

IL-12p40 gene-deficient BALB/c mice exhibit lower weight loss, reduced lung pathology and decreased sensitization to allergen in response to infection with pneumonia virus of mice

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ABSTRACT

Respiratory syncytial virus (RSV) is a major cause of bronchiolitis and pneumonia in infants and pneumonia virus of mice (PVM) causes similar disease. BALB/c mice are highly susceptible, while C57BL/6 mice are more resistant to PVM. IL-12 was significantly more up-regulated in response to PVM infection in BALB/c than in C57BL/6 mice. IL-12p40-deficient neonatal and adult BALB/c mice showed significantly less weight loss than wild-type mice after PVM challenge. The percentage of regulatory T cells, as well as IFN- β and IL-18 expression, was higher in the lungs of both neonatal and adult IL-12p40^{-/-} mice. Adult IL-12p40^{-/-} mice also showed enhanced TGF- β and IL-10 expression and reduced inflammatory responses. Furthermore, IL-12p40^{-/-} mice showed decreased sensitization to inhaled cockroach antigen after PVM infection when compared to wild-type mice. In conclusion, these data suggest that a depressed regulatory capacity in BALB/c mice to PVM infection results in enhanced immunopathology and sensitization to allergen.

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1. Introduction

Pneumonia virus of mice (PVM), a natural rodent pathogen of the family *Paramyxoviridae* and genus *pneumovirus* is an enveloped, negative-strand RNA virus (Dyer et al., 2012). PVM is closely related to RSV and replicates effectively to high titres from low inocula (Watkiss et al., 2013). PVM reproduces several severe clinical symptoms associated with RSV infection i.e viral bronchiolitis, pneumonia and respiratory dysfunction. The severity of RSV infection in children has been shown to be related to their genetic background. Almost all children have been infected with RSV by two years of age. While this leads to hospitalization of some of them, the majority recovers without requiring hospitalization (Tregoning et al., 2010). Similarly, the severity of PVM infection in mice is dependent upon the mouse strain (Tregoning et al., 2010; Watkiss et al., 2013). Our previous studies with PVM-15 in neonates, adults and elderly (BALB/c and C57BL/6) mice showed that BALB/c mice are highly susceptible and C57BL/6 mice are more

resistant to PVM-15 infection. PVM-15 induces earlier and enhanced secretion of pro-inflammatory cytokines and chemokines in BALB/c mice (Shrivastava et al., 2016; Watkiss et al., 2013). We also observed that after re-exposure to PVM BALB/c mice are protected and show no up-regulation of these pro-inflammatory cytokines and chemokines (Shrivastava et al., 2015; Shrivastava et al., 2016).

The IL-12 family is a cytokine family consisting of heterodimeric IL-12, IL-23, IL-27, IL-35, IL-12p40 monomer and IL-12 p40 homodimer (Brahmachari and Pahan, 2009; Vignali and Kuchroo, 2012). These cytokines have some structural similarities, yet they have diverse functions. IL-12 and IL-23 are pro-inflammatory cytokines belonging to this family, and they coordinate the development of T_H1 and T_H17 immunity. IL-12 can antagonize T_H2 responses by inhibiting IL-4 production (Wills-Karp, 2001) and by limiting the production of IL-2. IL-12 can also negatively affect natural regulatory T cells (nTregs) (Dickensheets et al., 2000; Villarino et al., 2007). IL-12 is composed of two disulfide linked subunits known as p35 and p40 (Watford et al., 2003). The p40 subunit is shared by IL-12 and IL-23 (Vignali and Kuchroo, 2012). IL-12p40 is primarily secreted by activated macrophages, dendritic cells and airway epithelial cells (Chakraborty et al., 2012), and can be secreted as a monomer (IL-12p40) or dimer (IL-12p80).

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According to several studies, IL-12p40 plays a pivotal role in initiating and regulating host immune responses in experimental models of asthma and infectious disease (Gutierrez et al., 2008; Kinjo et al., 2002; Onari et al., 2009; Wang et al., 2004). In fact, in a study performed with RSV authors showed that IL-12p40 deficiency increases lung inflammation and Th2 cytokine production, but has no effect on viral clearance and IFN- γ production (Wang et al., 2004). Recently, it was shown that IL-12p40 plays an important role in limiting human metapneumovirus infection (hMPV), but it did not have a substantial effect on viral replication in lungs (Chakraborty et al., 2012). Despite this body of information regarding the role of IL-12p40 in bacterial and viral infections, the effect of IL-12p40 during PVM infection has not yet been explored.

We hypothesized that IL-12 promotes a pathogenic response to PVM infection in BALB/c mice by mediating the development of an imbalanced Th1/Th2 response or excessive Th1 response. To test this hypothesis, we studied the role of IL-12p40 in disease, as well as inflammatory and immune responses, during PVM infection of wild-type and IL-12p40^{-/-} BALB/c mice. Both neonatal and adult IL-12p40^{-/-} BALB/c mice experienced less weight loss than wild-type mice. Furthermore, increased Treg influx into the lungs of both neonatal and adult IL-12p40^{-/-} PVM infected mice was observed, which suggests that in BALB/c mice PVM-15 infection induces excessive IL-12 production resulting in suppression of Tregs and an over-vigorous and pathological immune response.

2. Results

2.1. Infection with PVM-15 induces enhanced expression of IL-12 in adult and neonatal BALB/c mice when compared to C57BL/6 mice

Previously PVM-15 has been shown to cause more serious clinical disease in BALB/c than in C57BL/6 mice. Since we observed that in neonates IL-12 was one of the cytokines upregulated in BALB/c compared to C57BL/6 mice (Shrivastava et al., 2015), and IL-12 is a major multi-functional regulatory cytokine, the expression of IL-12 in lungs of BALB/c and C57BL/6 mice in response to PVM-15 was examined in more detail. Adult and neonatal mice of both strains were infected with 300 pfu of PVM-15. In addition, two groups of adult mice were inoculated with 3000 pfu PVM-15, which based on our previous studies, is lethal in BALB/c mice. In neonates no IL-12 production was detected on days 3 and 5 p.i., but both BALB/c and C57BL/6 neonates produced IL-12 on day 7, BALB/c at significantly higher levels (Fig. 1A). In adults inoculated with PVM-15, IL-12 was also produced at higher levels in the lungs of BALB/c mice than in those of C57BL/6 mice on day 7 p.i. (Fig. 1B). At a ten-fold higher dose of PVM-15, IL-12 was produced earlier in BALB/c mice, i.e. on day 5 post-infection, and again at significantly higher levels than in C57BL/6 mice (Fig. 1B). These data suggest that IL-12 may play a role in the enhanced disease observed in BALB/c mice.

