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Innate immune protection from pneumonia virus of mice induced by a novel immunomodulator is prolonged by dual treatment and mediated by macrophages

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ABSTRACT

Respiratory syncytial virus (RSV) is responsible for a large proportion of acute lower respiratory tract infections, specifically in children. Pneumonia virus of mice (PVM) causes similar lung pathology and clinical disease in rodents, and is therefore an appropriate model of RSV infection. Previously, we demonstrated that a single intranasal dose of P-I-P, a novel immunomodulator composed of the toll-like receptor 3 agonist poly(I:C), an innate defense regulator peptide and a polyphosphazene, confers protection in Balb/c mice for up to 3 days from lethal PVM-15 infection. In the present study a dual intranasal treatment with P-I-P was shown to extend the duration of the protection conferred by P-I-P from PVM-15 challenge. Balb/c mice treated twice with P-I-P showed higher survival rates and milder clinical signs when compared to animals that received a single P-I-P dose. While the mice treated with two consecutive doses of P-I-P experienced some weight loss, they all recovered. The dual P-I-P treatment mediated infiltration of several innate immune cells into the BALF and lung, including alveolar macrophages, neutrophils, and γδ T cells. Partial depletion of alveolar macrophages decreased survival rates and exacerbated clinical signs of mice subjected to the P-I-P dual treatment regime followed by PVM-15 challenge. This suggests that the alveolar macrophage is at least partially responsible for the protection elicited by this novel prophylactic treatment strategy.

1. Introduction

Respiratory syncytial virus (RSV) is a major human pathogen responsible for acute lower respiratory tract infections in infants, the elderly, and immunocompromised individuals (Griffiths et al., 2017; Jorquera and Tripp, 2017; Nair et al., 2010; Garg et al., 2014). There are numerous vaccine candidates, but these are yet to be licensed (Griffiths et al., 2017). Treatment strategies to manage RSV disease include respiratory support and hydration, as well as the use of palivizumab and/or ribavirin in high-risk patients. These treatments are either extremely costly or have poor performance with serious side effects (Griffiths et al., 2017; Graham and Anderson, 2013).

Current RSV animal models do not fully reproduce the pathogenesis of RSV infections in humans. However, the use of other pneumoviruses matched to their cognate hosts can provide a better depiction of RSV disease *in vivo* (Graham and Anderson, 2013). The pneumonia virus of mice (PVM) is used as a model of RSV infection as it belongs to the same virus family and causes similar lung pathology and clinical disease in rodents (Watkiss et al., 2013; Easton et al., 2004; Bem et al., 2011).

Alveolar macrophages (AMs) are the most abundant cell population in the airways under steady-state, accounting for more than 95% of all leukocytes in the lower respiratory tract (Rigaux et al., 2012; Makris et al., 2016). These cells play a central role in the maintenance of tissue homeostasis and repair, as well as immune recognition of pathogen-associated molecular patterns (PAMPs) (Rigaux et al., 2012; Kolli et al., 2014; Hussell and Bell, 2014). Upon activation, AMs increase their phagocytic activity and the production of both pro-inflammatory and immunomodulatory cytokines (Kolli et al., 2014). Therefore, these cells are known as gatekeepers of the lung milieu (Hussell and Bell, 2014). The role that these cells play in terms of pathogenesis and viral clearance can vary significantly within the Paramyxoviridae family. For instance, AMs enhanced clinical disease, airway obstruction and immunopathology in the context of human metapneumovirus infection in mice (Kolli et al., 2014). In contrast, in the RSV infection model, AMs are necessary for virus clearance, disease control and the promotion of an optimal antiviral innate immune response (Kolli et al., 2014).

Recently, we demonstrated that a single intranasal dose of P-I-P,

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composed of the toll-like receptor 3 (TLR-3) agonist poly(I:C), an innate defense regulator (IDR) peptide and a polyphosphazene, confers protection in Balb/c mice from lethal PVM-15 infection for up to 3 days (Martinez et al., 2016). In the present study, the duration of the protection from PVM infection was prolonged by administering two consecutive P-I-P treatments. This suggests acquisition of trained immunity by pulmonary innate immune cell population(s), likely alveolar macrophages, which played a role in the protection elicited by the dual prophylactic P-I-P treatment.

2. Materials and methods

2.1. Cell line and virus

The PVM-15 strain was propagated in Baby Hamster Kidney 21 (BHK-21, ATCC) cells in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 2% heat-inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 10 mM HEPES, and 50 μg/mL gentamicin (Thermo Fisher Scientific).

2.2. Animals, P-I-P formulations, and PVM-15 challenge

Five to six week-old female Balb/c mice (Charles River Laboratories) were treated intranasally with either 20 μl of phosphate-buffered saline (PBS, Thermo Fisher Scientific) or P-I-P, containing 20 μg poly(I:C) (Invivogen), 40 μg IDR peptide 1002 (VQRWLIVWRIRK; Genscript), and 20 μg poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) (Idaho National Laboratory) in PBS. P-I-P was administered on days 0 and/or 8. On day 14 mice were euthanized to collect lungs and bronchoalveolar lavage fluid (BALF). The protective efficacy of P-I-P was tested in the context of a lethal PVM-15 challenge. Mice were given intranasal P-I-P treatments 14, 6, or 14 and 6 days prior to intranasal challenge with 3000 pfu of PVM-15 in 50 μl. Five mice per group were scored and weighed daily according to a modified version of *Morton and Griffith's* guidelines (Morton and Griffiths, 1985). All animal trials were conducted in accordance with the Canadian Council on Animal Care. A full description of all treatment groups in this study is provided in Fig. S1.

