Research Article

Analgesia in Mice with Experimental Meningitis Reduces Pain without Altering Immune Parameters

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Summary

Intracranial lymphocytic choriomeningitis virus (LCMV) infection is a widely used animal model to study virus-induced cytotoxic T cell (CTL) mediated meningitis and immunopathology. This model causes severe pain and distress in mice, especially at later stages of the disease. Therefore, new treatment regimens to improve animal welfare have to be developed. In this study, we subcutaneously implanted ALZET® osmotic pumps continuously releasing buprenorphine to reduce pain in mice with LCMV-induced meningitis. Mice treated with buprenorphine demonstrated strongly reduced symptoms of pain. The LCMV-specific cytotoxic T cell response and the immune cell infiltration into the central nervous system (CNS) were unchanged in analgesia treated mice, indicating that the LCMV-induced immune response was not altered in these mice. Taken together, we demonstrate that in this animal model for meningitis continuous buprenorphine treatment improves animal welfare without affecting the immune response.

Keywords: 3R, refinement, meningitis, LCMV, pain

1 Introduction

Lymphocytic choriomeningitis virus (LCMV), a bisegmented ssRNA virus belonging to the Arenaviridae family, is considered an emerging human pathogen (Tyler, 2009). LCMV infection in its natural host, i.e., the mouse, is a widely studied model for virus host interactions that has contributed to the understanding of immune tolerance, immunodominance, MHC restriction, viral-immune interactions and the basis for viral persistence (Buchmeier et al., 1980; Kang and McGavern, 2008). Peripheral LCMV infection of mice has little clinical impact and results in a strong cytotoxic ($CD8^+$) T cell (CTL) response leading to clearance of the virus within 8-12 days post infection. In contrast, in adult immunocompetent mice, intracranial (i.c.) injection of LCMV results in the development of acute, fatal meningitis on day 6-8 post infection with characteristic seizures and mononuclear cell infiltrates in the meninges, ependyma and choroid plexus. Intracranial LCMV-infection has served for decades as a model of $CD8^+$ T cell-mediated viral meningitis (Kang and McGavern, 2008). Since the virus itself is noncytolytic (Hotchin and Weigand, 1961), pathogenesis and death are directly related to the influx of virus-specific CTLs (Doherty et al., 1990). The $CD8^+$ T cells produce cytokines and chemokines that mediate extravasation of myelomonocytic cells, which leads to vascular leakage and acute lethality (Kim et al., 2009).

The principles of the 3Rs (Replacement, Reduction and Refinement) were developed over 50 years ago as a framework for humane animal research (Russell and Burch, 1959). Refinement refers to methods that avoid or minimize the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved in experiments, thereby enhancing their wellbeing. Intracranial LCMV-infection is still a widely used mouse model to study CTL-mediated meningitis (Kremer et al., 2010; Bonilla et al., 2012; Li et al., 2013). However, this model causes severe pain and distress in mice (Kang and McGavern, 2008; Kremer et

Abbreviations

BBB, blood-brain-barrier; CNS, central nervous system; CTL, cytotoxic T cell; FACS, fluorescent activated cell sorting; GP, glycoprotein; i.c., intracranial; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility complex; NP, nucleoprotein; PFU, plaque forming unit; s.c., subcutaneous

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al., 2010). Hence, the development of treatment regimens to reduce pain and improve wellbeing of these animals is highly warranted.

In this study, we used subcutaneously implanted ALZET® osmotic pumps to apply the analgesic buprenorphine. We observed strongly reduced pain scores in diseased mice receiving analgesics, whereas the immune response was not altered in these mice. Hence, our study offers a new treatment option to improve wellbeing of mice used to study LCMV-induced meningitis without grossly altering immune parameters and will help to improve animal welfare in future studies using the LCMV-induced meningitis model.

