD-1/PD-L1 pathway blockade against PAE

Parasite and oral infection of mice

*Echinococcus multilocularis* eggs were isolated from a naturally *E. multilocularis*-infected dog euthanized at the Small Animal Clinic of the Vetsuisse Faculty due to a non-infectious health problem, infection was detected upon routine necropsy investigation by pathologists. In order to prepare the infection material for mice, the dog intestine was removed under appropriate safety precautions and cut into four pieces. After opening the dog intestine, the worm-containing mucus sections were scraped out, put into petri dishes containing sterile water. Subsequently, the mucosal suspension was serially filtered through a 500 μmø and then 250 μmø metal sieve, by concurrently disrupting the worms with an inverted 2 mL syringe top. This suspension was further filtered through a 105 μmø nylon sieve. The eggs were then washed by repeated sedimentation (1 x g, 30 minutes, room temperature) in sterile water containing 1% penicillin/streptomycin and stored in the same solution at 4°C. For primary infection of mice, 8- to 10-week-old female animals were receiving approximately 400 eggs suspended in 100 μL sterile water, upon use of a blunt-ended feeding tube. Each experimental group included six animals unless otherwise stated. Control mice (mock-treatment, see below) were sacrificed by CO₂-thanastasia at 2 weeks post infection (corresponding to the early, acute stage of infection). The number of liver lesions was then macroscopically assessed as described elsewhere.

PD-L1 blocking

All mice belonging to the PD-L1 blocking group (AE α PD-L1) received 200 μg of anti-PD-L1 MAb i.p. (BioXcell, clone 10F.9G2) dissolved in 100 μL PBS at 1 day before infection and maintained for 2 weeks, with a subsequent frequency of one injection/mouse every 4 days. All mice were daily monitored for survival and morbidity.

2.4 | Total RNA extraction and qRT-PCR

Total RNA was isolated from spleens using the Qiagen RNeasy MiniKit according to the manufacturer’s instructions. The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Only samples with a 260-nm:280-nm ratio between 1.9 and 2.1 and a 28S:18S ratio within 1.5 to 2 were further processed. cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany). SYBR-Green Mix-based qRT-PCR was carried out on a Rotor-Gene 6000 QPCR detection system (Corbett, Foster City, CA, USA) with the FastStart Essential DNA Green Master (Roche, Basel, Switzerland) following the manufacturer’s instructions. PCR cycling was performed in triplicates in final volumes of 20 μL containing 2 μL cDNA and 10 pmol/L of each primer (cycle scheme: initial denaturation at 95°C to 15 minutes, 45 cycles of 95°C to 15 seconds, 55°C to 30 seconds and 72°C to 30 seconds). Fluorescence was measured in every cycle, and a melting curve was analyzed after the PCR by increasing the temperature from 55 to 95°C in 0.5°C increments. The primers used were described earlier and mRNA levels of different cytokines were quantified relative to the mRNA level of housekeeping gene β-actin.

2.5 | Cell preparation and flow cytometry

Spleen cells from naïve (control) and *E. multilocularis*-infected mice were collected by splenic grinding, each step with 5 mL RPMI-1640. Cells were subsequently washed twice with and then resuspended in RPMI-1640 (Gibco) for cell staining.

Aliquots of 10⁶ cells/100 μL of staining buffer per well were incubated each with 1 μg of purified anti-CD16/CD32 for 20 minutes in the dark, in order to block non-specific binding of antibodies to the FcγII and FcγII receptors. Subsequently, these cells were separately stained with the following surface markers for 15 minutes with 1 μg of primary antibodies: PE-labelled CD3, PE-Cy5-labelled NK1.1 and APC-labelled anti-CD4. All antibodies were from eBioscience (San Diego, CA, USA). For intracellular staining, spleen cells were first incubated with Inside Fix (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes at room temperature and subsequently stained with PE-labelled anti-Foxp3, anti-IL-10, anti-IL-4 and anti-IFN-γ (eBioscience) in Inside Perm (Miltenyi Biotec) for 15 minutes in the dark. Corresponding fluorochrome-labelled isotype control antibodies were used for staining controls. Cells resuspended in 300 μL of buffer (0.15 mol/L NaCl, 1 mmol/L NaH₂PO₄, H₂O, 10 mmol/L Na₂HPO₄, 2H₂O and 3 mmol/L NaN₃) were analysed in a flow cytometer (Becton Dickinson, Heidelberg, Germany) using the corresponding CELL QUEST software.

