ER stress Protein PERK promotes inappropriate innate immune responses and pathogenesis during RSV infection

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Running Title: PERK regulates DC function

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Abstract

The activation of dendritic cells (DC) during respiratory viral infections is central to directing the immune response and the pathologic outcome. In these studies the effect of RSV infection on development of ER stress responses and the impact on innate immunity was examined. The upregulation of ER stress was closely associated with the PERK

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pathway through the upregulation of CHOP in RSV infected DC. The inhibition of PERK corresponded with decreased EIF2a phosphorylation but had no significant effect on Nrf2 in DC, two primary pathways regulated by PERK. Subsequent studies identified that by blocking PERK activity in infected DC an altered ER stress response and innate cytokine profile was observed with the upregulation of IFNβ and IL-12, coincident to the down regulation of IL-1β. When mitochondria respiration was assessed in PERK deficient DC there were increased dysfunctional mitochondria after RSV infection that resulted in reduced oxygen consumption rates (OCR) and ATP production indicating altered cellular metabolism. Use of a CD11c targeted genetic deleted murine model, RSV infection was characterized by reduced inflammation and diminished mucus staining as well as reduced mucus-associated gene *gob5* expression. The assessment of the cytokine responses showed decreased IL-13 and IL-17 along with diminished IL-1β in the lungs of PERK deficient infected mice. When PERK-deficient animals were assessed in parallel for lung leukocyte numbers, animals displayed significantly reduced myeloid and activated CD4 and CD8 T cell numbers. Thus, the PERK activation pathway may provide a rational target for altering the severe outcome of an RSV infection through modifying immune responses.

Introduction

Respiratory syncytial virus (RSV) infection remains a leading cause of hospitalization in infants and older adults (1). While there is no effective vaccine to prevent RSV infection, prophylactic anti-RSV monoclonal antibody treatment is often administered to vulnerable infant populations such as those born prematurely, or with congenital heart defects (2, 3). Infants hospitalized for RSV infection present with severe inflammation and airway constriction that stems from an aberrant immune response to the virus (4-6). During severe RSV infection an inappropriate immune response skews toward Th2 and Th17 responses, which promotes significant mucus production and goblet cell hypertrophy leading to

pathogenic sequelae (7-11). Further, hospitalization due to RSV infection is associated with an increased susceptibility to asthma (12-16), suggesting that the effects of the skewed immune response are not limited to infancy, but rather cause long lasting airway changes and immune system alteration. Understanding the molecular mechanisms of RSV induced inflammation is key to identifying effective therapeutic targets during early life disease that may have long-term consequences.

It is known that cellular stress, such as endoplasmic reticulum (ER) stress and oxidative stress in the form of reactive oxygen species (ROS) production can promote inflammatory pathways that result in pathogenic responses (17-19). Although other studies have established the impact of ROS on RSV (20), the mechanisms in which ER- and oxidative stress themselves affect innate immunity to RSV are less clear (21). Two mediators of the ER stress response are PKR-like endoplasmic reticulum kinase (PERK, gene eif2ak3), which is activated by ROS production, and inositol-requiring enzyme 1 (IRE-1), which is activated upon RNAse activity. RSV infection leads to ROS production and can influence the intensity of the responses (20, 22, 23). PERK phosphorylates EIF2a, which signals for a reversible halt in translation, and Nrf2, a transcriptional repressor that constantly undergoes degradation when dephosphorylated. Interestingly, Nrf2 in particular may have protective roles during RSV infection but has several pathways that can activate its function (24-28). Our results indicate that during RSV infection ER stress is induced and PERK is activated in dendritic cells, and appears to contribute to the pathogenic RSV immune response in several ways: 1) transcriptional regulation of key innate cytokines; 2) enhancement of EIF2a-phosphorylation signaling that leads to altered APC function; and 3) induction of CD4 T cell recruitment to the lung associated with IL-13 and IL-17 production. Our findings support these latter concepts and we present data that suggest that blocking the PERK activation pathways during RSV responses would alter pathogenesis in the lung.

Materials and Methods

Mice

C57BL/6J (BL6), B6; $PERK_{f/f}$ and C57BL/6J-Tg (Itgax-Cre-EGFP) 4097Ach/J (CD11c-Cre-GFP) mice were purchased at 6-7 wk of age from The Jackson Laboratory (Bar Harbor, ME). $PERK_{f/f}$ mice were crossed to CD11c-Cre-GFP transgenic mice. Thus, Cre+ mice lack a functional PERK in $CD11c^{high}$ cells. $PERK^{f/f}$-CD11c-Cre mouse breeding took place in-house at the University of Michigan (Ann Arbor, MI) with Cre- mice used as the wildtype control expressing normal PERK levels. All work involving animals was reviewed and approved by the University of Michigan Committee on Care and Use of Animals.

