The Genetic Knockdown of IRF9 Leads to the Reduction of Antiviral Resistance in Therapeutically Induced Lung Epithelial Cells

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Introduction

- Pneumonia is the most common cause of infection in cancer patients due to malignancy and treatment imparting some degree of immunosurpression.[1]
- Stimulation of lung epithelial cells with the combination treatment of Toll-like receptor agonists, Pam2CSK4 (“Pam2”;TLR2/6) and ODN M362 (“ODN”;TLR9) confers inducible resistance against infectious pneumonias via a reactive oxygen species (ROS) dependent-mechanism.[2,3]
- Ongoing projects are focused on studying the biochemical mechanisms by which Pam2-ODN induces ROS-mediated pathogen killing.
- Activation of the Interferon-Stimulated Gene Factor 3 (ISGF3) transcription factor complex – composed of STAT1, STAT2, and IFR9 – leads to the expression of the Dual Oxidase 2 (DUOX2) NADPH oxidases responsible for ROS-mediated antiviral immune response in a non-canonical STAT2/IRF9-dependent activation pathway.[4]

Background

- Knockdown of either DUOX gene significantly reduced H_2O_2 production but a small significant increase is still observed when cells were treated with Pam2-ODN. Additionally, the antiviral defense induced by Pam2-ODN is robust but returns to baseline when cells are treated with an ROS scavenger (catalase).

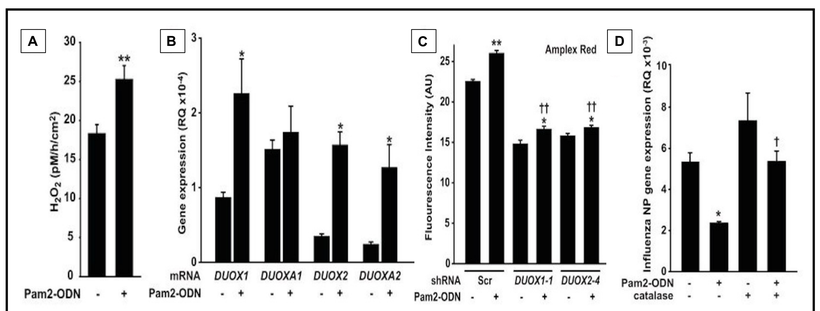

Fig. 1. (A) HBEC3kt cells treated with Pam2-ODN generated significantly more H_2O_2 than the control. **(B)** Expression of DUOX-related genes significantly increased 2 h after Pam2-ODN treatment. **(C)** H_2O_2 production significantly decreases when DUOX-related genes are knocked down. **(D)** Antiviral response is attenuated when cells are subjected to a ROS scavenger but is recovered when treated with Pam2-ODN[3]

- Microarray data reported the upregulation of IRF9 by Pam2ODN in the cell lines culture and highlighted the requirement of Pam2ODN-induced *Duox2* and ROS to protect mice against viral and bacterial infections.
- Knockdown of STAT1, STAT2, and IRF9 gene expression utilizing small interfering (siRNA) demonstrated a significant decrease in DUOX2 expression in Pam2-ODN treated cells when STAT2 and IRF9 were silenced with no significant effect being observed when STAT1 was silenced. This indicates that DUOX2 expression is mediated by STAT2 and IRF9.