2.3. Collection of BALF and lung samples

The lungs of 5–8 mice were lavaged with 700 μl of PBS supplemented with 2% FBS and 3 mM ethylenediaminetetraacetic acid. The resulting BALFs were pooled per group. The single-lobed lungs were collected and pooled in gentleMACS C tubes (Miltenyi Biotec Inc.) containing Hank's balanced salt solution (Gibco, Life Technologies), 5% FBS (Life Technologies), collagenase Type IA (0.5 mg/ml, Sigma-Aldrich) and deoxyribonuclease I Type IV (20 μg/ml, Sigma-Aldrich). Lung single-cell suspensions were obtained as previously described (Watkiss et al., 2013; Shrivastava et al., 2015). The multi-lobed lungs were individually collected, weighed, homogenized in a mini bead-beater (BioSpec Products Inc.), centrifuged at 4 °C to remove gross debris, aliquoted, flash-frozen in liquid nitrogen and stored at −80 °C.

2.4. Virus titrations

Serial dilutions of the collected lung homogenates were transferred in duplicates onto 80% confluent BHK-21 cell monolayers. After 72 h of incubation at 37 °C and 5% CO_2, the cells were fixed with 25% acetic acid/75% ethanol for 20 min at room temperature. The plates were washed three times with PBS, blocked and permeabilized with PBS containing 20% FBS and 0.2% Triton X-100 (Sigma-Aldrich). Plaques were visualized with a rabbit polyclonal anti-PVM nucleoprotein antibody (in-house) followed by alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG, and developed with an AP substrate kit (Bio-rad).

2.5. Multiplex cytokine ELISAs of BALF and lung homogenate supernatants

BALF samples were centrifuged at 1200 rpm for 5 min at 4 °C, and the supernatants were collected, aliquoted with 1X SIGMAFAST™ Protease Inhibitor solution (Sigma-Aldrich) and flash-frozen in liquid nitrogen. The multi-lobed lung homogenate supernatants were treated in a similar fashion as described above. The MSD Mouse U-PLEX T-Cell Combo 14-plex and the MSD Mouse V-PLEX Pro-inflammatory Custom Panel (IL-1β, IL-6, IL-12p70) were used to quantify the various cytokines in the BALF and lungs according to manufacturer's instructions (Meso Scale Discovery).

2.6. Antibodies and flow cytometry

BALF cell pellets and lung single-cell suspensions were blocked with TrueStain fcX anti-mouse CD16/CD32 antibody (Biolegend), and then stained with fluorochrome-conjugated antibody cocktails (Table S1). Flow cytometry was performed using a FACS Calibur machine (BD Biosciences). Data analysis was performed using Kaluza software (Beckman Coulter Inc).

2.7. Depletion of alveolar macrophages from murine airways

One day before virus challenge, mice treated with either PBS or two consecutive P-I-P doses 8 days apart received 50 μl of 5 mg/ml liposomal clodronate (dichloromethylene diphosphonate, Cl_2MDP) or Encapsosome, a control liposome suspension (Encapsula nanosciences), intranasally. Twenty-four hours later, BALF and lung samples were collected from 3 to 5 mice per group for analysis by flow cytometry. The remaining animals were infected intranasally with 3000 pfu of PVM-15 in 50 μl. Clinical signs and survival rates were monitored during the administration of liposomes, on the day of virus challenge, and for 14 days following infection.

2.8. Statistical analysis

GraphPad Prism version 7 was used to analyze all the data (GraphPad Software, Inc.). Differences among groups were assessed using Student t-tests, one-way ANOVAs and the Newman-Keuls method for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Two consecutive doses of P-I-P elicit prolonged immune protection against lethal PVM infection in mice

The protection against lethal PVM challenge resulting from a single intranasal P-I-P treatment in Balb/c mice lasted up to 3 days as described by Martinez et al. (2016). When this treatment was delivered 6 days before viral exposure, 30% of the animals survived the challenge (Martinez et al., 2016). This indicates that the longer the interval between prophylactic P-I-P treatment and PVM challenge, the less effective its protective effect. Recently, innate myeloid cells, such as macrophages and monocytes, were found to build memory-like responses to secondary stimuli in a non-specific fashion termed trained immunity (Placek et al., 2019). In addition, P-I-P treatment was associated with higher AM numbers in the lungs during PVM-15 infection (Martinez et al., 2016). To extend the duration of protection mediated by P-I-P, we aimed to improve the outcome of the treatment by implementing the concept of trained immunity and boosting the innate immune response with a second intranasal P-I-P dose. Mice were given intranasal P-I-P treatments 14 and 6 days prior to PVM challenge as described previously (Martinez et al., 2016). Interestingly, although the mice displayed mild clinical signs and weight loss (Fig. 1A and C), all animals recovered rapidly and survived the challenge (Fig. 1B). Overall, this shows that the dual treatment with P-I-P is superior to a single P-I-P treatment as it increases survival rates from 30% to 100%; thus, the innate immune protection against PVM infection can last for ~1 week.