BMDC culture

Bone marrow-derived DCs (BMDCs) were isolated from whole bone marrow of naive C57BL/6 mice. Bone marrow cells were seeded into tissue culture flasks containing RPMI 1640-based complete medium supplemented with 15ng GM-CSF/ml (R&D Systems, Minneapolis, MN). C57BL/6 mouse-derived BMDCs were fed on days 3, 5, 7, 9 and harvested on day 10, a time point by which cells were ≥ 85% CD11b+ CD11c+ BMDCs by flow cytometric analysis. In some experiments control and RSV infected BMDC were treated with PERK inhibitor (PERKi (5uM, GSK2606414)) to block the downstream signaling of target molecules.

RSV Growth

Line 19 RSV (antigenic subgroup A), originally obtained from a sick infant at the University of Michigan Hospital System, was shown in animal models to mimic human infection by eliciting airway mucus production upon inoculation with 1-3 × 10^5 PFU RSV. RSV was

propagated in our laboratory in HEp-2 cells (American Type Culture Collection). Mice were infected intratracheally with 1.5×10^5 PFU RSV as previously described (29).

Quantitative PCR

Total RNA was isolated from BMDC culture using TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Grand Island, NY). RNA was reverse transcribed, and cytokine gene expression was assessed using TaqMan Gene Expression Assay primer/probe sets on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Custom primers were used to assess transcription levels of RSV-G, and RSV-F, as previously described (30). Fold change expression was calculated from gene expression values normalized to 18s RNA.

Immunoblot Analysis

Total cells lysates were prepared using 1X Cell Lysis Buffer (Cell Signaling). Same amount of 3~10 ug of total proteins were separated by Nu-PAGE (Invitrogen) and transferred on nitrocellulose membrane. The primary Abs were diluted in 5% BSA in 1X TBST.

Histopathology and Mucus Assessment

Serial 6 um sections were cut from paraffin-embedded fixed lungs and stained by periodic acid-Schiff (PAS) staining. PAS stained slides were blindly scored for goblet cell hyperplasia and mucus plugging by light microscopy. The following scoring system was used: 1, absent; 2, staining in multiple airways; 3, staining in multiple airways with mucus plugging; 4, severe mucus plugging in multiple airways.

Flow Cytometry

Cells were isolated from the left lungs by digestion in 200µg/ml Liberase TM (Roche Applied Science, Indianapolis, IN) and 200U/ml DNase I (Sigma-Aldrich) at 37C. After lysing RBCs,

FcR-blocking was used to limit nonspecific staining. Cells were stained with Live/Dead Fixable Yellow (Invitrogen), followed by fluorescent antibodies for 30 min. Total number of cells for each population in individual lungs was calculated using gating percentage multiplied by total number of cells in each lung preparation. Analysis was performed using FlowJo software (TreeStar, Ashland OR).

Seahorse Assay

The Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to measure the mitochondrial function of BMDC. Briefly, $1x10^5$ cells per well, cultured in RPMI complete media supplemented with 10% (v/v) heat-inactivated FBS were plated in a 96 well Seahorse plate. BMDC were infected with RSV for 2h, then washed twice with Seahorse assay media (Agilent Technologies, Santa Clara, CA, USA) and incubated for 30 min in a CO_2 free incubator at 37°C. Oxygen consumption rate (OCR) was determined using a cell Mito Stress Test kit (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. Oligomycin (2mM); FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) (1.5mM); Rotenone (0.5 mM), and Antimycin (0.5 mM) were used in the assay. *Oligomycin is used to block ATP synthase to deplete the cells of their capacity to make ATP. FCCP is then added and allows the inner mitochondrial membrane to be permeable for protons and maximum electron flux through the electron transport chain to promote maximal respiration. Addition of Rotenone and antimycin A inhibit complex I and III, respectively, to measure spare respiratory capacity. Calculation of the responses by Seahorse allows the measurement of baseline and maximum mitochondrial respiration and ATP synthesis.*

Statistical analysis

Data were analyzed and graphed using the GraphPad Prism 7 software (San Diego, CA). Statistical significance was determined by one-way analysis of variance and Students post-test to obtain p-values. Adjusted *p*-values below 0.05 are considered statistically significant.

Results

RSV infection induces PERK activity and downstream effects on cytokine gene expression in dendritic cells

Previous studies in our laboratory and others have indicated that dendritic cells (DC) are central to driving the pathogenic responses in the lungs of RSV infected animals (31-45) and therefore a better understanding of their activation may provide new therapeutic direction. ER stress responses during infectious processes have been suggested to enhance immunopathology. DCs infected with RSV for 1-24 hours showed little upregulation of IRE1 or PERK (Eif2ak3) mRNA (Figure 1). However, when we examined the activation of critical downstream targets of ER stress activation, CHOP (Ddit3) and XBP1, we observed a significant upregulation of only CHOP but not XBP1, suggesting that the PERK pathway was a primary pathway initiated by RSV infection. These results provided initial data suggesting ER stress was increased during RSV infection of DC and may promote additional activation of the DC.