2 Animals and methods

Mice, housing conditions and ethics statement

Animal experiments were approved by the review board of Regierungspräsidium Freiburg (G-11/110) in accordance with German Animal Protection Law. The study went through a process of ethical review (Regierungspräsidium Freiburg) prior to study commencement. Although some pain was induced in the control group, this study will help to improve animal welfare in future studies using the LCMV-induced meningitis model. The potential for application of the 3Rs was rigorously researched prior to starting, and every opportunity was taken during the course of the study to implement each of them. All individuals involved in the use of animals were well trained and experienced with the anaesthesia, analgesia and euthanasia carried out in this study. Animal husbandry and care was in accordance with contemporary best practice and all individuals involved with the care and use of animals were trained and skilled to an acceptable level of competency. Appropriate methods were used to minimize pain and distress, and humane endpoints were defined (see pain score).

C57BL/6 mice (H-2^b) were originally purchased from Charles River, Germany. Mice were kept in a specific pathogen-free facility. Immune and health status were regularly checked. Mice were positive for *Trichomonas spp*. All the animals were housed in groups of five animals in Eurotype II long clear-transparent plastic cages with autoclaved dust-free sawdust bedding. They were fed a pelleted and extruded mouse diet (wheat, soybean meal (NGMO), flacked oats, wheat middlings, herring meal, barley, sunflower cake, corn (NGMO), linseed meal, wheat germ, poultry meal, brewer's dried yeast, molasses, whey powder, soybean oil, sucrose, minerals, vitamins, amino acids; product number 3800; KLIBA NAFAG, Switzerland) *ad libitum* and had unrestricted access to drinking water. The light/dark cycle in the room consisted of 12/12 h with artificial light. Female mice were used at 8 weeks of age. Prior to the experiment mice were naïve and not previously used for other purposes. The number of mice for the comparison of the PBS and the buprenorphine group was determined using G*Power 3.0.10 (comparison of two independent groups, effect size d: 0,2372, α err prob: 0.05) (Faul et al., 2007). A total number of 12 animals (5 mice in the PBS group; 5 mice in the buprenorphine group; 2 mice in the naïve group) was used. Prior to the experiment mice were randomly assigned to each group. Ear holes produced by an ear punch device were used to mark the mice. The average weight per mouse was 20.1 g ± 0.8 g (mean ± SD). Due to improper release of buprenorphine from the osmotic pump, one mouse had to be excluded from the study.

ALZET® osmotic pump implantation

Before implantation, all mice were treated with 0.05 mg/kg s.c. buprenorphine (TEMGESIC®, Reckitt Benckiser Healthcare (UK) Ltd.) to suppress post-surgical pain. Buprenorphine was chosen because, compared to other opioids, it was shown to have the least immune-modulatory effects (Al-Hashimi et al., 2013). Osmotic pump implantation was performed under continuous isoflurane anesthesia using the XGI-8 Gas Anesthesia System (PerkinEmer). ALZET® osmotic pumps (model 1007D; releases approximately 0.5 μl/h for 1 week) were subcutaneously implanted (2-4 p.m.) in the left flank. The use of these pumps ensures a continuous drug release. Buprenorphine containing pumps were prepared according to the manufacturer's lot specific instructions to release 0.15 mg/kg buprenorphine per day. Control osmotic pumps were filled with PBS.

Virus

LCMV (strain WE) was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Mice were intracranially infected with 30 PFU LCMV using a 26G x1/2" syringe with a cap to restrict needle penetration into the skullcap to 0.5 mm. Infection was done directly after pump implantation under isoflurane anesthesia using the XGI-8 Gas Anesthesia System (PerkinEmer).

Pain score

Intracranially LCMV-infected mice were scored twice a day (day 0 - day 4) or every 4 hours (day 5 - day 7) in a random order. The PBS, the buprenorphine, and the naïve group were held in separate cages. The degree of pain was scored based on the mouse grimace scale according to Langford et al. (2010) with the following modifications: The evaluated categories 1) orbital tightening, 2) nose bulge, 3) hunchback and 4) locomotion constraint were separately graded as follows: 0, not present; 1, moderate; and 2, severe. The sum of the scores for each of the four categories yielded the final pain score. The following end points were defined: Animals would have to be euthanized if the sum of the scores reached 6 or if 2 out the 4 scored categories reached a score of 2. No animal reached the end points. 158 h post infection, mice were euthanized with CO_2 and perfused with PBS via the left ventricle of the heart. Spleen and brain were removed from every mouse.