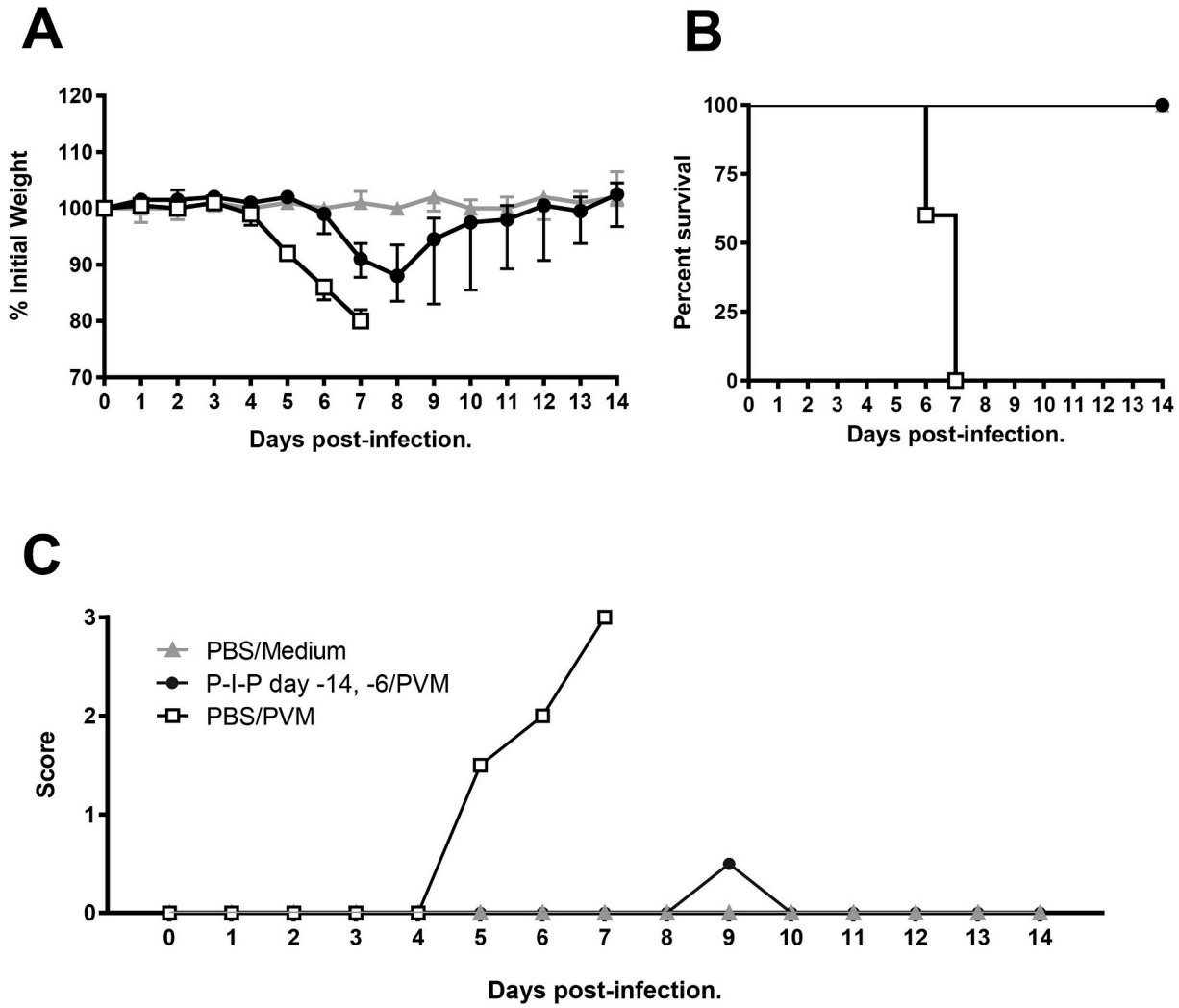

Fig. 1. Weight change, clinical and survival scores of P-I-P- and PBS-treated Balb/c mice. Mice were given an intranasal treatment of P-I-P or PBS on days −14 and −6 prior to PVM challenge (Fig. S1). Animals were weighed and scored on the day of challenge (i.e. day 0), and for 14 days after infection. Panel A illustrates weight loss of mice following P-I-P treatment and PVM infection as a median percentage of the starting weight with error bars indicating the interquartile range. Panel B represents survival rates as the median percentage of the total number of animals per group during the 14 days following infection. Panel C shows clinical scores for weight loss and appearance represented by the median value for each group throughout the entire animal trial. Each experiment was performed twice, with an average number of mice per group of n = 5, and a total number of animals equal to 10 per group.

3.2. Reduction in PVM virus titers is not correlated to the number of P-I-P doses

Since the mice were protected from PVM disease, we determined if the P-I-P treatments lead to lower virus titers in the lungs. On both days 3 and 5 post challenge, the group that received the dual P-I-P treatment had lower virus titers when compared to the PBS group. Furthermore, the titers of the dual dose group were lower than those of the day −14 P-I-P group, while there was no difference between the day −6 P-I-P treatment and the dual treatment groups (Fig. 2). This suggests that the dual P-I-P treatment regime did not have a direct effect in terms of virus replication. While the dual P-I-P and day −6 P-I-P treatment groups displayed different clinical outcomes, there was no significant difference in PVM replication between these two groups. This agrees with the conclusion drawn by Martinez et al. that in terms of disease, PVM does not exert its damage via replication but rather by immunopathology (Martinez et al., 2016).