The PERK activation pathway initiates downstream signaling via phosphorylation of Eif2ak3 as well as increased Nrf2 levels that modify gene expression, both of which were observed at 2 hours post-RSV infection (Figure 2A). When PERK was inhibited (PERKi (5uM, GSK2606414)) phosphorylation of Eif2α compared to total Eif2α was significantly decreased, while Nrf2 levels were also decreased with PERK inhibition but did not reach significance. Nrf2 levels are controlled by ubiquitin-mediated degradation such that there is

high turnover due to its chaperone Keap1 and may be beneficial for infectious disease pathways induced by multiple pathways (46-48). Thus, PERK independent pathways likely promote the activation of Nrf2 stabilization suggesting why PERK inhibition may not be specific for Nrf2. When RSV-infected DCs were treated with the PERK inhibitor (5uM, GSK2606414), the innate cytokine gene expression profiles were also altered (Figure 2B). PERK inhibition during RSV infection resulted in decreased expression of downstream ER stress proteins, atf6 and Chop, with increased expression of critical anti-viral innate cytokines IL-12 and Type-I interferon IFN-β and reduced expression of IL-1β. This altered cytokine expression profile indicated a role for ER stress and PERK in regulating the innate immune response to RSV infection modifying the anti-viral responses.

PERK-deficient DC have altered mitochondrial respiration during RSV infection

In order to further understand how PERK was regulating the innate immune response isolated DC from PERK$^{f/f}$-CD11c Cre- and PERK$^{f/f}$-CD11c Cre+ were grown *in vitro* and assessed for their cellular metabolic differences. Initial analyses assessed whether there were differences in the reactive oxygen species (mROS) that are generated during stress and shown to increase after RSV infection (49, 50). Using Flow cytometry-based Mitosox staining, indicative of mitochondrial dysfunction, increased staining was observed in RSV infected PERK$^{f/f}$-CD11c Cre- (WT) DC and were further significantly increased in PERK$^{f/f}$-CD11c Cre+ (PERK-deficient) DC compared to the WT RSV infected DC (Figure 3A). This indicates that PERK signaling may partially preserve mitochondria function in RSV-infected DCs.

A primary source of ROS generation is the mitochondrial electron transport chain and as it is indicative of dysfunctional mitochondria, we examined mitochondria function in the PERK-deficient DC population compared to WT during RSV infection. Using the Seahorse-based cell Mito Stress Test we found that the oxygen consumption rate (OCR) was

significantly reduced in the PERK-deficient DC at maximal respiration when OXPHOS was uncoupled with FCCP treatment. Relative to WT DCs, maximal respiration OCR in PERK-deficient DCs was further reduced upon RSV infection (Figure 3B & 3C). Importantly, oxygen consumption rate (OCR), a measure of mitochondrial oxidative phosphorylation, was reduced in PERK-deficient DCs relative to WT at baseline only upon RSV infection but not at baseline. This suggests that RSV infection prompted the defective mitochondrial response in PERK-deficient cells. The significant decreased OCR (directly reflective of ATP production capacity to provide for increased cellular use) in the PERK deficient DC resulted in decreased ATP production (Figure 3D). Thus, the absence of PERK to alleviate the ER stress appears to promote increased dysfunctional mitochondria upon RSV infection that results in altered energy balance in DC while at the same time initiates a modified cytokine activation profile.

RSV infection results in PERK-mediated inflammatory cytokine response and mucus production *in vivo* associated with CD4 T cells

In order to more specifically examine the role of PERK in DC and immune activation during RSV infection $PERK^{f/f}$-CD11c Cre- and $PERK^{f/f}$-CD11c Cre+ mice were infected with RSV for eight days. Lung tissue was harvested for histopathology and mRNA expression analyzed. PERK deficient (Cre+) animals exhibited decreased immunopathology, with lung tissue showing less mucus staining and decreased inflammatory cell infiltration compared to PERK sufficient (CRE-) mice (Figure 4A). A blinded assessment of mucus presence in the PAS stained histology slides showed decreased presence of mucus in the PERK deficient animals that is reflective of disease severity with RSV infection (Figure 4A). In addition, when mRNA analysis was performed on whole lung mRNA a reduced expression of IL-1β, IL-13, and IL-17, as well as mucus associated gene *gob5* was observed (Figure 4B). These

data demonstrate that inhibiting PERK activation reduced pathogenesis during RSV infection related to altered cytokine environments.

The local immune environment of the lung was significantly modified in the PERK deficient mice as indicated by both the intensity of the cytokine responses and the presence of mucus. Flow cytometry analysis was used to examine the intensity and severity of the inflammatory response. To identify the immune cell types affected by PERK signaling inhibition in CD11c+ cells during RSV infection, wild type and $PERK_{fl/fl}$-CD11c-Cre mice were infected with RSV for 8 days. Lung tissue was harvested and dispersed for flow cytometry analysis to examine changes in myeloid and T cell populations that have been associated with the severity of RSV-induced pathology (51-55). The examination of DC and granulocyte populations indicated that the inflammatory DC (CD11b+CD11c+) were significantly reduced with the CD103+ DC showing a trend toward reduction (Figure 5A). When examining CD4+ and CD8+ activated T cell numbers there was a marked reduction in both the CD69+ T cell populations in $PERK_{fl/fl}$-CD11c-Cre compared to wildtype mice (Figure 5B). In addition, the ILC2 population that has been associated with increased pathology during RSV infection were reduced but not significantly. In addition, IL-17 and IFN production from the CD4 T cells by intracellular cytokine staining was assessed to examine the outcome of the immune responses. While the number of activated CD4+CD69+ T cells were reduced in the $PERK_{fl/fl}$-CD11c-Cre mice, there was also a shift in the CD4 phenotype with a significant reduction in the IL-17+ and a relative increase in IFNg+ CD4 T cells (Figure 5C). Thus, there are changes in lymphoid cell populations that drive immunopathogenic responses that are differentially regulated by PERK-mediated pathways from DCs that reflect a more appropriate anti-viral immune environment.