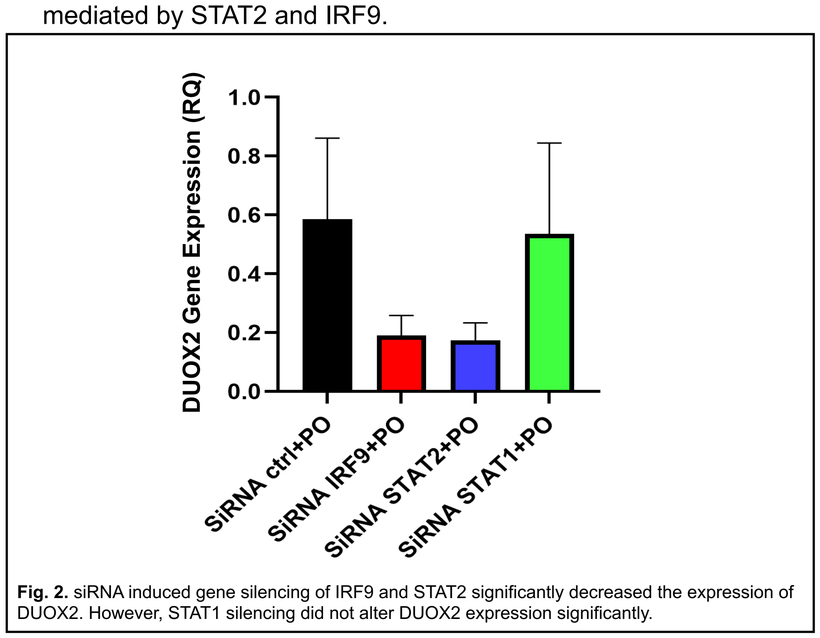

Fig. 2. siRNA induced gene silencing of IRF9 and STAT2 significantly decreased the expression of DUOX2. However, STAT1 silencing did not alter DUOX2 expression significantly.

Objectives

- General: investigate the role ISGF3 complex in the Pam2-ODN inducible antiviral immune response.
- Specific: analyze the role of IRF9 in the Pam2-ODN inducible antiviral immune response using MLE-15 cells.

Methods

- IRF9-knockdown (IRF9KD) murine MLE-15 lung epithelial cells were generated utilizing lentivirus and molecular cloning techniques to study the relationship between IRF9 and ROS production. IRF9KD cells were characterized by visualizing the expression of green fluorescent protein.

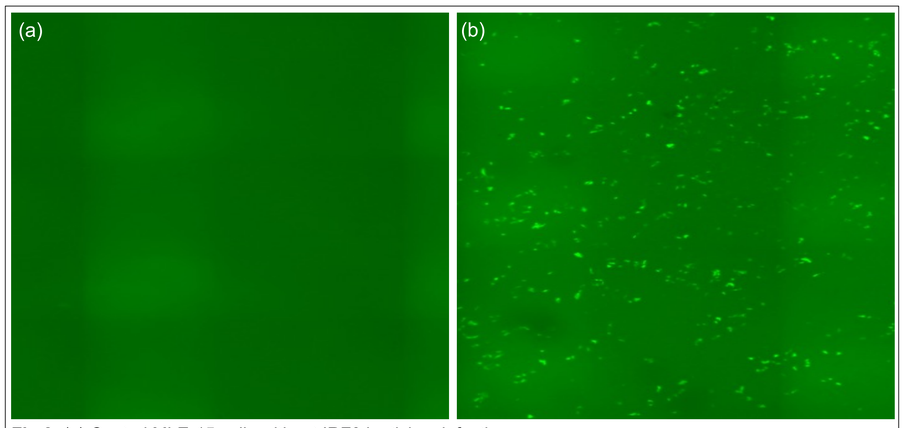

Fig 3. (a) Control MLE-15 cells without IRF9 lentivirus infection and (b) GFP expression in MLE-15 cells treated with IRF9 lentivirus infection.[5]

- Wildtype (*wt*) and IFR9KD MLE-15 cells were grown and incubated overnight. The cells were then treated with Pam2-ODN 4 hours prior to viral challenge. After treatment, the cells were challenged with the Influenza A virus (IAV) and were incubated for 24 hr.
- After 24 hr, the cells were harvested which was followed by RNA extraction. cDNA was then created utilizing the RNA templates. Utilizing qPCR, the amplification of the DNA sequences for the IAV Nucleoprotein (NP) was utilized as a proxy for viral replication and 18s rRNA was utilized as the internal control.

Results

- Genetic knockdown of IRF9 in murine MLE-15 lung epithelial cells resulted in a significant decrease in the ability to produce an antiviral response against IAV regardless of treatment with Pam2-ODN (PO). Expression of NP is rescued in the IRF9KD cells suggesting that IRF9 is critical in allowing the cell to mount a robust antiviral response.