2.6 | Statistical analyses

All data were analysed by SPSS 17.0. The results are presented as means ± SD. Normality of data was assessed by D’Agostino & Pearson and Shapiro-Wilk test. For normally distributed groups of data, one-way ANOVA followed by Bonferroni’s post-test was used to compare the differences between groups. Significance was defined as *P* < 0.05 for all tests, except those subsequently corrected by Bonferroni.

3 | RESULTS

3.1 | Part I: PD-1/PD-L1 pathway plays a critical role in the overall infection control against secondary AE (intraperitoneal infection mode leading to chronic stage of AE)

3.1.1 | Significantly decreased parasite load in SAE mice with PD-1/PD-L1 pathway blockade

Experiment 1 focused on the potential role of PD-1/PD-L1 ligation in the immunological fight against secondary AE. Abrogation of PD-1/
PD-L1 pathway was obtained by anti-PD-L1 MAb administration (intraperitoneal injection, 200 μg/mouse/injection), initiated 1 day before infection and subsequently maintained for 4 weeks (with 1 application/4 d). Parasite load was determined by wet weight measurement at 4 wk post secondary E. multilocularis infection and necropsy. Data represent individual mice from three independent experiments of a total of six mice in each group. Comparison between groups was performed using a one-way ANOVA for statistical analysis. *P < 0.05. B, IL-2; C, IL-12; D, IFN-γ; E, IL-17A; F, Foxp3 mRNA expression levels in the spleen of mice following secondary E. multilocularis infection (measured by qRT-PCR). Data represent mean ± SD of a total of six mice in each group. Comparison between groups was performed using a one-way ANOVA with Bonferroni’s multiple comparison post-test for statistical analysis. *P < 0.006. Control: non-infected wild-type mice; SAE-WT: E. multilocularis secondarily infected wild-type mice; SAE-α PD-L1: E. multilocularis secondarily infected wild-type mice treated with anti-PD-L1 MAb

3.1.2 | Increased Th1 and decreased Treg immune response in SAE mice treated with PD-1/PD-L1 pathway blockade

By using qRT-PCR, a significant increase of gene expression levels for IL-2, IL-12 and IFN-γ was observed in the spleen from SAE-WT mice treated with anti-PD-L1 MAb, when compared to the untreated infected SAE-WT mice (Figure 1B-D; for all P < 0.006). IL-17A mRNA levels showed no significant difference in the spleen from SAE-WT mice treated with anti-PD-L1 MAb, when compared to the infected SAE-WT mice and to the non-infected controls (Figure 1E). Foxp3 gene expression level was significantly decreased in the spleen from SAE-WT mice treated with anti-PD-L1 MAb, when compared to the SAE-WT mice (Figure 1F; P < 0.006).

3.2 | Part II: Early blockade of PD-1/PD-L1 pathway is very critical in the overall infection control against primary AE (peroral infection mode leading to acute or early stage AE)

3.2.1 | Significantly lower liver lesion numbers in PAE mice upon PD-1/PD-L1 pathway blockade

Experiment 2 focused on the potential role of PD-1/PD-L1 ligation in the immunological fight against primary AE. Abrogation of PD-1/PD-L1 pathway was obtained by anti-PD-L1 MAb treatment (intraperitoneal injection, 200 μg/mouse/injection), initiated 1 day before infection and subsequently maintained for 2 weeks (with 1 application/4 d). In the natural (peroral) infection system, anti-PD-L1 MAb administration...
resulted in a significantly lower number of liver lesions, as compared to the infected non-treated group (PAE-WT; P < 0.05; Figure 2A).

### 3.2.2 Decreased Treg immune response in PAE mice with PD-1/PD-L1 pathway blockade

Peroral infection was associated with an increase of CD4+ Foxp3+ and CD4+ IL-10+ cells in the spleen, when compared to the non-infected WT controls (Figure 2B-D; P < 0.008). There was a significant decrease of both CD4+ Foxp3+ and CD4+ IL-10+ frequency in the spleen from PAE mice treated with anti-PD-L1 MAb, when compared to PAE-WT mice (Figure 2B-D; P < 0.008). qRT-PCR yielded for Foxp3 and IL-10 gene expression level of 2.0 AU (mean arbitrary units) and 1.0 AU (mean) in the spleen of PAE-WT mice, while in PAE-WT mice treated with anti-PD-L1 MAb the mRNA expression levels were 0.7 and 0.05 AU, respectively (Figure 2E).