Discussion

The present study identifies that DC activation induced by RSV is altered by the ER stress signaling molecule PERK leading to altered cytokine response and immunopathology. Previous studies have shown that viral infection can promote ER stress that leads to the alteration of cellular metabolism (21, 33, 56, 57). In these studies mice with CD11c-specific PERK deficiency have an altered immune phenotype with decreased IL-13 and IL-17 along with reduced mucus production in the lungs of RSV infected mice. IL-13 and IL-17 have been shown to be pathogenic and promote enhanced damage in the lungs of RSV infected mice and associated with disease in infants (9, 58-63). The reduction in IL-13 and IL-17 gene expression in DC targeted PERK-deficient animals is consistent with the reduction in CD4+ T cells in the lungs of infected mice. The diminished IL-17 responses also follow the IL-1 production reduction that can drive Th17 cell differentiation (64, 65). One of the primary mechanisms for ER stress-induced inflammasome activation and inflammatory cytokine production is through NLRP3 activation (57, 66, 67). NLRP3 activity is downstream of PERK associated ER stress and would lead to increased IL-1 that correlates to multiple aspects of these findings (68) (57, 69). IL-1 is a known mediator of Th17 differentiation (64) and promotes inflammation and cytokine production (65, 70, 71). Thus, these studies help to identify several integrated mechanisms during RSV-induced pathology, including 1) the induction of ER stress in CD11c+ DC promotes pathogenic responses, 2) inhibition of PERK attenuates pathogenic innate and acquired immune responses, and 3) the activation of PERK plays a pivotal role in generation of pathogenic T cell immune responses. Importantly, the reduced inflammation and pathology is accompanied by a shift in T cell cytokines that suggest a more appropriate anti-viral response.

ER stress can be induced in a number of scenarios including the overproduction of ROS, bacterial toxins, unfolded protein response (UPR), and during viral infections. The ER

stress response attempts to bring the cellular process back to homeostasis by activating a number of protein pathways to address different aspects of the dysregulation, including IRE1 and PERK. Our data have previously shown that under conditions where autophagy is blocked (with LC3b-/- mice) the IRE1 pathway appears to be dominant and promotes the activation of similar pathologic processes as those identified as targets of PERK, Th2/Th17 and inflammasome-induced IL-1 (33). Targeting IL-1 (with soluble IL-1Ra receptor antagonist) in those latter studies reduced the Th2/Th17 acquired immune response and attenuated lung pathology. In the present study PERK, which activates *nrf2* and *elf2a*, appears to be a prominent inducer of the downstream activation pathways that promote disease when autophagy is functional. The activation of these targets by PERK is responsible for translational arrest associated with unfolded protein responses (UPR) and allows the suppression of a number of innate cytokines (75). Interestingly, in our *in vitro* studies the inhibition of PERK led to increased production of a number innate cytokines, including IFNβ and IL-12 that are critical anti-viral innate cytokines, while IL-1 expression was decreased. The outcome was reduced pathology and a relative shift to fewer IL-17 and more IFN producing CD4 T cells in the lungs. Thus, the effects observed *in vivo* with the CD11c specific PERK deficient animals is consistent with the *in vitro* inhibition experiments. PERK-induced IL-1 is likely associated with inflammasome activation through eif2α phosphorylation. Phosphorylation of eif2α leads to the dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin (a hallmark event of oxidative stress), and thereby facilitates ER stress-mediated inflammasome activation (74, 75). Importantly, without PERK there is a reduction in mitochondria function and ATP production that disrupts the metabolism of RSV-infected DC, thereby altering innate cytokine responses. Interestingly, these findings demonstrate that while PERK is required for alleviating ER stress and optimizing mitochondrial ATP production, the PERK signaling pathway eif2α may also drive

unwanted changes in DC. Further studies are needed to explore the mitochondrial and metabolic changes in DCs during each stage of RSV infection, and the mechanisms that link DC metabolism to ER stress responses and innate immune regulation.