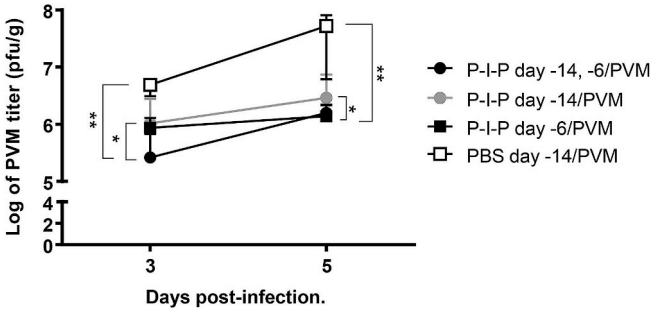

Fig. 2. PVM titers in the lungs of P-I-P treated Balb/c mice on days 3 and 5 p. i. Mice were treated as described in Fig. 1. The multi-lobed lungs of five mice per group were weighed, homogenized in DMEM, and used for virus titrations. The log of PVM titers in the lung tissue expressed in pfu/g is shown. Data are represented as the median with interquartile range of five biological replicates per group. Each experiment was repeated twice. *$p < 0.05$, **$p < 0.01$.

3.3. Mice treated with two consecutive doses of P-I-P have higher protein concentrations of TNF-α in the BALF and IL-17E in the lung tissue

To better understand the mechanism of action of the dual P-I-P treatment, we investigated if its protective effect can be attributed to soluble mediators in the BALF and lung tissue of Balb/c mice. The nomenclature used to describe P-I-P treatments in these experiments is different

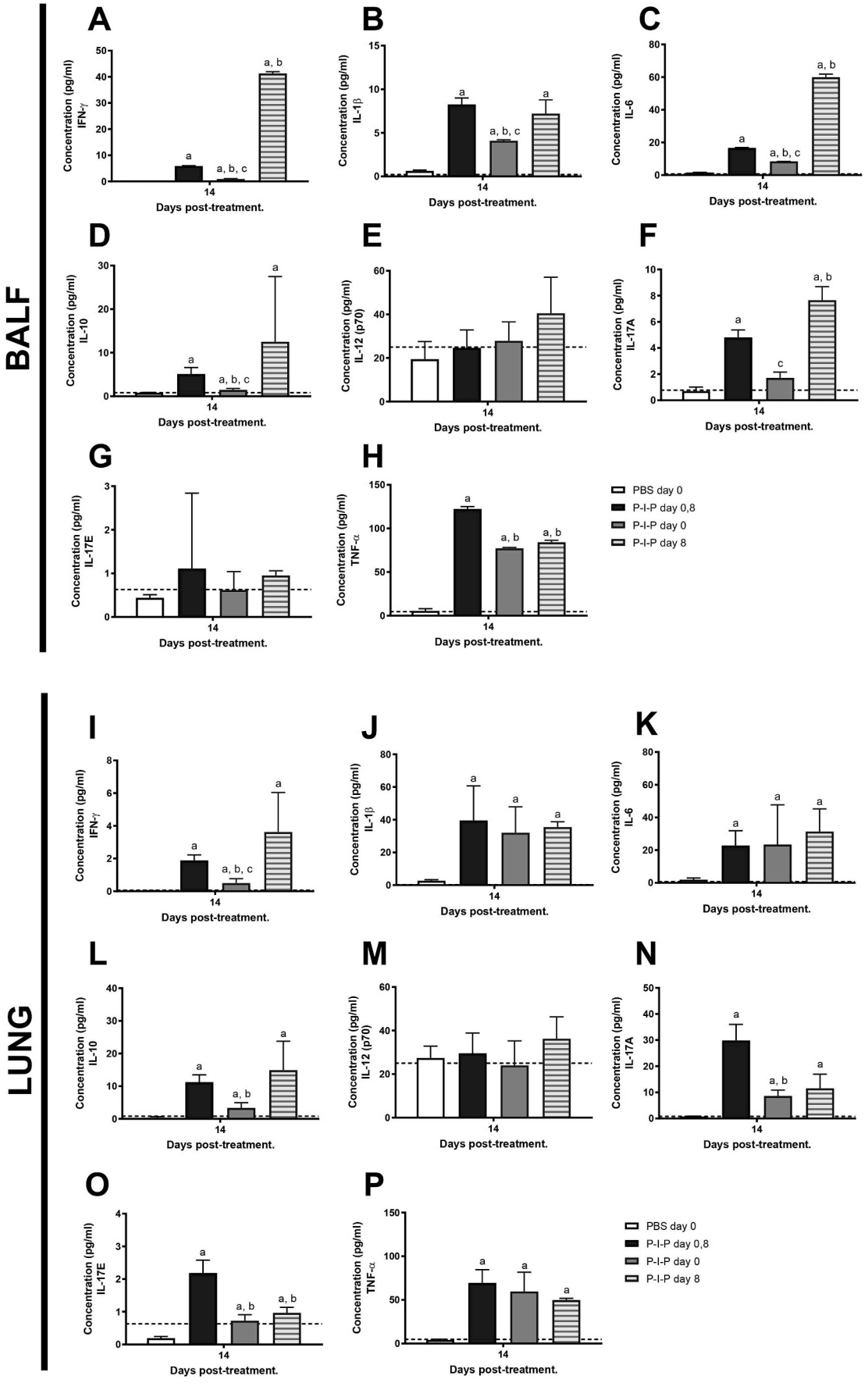

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Fig. 3. Protein expression levels of cytokine genes of P-I-P treated Balb/c mice. Five mice per group were given an intranasal treatment of P-I-P on day 0, on day 8 or on both day 0 and day 8. On day 14 the BALFs and the multi-lobed lungs were collected separately for each individual animal. Protein expression was assayed by electrochemiluminescence-based multiplex ELISAs. Panels A to H and I to P represent protein expression in pg/mL for IFN-γ, IL-1β, IL-6, IL-10, IL-12, IL-17A, IL-17E, and TNF-α in the BALFs and lungs, respectively. Data are shown as median with interquartile range of five biological replicates. An "a" indicates significant differences between the PBS group and the P-I-P treated groups (i.e. P-I-P day 0, 8; P-I-P day 0; and P-I-P day 8); "b" indicates significant differences between the dual dose P-I-P group and the single dose P-I-P groups (i.e. P-I-P day 0; and P-I-P day 8), and "c" indicates significant differences between the single dose P-I-P day 8 group and the single dose P-I-P day 0 group ($p < 0.05$).