### 3.2.3 Decreased Th2 immune response in PAE mice with PD-1/PD-L1 pathway blockade

A significant increase of CD4+ IL-4+ frequency in the spleen was observed at 2 weeks post oral infection with *E. multilocularis* eggs.
WANG et al. (PAE mice), when compared to the non-infected WT controls (Figure 3A,B; \(P < 0.025\)). There was a significant decrease in CD4\(^+\) IL-4\(^+\) frequency in the spleen from PAE mice treated with anti-PD-L1 MAb, when compared to PAE-WT mice (Figure 3A,B; \(P < 0.025\)). However, there was no difference in CD4\(^+\) IFN-\(\gamma\)+ frequency in the spleen between PAE-WT mice with and without anti-PD-L1 MAb treatment (Figure 3C,D; \(P < 0.025\)). qRT-PCR showed that IL-4 gene expression levels were at 3.0 ± 1.0 AU in AE-WT and at 1.95 ± 0.66 in the spleen from anti-PD-L1-treated PAE mice (Figure 3E; \(P < 0.025\)). No difference for IFN-\(\gamma\) expression levels was observed in the spleen from PAE-WT mice treated with anti-PD-L1 MAb, when compared to untreated PAE-WT mice (Figure 3F; \(P < 0.025\)).

3.2.4 | Innate immune responses are activated in PAE mice but not altered with PD-1/PD-L1 pathway blockade

By flow cytometry, a significant increase of NK cell number in the spleen was observed in PAE mice compared to infection-free WT controls (Figure 4A-C; \(P < 0.025\)). No difference in NK T cell number in the spleen was found between the study groups (Figure 4A-C; \(P < 0.025\)). The NK cell or NK T cell numbers were similar in the spleen from PAE-WT mice treated with anti-PD-L1 MAb and those PAE-WT and untreated WT animals (Figure 4A-C; \(P < 0.025\)).
DISCUSSION

The presence of PD-1 and PD-L1 has a major role in the inhibition of effector T cell function. Clinical studies have indicated that antibodies blocking PD-1 and PD-L1 have a reliable effect on many advanced malignancies. PD-1 and PD-L1 targeting is an efficient way to maintain the function of effector T cells. Monoclonal antibodies (MAbs) are a class of drugs called checkpoint inhibitors that inhibit the interaction of PD-1 and PD-L1 and overcome the disadvantages of conventional anticancer therapy. In vitro and in vivo studies that were done by Lussier et al showed that blocking PD-1 using an antibody could partially increase T cell function. MAbs-based immunotherapy can, when compared to chemotherapy, significantly work under reduced toxicity within usually tolerable limits, while being able to shrink solid tumours, suppress advanced tumours and metastasis, and overall improve patient survival.

In the present study, it was shown that (a) a significantly decreased parasite load in mice following intraperitoneal infection and concomitant PD-1/PD-L1 pathway blockade, which associated with an increased Th1/Th17 and decreased Treg immune response; (b) significantly fewer liver lesions in oral-infected AE mice and concomitant PD-1/PD-L1 pathway blockade, which associated with a decreased Treg/Th2 immune response; and (c) PD-1/PD-L1 pathway appears thus as a potential immunotherapeutical target against both primary and secondary AE.

Tregs, which over-express a subset of regulatory cytokine genes including those coding for IL-10 and TGF-β, play an important role in promoting immune tolerance in a number of parasitic disease models. CD4+CD25+ Tregs were upregulated in PECs from wild-type E. multilocularis-infected mice (i.p. infection, SAE), when compared to non-infected littermates. With inducible depletion of Foxp3+ Tregs, the metacestode growth yielded a significantly lower
parasite load (i.p. infection, SAE) not only when Foxp3⁺ Tregs were depleted preventively before E. multilocularis infection, but also when they were depleted therapeutically when the infection was already established (oral infection, PAE). The significantly smaller average lesion size in the liver due to Foxp3⁺ Tregs depletion in PAE was associated with a higher Th1 immune response, a lower IL-10 production and upregulation of APC activation. The late infection or PAE was characterized by a strong Foxp3 expression and weak expression of most mediators, suggesting that their production is suppressed by Tregs. Understanding how Tregs regulate the immune process in AE could thus help finding new immunotherapeutical targets, that is, PD-1/PD-L1 ligation. In the present study, in both infection mouse models, Treg immune response was decreased upon anti-PD-L1 MAb application. Tregs (CD4⁺ Foxp3⁺) create a highly immunosuppressive tumour environment through maintenance of the expression of PD-1 on its surface.