The CD11c-specific PERK deficiency appeared to have broad consequences on cytokine gene expression profiles, even though this was a DC/CD11c targeted deficiency rather than a full body knockout. This indicates that PERK is likely upstream from a number of immune signaling/activation processes. The lung cell analysis studies demonstrated that DC-targeted PERK-deficient animals had reduced accumulation of activated T cells. In addition, there are indirect pathways implicated in other studies of PERK signaling and immunity. For example, PERK deficiency can decrease the degradation of the type I interferon receptor, IFNAR1, thus increasing type I interferon signaling leading to more efficient viral clearance (78). RSV infection hampers the anti-viral immune response by dampening type I interferon signaling (79, 80), possibly through the induction of ER stress responses. Thus, by targeting PERK-induced ER stress the resulting increased type I IFN and IL-12 could promote a more beneficial immune environment exemplified by decreased IL-17 and increased IFNg producing T cells in the lung. This latter effect was observed both *in vitro* and *in vivo* with isolated DC that are a driving mechanism to the *in vivo* effect on T cells in the CD11c targeted deletion of PERK. Together, our studies demonstrate that the inhibition of PERK in dendritic cells results in a reduction of inflammation and mucus production in the lung. The inhibition of this pathway may provide a novel therapeutic target for mitigating the damaging airway inflammation induced by RSV infection and possibly subsequent disease sequelae through management of the overall immune responses.

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Figure legends

Figure 1- Bone marrow-derived DC (BMDC) infected by RSV upregulate CHOP expression related to the PERK pathway. BMDC were infected by RSV (MOI=1.0) and mRNA was isolated in a temporal fashion to examine the expression of ER stress related proteins and their downstream targets XBP-1 and CHOP for IRE-1 and PERK, respectively. Quantitative PCR was used to assess the significant increase each of mRNA targets and compared to time 0 expression levels at each of the 5 time points. Data represent the mean ± SE of 3 repeated independent BMDC cell lines.

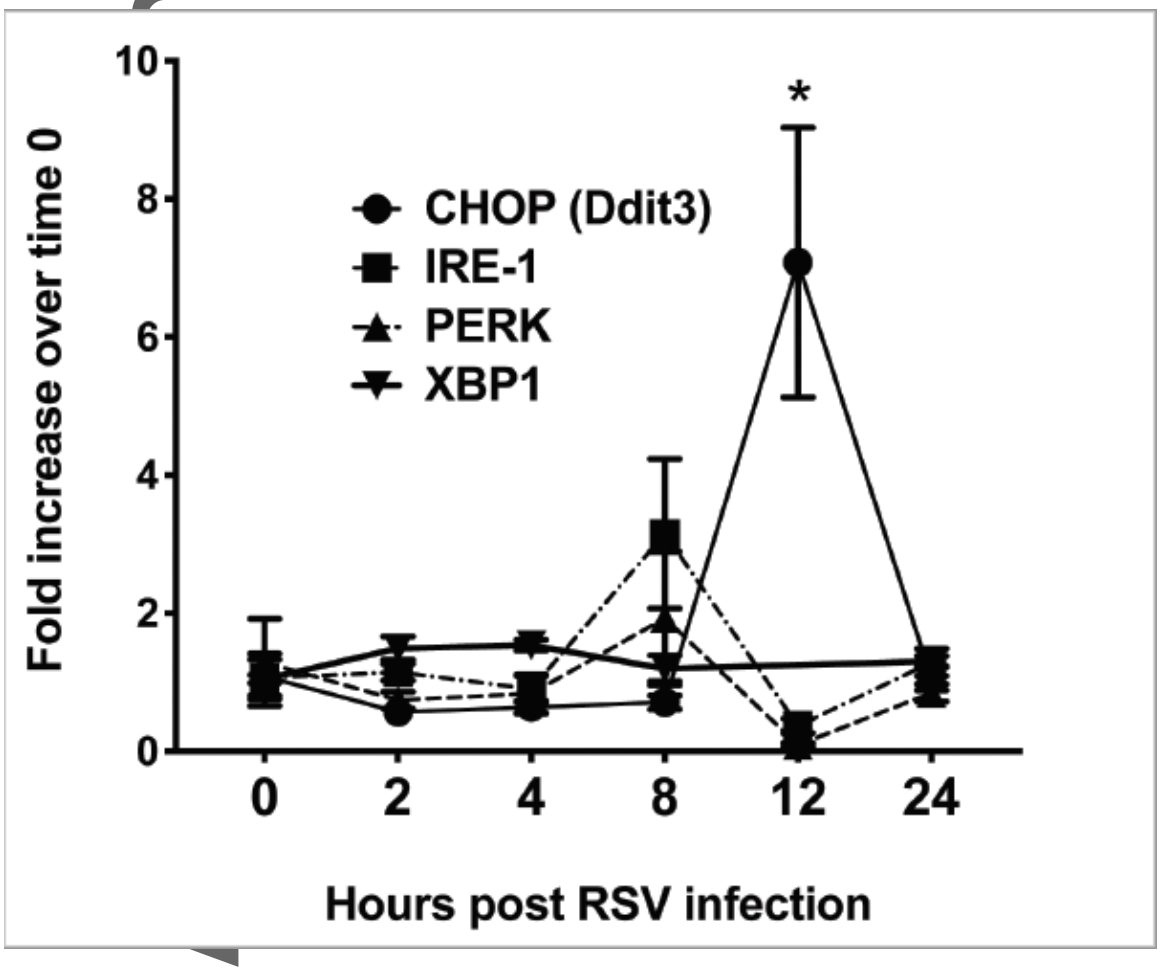


Figure 2- RSV infection induces phosphorylation of eif2a and increased Nrf2 in BMDC cultures: PERK inhibition alters innate cytokine expression. (A) BMDC grown from naïve mice were infected with RSV (MOI= 1.0) with or without PERK inhibitor (5uM, GSK2606414) were examined by Western blot for the ratio of phosphorylation of eif2α/total eif2α and ratio of Nrf2/βActin after 24 hrs of infection. Data are expressed in bar graphs as mean ± SE from the 3 repeat cultures of 10^6 BMDCs. (B) The expression of downstream ER stress proteins and cytokines in RSV infected DC are altered by inhibition of PERK correlating to eif2a phosphorylation. Data represent mean ± SE from 3 repeat cultures.