from the previous trials as there is no PVM infection involved. Mice were given P-I-P treatments on days 0 and/or 8. On day 14 animals were euthanized and the BALF and lung tissue were collected and processed. IFN-γ, IL-1β, IL-6, IL-10, and IL-17A protein concentrations in the BALF and IFN-γ, IL-10 and IL-17A concentrations in the lung homogenates of the day 0 P-I-P group were significantly lower than those detected either in the day 8 P-I-P or dual P-I-P treatment groups (Fig. 3A to D, 3F, 3I, 3L, 3N). IL-12p70 and IL-17E protein concentrations in the BALF and IL-12p70 protein concentrations in the lung homogenates were not different between any of the groups (Fig. 3E, 3G, 3M). TNF-α protein concentrations in the BALF were significantly higher in the dual P-I-P treatment group when compared to the single P-I-P treatment groups (Fig. 3H). In addition, IL-17E was found in higher concentrations in the lung homogenates of the dual P-I-P treatment group compared to the single treatment groups (Fig. 3O). These cytokine profiles do not necessarily predict the outcome of P-I-P-mediated protection in the context of lethal PVM challenge. For instance, the day 8 P-I-P group appeared to have an advantage over the dual P-I-P treatment group as there were higher levels of inflammatory cytokines, such as IFN-γ, IL-6, and IL-17A, in the BALF prior to challenge (Fig. 3A, 3C, 3F). However, survival rates were much lower for mice treated 6 days prior to PVM challenge compared to those who received two P-I-P doses. Similarly, the protection conferred by the dual P-I-P treatment was not correlated to changes in cytokine production after PVM infection (data not shown). Thus, the protective effect of the dual P-I-P treatment appears not to be directly linked to a specific cytokine, but rather to an individual cell type as clinical outcomes did not match the cytokine profiles identified in this study.

3.4. The dual P-I-P treatment mediates the recruitment of various innate immune cells into the BALF and lungs of Balb/c mice

As cytokines do not seem to be directly involved in the mechanism of action of the dual P-I-P treatment regime, we subsequently studied the influx of various innate immune cells into the respiratory tract of mice. As observed in Fig. 3 animals treated with the dual P-I-P dose displayed increased levels of IFN-γ and the IL-17 family of cytokines in both BAL and lung. Traditionally, the major producers of these cytokines are specialized αβ T cells, commonly associated with adaptive immunity. P-I-P being an antigen-free formulation, we investigated γδ T cells, another potential source of these cytokines at the innate level. While making up a small percentage of total T lymphocytes in both human and mouse, γδ T cells are a major population of innate resident T cells in the mucosal surfaces such as lung, skin, gut and reproductive tract (Lalor and McLoughlin, 2016; Jin and Dong, 2013). In the BALF, AMs, total and $V\gamma2^+$ γδ T cells were present in significantly higher numbers in the dual P-I-P treatment group when compared to the single P-I-P treatment groups (Fig. 4A, 4C, 4D). However, the neutrophil cell count was significantly higher for the day 8 P-I-P group when compared to the dual P-I-P treatment group (Fig. 4B). In the lung compartment, total interstitial macrophage and neutrophil cell numbers were significantly higher for the dual P-I-P treatment group when compared to the single P-I-P treatment groups (Fig. 4E, 4F), while total and $V\gamma2^+$ γδ T cell populations were present at significantly higher numbers in the day 0 P-I-P treatment group (Fig. 4G, 4H). Overall, the common cell type present in the airways of mice treated twice with P-I-P was the macrophage. Thus, the macrophage, either alveolar or interstitial, may contribute to the protection mechanism mediated by this treatment regime *in vivo*.

3.5. Alveolar macrophages are partially responsible for the dual P-I-P treatment-mediated protection against PVM infection

For the final sets of experiments, we focused on the role of AMs in the innate immune protection against PVM infection in Balb/c mice treated twice with P-I-P. Alveolar macrophages are pivotal players in mediating innate immune responses in the lung milieu (Zaslona et al., 2014). In addition, these cells are involved in virus clearance, disease progression, and immunopathology in both RSV and influenza virus infection models (Kolli et al., 2014; Reed et al., 2008; Schneider et al., 2014). Hence, we subjected mice that received two consecutive doses of P-I-P to a single intranasal liposomal clodronate or Encaspsome (empty) treatment one day before PVM inoculation. Mice that received the dual P-I-P regime and intranasal clodronate liposome treatment had significantly reduced AM counts (47.3%) 24 h post-depletion when compared to the animals that received Encapsosome (Fig. 5A). As expected, clodronate liposome treatment did not have an effect on myeloid DC and neutrophil populations (data not shown). Following PVM infection, mice that received a dual P-I-P treatment and had a lower AM cell count in the BALF displayed survival rates of 40%, while 80% of the P-I-P and Encapsosome-treated animals survived (Fig. 5B). Even the Encapsosome can induce inflammatory responses in mice, explaining why the dual P-I-P treatment group that received Encapsosome had a small drop in survival. In addition, the mice that received clodronate liposomes had higher clinical scores than the mice that received Encapsosome (Fig. 5B). Overall, this suggests that AMs play a central role in the mechanism of protection exerted by the dual P-I-P treatment regime.