2.2. Reduced weight loss in response to PVM-15 infection in IL-12p40^{-/-} BALB/c mice

In order to determine the role of IL-12 in the pathogenesis of PVM-15, wild-type and IL-12p40^{-/-} neonatal and adult BALB/c mice were infected intranasally with 300 pfu of PVM-15. Weight change was examined as one of the main clinical parameters. Wild-type neonates exhibited reduced weight gain in response to PVM-15 infection and deviated from medium-challenged control mice from days 1 to 7 p.i. with significant differences between days 5 and 7 (Fig. 2A). Interestingly, PVM-15 challenge of IL-12p40^{-/-} neonatal mice had no impact on weight; they continued to gain at a similar rate as medium-exposed IL-12p40^{-/-} mice (Fig. 2B). PVM-

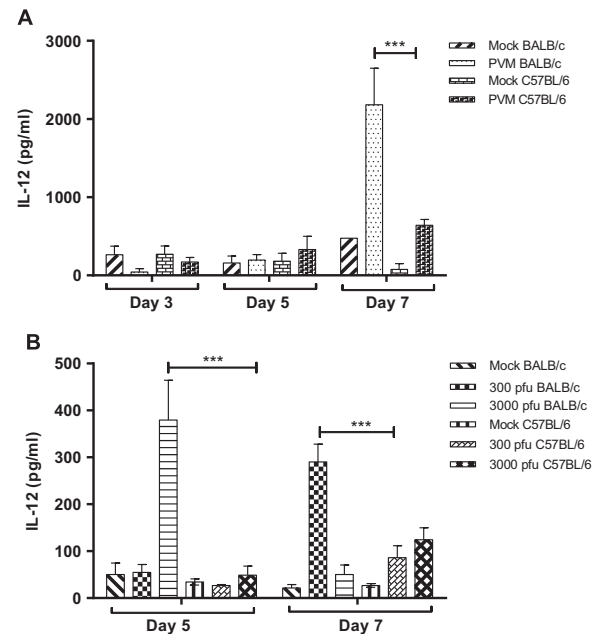


Fig. 1. Production of IL-12 by BALB/c and C57BL/6 mice in response to PVM-15 infection. (A) Seven day-old BALB/c and C57BL/6 neonates were challenged with 300 pfu PVM-15, or treated with PVM culture medium (Mock). IL-12 levels in the lung homogenates were quantified on days 3, 5 and 7 p.i. (B). Six to eight week-old adult BALB/c and C57BL/6 mice were infected with 300 or 3000 pfu PVM-15, or treated with PVM culture medium (Mock). IL-12 levels in the lung homogenates were quantified on days 5 and 7 p.i. Data are representative of two independent experiments with 6 neonatal and 5 adult mice per group per time point. Significance of differences is shown as: *** $P < 0.001$.

15 challenged wild-type and IL-12p40^{-/-} adult mice behaved in a similar manner up to day 6 p.i., after which a sudden weight loss of 10% was observed in wild-type mice (Fig. 2C). While IL-12p40^{-/-} mice also lost some weight on day 7 p.i., this was significantly less than the loss in wild-type adult mice. We did another trial in which we found the weight of the IL-12p40^{-/-} mice to increase from day 8 onwards, returning to 105% of their original weight by day 13; during that period of time the wild-type mice consistently weighed significantly less (5–8% less) than the IL-12p40^{-/-} mice. Virus quantification was also performed on days 3, 5 and 7 p.i. by plaque assay. We recovered the same amount of virus from the lungs of wild-type and IL-12p40^{-/-} neonatal mice at all time points, and while the virus titres tended to be elevated in wild-type adult mice in comparison to IL-12p40^{-/-} mice, this was not significant (Fig. 2D).

2.3. Reduced lung inflammation in response to PVM-15 infection in IL-12p40^{-/-} adult mice

To further assess the impact of IL-12 on PVM pathogenesis, the pathology in the lungs was assessed in adult wild-type and IL-12p40^{-/-} mice on days 3, 5 and 7 after PVM infection. Although there was no significant difference in the lung score between wild-type and IL-12p40^{-/-} mice, there was a trend towards increased pathology in wild-type mice (Fig. 3). The average score in the wild-type group was 2.3 on day 5 and 2.7 on day 7. As a score of 3 indicates broadly dispersed lesions, edema, mononuclear cell infiltration in alveolar spaces and blood vessels, multifocal hemorrhage, alveolitis and severe inflammation, this represents substantial pathology (Fig. 3D). In contrast, one IL-12p40^{-/-} mouse had a score of 2 indicating multiple lesions or more severe single extensive lesions with cellular infiltration on day 5 (Fig. 3C). On day 7, one IL-12p40^{-/-} animal had a score of 3, while the other animals scored 1, which indicates a localized and mild perivascular