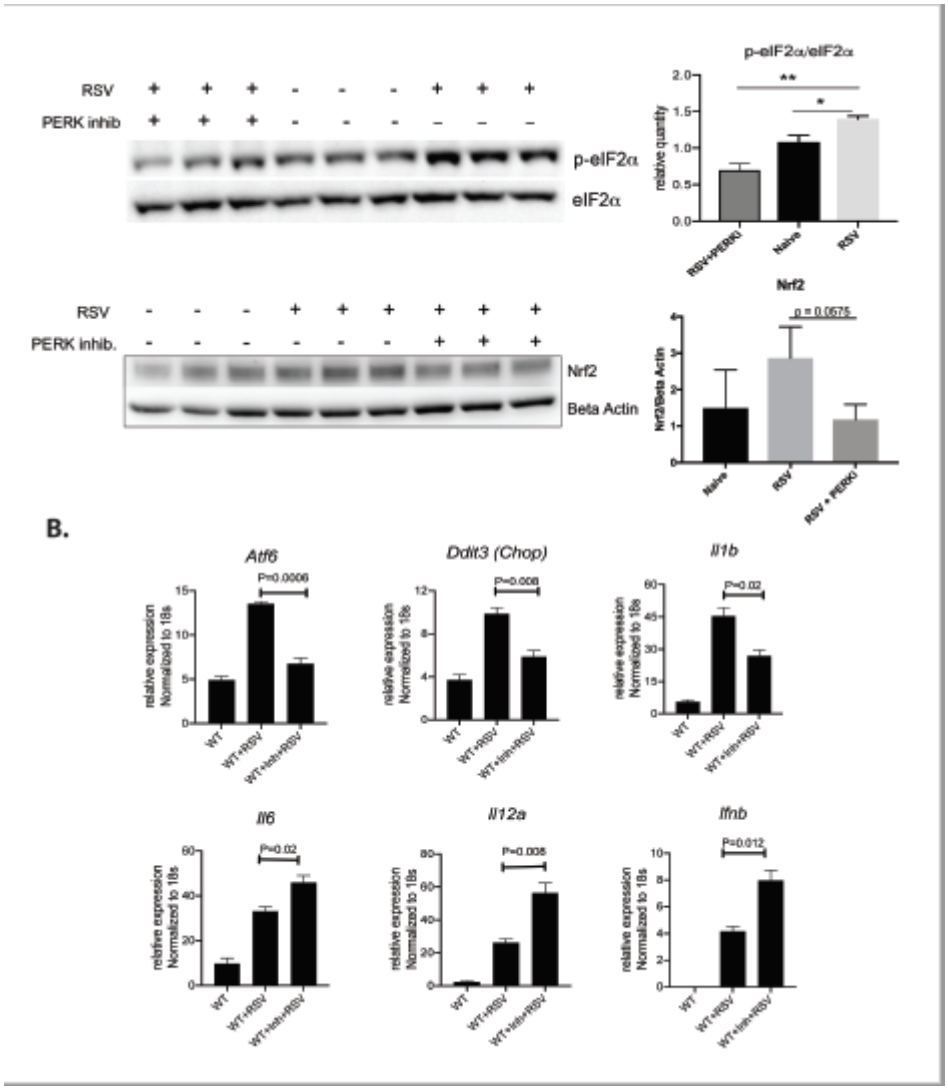


Figure 3- The deletion of PERK in RSV infected BMDC alters mitochondria stabilization and function. (A) BMDC from PERK$^{f/f}$-CD11c Cre+ mice (PERK-/-) were stained for ROS expression using Mitosox and assessed by Flow cytometry for increased fluorescence. (B-D) Seahorse-based cell Mito Stress Test was used to assess WT or PERK-/- BMDC with or without RSV infection at baseline and with maximum mitochondrial stress (FCCP). (E) ATP production was calculated based upon the Seahorse analysis with or without RSV infection in the WT and PERK-/- BMDC. Data represents mean ± SE from 3 repeated cell lines.