Cell preparation

Single cell suspensions were generated from spleens by mashing the whole organ with a plunger through a metal mesh. Mononuclear cells from CNS were isolated by enzymatic digestion of brains followed by Percoll® gradient centrifugation as previously described (Basler et al., 2014). Briefly, brains were enzymatically digested with 0.2 mg/ml collagenase D (Roche Diagnostics GmbH, Roche Applied Science, Germany) and

0.2 mg/ml DNase I (Roche Diagnostics GmbH, Roche Applied Science, Germany) and homogenates were resuspended in 30% Percoll® (Sigma-Aldrich, Germany) and centrifuged at 2700 rpm and 4°C for 35 min, without brake. The interface containing mononuclear cells was washed and analyzed by flow cytometry.

Flow cytometry

Flow cytometry was performed as previously described (Basler et al., 2011). Shortly, splenocytes or CNS mononuclear cells were incubated for 30 min with antibodies against CD4 (GK1.5, eBioscience), CD8 (53-6.7, eBioscience), CD45 (clone 30-F11, eBioscience), CD19 (1D3, eBioscience), CD11b (M1/70, eBioscience) or NK1.1 (NK1.1, eBioscience) at 4°C. After two washes, cells were analysed using the Accuri 6 flow cytometer system (BD Biosciences).

For tetramer staining, tetrameric complexes of APC conjugated H-2D^b-LCMV-GP33-41 and H-2D^b-LCMV-NP396-404 were purchased from TCMetrix (Epalinges, Switzerland). Cells were incubated with tetramer (1:150) in 50 µl FACS buffer (PBS containing 2% FCS, 2 mM NaN_3 and 2 mM EDTA) for 30 min at RT. Cells were washed and stained with anti-CD8 antibodies as described above. Samples were washed twice and analyzed by flow cytometry.

For intracellular cytokine staining, splenocytes were incubated in round-bottom 96-well plates with 10^{-6} M of the specific peptide in IMDM (Iscove's Modified Dulbecco's Media) 10% containing brefeldin A (10 μg/ml) for 5 h at 37°C. The synthetic peptides GP33–41 (KAVYNFATC), GP276–286 (SGVENPGGYCL) and NP396–404 (FQPQNGQFI), were obtained from P. Henklein (Charité, Berlin, Germany). Staining, fixation and permeabilization of the cells were performed exactly as previously detailed (Basler et al., 2004).

Statistical analysis

The statistical significance was determined using Student's t test. All statistical analyses were performed using GraphPad Prism Software (version 6.04) (GraphPad, San Diego, CA). Statistical significance was achieved when $p < 0.05$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s. not significant. Due to the low number of mice in the naïve group (n = 2), no statistical analysis was performed between the naïve control group and other groups.

3 Results

3.1 Reduced disease score in buprenorphine treated mice with experimental meningitis

Intracranial LCMV infection in mice results in fatal virus-induced meningitis. To test whether the pain induced in the diseased mice can be reduced, ALZET® osmotic pumps containing the analgesic agent buprenorphine were subcutaneously implanted. The use of these pumps ensures continuous drug release. Mice were intracranially infected with 30 PFU LCMV-WE. A total number of 12 naïve animals (5 mice in the PBS group; 5 mice in the buprenorphine group; 2 mice in the naïve group) was used. Immune and health status of the mice were regularly checked and revealed that mice were positive for *Trichomonas spp*. The average weight per mouse was 20.1 g ± 0.8 g (mean ± SD). The degree of pain was regularly scored based on the mouse grimace scale according to Langford et al. (2010) with the following modifications: The evaluated categories encompassed orbital tightening, ear position, hunchback and locomotion constraint.

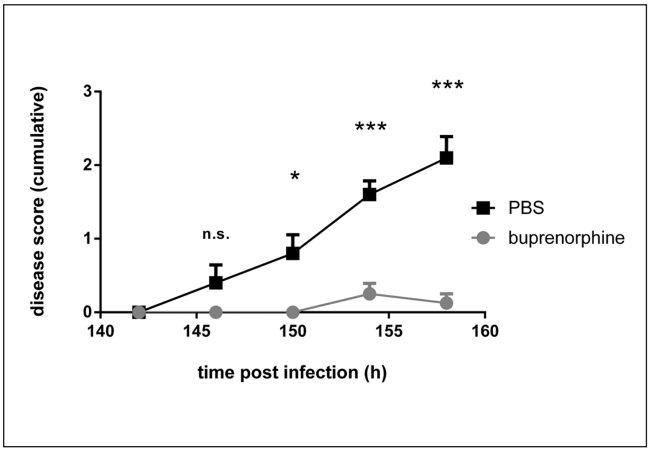

Fig. 1: Strongly reduced meningitis development in mice treated with buprenorphine