4. Discussion

In the present study, we evaluated the efficacy of dual P-I-P treatment in protecting mice from a lethal PVM-15 infection. Additionally, we studied the role that AMs play in the mechanism of action of this formulation *in vivo*. Intranasal dual P-I-P treatment conferred complete protection to Balb/c mice as reflected by higher survival rates and milder clinical signs when compared to animals that received P-I-P as a single dose. While mice subjected to the dual P-I-P treatment experienced mild weight loss, they all fully recovered. Overall, by administering two consecutive P-I-P doses, we extended the duration of protection from PVM-15 challenge to ~1 week. The dual P-I-P treatment mediated infiltration of several innate immune cells into the BALF and lung tissue, including AMs, neutrophils, and γδ T cells.

Partial AM depletion decreased survival rates and exacerbated clinical signs of mice subjected to the dual P-I-P treatment and then challenged with PVM. Therefore, the AM played a role in the protection induced by this prophylactic treatment. The essential function of AMs in terms of innate immune protection against respiratory pathogens has been demonstrated in New Zealand Black (NZB) mice, which have a natural deficiency in macrophage function (Reed et al., 2008). Intranasal inoculation of the RSV A2 strain in NZB mice resulted in enhanced disease, airway occlusion and immunopathology (Reed et al., 2008). These results are in agreement with studies using liposomal clodronate to deplete AMs in wild-type Balb/c mice prior to RSV infection (Kolli et al., 2014; Reed et al., 2008). Macrophages phagocytose and remove cellular debris to maintain tissue homeostasis (Schneider et al., 2014), so a detrimental consequence of impaired AM function *in vivo* is the excess buildup of cellular debris within the lung. Abnormal