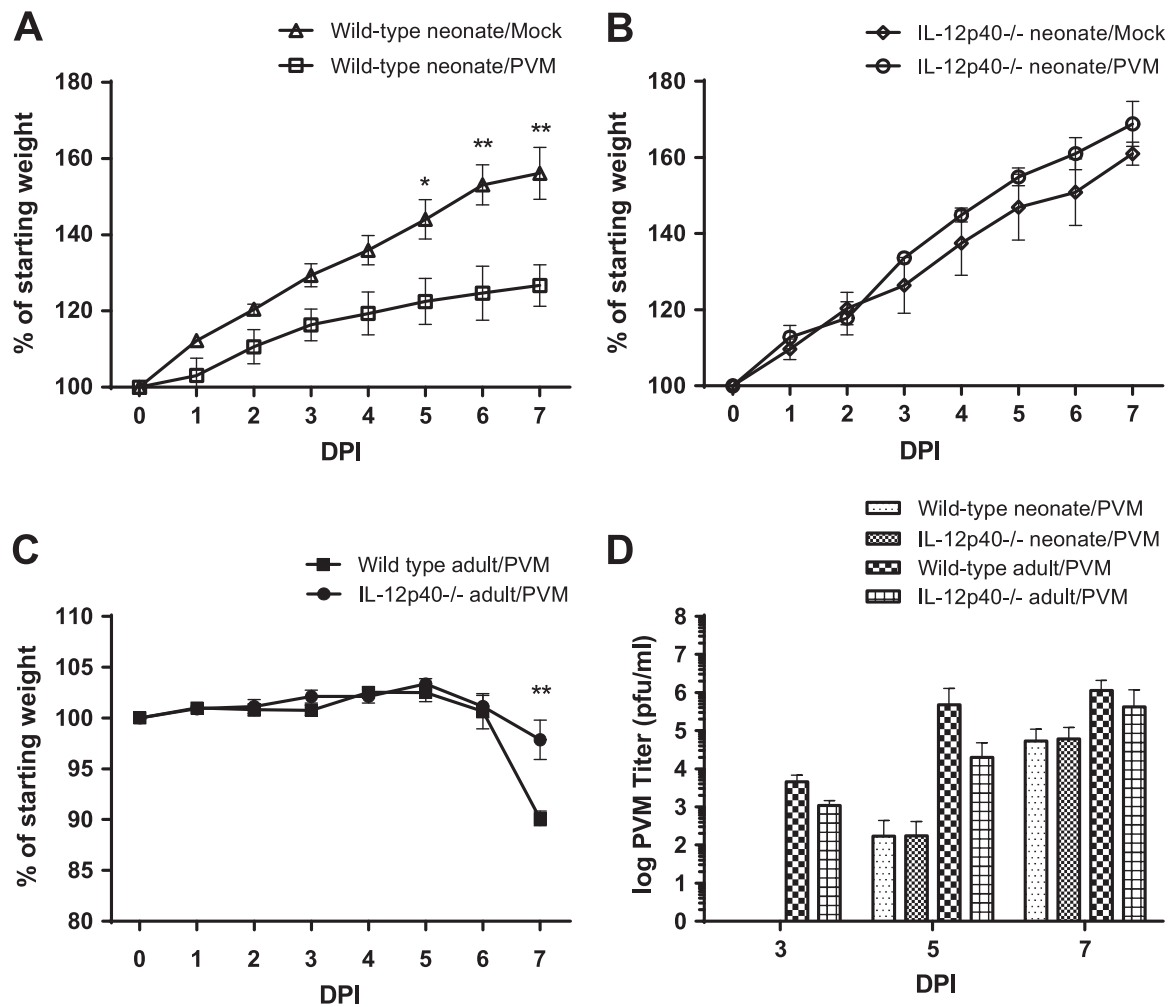


Fig. 2. Weight change and virus replication in lungs of adult and neonatal wild-type and IL-12p40^{-/-} mice after infection with PVM-15. (A and B) Seven day-old wild-type and IL-12p40^{-/-} BALB/c neonates were challenged with 300 pfu PVM-15 or treated with PVM culture medium (Mock). Neonates were weighed daily from day 0 till day 7 p.i. (DPI) as litters. (C and D) Six to eight week-old adult wild-type and IL-12p40^{-/-} BALB/c mice were challenged with 300 pfu PVM-15. Adult mice were weighed daily from day 0 till day 7 p.i. Analysis of weight loss was performed as a measure of disease severity, and change is expressed as percentage of starting weight. Data are representative of two independent experiments and represent means $\pm$ SEM of 6 mice per group. (D) Viral burden in lungs after infection with PVM-15 in neonates and adult mice. Data are representative of two independent experiments with 6 neonatal and 5 adult mice per group per time point. Significance of differences is shown as: * $P < 0.05$; ** $P < 0.01$.

inflammation (Fig. 3B). The cellular inflammatory response tended to be progressive in wild-type mice from days 5–7, while IL-12p40^{-/-} mice maintained the same score (Fig. 3E).

2.4. Reduced cellular infiltration in mediastinal lymph nodes and increased influx of regulatory T cells in lungs of adult IL-12p40^{-/-} mice

To further elucidate the role of IL-12p40 in the regulation of PVM-15 infection in BALB/c mice, we examined the influx of CD4 and CD8 T cells in the MLNs of adult wild-type and IL-12p40^{-/-} mice. On days 5 and 7 post PVM-15 infection the total number of cells in the MLNs was significantly higher in adult wild-type mice when compared to IL-12p40^{-/-} mice (Fig. 4A). Furthermore, the percentage of CD4 and CD8 T cells in the mediastinal lymph nodes (MLNs) of adult IL-12p40^{-/-} mice was significantly lower than that of wild-type mice on days 5 and 7 post-infection (Fig. 4B and C). These data indicate that a more potent immune response to PVM-15 in BALB/c mice may contribute to immunopathology.

As T regulatory cells (Tregs) play a pivotal role in maintaining the balance between an excessive immune response and immunosuppression and may be down-regulated by IL-12, we performed intracellular staining of FOXP3 along with surface staining

of CD4 and CD25 to identify Treg populations in lungs of adult wild-type and IL-12p40^{-/-} mice. Mice deficient in IL-12p40 had an increased influx of total CD4 T cells (Fig. 5A) and Tregs (Fig. 5B) in their lungs indicating that IL-12p40 may have an inhibitory effect on Treg populations in the lungs.

Furthermore, adult IL-12p40^{-/-} mice showed transient up-regulation of IFN- β and IL-18 on day 5 p.i., while IFN- β mRNA levels increased gradually and IL-18 expression was overall lower in the wild-type mice (Fig. 5C–D). TGF- β appeared to be down-regulated after PVM-15 infection in wild-type adult mice, but not in IL-12p40^{-/-} mice on days 5 and 7 p.i. (Fig. 5E). IL-10 expression appeared to increase from day 3 p.i. onwards in IL-12p40^{-/-} mice, while this did not happen until days 5 and 7 in wild-type mice; on day 5 the IL-10 mRNA levels were significantly higher in IL-12p40^{-/-} mice than in wild-type mice (Fig. 5F). This agrees with the increased Treg populations in IL-12p40^{-/-} mice.

2.5. Decreased sensitization to inhaled cockroach antigen in adult IL-12p40^{-/-} mice

Cockroach allergens are an important cause of asthma exacerbations worldwide, and mucosal exposure to cockroach extract induces allergic sensitization in mice (Arizmendi et al., 2011). The