ALZET® osmotic pumps containing buprenorphine or PBS were subcutaneously implanted into C57BL/6 wild type mice. Mice were intracranially infected with 30 PFU LCMV-WE and the disease score (y-axis) was assessed as outlined in Animals and Methods and regularly graded at the indicated time intervals (in hours) post infection. Data points represent mean ± SEM of five (PBS group) or four (buprenorphine group) mice; n.s. not significant.

146 h post infection the first disease symptoms were observed in control mice and continuously increased during the next 12 h (Fig. 1). Mice were euthanized 158 h post infection to reduce suffering in non-analgesized mice. Due to improper release of buprenorphine from the osmotic pump, one mouse had to be excluded from the study. In contrast to untreated mice, mice treated with buprenorphine had significantly reduced pain scores with no obvious signs of pain.

3.2 The peripheral T cell response is not altered in buprenorphine treated mice with experimental meningitis

To test whether continuous buprenorphine treatment affects the viability of immune cells, T cells (CD8$^+$ and CD4$^+$), B cells (CD19$^+$) and natural killer cells (NK1.1$^+$) from spleens (158 h post infection) were analyzed by flow cytometry (Fig. 2). Neither the percentage nor the absolute numbers of CD8$^+$, CD4$^+$, NK1.1$^+$ and CD19$^+$ cells were altered between PBS and buprenorphine treated mice. The increased percentage and absolute numbers of CD8$^+$ cells and the reduced percentage of CD4$^+$ cells in infected mice (PBS and buprenorphine treated mice) compared to naïve mice indicates the induction of an LCMV-induced cytotoxic T cell response.