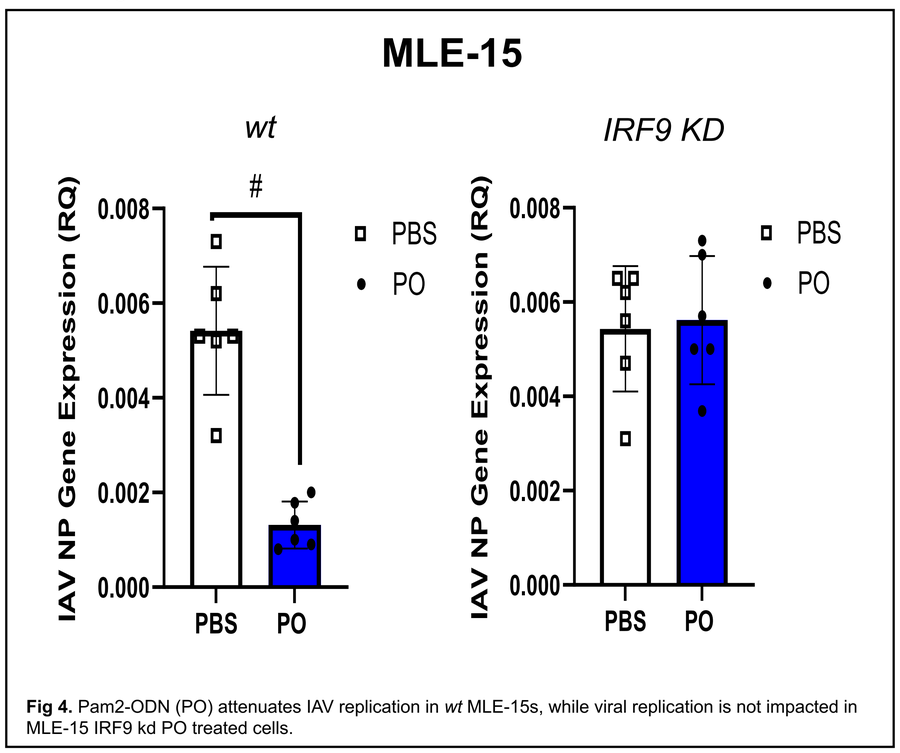

Fig 4. Pam2-ODN (PO) attenuates IAV replication in *wt* MLE-15s, while viral replication is not impacted in MLE-15 IRF9 kd PO treated cells.

Conclusion

- ROS production plays an important role in developing an antiviral response
- Activation of the ISGF3 complex leads to the expression of DUOX-related genes through a STAT2/IRF9-dependent activation pathway attenuated antiviral capabilities and ROS production.
- Cells treated with IRF9 siRNA and STAT2 siRNA showed a decreased capacity to express DUOX2
- Lentiviral knockdown of IRF9 in MLE-15 cells resulted in the inability to produce antiviral immune response in Pam2-ODN treated cells, suggesting that IRF9 plays a critical role in the DUOX2-induced ROS antiviral immune response.

Future Directions

- Complete STAT1 and STAT2 lentiviral knockdown in lung epithelial cell lines to clarify the ISGF3 pathway associated with Pam2-ODN antiviral immune response.
- Complete supplemental assays to further characterize the requirement of the components of the ISGF3 in the DUOX2 induction and ROS production.
- *In vivo* experiments using STAT1, STAT2 and IRF9 knockout mice
- Investigate the role of Pam2-ODN-induced ISGF3 in the bacterial and fungal infections that also have an increased prevalence in cancer patients.

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- S.E.E. is an author on U.S. patent 8,883,174, “Stimulation of Innate Resistance of the Lungs to Infection with Synthetic Ligands.” M.J.T., B.F.D., and S.E.E. own stock in Pulmotect, Inc., which holds the commercial options on these patent disclosures. This project is supported by NIH Grant R35 HL144805 to S.E.E. All other authors declare that no conflict of interest exists.