In the presence of CD3 and TGF-β, the PD-1 receptor of Treg cells was found to increase the de novo transformation of naive CD4⁺ T cells to Treg cells, thus attenuating immune effector responses. This conversion increases Treg expression and immune suppressive function of CD4⁺ T cell through inhibition of mammalian target of rapamycin (mTOR)-Akt signalling cascade. Thus, the presence of PD-1 expression not only suppresses effector T cell function but also increases the conversion of the immunosuppressive Treg cell population.

From the major findings accumulated in the last two decades, in E. multilocularis metacestode infection, an initial acute inflammatory Th1 response is gradually converting into a mixed Th1/Th2 response during the chronic phase of AE. This relatively simple Th1/Th2 paradigm has been continually revised, and alternative T cell lineages have been proposed to fine-tune the host immune response. PD-1 blockade resulted in an increased Th1 effector response, and increased levels of IL-2, IFN-γ and TNF-α after PHA or Candida stimulation of blood cells from both healthy controls and HIV-infected subjects were demonstrated. Anti-PD-L1 MAb treatment also led to an increased Th1 response and reduced Th2 response during echinococcosis. This might suggest that PD-1/PD-L1 pathway blockade could possibly promote a curative immune response against AE through tipping the Th1/Th2 balance and through inhibition of Treg cells. Future studies will thus have to address the question if long-term modulating of the PD-1/PD-L1 pathway may contribute to cure AE immunologically.

Natural killer (NK) and NK T cells are an innate-like lymphocyte population. They express markers associated with both T cells and NK cells. NK/NKT cells can regulate diverse immune responses and produce large quantities of cytokines following activation. A lower NK cell cytotoxicity was found in PBMC of patients with AE in comparison with patients with non-parasitic biliary disease, and the low number of NK cells was associated with the lack of NKG2D expression on CD8⁺ T cells in liver sections of patients with AE. A most recent study showed that exposure to E. multilocularis-vesicle fluid had a significant bearing on activation and proliferation of NK cells in human PBMC, suggesting that NK cells may play an important role in the immune response of the host against E. multilocularis. Albeit widely studied during viral, bacterial and protozoan parasite infections, however, the role of NK T cells during metazoan parasite infections especially E. multilocularis infection remains still largely unexplored. In the present study, a significant increase of NK cell number but no difference in NK T cell number was observed in PAE mice compared to infection-free WT controls, suggesting NK cells play an important role against acute AE. However, there was no difference in NK cell or NK T cell numbers treated with or without anti-PD-L1 MAb. The mechanism how PD1/PD-L1 ligation regulates NK/NK T cells needs to be further studied.

Overall, the present results highly suggest that a PD-1/PD-L1 pathway blockade is very promising in contributing and immunotherapeutical support to treat and control larval E. multilocularis infection. An anticipated understanding of the mechanism by which PD-1/PD-L1 pathway blockade regulates the immune process in AE may also help to find new alternative immunotherapeutical targets. Up to now, IFN-γ and IL-12 are the only cytokines that have been experimentally tested for their therapeutic effects and were shown to be partially or totally effective for controlling parasite growth; IFN-γ was able to effectively suppress AE occurrence via an enhanced Th1 and a lower Th2 immunity in the mouse model, and IFN-γ administration was reported to reduce AE lesion size in one patient co-infected with HCV. However, it was never tested as therapeutic agents whether this was applicable to larger numbers of human AE patients. Alveolar echinococcosis still remains a severe life-threatening disease without a fully curative therapy, and new treatment options are urgently needed.

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CONFLICT OF INTEREST

The authors declare no commercial or financial conflict of interest.

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