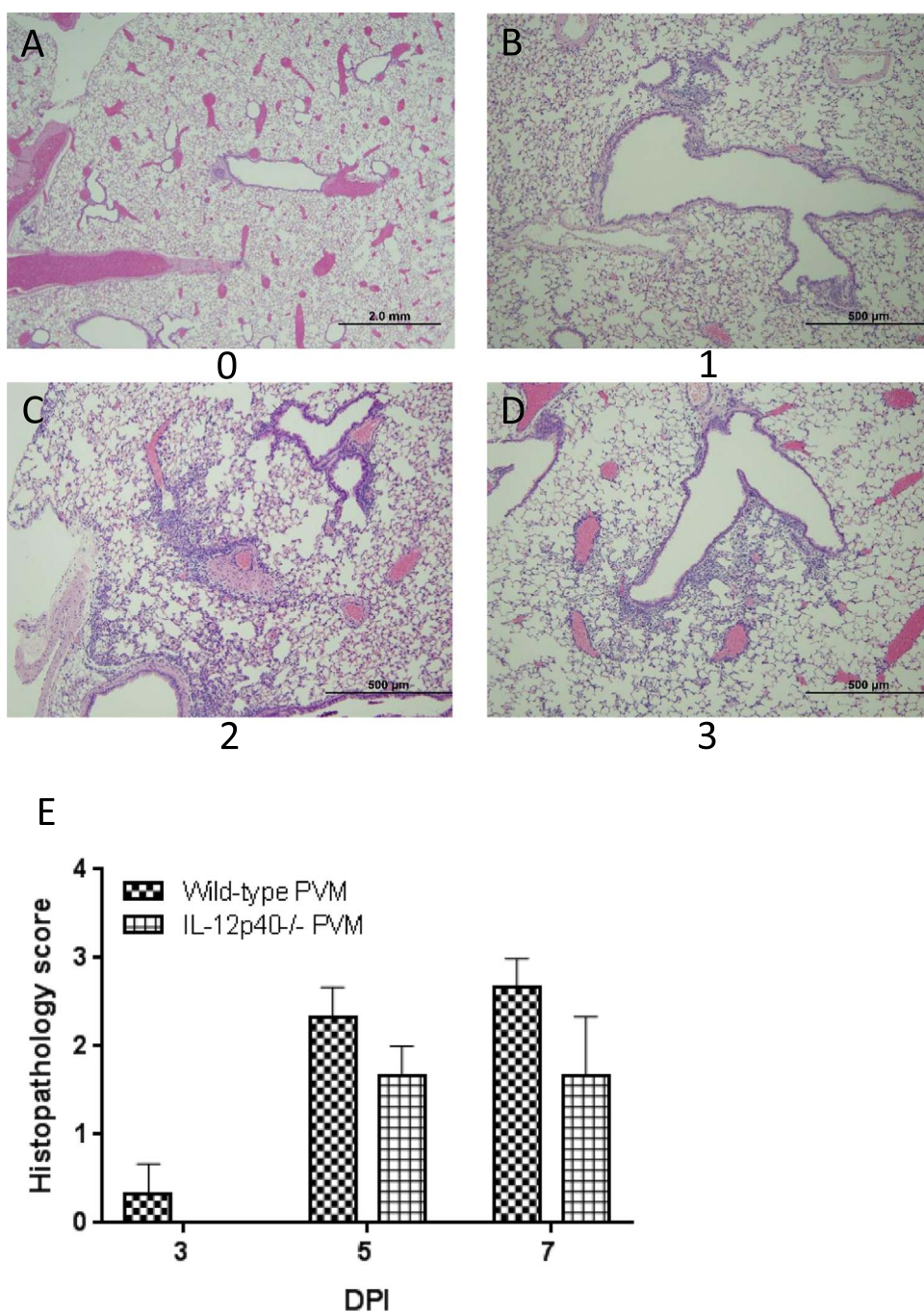


Fig. 3. Histopathology in lungs from adult wild-type and IL-12p40^{-/-} mice after infection with PVM-15. Six to eight week-old adult wild-type and IL-12p40^{-/-} BALB/c mice were challenged with 300 pfu PVM-15. Three mice per group were sacrificed on days 3, 5 and 7 p.i. for histopathology. The mean histopathological scores in lungs were determined based on the following parameters: (i) alveolitis (ii) perivascularitis (iii) edema (iv) mononuclear cell infiltration. (A) normal lung morphology for 6–8 week old mice; (B) representative lung section for animals scoring 1; (C) representative lung section for animals scoring 2; (D) representative lung section for animals scoring 3; (E) Lung histology scores in adult wild-type and IL-12p40^{-/-} mice.

majority of acute asthmatic episodes in humans are precipitated by a viral infection. RSV infection of mice prior to exposure to cockroach antigen has been shown to induce airway hyperresponsiveness (John et al., 2005). To determine whether PVM-15 infection in the presence or absence of IL-12p40 promotes allergic sensitization, adult IL-12p40^{-/-} and wild-type mice were first infected with 30 or 300 pfu PVM-15 and were then given cockroach antigen intranasally on days 4, 10, 17 and 28 post-infection. When wild-type or IL-12p40^{-/-} adult mice were infected with 300 pfu of PVM-15 and then inoculated with cockroach allergen on day

4 post-infection, they started losing weight the next day and dropped to a significantly lower weight of 85% on day 6; these mice all died on day 7 (data not shown). IL-12 p40^{-/-} mice infected with 30 pfu PVM-15 did not show any significant weight loss after intranasal challenge with cockroach allergen; however, wild-type mice started losing weight by day 5 and dropped to a median of 85% and 83% of their starting weight on days 7 and 8, respectively (Fig. 6A). While 50% of wild-type mice were dead by day 10 (Fig. 6B), all the IL-12p40^{-/-} mice survived the subsequent allergen challenge administered on days 10, 17 and 28.

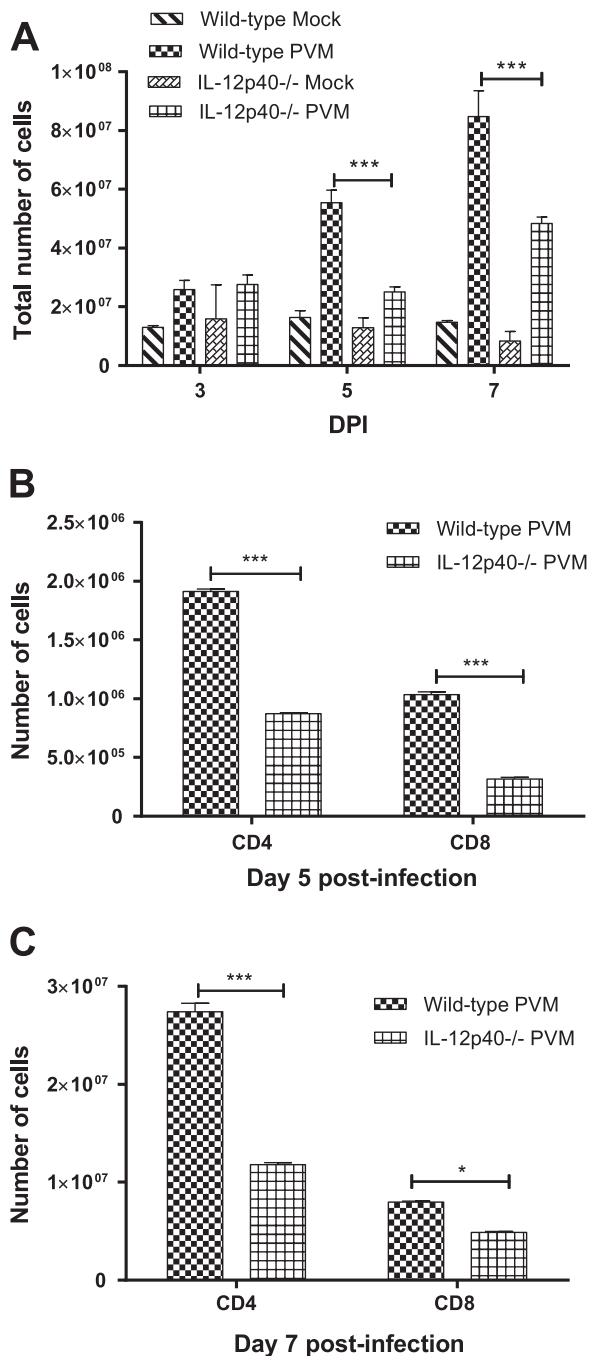


Fig. 4. Cellular infiltration in lung draining lymph nodes of adult wild-type and IL-12p40^{-/-} mice after infection with PVM-15. (A) Total cell numbers were measured in MLNs in adult wild-type and IL-12p40^{-/-} mice after infection with PVM-15 and in medium-exposed animals (Mock). (B and C) Total numbers of CD4⁺ and CD8⁺ T cells in MLNs on days 5 and day 7 p.i., respectively. Data are representative of two independent experiments with 5 mice per group per time point. Data represent means $\pm$ SEM. Significance of differences is shown as: * $P < 0.05$; *** $P < 0.001$.

2.6. Increased influx of regulatory T cells in lungs of neonatal IL-12p40^{-/-} mice

Since neonatal IL-12p40^{-/-} BALB/c mice gained significantly more weight than newborn wild-type mice, the numbers of CD4 T cells and Tregs in their lungs were also examined. As shown in Fig. 7A and B, neonatal IL-12p40^{-/-} mice experienced significantly more influx of CD4 T cells and Treg cells into the lungs than wild-type mice on days 3 and 5 after PVM challenge.

In view of the difference between neonatal PVM-infected wild-type and IL-12p40^{-/-} mice in weight gain and cellular influx in the lungs, we investigated the production of the cytokines that were affected in the adult mice. Highly significant up-regulation of IFN- β mRNA and moderate, but significant increase in IL-18 transcript was observed on day 5 after PVM-15 infection (Fig. 7), but there was no difference in TNF- β or IL-10 expression (data not shown).

