

# THE EXPRESSION OF PPAR- $\gamma$ RECEPTOR IN ADULT AND NEONATAL LUNGS DURING RESPIRATORY VIRAL INFECTION

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## Abstract:

*Respiratory viral infection is a significant cause of morbidity and mortality worldwide. Severe illness during a viral lung infection may be due to inflammation as a result of the host immune response, rather than direct damage by the virus itself. Previously, a mouse model of respiratory viral infection demonstrated that there was significantly reduced inflammation in neonatal lungs compared to adults. This occurred even though the viral titer and viral clearance was similar in both adults and neonates. The reduced inflammation in neonates was associated with significantly higher levels of Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>). PGD<sub>2</sub> is converted into prostaglandin J<sub>2</sub> (PGJ<sub>2</sub>), which binds to the receptor PPAR- $\gamma$ . Importantly, studies using PPAR- $\gamma$  agonists in adult lungs during infection reduced inflammation. In contrast, treatment of neonates with PPAR- $\gamma$  antagonists increased susceptibility to infection. These studies suggested that the anti-inflammatory effects of PGD<sub>2</sub> might be through its conversion to PGJ<sub>2</sub> and subsequent binding to the PPAR- $\gamma$  receptor. To date, the expression of PPAR- $\gamma$  in adult and neonatal lungs during respiratory viral infection has not been determined. Adult and neonatal C57BL/6 mice were infected with 500 pfu/g body weight Sendai virus (SeV) and lungs were fixed in formalin at various times after infection. Five  $\mu$ m lung sections were stained with an anti-PPAR- $\gamma$  antibody. Results showed that PPAR- $\gamma$  was expressed in the airway epithelium of uninfected adults and neonates. This expression decreased in both after viral infection. To analyze PPAR- $\gamma$  expression in isolated airway epithelial cells, tracheas from uninfected C57BL/6 adult mice were treated with pronase and the epithelial cells were cultured in an air-liquid interface. These cells were infected with SeV (MOI=0.1) and RNA was isolated at 24, 48 and 72 hours post-infection. PPAR- $\gamma$  mRNA expression was measured by qPCR. The results showed no difference in PPAR- $\gamma$  expression between uninfected and infected cultured airway epithelial cells. This suggests that viral infection of epithelial cells does not directly affect PPAR- $\gamma$  mRNA expression. Therefore, the mechanism of reduced PPAR- $\gamma$  protein expression in vivo will need to be determined.*

## Introduction

Lower respiratory infections were classified as the fourth leading cause of death back in 2016 and the deadliest communicable diseases, causing 3 million deaths worldwide [1]. Examples of viruses that cause lower respiratory viral infections include respiratory syncytial virus (RSV) and influenza. In order to study respiratory viral infection, we use a mouse model of

infection with Sendai virus. This virus is a natural mouse pathogen that causes a bronchiolitis similar to RSV infection in humans.

Previous studies showed that neonatal mice were able to clear Sendai virus from the lungs in the absence of inflammation compared to adult mice [2]. This reduction in inflammation was associated with

increased levels of a lipid molecule called prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) [3]. Prostaglandin D<sub>2</sub> is a lipid molecule that is derived from arachidonic acid. Arachidonic acid is released from membrane phospholipids and is then converted by a series of enzymatic reactions to PGD<sub>2</sub>. It can then be further converted by dehydration to PGJ<sub>2</sub>.

The PGD<sub>2</sub> and PGJ<sub>2</sub> molecules are able to bind receptors DP1 and DP2 on the plasma membrane, but PGJ<sub>2</sub> also can bind PPAR-γ in the cytoplasm. The effects of PGD<sub>2</sub> or PGJ<sub>2</sub> depend on the cells that are expressing the receptors. In some cases, these molecules could induce inflammation and in other cases they could reduce inflammation. The PGD<sub>2</sub> ligand binds to the receptors DP1 (a protein-coupled receptor that is encoded by the PTGDR1 gene), and DP2; PGD<sub>2</sub> may also be converted into PGJ<sub>2</sub> that binds to receptors DP1, DP2, and PPARγ. DP1 is expressed by cells that are involved in mediating allergic and inflammatory reactions [4]. Through the process of cell signaling, the PGD<sub>2</sub> or PGJ<sub>2</sub> molecule may bind to its receptor and cause a reduction in inflammation. If that signal is blocked with an antagonist drug, then that may increase inflammation.