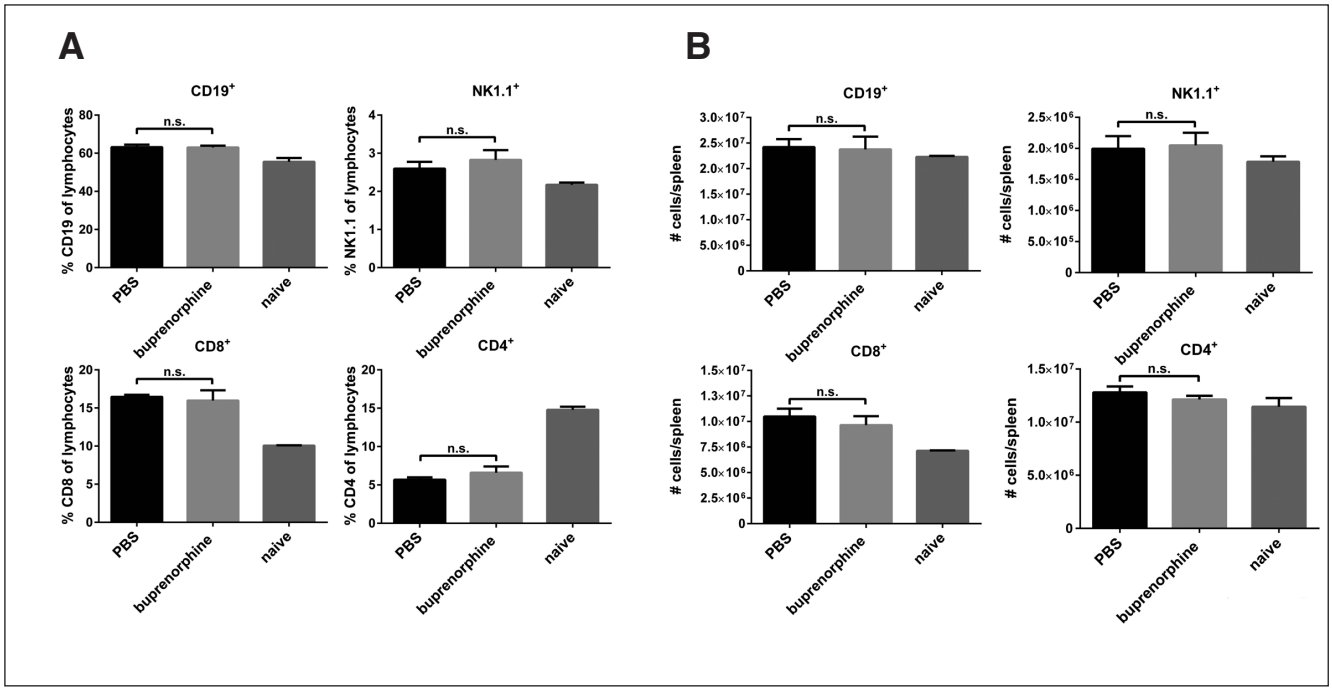

Fig. 2: Percentages and absolute cell numbers of CD8$^+$, CD4$^+$, NK1.1$^+$ and CD19$^+$ cells are not altered in the spleen of mice continuously treated with buprenorphine

ALZET® osmotic pumps containing buprenorphine or PBS were subcutaneously implanted into C57BL/6 wild type mice. Mice were intracranially infected with 30 PFU LCMV-WE. 158 h post infection, the proportions (A) or cell numbers per spleen (B) of CD8$^+$, CD4$^+$, NK1.1$^+$ and CD19$^+$ splenocytes derived from PBS or buprenorphine treated mice were determined by flow cytometry. Values are the means ± SEM of five (PBS group), four (buprenorphine group) or two (naïve) mice; n.s. not significant.

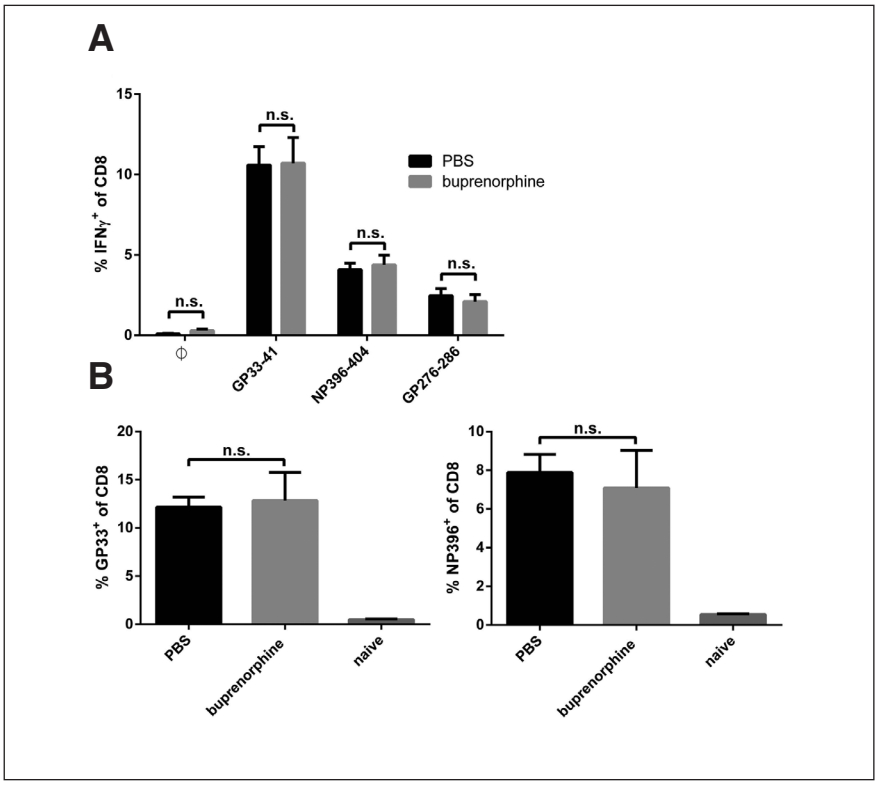

Fig. 3: The peripheral LCMV-specific CTL response is not altered in mice continuously treated with buprenorphine