3. Discussion

Pneumoviruses are highly species-specific, and the response to PVM infection even varies depending on the mouse strain. As shown earlier, C57BL/6 mice are more resistant to PVM-15 infection than BALB/c mice. Our previous work also indicates that an early and imbalanced pro-inflammatory response in BALB/c mice results in an insufficient protective immune response (Watkiss et al., 2013). IL-12 is a major cytokine involved in initiating and regulating the immune response. It bridges the innate and adaptive immune response, promotes Th1 cell development, and may down-regulate Tregs (Hamza et al., 2010). During the early stages of the immune response to PVM infection, neonatal and elderly BALB/c mice produced significantly enhanced IL-12 levels (Shrivastava et al., 2015; Shrivastava et al., 2016; Watkiss et al., 2013). In addition, re-infection of adult and neonatal BALB/c mice with a lethal dose of PVM-15 resulted in complete protection without up-regulation in levels of pro-inflammatory cytokines and chemokines including IL-12 (Shrivastava et al., 2015; Shrivastava et al., 2016). Given the importance of IL-12 in promoting and modulating immune responses, we used IL-12p40^{-/-} gene deleted mice to determine its role in PVM-induced pathogenesis in BALB/c mice shown to be highly susceptible to PVM infection. PVM-15 challenged IL-12p40 deficient adult BALB/c mice showed significantly less weight loss and IL-12p40 deficient neonatal mice more weight gain than wild-type mice. Interestingly, increased Treg influx into the lungs of both adult and neonatal IL-12p40^{-/-} PVM-infected mice was observed. Furthermore, the response to inhaled cockroach antigen after PVM infection was significantly less pronounced in IL-12p40^{-/-} mice, which is in agreement with the enhanced Treg numbers in IL-12p40^{-/-} mice.

Multiple regulatory mechanisms exist to limit pulmonary pathology, but at the same time eliminate the pathogen during the course of an inflammatory response to a respiratory pathogen. Pulmonary Tregs regulate the immune response to respiratory pathogens (Fulton et al., 2010) and are very important in preventing lung inflammation (Kim et al., 2007; Rubtsov et al., 2008), creating mucosal tolerance to antigen (Curotto de Lafaille et al., 2008; Curotto de Lafaille and Lafaille, 2009) and inhibiting allergic and asthmatic processes (Wohlfert and Belkaid, 2008). Previous studies with Treg-depleted mice have shown that during RSV infection Tregs perform a vital anti-inflammatory function by suppressing pathogenic T cell responses and inhibiting lung eosinophilia. The authors also suggested that dysregulation of normal immune responses to RSV may contribute to severe RSV disease (Durant et al., 2013). It has been shown that following RSV infection, there is an expansion of Tregs in the lungs which coincides with increased numbers of CD4 and CD8 T cells. There are several reports that show evidence of important contributions of pulmonary Tregs in limiting immunopathology and resolving disease during severe RSV infection (Christiaansen et al., 2014; Fulton et al., 2010; Lee et al., 2010). Interestingly, Tregs were recently shown to modulate the B-cell activity to control RSV-mediated exacerbated disease. Depletion of Tregs prior to RSV infection resulted in decreased F-specific antibody production, neutralizing antibody levels and viral clearance, and enhanced IgG2a and pulmonary CD8 T cell influx. The N-specific antibody levels, however,

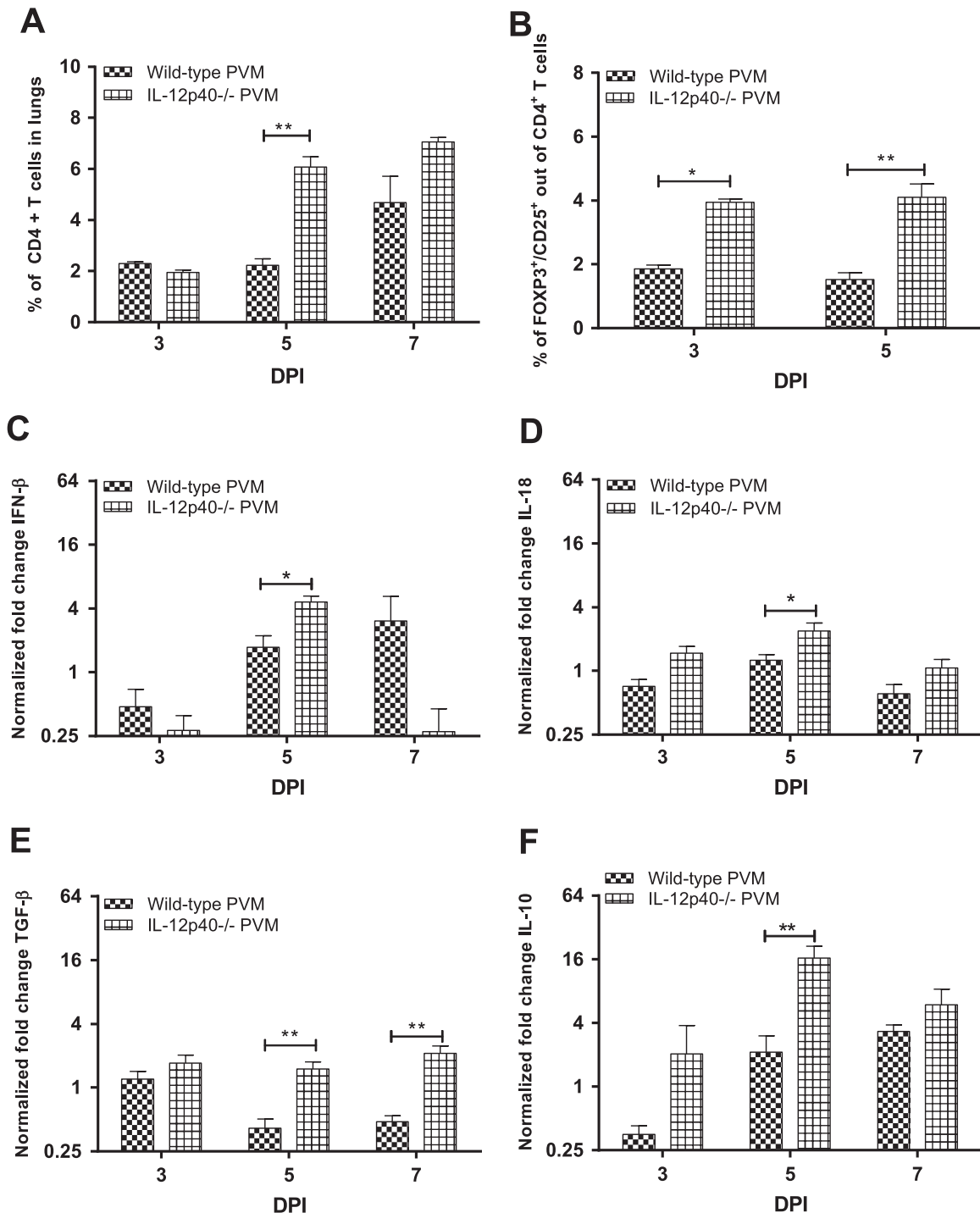


Fig. 5. CD4 T cells, regulatory T cells and cytokine expression in lungs of adult wild-type and IL-12p40^{-/-} mice after infection with PVM-15. Six to eight week-old wild-type and IL-12p40^{-/-} mice were infected with 300 pfu PVM-15 or treated with culture medium (Mock). Cell numbers were quantified by flow cytometry and lung RNA was isolated from PVM-15 infected mice. (A) Percentage of CD3⁺CD4⁺ T cells in the lungs. (B) Percentage of FoxP3⁺, CD25⁺ CD4⁺ (T-reg) cells in the lungs. (C–F) Expression levels of IFN-β (C), IL-18 (D), TGF-β (E) and IL-10 (F) transcripts. Data were normalized against GAPDH housekeeping gene and are expressed as normalized fold-change over mock-infected animals. Data are representative of three independent experiments with 5 mice per group per time point. Bars represent means ± SEM. Significance of differences is shown as: *P < 0.05; **P < 0.01.

were increased, and enhanced inflammation in the lung was observed (Shao et al., 2015).