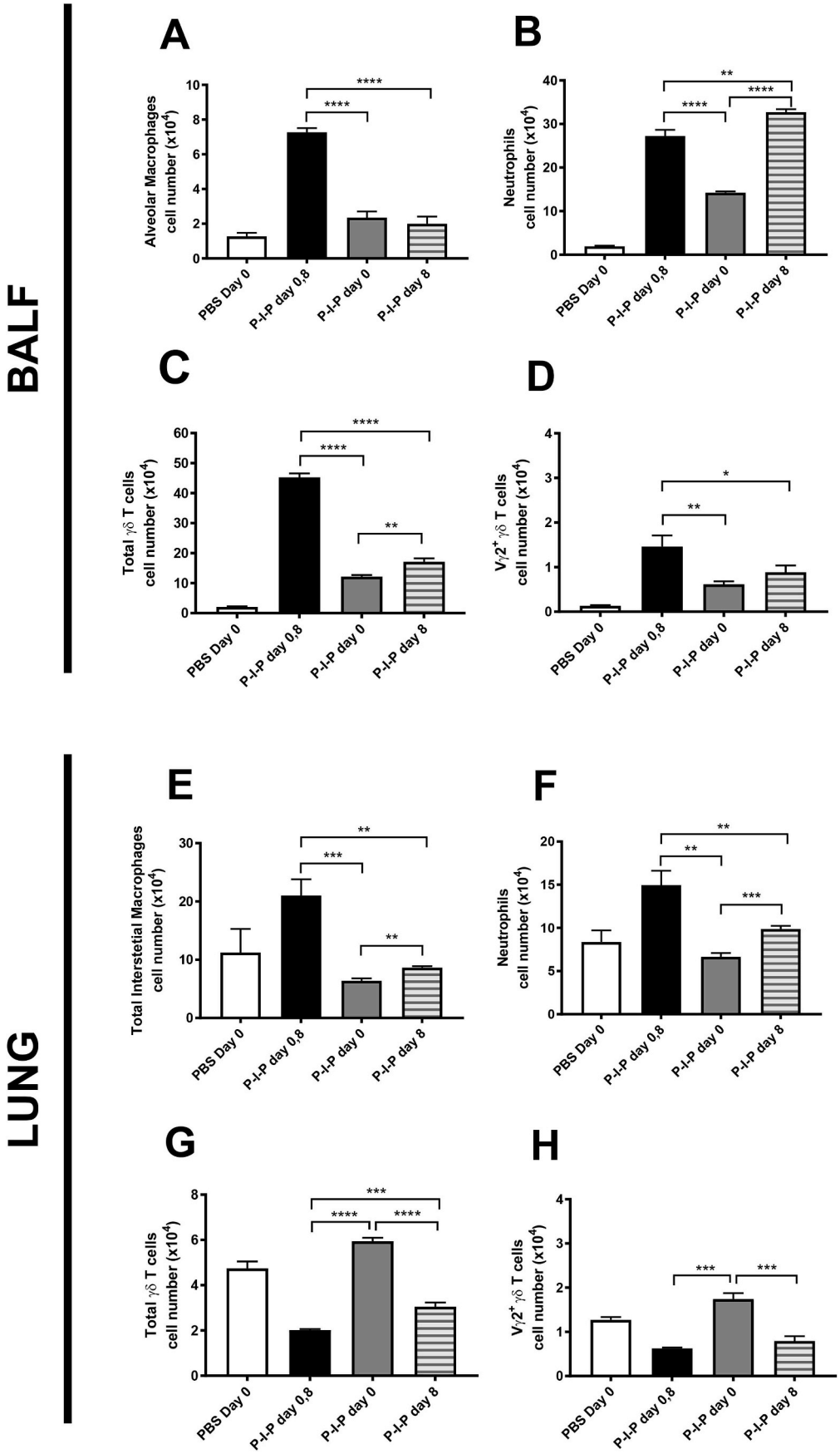

Fig. 4. Infiltration of immune cell populations into the lungs of P-I-P treated Balb/c mice. Mice were given an intranasal treatment of P-I-P on day 0, on day 8 or on both day 0 and day 8. On day 14 the BALFs and lungs of 5–8 mice per group were collected, pooled, and processed at the time points indicated. Following physical and chemical digestion of the tissue, single-cell suspensions were acquired. Total numbers of alveolar macrophages (A), neutrophils (B), γδ T cells (C) and V12+ γδ T cells (D) in the BALFs (A-D) and the lungs (E-H) were analyzed by flow cytometry. Results are presented as cell number x 10^4 per million cells. Data are shown as the median with range of three technical replicates per cell type assayed. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