ALZET® osmotic pumps containing buprenorphine or PBS were subcutaneously implanted into C57BL/6 wild type mice. Mice were intracranially infected with 30 PFU LCMV-WE and spleen cells were harvested 158 h later. (A) Splenocytes were stimulated *in vitro* with indicated peptides for 5 h. Shown are the percentages of IFN-γ-positive CD8$^+$ cells as determined by flow cytometry. Ø represents splenocytes without *in vitro* peptide stimulation. The values are shown as the mean ± SEM of 5 (PBS) or 4 (buprenorphine) mice. (B) The CTL response was analyzed by staining with GP33-H-2D^b- (left side) or NP396-H-2D^b (right side)-specific tetramers and flow cytometry. The y-axis shows the percent tetramer positive of all CD8$^+$ cells. The values are shown as the mean ± SEM of 5 (PBS), 4 (buprenorphine) or 2 (naïve) mice; n.s. not significant.

To compare the anti-LCMV cytotoxic T cell response of buprenorphine treated and control mice, splenocytes derived from these mice were assayed 158 h after infection for responses to three well-defined LCMV epitopes by intracellular cytokine staining for IFN-γ (Fig. 3A). CTL responses to the dominant epitopes GP33 and NP396 and the subdominant epitope GP276 were similar. To confirm this result with an additional method, we performed double staining of splenocytes from buprenorphine treated and control mice for CD8 and for GP33- or NP396-specific TCR (MHC tetramer staining) 158 h post infection with LCMV-WE (Fig. 3B). Similar results as shown in Fig. 3A were obtained with this assay.

3.3 No influence of continuous buprenorphine treatment on CNS entry of immune cells

No difference in the peripheral CTL response could be observed between mice continuously treated with buprenorphine and control mice. In LCMV-induced meningitis, CTLs mediate immunopathology by recruiting monocytes and neutrophils to the infected CNS, which results in loss of meningeal blood vessel integrity, breakdown of the blood-brain-barrier (BBB) and fatal CNS vascular injury (Kim et al., 2009). Therefore, infiltration of immune cells into the brain 158 h post infection was determined by flow cytometry. Continuous buprenorphine treatment did not significantly alter the infiltration of $CD4^+$ T cells, $CD8^+$ T cells, activated lymphocytes ($CD45^{high}CD11b^-$) or activated myeloid cells ($CD45^{high}CD11b^+$) (Fig. 4A). Moreover, the CNS resident microglia ($CD45^{int}CD11b^+$) were not affected.

To evaluate whether continuous buprenorphine treatment affects the infiltration of LCMV-specific cytotoxic T cells into the brain, $CD8^+$ T cells infiltrating the brain were analyzed for LCMV-specificity. Staining with GP33- and NP396-MHC-I-tetramers revealed no alteration in the percentage of GP33- or NP396-specific CTLs infiltrating the brain (Fig. 4B).