We found increased numbers of CD4 T cells in the lungs of both neonatal and adult IL-12 p40^{-/-} mice, when compared to wild-type mice. Interestingly, the increase in CD4 T cell numbers in the lungs was correlated to an increased Treg population. In adult IL-12p40^{-/-} mice an increase in IFN-β, IL-18, TGF-β and IL-10 transcripts levels was also found. Of note, IFN-β has been implied in

induction of Tregs (Metidji et al., 2015), while IL-18 can regulate Treg function (Harrison et al., 2015). Furthermore, while multiple mechanisms may contribute to Treg-mediated immunosuppression, the secretion of TGF-β and IL-10 has been implicated as one of potential immunosuppressive factors (Chaudhry et al., 2011; Tran, 2012). In line with this work, studies on RSV have shown the importance of IL-10 in controlling disease and inflammation during acute infection (Loebbermann et al., 2012). IL-10 plays an

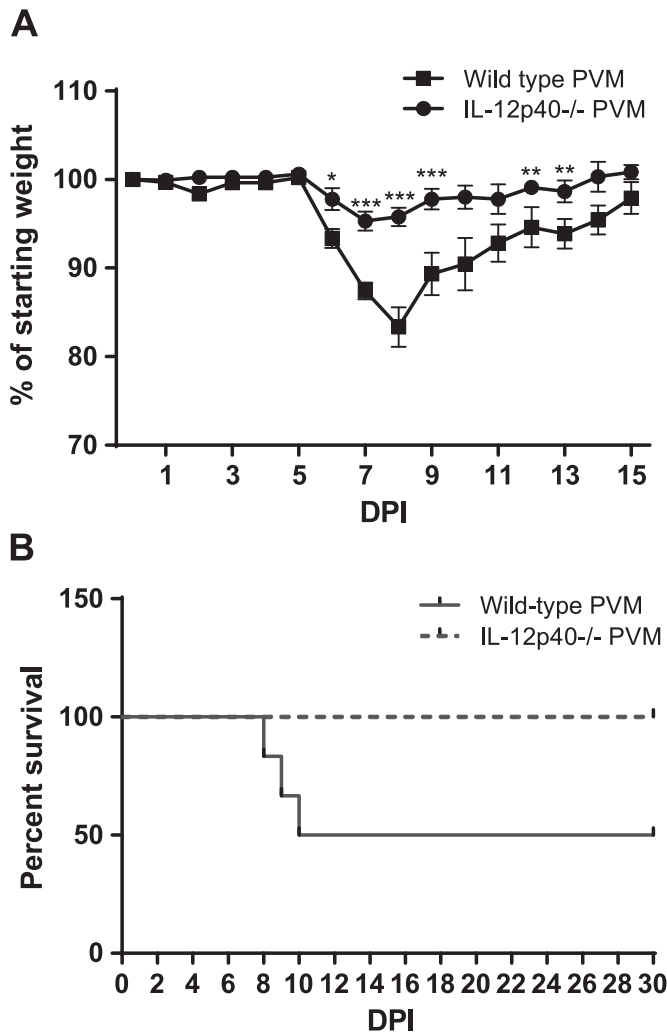


Fig. 6. Weight change in adult wild-type and IL-12p40^{-/-} mice after PVM-15 infection followed by allergen challenge. Six to eight week-old wild-type and IL-12p40^{-/-} mice were infected with 30 pfu PVM-15. Protein extract of cockroach antigen was given on days 4, 10, 17 and 28 post-infection. (A) Weight change in response to PVM-15 infection followed by allergen challenge in wild-type and IL-12p40^{-/-} mice. (B) Survival in response to PVM-15 infection followed by allergen challenge in wild-type and IL-12p40^{-/-} mice. Data are representative of three independent experiments with 5 mice per group. Data in A are shown as means $\pm$ SEM. Significance of differences is shown as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

important part in limiting inflammation at environmental interfaces, in particular, in the gut and lung mucosa (Groux, 2003; Sun et al., 2009). In this study IL-12p40^{-/-} neonates had increased Tregs in the lungs, but no increased transcripts of IL-10 and TGF- β . However, we found significantly increased expression of IFN- β and IL-18 in IL-12p40^{-/-} neonates. Specifically the increased IFN- β levels might be a correlate to the reduced disease observed in neonatal mice, as it was recently shown that a limited type-1 IFN and plasmacytoid dendritic cell response in neonatal RSV infection promotes immunopathogenesis upon re-infection (Cormier et al., 2014).

Here we speculate that a misguided, exacerbated and uncontrolled pro-inflammatory response in BALB/c mice results in inhibition of type-1 IFN secretion, Treg development and IL-10 and TGF- β production, thus causing increased clinical disease and immunopathology. In contrast to our observation, in BALB/c mice infected with RSV and C57BL/6 mice infected with hMPV lack of IL-12p40 exacerbated the disease severity, but this did not alter the viral replication (Chakraborty et al., 2012; Ehl et al., 2004; Wang et al., 2004). hMPV infection in adult IL-12p40^{-/-}/C57BL/6 mice

resulted in significant body weight loss and delayed recovery; however, in adult IL-12p40^{-/-} BALB/c mice infected with RSV no weight loss was observed (Chakraborty et al., 2012; Ehl et al., 2004; Wang et al., 2004). Along similar lines IFN- γ production was down-regulated in hMPV-infected adult IL-12p40^{-/-} C57BL/6 mice, but IFN- γ levels were unchanged in RSV-infected IL-12p40^{-/-} mice (Chakraborty et al., 2012; Ehl et al., 2004; Wang et al., 2004). The disparity between the results obtained in IL-12p40^{-/-} mice for different viruses can be associated to the different characteristics of the viral pathogens studied, in particular as the above mentioned viruses are human pathogens.

Interestingly, infection with 30 pfu PVM-15 followed by cockroach allergen challenge resulted in death of 50% of the wild-type mice, while all IL-12p40^{-/-} mice survived. In a previous report, blockage of IL-12 was reported to have a different effect dependent on the timing. IL-12 was shown to counteract Th2 sensitization, while it contributed to allergic airway disease upon allergen exposure in the post-sensitization phase in mice. Neutralization of IL-12 during the course of repeated allergen challenge of previously sensitized mice attenuated the development of allergic airway disease (Meyts et al., 2006). In infants with RSV bronchiolitis, IL-12p40 was correlated to the development of recurrent wheezing later in life, and both IL-12p40 and IL-12p70 were elevated in the bronchoalveolar lavage fluids of RSV-infected children that developed asthma during the follow-up period later in life; the authors stated that IL-12 might be one of the molecular predictors for asthma following RSV infection (Bertrand et al., 2015).