PPARγ (peroxisome proliferator-activated receptor-γ) is a form of transcription factor essential to regulating the immune response in the lung. This receptor may be expressed on multiple cell types with specific roles in controlling the development and functions of the lungs in neonatal and adult mice during respiratory viral infections. PPAR-γ requires the binding of lipid ligand and dimerization with a retinoid acid receptor. The receptor then binds with a high affinity to a PPAR response element of a targeted gene promoter [5]. There are numerous endogenous lipid ligands that bind to the PPARs, and they typically include

polyunsaturated fatty acids and conversion products of eicosanoids. Particularly, PPAR-γ is activated by oxidized fatty acids that are found in oxidized low-density lipoprotein. Besides binding with targeted genes, PPAR-γ can act as a transcriptional repressor of the pro-inflammatory transcription factor NFκB by inducing the proteasomal degradation of the NFκB subunit p65 directly [6].

Previous studies have compared the adult and neonatal response to Sendai virus. The virus was diluted in saline solution and one drop of Sendai virus (500 pfu/g body weight) was applied to the nose of each mouse. Figure 1.0 shows that the virus replicated the same in both adults and neonates on day 3. Both adults and neonates were able to clear the virus from the lungs by days 10 and 14. So, adult and neonatal mice were able to clear the virus in a similar

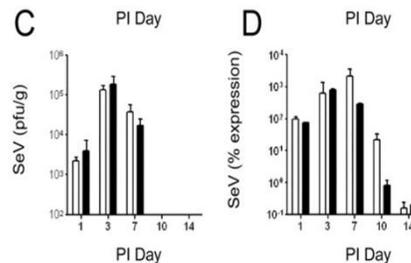
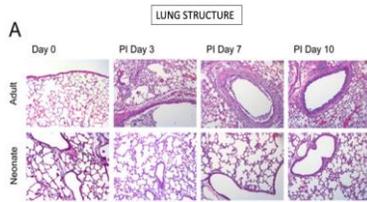


Figure 1.0. White bars represent the adults and the black bars represent the neonates. Graph on the left shows results from a viral plaque assay. Graph on the right shows results from qPCR for viral RNA

fashion (Figure 1.0)

Hematoxylin and eosin staining was performed to determine the amount of inflammation in the lungs of adults and neonates that were infected with Sendai Virus. The hematoxylin stained the nuclei a purple color, so where we see more purple indicates that there are more immune cells coming into the lungs. The large white spaces shown in the lung samples are large

airways, and the sponge-like structures are the alveolar spaces (Figure 2.0) [2].



Reference: Bhattacharva et al. Virology Journal 2011

Figure 2.0. Hematoxylin and eosin staining of lung structures from adults and neonates after infection with Sendai virus (500 pfu / g body weight).

The reduction in inflammation in the neonates was associated with increased  $PGD_2$  in the neonatal lungs compared to adults during infection (Figure 3.0). This suggested that  $PGD_2$  may be involved in the reducing inflammation observed in neonatal lungs.

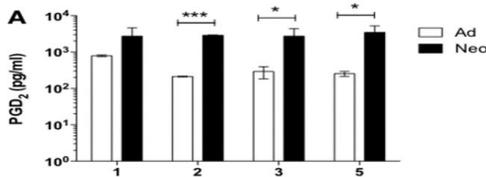


Figure 3.0 Adult and neonatal mice were infected with Sendai virus (500 pfu/g body weight). Bronchoalveolar lavage fluid was collected on the indicated days post-infection.  $PGD_2$  levels were determined by ELISA.

The next experiment in the lab was to determine if inflammation would increase in neonatal mice during respiratory viral infection if we blocked the action of  $PGD_2$  from binding to the receptor sites with a PPAR- $\gamma$  antagonist drug. Neonatal mice that received the PPAR- $\gamma$  antagonist were more susceptible to viral infection (Figure 4.0).