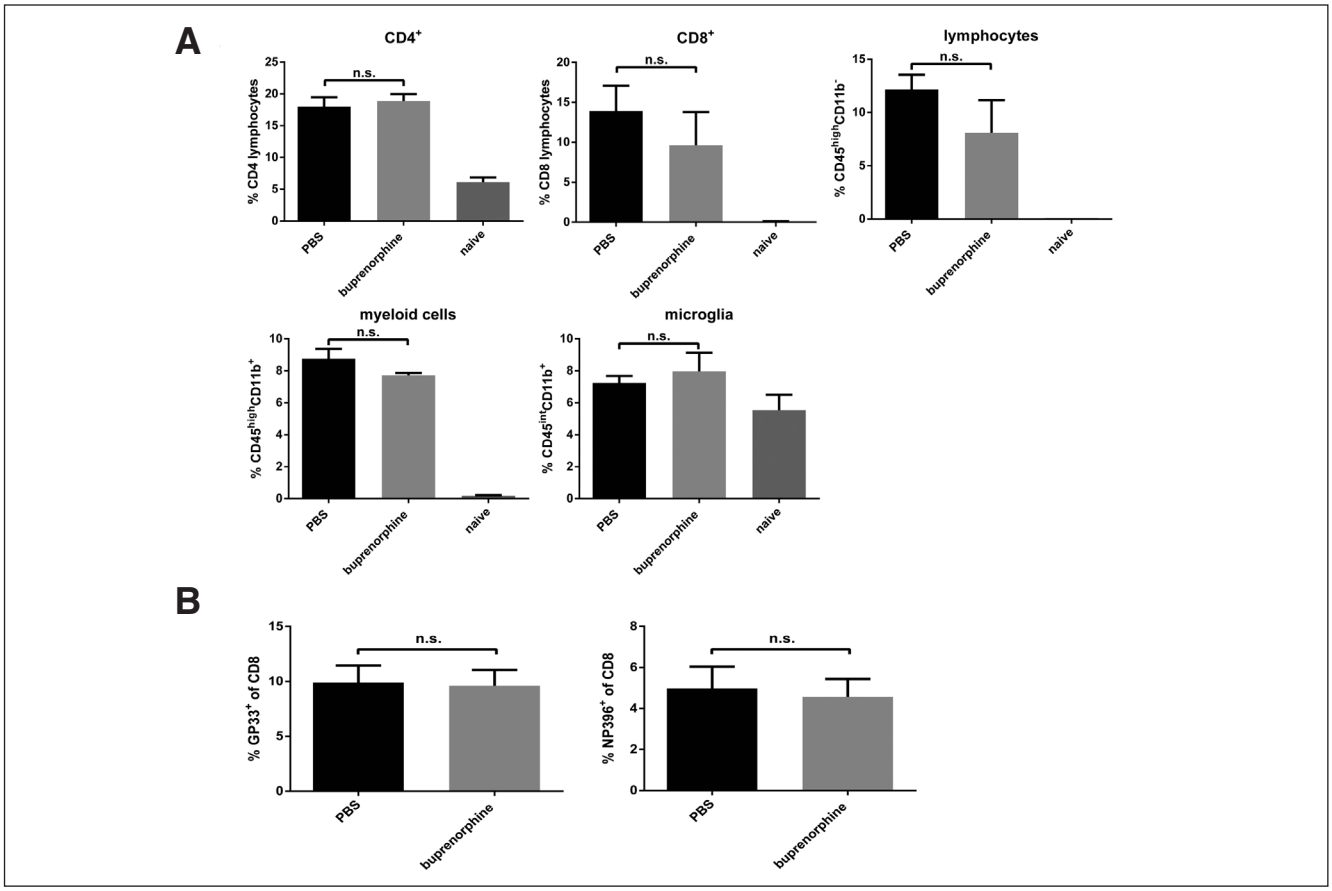

Fig. 4: Inflammatory CNS infiltrates in mice continuously treated with buprenorphine

ALZET® osmotic pumps containing buprenorphine or PBS were subcutaneously implanted into C57BL/6 wild type mice. Mice were intracranially infected with 30 PFU LCMV-WE. 158 h post infection, brains of buprenorphine or PBS treated mice were perfused with PBS and mononuclear cells isolated from the brain. (A) Mononuclear cells were stained for CD4, CD8, CD45 and CD11b. Graphs show mean percentage ± SEM of $CD4^+$, $CD8^+$, $CD45^{high}CD11b^-$ invading lymphocytes, $CD45^{high}CD11b^+$ invading myeloid cells and $CD45^{int}CD11b^+$ CNS resident microglia in the brain (n = 4 per group and n = 2 naïve mice). (B) LCMV-specific cytotoxic T cells infiltrating the brain were stained with GP33-H-$2D^b$- (left side) or NP396-H-$2D^b$ (right side)-specific tetramers and analyzed by flow cytometry. The y-axis shows the percent tetramer positive of all $CD8^+$ cells. The values are shown as the mean ± SEM of 5 (PBS) or 4 (buprenorphine) mice; n.s. not significant.

4 Discussion

The LCMV-induced murine meningitis model is frequently used to study different aspects of immunology (Lin et al., 2010; Kremer et al., 2010; Bonilla et al., 2012; Li et al., 2013). However, this mouse model is considered to induce severe pain in diseased mice, especially at later stages of the disease. Therefore, treatment options to improve the welfare of mice used in this animal model are required. In this study, we subcutaneously implanted ALZET® osmotic pumps releasing the analgesic agent buprenorphine. Continuous delivery with osmotic pumps ensures constant compound levels for maximized therapeutic efficacy and reduced adverse effects. Additionally, unnecessary stressful animal handling due to repeated injection is not required. With a price of approximately 40 € per pump (model 1007D) the ALZET® osmotic pumps are affordable and could principally be reused. Implantation is straightforward but needs some training to minimize incision size. Buprenorphine is among the class of opiate drugs that bind to opiate receptors and prevent the sensation of pain produced by brain chemicals (Cowan, 2007). In the treatment of laboratory rodents and in veterinary medicine, buprenorphine has become a commonly used analgesic drug.