The subunit p40 of IL-12 is also shared with IL-23, another member of the IL-12 family. IL-23 is said to be associated with the generation, expansion and/or maintenance of Th17 cells and IL-17 production (Lyakh et al., 2008). In a study with IL-23KO and IL-23R transgenic mice IL-23 deficiency was shown to alleviate airway inflammation by decreasing eosinophil recruitment and Th2 cytokine production; however, allergen-specific Th17 responses were not altered in IL-23-deficient or IL-23 Tg mice compared with their controls, thus suggesting that a Th17-independent mechanism might exist in IL-12p40^{-/-} mice to control inflammation after allergen challenge (Peng et al., 2010). In our study we did not find any difference in expression of IL-17 transcript in IL-12p40^{-/-} and wild type mice (data not shown).

In a previous study the capacity of monocyte-derived dendritic cells (moDCs), generated from children of healthy and allergic mothers, to express genes encoding subunits of IL-12 family cytokines was examined (Hrdy et al., 2014). Interestingly, the authors found significantly increased gene expression of IL-12 family members in children of allergic mothers, in comparison with children of healthy mothers, although they did not observe significant changes in the protein levels of IL-12 and IL-23; however, the IL-35 and IL-27 protein levels were significantly reduced in children of allergic mothers. As IL-35 is said to suppress inflammatory responses of immune cells, the authors speculated that formation of this cytokine could be defective in allergy-prone newborns. In our study decreased sensitivity of cockroach allergen in IL-12p40^{-/-} mice might be due to the absence of IL-12 or IL-23 or it can be argued that absence of IL-12p40 potentiates suppressive ability of IL-35, which could dampen inappropriate allergic responses.

IL-12 can mediate suppression of Tregs; treatment with IL-12 has been shown to induce IFN- γ production in both T cells and Tregs, and reduced Treg frequency and FOXP3 expression (Zhao et al., 2012). It is probable, therefore, that in our experimental model of PVM-15 infection in BALB/c mice excessive production of IL-12 results in suppression of Tregs, leading to an over-vigorous and pathological immune response, and death following exposure to cockroach antigen. In summary, it is likely that a depressed regulatory capacity in BALB/c mice to PVM infection results in loss of control and enhanced immunopathology.

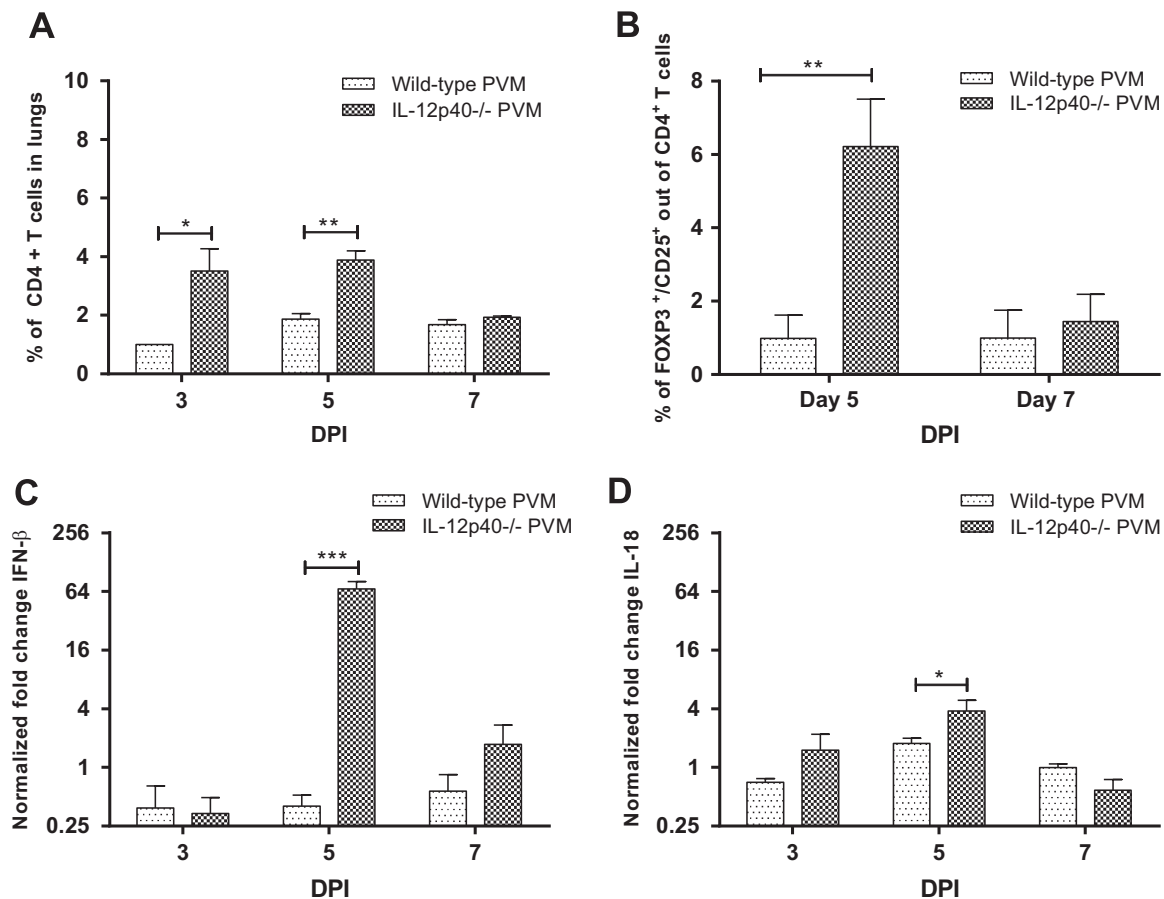


Fig. 7. CD4 T cells, regulatory T cells and cytokine expression in lungs of wild-type and IL-12p40^{-/-} neonatal mice after infection with PVM-15. Seven day-old wild-type and IL-12p40^{-/-} mice were infected with 300 pfu PVM-15 or treated with culture medium (Mock). Cell numbers were quantified by flow cytometry and lung RNA was isolated from PVM-15 infected mice. (A) Percentage of CD3⁺CD4⁺ T cells in the lungs. (B) Percentage of FoxP3⁺, CD25⁺ CD4⁺ (T-reg) cells in the lungs. (C–D) Expression levels of IFN- β (C) and IL-18 (D) transcripts. Data were normalized against GAPDH housekeeping gene and are expressed as normalized fold-change over mock-infected animals. Data are representative of three independent experiments with 6 mice per group per time point. Bars represent means $\pm$ SEM. Significance of differences is shown as: *P < 0.05; **P < 0.01; ***P < 0.001.