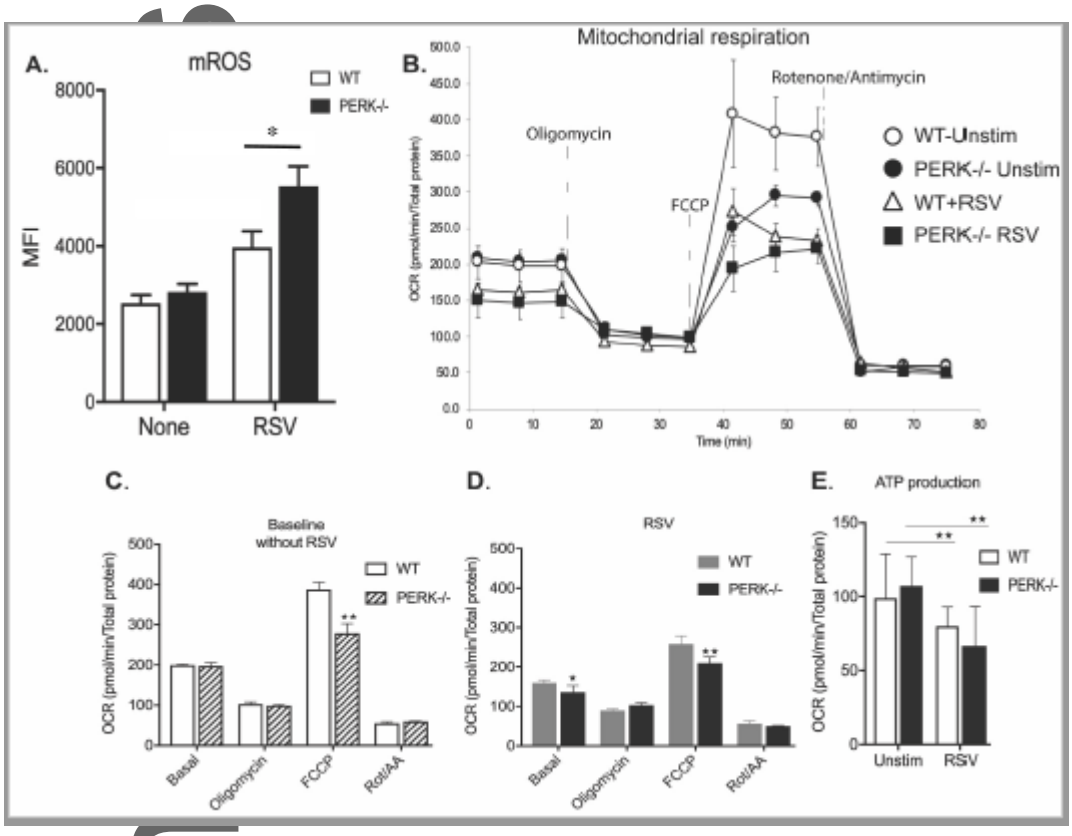
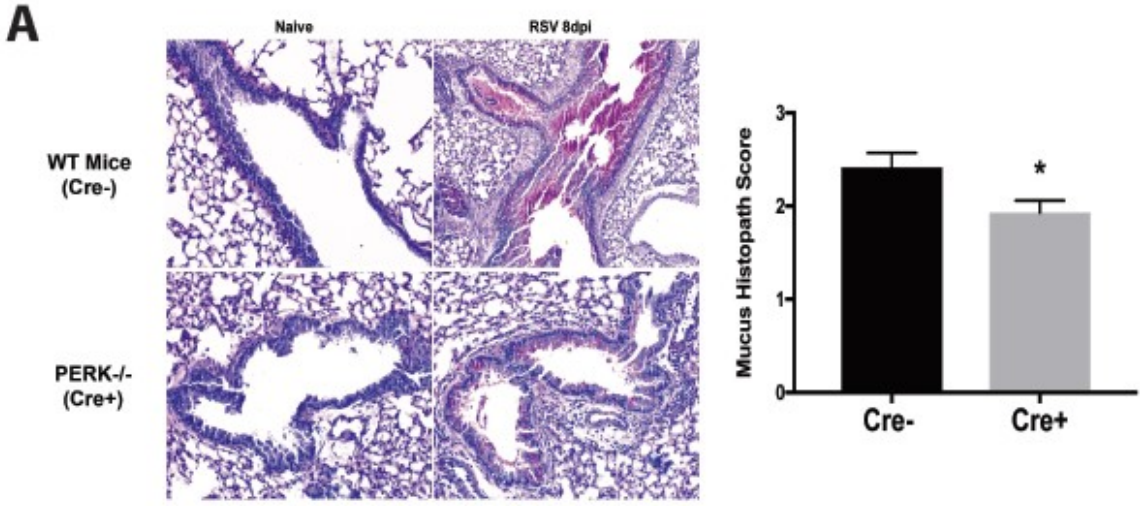


Figure 4- PERK deficient animals have reduced RSV-associated lung pathology and altered cytokine responses. (A) WT ($PERK^{f/f}$-CD11c Cre-) or PERK -/- ($PERK^{f/f}$-CD11cCre+) mice were infected with RSV (2 x 10^5 pfu) and harvested on day 8 of infection with lungs stained for histology with periodic acid Schiff (PAS) stain (red) for mucus. Sections are representative of 5 mice/group. As an indication of the mucus production, a blinded assessment of the slides was done based upon a 4 point scale with 1 being no mucus to 4 being completely filled airways (see Materials and Methods). (B) The left lobe of the lung was processed for mRNA analysis for key cytokine genes, IL-13, IL-17 and IL-1 along with goblet/mucus associated gob5 expression. Data represent mean ± SE of fold increase relative to uninfected mice of 5 mice/group.