The results of the study suggested that there was more inflammation when the PPAR- $\gamma$  receptor was blocked during Sendai virus. The white circles showed that all neonates infected with Sendai virus died by day 10. Neonates that received DP1 or DP2 antagonists had similar survival to the virus only group, but the group treated with

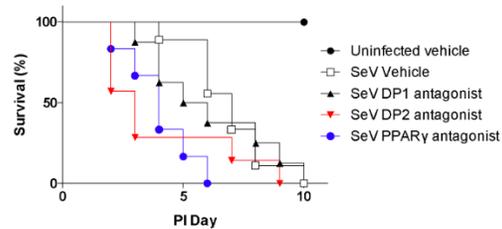


Figure 4.0 Survival of neonatal mice after Sendai virus infection. Some the neonates were treated with an antagonist drug that blockd  $PGD_2$  or  $PGJ_2$  from binding to the receptors DP1, DP2 and PPAR- $\gamma$  receptors sites

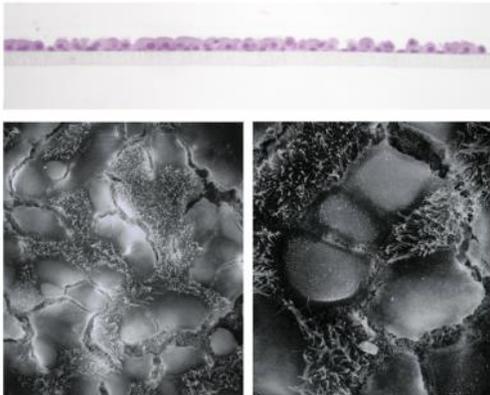
PPAR- $\gamma$  antagonist did worse; there were no survivors by day 6.

Together, these experiments suggest that treatment with PPAR- $\gamma$  agonists may be a way to reduce inflammation in adult lungs during viral infection. To better understand the role of PPAR- $\gamma$ , we wanted to determine how is it expressed in the lungs. We conducted two methods to determine PPAR- $\gamma$  expression in adult and neonatal lungs during viral infection. We used real time PCR to measure PPAR- $\gamma$  messenger RNA levels in neonatal and adult lungs, tracheas and cultured airway epithelial cells. We also determined PPAR- $\gamma$  protein expression in lung sections using immunohistochemistry. By using these two methods, we wanted to be able to find a comparison between the expression of PPAR- $\gamma$  receptor in adult and neonatal mice during respiratory viral infection.

## Methods

**Viral infection** Six – eight week old adult and two day old neonatal C57BL/6 mice were infected intranasally with Sendai Virus (500 pfu/g body weight). Mice were observed daily for general health and body weight measurements as previously described [7].

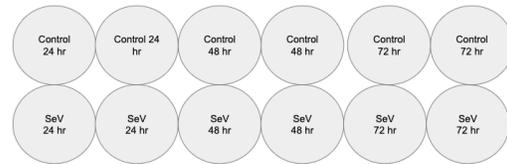
**Immunostaining.** On different days post-infection, mice were euthanized and the lungs were inflated with 10% neutral buffered formalin. The lungs were dehydrated and embedded in paraffin. Five micron sections were used for immunostaining. The lung sections were stained with a rabbit antibody for PPAR- $\gamma$ . The primary antibody was then detected with a secondary antibody tagged with an alkaline phosphatase enzyme and was visualized with a red substrate. Then the slides were counterstained with hematoxylin. We performed a comparison analysis between the adult and neonate lungs undergoing Sendai Virus from days 0 to day 7.



*Figure 5.0. The top panel shows a cross-section of tracheal epithelial cells grown on a Transwell™. The bottom left micrograph shows a mouse trachea and the panel on the right shows a culture of tracheal epithelial cells.*

## Primary cultures of airway epithelial cells

To discover if there would be a decrease of the expression of PPAR- $\gamma$  in cultured airway epithelial cells during viral infections, we cultured the airway epithelial cells from adult mice by isolating the cells from the tracheas [8]. Tracheas were placed in solution with pronase overnight at 4°C. The tubes were then gently inverted to release epithelial cells, so that we could spin the cells into pellets. Lastly, the cells are cultured in Transwells™. The tracheal epithelial cells are grown in grown in small Transwell™



*Figure 6.0. Cells were infected with Sendai Virus MOI= 0.1. RNA was isolated at the indicated times after infection.*

in an air-liquid interface. This method allowed the cells to have liquid on one side and air on the other side just like in the lungs (Figure 5.0).