4. Materials and methods

4.1. Animals and virus inoculation

IL-12p40^{-/-} mice on a BALB/c background were purchased from the Jackson Laboratory (Bar Harbor, MN, USA); they were bred under pathogen-free conditions in the animal care facility of VIDO-InterVac, Saskatoon. Wild-type adult and pregnant mice were purchased from Charles River Laboratories (Wilmington, MN, USA). Seven day-old wild-type C57BL/6 and BALB/c, and IL-12p40^{-/-} BALB/c neonates were inoculated intranasally with 300 pfu PVM-15 in a volume of 10 μ l. Six to eight week-old wild-type C57BL/6 and BALB/c adults, and IL-12p40^{-/-} BALB/c adults were inoculated intranasally with 300 pfu PVM-15 in 50 μ l. Some wild-type BALB/c and C57BL/6 adults were given 3000 pfu PMV-15. Control neonatal and adult mice received culture medium (mock). Six neonates and five adults per group were euthanized on days 3, 5 and 7 p.i. to collect lungs and MLNs. Mice were bled out by cardiac puncture. In some trials mice were inoculated with 30 or 300 pfu PVM-15, and then exposed to 1 μ g in 20 μ l inhaled cockroach antigen (Greer Laboratories, Lenoir, NC) on days 4, 10, 17 and 28 after PVM infection, and the surviving mice were euthanized on day 30. Experiments were performed according to the guidelines established by the University Committee on Animal Care and Supply at the University of Saskatchewan in accordance with the Canadian Council for Animal Care.

4.2. PVM quantification

PVM was quantified using a standard immunofluorescent plaque assay as previously described (Watkiss et al., 2013). Briefly, tenfold serial dilutions of lung homogenates were made and transferred in duplicate onto 80% confluent BHK-21 cell monolayers. After 72 h incubation at 37 $^{\circ}$ C, cells were fixed with an ice-cold solution of (4:1) acetone and methanol, and dried at RT. Plates were blocked with PBS containing 5% goat serum (Thermo Fisher Scientific, Waltham, MA, USA), followed by a 2–3 h incubation with a 1:500 dilution of a PVM N protein-specific polyclonal rabbit antibody (made in-house) in PBS containing 1% goat serum. After the incubation, plates were washed and incubated in the dark with an Alexafluor488-conjugated goat anti-rabbit antibody. Plaques were counted with a fluorescence microscope (Zeiss, Oberkochen, Germany).

4.3. IL-12 ELISA

IL-12 was detected in cell-free supernatants of lung homogenates with the meso scale discovery (MSD) Multi-Array Mouse Cytokine Ultra-sensitive Assay for IL-12, and an electrochemiluminescence (ECL) detection-based MSD Sector Imager 2400 (MSD, Gaithersburg, MD, USA). The assay was performed according to the manufacturer's instructions. The MSD Discovery Workbench Software was used to analyze the results.

Table 1

List of primers used in qPCR experiments.

Gene	Forward Primer	Reverse Primer	Annealing Temp. (°C)
IFN- β	ATCATGAACAACAGGTGGATCCTCC	TTCAAGTGGAGAGCAGTTGAG	63.9
IL-18	TGGTCCATGCTTCTGGACTCCT	TTCCTGGGCAAGAGGAAGTGATT	56.3
TGF- β	AGGGCTACCATGCCAACTTC	CCACGTAGTAGACGATGGGC	57.5
IL-10	GCTGCTGCTCTTACTGACT	CTGGGAAGTGGGTGCAGTTA	57.5
GAPDH	AACCTTGGCATTGTGGAAGG	ACACATTGGGGTAGGAACA	57.5

4.4. Lung histology

For histology the multi-lobed right lung was perfused with 1 ml of 10% neutral buffered formalin (VWR, Radnor, PA, USA) and collected on days 3, 5 and 7 p.i. The lungs were fixed in formalin after being placed in a cassette. The perfused lungs were then processed as described previously (Watkins et al., 2013) and scored in a blinded manner by a veterinary pathologist. Scores were given based on the presence and severity, as well as the dissemination of lesions, characterized by cellular infiltrates and edema in the tissue surrounding the bronchioles and blood vessels visible in lung sections. A score of 0 denotes a normal lung, 1 indicates signs of perivascular edema and mild perivascularitis, limited to small foci that were not widespread, while a score of 2 denotes moderate levels of perivascularitis, vasculitis, and multifocal perivascular edema. Finally, a score of 3 represents animals with extensive, severe, multifocal perivascularitis, vasculitis, and vascular edema, and/or necrotic and fibrous areas.

4.5. Flow cytometry

Single cell suspensions of perfused lungs and MLNs were made as previously described (Shrivastava et al., 2016; Watkins et al., 2013). Trypan blue exclusion was used to estimate cell viability. Total cell numbers were counted using a Beckman coulter counter (Beckman Coulter Inc., Pasadena, CA, USA). Cell staining was performed as previously described (Shrivastava et al., 2016; Watkins et al., 2013). For surface staining, the following antibodies were used; anti-CD3 (cat no.555275), anti-CD4 (cat no.553729), anti-CD8 (cat no. 553031), and anti-MHC II (cat no 553610) (BD Pharmingen, San Diego, CA, USA); anti-CD11c (cat no. 117308), anti-CD45R/B220 (cat no.103205) and anti-CD25 (cat no.102011) (BioLegend, San Diego, CA, USA), and m-PDCA-1 (cat no. 5120327174) (MiltenyiBiotec, San Diego, CA, USA). For intracellular staining anti-FOXP3 (cat no.126403) (BioLegend) was used according to BioLegend's protocol (catalogue no. 421403). Flow cytometry was performed with a FACS Calibur (BD Biosciences, San Jose, CA, USA). Cells were gated for live cells, singlets and lymphocytes, and then analyzed. Analysis of flow cytometry data was performed using Kaluza software (Beckman Coulter).

4.6. RNA isolation, cDNA synthesis and quantitative real-time PCR

Lung samples were collected from each animal in TRIzol Reagent (Life Technologies, Burlington, ON, Canada) and homogenized for 10 s at 4800 rpm in a mini bead-beater (BioSpec Products Inc., Bartlesville, OK, USA). Total RNA was isolated from the lung samples, and the quality of the RNA was checked in an Agilent Bioanalyzer. cDNA was prepared using a QuantiTect Reverse Transcription kit (Qiagen, Venlo, Limburg, Netherlands). Quantitative real-time PCR was performed by a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System using FastStart SYBR Green Master (Roche, Basel, Switzerland). The reaction conditions consisted of: 1 cycle at 95 °C for 10 min for activation of FastStartTaq DNA Polymerase, 40 cycles at 95 °C for 15 s for initial denaturation, 55 °C – 65 °C (primer dependent) for 30 sec for primer annealing

and 72 °C for 30 s for primer extension. To check the specificity of the amplicon, a melt curve was generated by setting the initial temperature at 55 °C ramping to 95 °C with 0.5 °C increase each 10 s for 80 cycles. The primer sequences of the IFN- β , TGF- β , IL-10, IL-18 and GAPDH genes are listed in Table 1. Normalized fold-change was calculated over mock-infected animals.

4.7. Statistical analysis

Statistical software (Graph Pad Prism Version 6.00) was used to analyze all data. One-way ANOVA with Newman–Keuls post-test was used to compare differences between multiple groups. Mann–Whitney *U* test was used to compare individual groups, if significance was found among the groups. Differences were considered significant if $P < 0.05$.

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