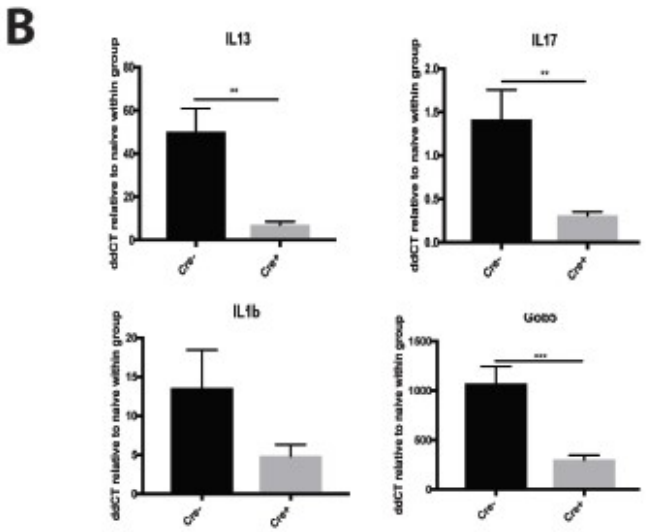
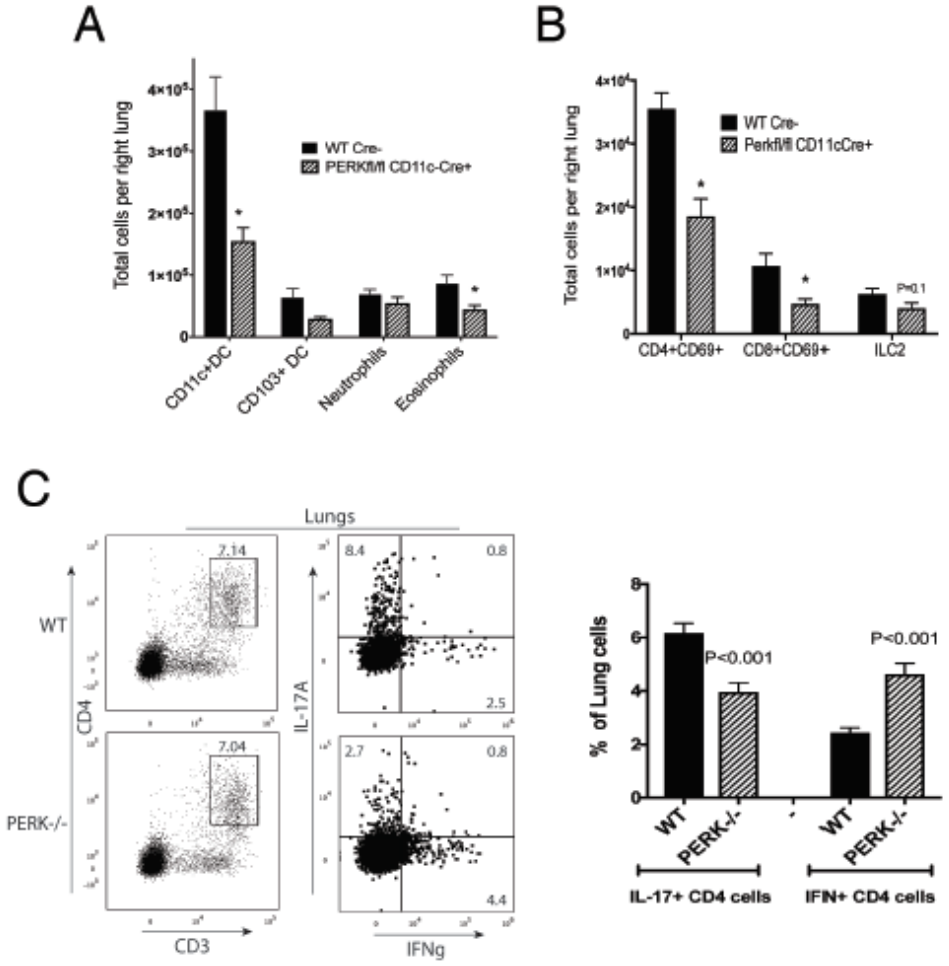


Figure 5- Accumulation of activated T cell populations are reduced in PERK-/- mice infected with RSV. Uninfected and RSV infected WT and PERK-/- mice were examined for accumulation of specific innate and acquired cell populations. Lungs from 8-day infected mice were dispersed by enzyme digestion into single cell suspensions and stained for specific cell populations for flow cytometry analysis using antibodies as described in the Materials and Methods. A) Myeloid cell populations were examined by flow cytometry including primary DC populations and granulocyte. B) Activated CD4 and CD8 T cell populations as well as ILC2 were assessed by flow cytometry as described in the Methods. C) Intracellular staining for IL-17 and IFNγ in CD4+ T cells from lungs of RSV-infected mice. Data represents mean ± SE from 5 mice/group.



Graphical Abstract