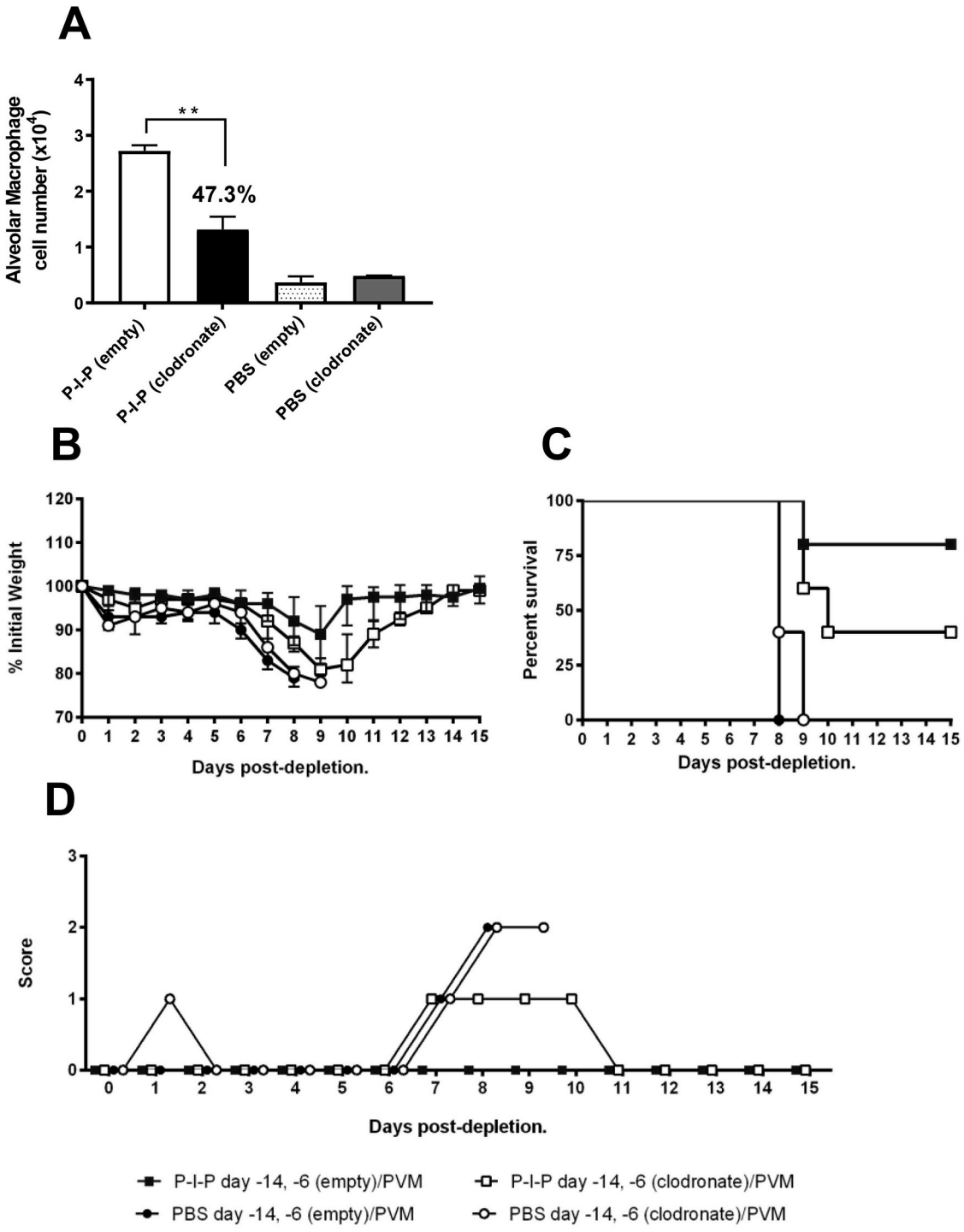

Fig. 5. Macrophage depletion, weight change, and clinical and survival scores of P-I-P- or PBS-treated Balb/c mice. Mice were given an intranasal treatment of P-I-P or PBS 14 days (day - 14) and 8 days (day – 8) prior to challenge with 3000 pfu PVM-15. Panel A: One day prior to virus challenge, mice previously treated with either PBS or the dual P-I-P regime received 50 μl of 5 mg/ml liposomal clodronate or Encapsosome (empty) intranasally. Twenty-four hours later, BALF and lung samples were collected to be analyzed by flow cytometry. Panel B illustrates weight loss of mice following P-I-P treatment and PVM infection as a median percentage of the starting weight with error bars indicating the interquartile range. Panel C represents survival rates as the median percentage of the total number of animals per group during the 14 days following infection. Panel D shows clinical scores for weight loss and appearance represented by the median value for each group throughout the entire animal trial. Each experiment was performed twice, with an average number of mice per group of $n = 5$, and a total number of animals equal to 10 per group. $^{**}p < 0.01$.

development and impaired function of AMs in GM–CSF–deficient mice results in severe mortality, hypoxia and decreased lung function in the context of a sublethal infection with influenza virus PR8 strain (Schneider et al., 2014). Similarly, selective ablation of AMs in CD169-DTR Balb/c mice infected intranasally with a low dose of influenza virus PR8 lead to excess inflammation, enhanced lung pathology, high virus titers, and death (Purnama et al., 2014).

Due to their complex nature, AMs are known to be a double-edged sword, possessing the capacity to both promote and dampen inflammatory responses within the respiratory tract (Hussell and Bell,

2014). Lethal PVM-15 infection in wild-type Balb/c mice results in similar disease and immunopathology as that described for influenza or RSV infections in AM-depleted mice (Kolli et al., 2014; Reed et al., 2008; Schneider et al., 2014; Purnama et al., 2014). When the dual P-I-P treatment was given before PVM-15 challenge, AM counts increased, followed by reduced clinical disease and increased survival rates, which is in agreement with the findings described above. Based on data from previous experiments, we can conclude that a low AM count in the lung prior to challenge is insufficient to mediate innate immune protection against PVM infection (Martinez et al., 2016). In PBS-treated mice that received either clodronate liposomes or Encapsosomes, lethal PVM-15 infection lead to death. In these conditions, AM numbers were low before and after depletion. Only a higher AM cell count prior to PVM-15 infection would lead to complete protection from the virus. The dual P-I-P treatment increased AM cell numbers in the airways, ensuring complete protection and survival of the animals.

In the context of RSV, γδ T cells are known to express endosomal TLRs such as TLR3 and TLR7, essential for early virus detection and antiviral immunity (Jin and Dong, 2013; McGill and Sacco, 2016). In addition, γδ T cells can significantly shape the nature and magnitude of the immunopathology associated with RSV infections (McGill and Sacco, 2016; Dodd et al., 2009). Recently, γδ T cells have also been associated with trained immunity in the context of viral infections. Murine cytomegalovirus induced memory γδ T cells *in vivo*, and the adoptive transfer of these memory γδ T cells to immunodeficient mice reduced viral load in infected animals and provided long term protection (Placek et al., 2019). The γδ T cells may also play a role in protection from PVM conferred by dual P-I-P treatment.

We selected the time points for the dual P-I-P treatment such that the individual treatments fail to provide protection against PVM-15 infection to most of the animals. Thus, the 1st and 2nd dose of P-I-P were separated by an 8-day interval, because the 1st dose would not provide protection on its own by the time the 2nd dose of P-I-P enters the system (Fig. S2). According to the traditional view of the innate immune system, and P-I-P being an antigen-free formulation, the 2nd dose of P-I-P should behave in a similar fashion as a single dose of P-I-P due to the lack of a memory response (Gardiner and Mills, 2016). This would imply that after the 2nd dose of P-I-P, survival rates should be ~30% as shown by single P-I-P treatment 6 days prior to PVM infection (Martinez et al., 2016). In contrast, we observed 100% survival when P-I-P was used as a dual treatment regime (Fig. 1B), supporting the contention that there is evidence of trained immunity (Gardiner and Mills, 2016). Macrophages can be trained to become more responsive to infectious stimuli following repeated exposure to certain PAMPs (Gardiner and Mills, 2016). For instance, immunization with the Bacillus Calmette-Guérin vaccine provides non-specific protection to pathogens other than *Mycobacterium tuberculosis* (Gardiner and Mills, 2016; Netea and van Crevel, 2014). P-I-P contains a TLR3 ligand, an immunomodulatory cationic peptide, and a polyphosphazene in its formulation. It is possible that the repeated exposure of pulmonary innate immune cells to P-I-P in the dual treatment regime induces epigenetic changes within these cells, therefore leading to a state of trained immunity.

The populations that might benefit from the dual P-I-P dose regime are those with weak immune systems, including the elderly, immunocompromised individuals such as transplantation patients, and adults with chronic heart/lung disease. P-I-P could be delivered as a single- or dual-dose spray with a device similar to that used for FluMist influenza vaccine and Imitrex or Zomig nasal sprays for migraine treatment (Djupesland, 2013). The dual P-I-P dose treatment might be used as part of the infection control measures during RSV (and/or other pneumoviruses) outbreaks in long-term care facilities such as nursing homes. In view of the high transmission rate of RSV, the dual P-I-P regime could be administered prophylactically to clinical staff that provides care to patients with high risk of developing RSV disease/complications, patients newly admitted or transferred from another facility where RSV cases are evident or suspected, and patients that live in close proximity to a potentially RSV-infected person.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104594.

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