Indeed, continuous release of buprenorphine in mice with LCMV-induced meningitis strongly reduced pain in these mice (Fig. 1). In contrast, behavioral signs of pain and distress were observed in mice without analgesic treatment, which led to constraint locomotion and hunchback formation. Hence, ALZET® osmotic pumps constantly releasing buprenorphine can be used to reduce pain in mice with LCMV-induced meningitis.

In addition to their effects on neuronal functions, opioids modulate other body functions in the periphery, including the immune system (Al-Hashimi et al., 2013). Thus, treatment with opioids often results in immunosuppression and induces anti-inflammatory effects. Buprenorphine, compared to other opioids, was shown to have the least immune-modulatory effects (Al-Hashimi et al., 2013). In a recent study, Borner and Kraus demonstrated that opioids inhibited NF-κB, a crucial transcription factor in T cell activation, in T cells (Borner and Kraus, 2013). The LCMV-induced meningitis model is strictly dependent on cytotoxic T cells (Kang and McGavern, 2008). Hence, we investigated whether the reduced pain score observed in Figure 1 was due to a buprenorphine-mediated suppression of the immune response in these mice.

We tested different immune parameters in intracranially infected mice continuously treated with buprenorphine (Fig. 2, 3, 4). First, we analyzed the immune response in the spleen (Fig. 2, 3). The percentage and absolute numbers of T cells ($CD8^+$ and $CD4^+$), B cells ($CD19^+$) and natural killer cells ($NK1.1^+$) in the spleen was not altered (Fig. 2), indicating that continuous buprenorphine treatment does not eliminate these immune cells. In contrast to naïve mice, a similar LCMV-induced CTL expansion could be observed in buprenorphine treated and control mice. Additionally, the LCMV-specific CTL response, as determined by intracellular cytokine staining and MHC tetramer staining, was not altered in mice continuously treated with buprenorphine (Fig. 3).

Disease progression of LCMV-induced meningitis is characterized by an inflammatory infiltration of immune cells into the brain, leading to neurodegeneration and eventually to death of infected mice. Since the virus itself is non-cytolytic, pathogenesis and death are directly related to the influx of virus-specific $CD8^+$ T cells into the CNS (Doherty et al., 1990). Therefore, we analyzed the infiltration of LCMV-specific T cells into the brain of diseased mice (Fig. 4B). No difference in the influx of LCMV-specific GP33- or NP396-specific T cells could be observed between buprenorphine and PBS treated mice. Cytotoxic T cells recruit monocytes and neutrophils to the infected central nervous system which results in the breakdown of the blood-brain-barrier (BBB) (Kim et al., 2009). Therefore, cellular infiltrates into the brain were analyzed in intracranially LCMV-infected buprenorphine and control mice. A massive influx of different immune cells could be observed in both groups (Fig. 4A). Infiltration of $CD4^+$ T cells, $CD8^+$ T cells, activated lymphocytes ($CD45^{high}CD11b^-$), activated myeloid cells ($CD45^{high}CD11b^+$) and also the CNS resident microglia ($CD45^{int}CD11b^+$) were not affected by buprenorphine treatment. Nevertheless, we cannot exclude that buprenorphine treatment influences immune parameters at very late stages of the disease.

Taken together, we have demonstrated that continuous buprenorphine treatment strongly reduced pain in LCMV-induced meningitis without altering the LCMV-induced immune response and cellular infiltration into CNS. This method can now be applied to improve welfare of mice in this model and could be implemented to other immune-mediated CNS inflammation models like infection with mouse hepatitis virus or tick-borne encephalitis virus (summarized in Amor et al., 2010).

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Conflict of interest

The authors declare no conflict of interest.

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