The cultured airway epithelial cells were infected with Sendai Virus at a Multiplicity of Infection (MOI)=0.1. RNA was isolated at indicated times after infection and cDNA synthesis was performed (Figure 6.0).

To measure the amount of PPAR- $\gamma$  mRNA, we used the quantitative PCR method. The first step was conducting primer optimization to ensure that the primers amplified the correct sized band, that there were no primer-dimers formed and to determine the correct primer concentration. We then used Applied Biosystems Power Up SYBR Green Master Mix for real-time PCR to measure how much DNA has been amplified. To calculate the math for this method, we

used the Delta Delta Ct analysis and normalized to the GAPDH housekeeping gene [9]. The results of primer optimization method is illustrated with a melt curve plot (Figure 8.0).

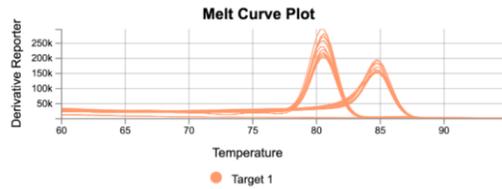


Figure 8.0. Melt Curve Plot displaying the results of measuring the amount of mRNA from the cultured airway epithelial cells. The higher peak represents the SeV group, and the control group is represented by the smaller peak.

The results of the real-time qPCR method for the melt curve plot displayed that there was only peak for each gene, no primer dimers, and no signal detected in the no cDNA control (Figure 8.0). The results of PPAR-g realtime PCR on infected airway epithelial cells in vitro are shown in Figure 7.0.

### Results

By immunostaining, we found that PPAR- $\gamma$  was expressed in airway epithelial cells in both uninfected adult and neonatal lungs. It was not expressed in the alveolar epithelial cells. After viral infection, the

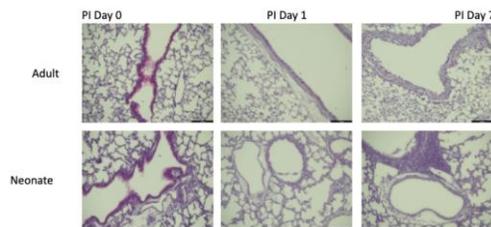


Figure 7.0 Expression of PPAR- $\gamma$  in adult and neonatal lungs was detected with an anti-PPAR- antibody and an alkaline phosphatase red substrate.

levels of PPAR- $\gamma$  protein decreased in

both adult and neonatal lungs (Figures 7.0 & 8.0).

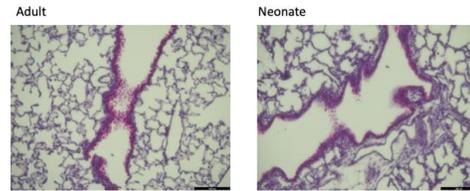


Figure 8.0 PPAR- $\gamma$  expressed in the airway epithelial cells in both uninfected adult and neonatal lungs. Cells that are positive for PPAR- $\gamma$  are bright pink.

The results from the PCR for PPAR- $\gamma$  showed that the expression of PPAR- $\gamma$  in adult cells did not change with Sendai

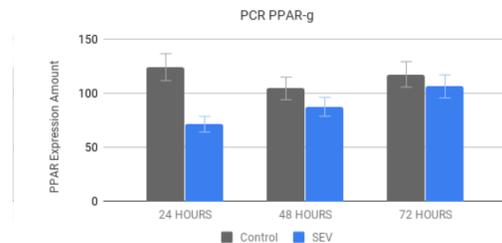


Figure 9.0. The bar graph shows the results of the PPAR- $\gamma$  PCR method from the cultured airway epithelial cells from the adult mice that were infected with SeV. Grey bars represent the control group and the blue bars represent the group that is infected with SeV.

virus infection *in vitro* (Figure 9.0).

### Conclusion

PPAR- $\gamma$  protein was expressed in the airway epithelium in uninfected adult and neonatal lungs during viral infection. The expression of PPAR- $\gamma$  was decreased in adults and neonates after infection. In the cultured cells, there was no difference in PPAR- $\gamma$  mRNA expression between infected and uninfected cells. Further studies will need to be performed to understand the differences between protein

and mRNA PPAR- $\gamma$  expression and between the *in vivo* and *in vitro* results.

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