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Defining Lipidomic Responses to Coronavirus Infection

September 2022

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U.S. DEPARTMENT OF ENERGY

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Abstract

Highly pathogenic human coronavirus infection can cause a severe atypical, rapid onset pneumonia with a mortality rate of up to 10% for severe acute respiratory syndrome coronavirus 1 (SARS-CoV 1), 35% for Middle East respiratory syndrome coronavirus (MERS-CoV), or 1% for severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2 causative agent of COVID 19). While medical countermeasures successfully controlled the worldwide SARS-CoV epidemic, the MERS-CoV epidemic is still ongoing and continues to be a concern for travelers in the Middle East and the multi-year SARS-CoV 2 pandemic underscore the importance of defining biomarkers that are diagnostic and/or predictive of severe disease outcomes for respiratory viruses. Systems biology approaches provide global snapshots of infection induced changes in host cells/tissues and provide extremely rich datasets for understanding host pathogen interactions. Metabolites, especially lipids, are critical for viral replication but less is understood about infection induced changes to lipids due to limits in lipid species detection and identification. To characterize how individual lipid species and proteins with lipid associated functions contribute to highly pathogenic human coronavirus replication and disease severity, existing datasets were probed to determine cell type specific lipid responses to MERS-CoV infection and verification studies were performed to determine if modification of the host lipid signature (by inhibiting enzymatic functions that produce specific lipid species) can perturb CoV replication in human lung cells. MERS-CoV infects human lung epithelial, endothelial and fibroblast cells. All three cell types were infected with MERS-CoV and samples collected to analyze lipids, proteins, metabolites, and transcripts from 12 to 48 hours post infection. Time matched mock-infected cells were collected in parallel for each cell type. Following sample and statistical analysis, functional enrichment and bioinformatic analysis was performed to identify differentially expressed lipids and proteins. Two lipid species were found to be significantly upregulated following MERS-CoV infection, ceramides, and triacylglycerol both of which are indicative of cells undergoing apoptotic cell death. In contrast, sphingomyelins (lipid molecules that can serve as a precursor for one pathway for ceramide synthesis) had significantly decreased expression in MERS-CoV infected cells. An inhibitor of acid sphingomyelinase (that converts sphingomyelin to ceramides and phosphorylcholine) reduced MERS-CoV replication suggesting that production of ceramides is key for successful viral replication and transmission. Acyl-CoA-synthetase 3 (ACSL3), the only protein whose function is lipid associated and had increased differential expression in our dataset, regulates the synthesis of triacylglycerol (increased expression). Inhibitors that directly block ACSL3 (Triacsin C) but not steps later in the triacylglycerol synthesis pathway (Etomoxir) inhibit MERS-CoV replication suggesting that triacylglycerol production and/or ACSL3 activity is also key for viral replication. ACSL3 expression is also upregulated in lung cancer cells and is predicted to promote continued cell viability which would also enhance viral replication. The differentially expressed lipid and lipid-associated protein species identified in our studies suggest that MERS-CoV infection is activating cellular death pathways to limit the number of cells that become infected but also stimulating the production of lipid-associated enzymes that can prolong host cell viability and the amount of time progeny virions can be produced and released. As the inhibitors that worked against MERS-CoV infection were also efficacious against SARS-CoV 2 infection, countermeasures that target these host pathways may provide novel ways to block highly pathogenic human coronavirus infection and/or prevent severe disease outcomes.

Acknowledgments

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1.0 Introduction

Highly pathogenic human coronaviruses cause a severe atypical, rapid onset pneumonia in infected patients with a mortality rate of up to 10% for severe acute respiratory syndrome coronavirus 1 (SARS-CoV 1), 35% for Middle East respiratory syndrome coronavirus (MERS-CoV, (Melo-Filho et al. 2022)), or 1% for severe acute respiratory syndrome coronavirus 2 (SARS-CoV causative agent of COVID 19). While medical countermeasures successfully controlled the worldwide SARS-CoV epidemic, the MERS-CoV epidemic is still ongoing and continues to be a concern for travelers in the Middle East and the multi-year SARS-CoV 2 pandemic underscore the importance of defining biomarkers that are diagnostic and/or predictive of severe disease outcomes for respiratory viruses. Improved surveillance methods have identified an ever-growing number of SARS- and MERS-like-CoV strains in a variety of bat species (Ruiz-Aravena et al. 2022) and recent studies have identified several strains with expanded host range that can replicate in primary human respiratory cells suggesting that multiple, novel highly pathogenic human coronavirus could be poised to jump into humans under the right conditions. Strains of MERS-CoV-like viruses are found in camels throughout the Middle East, even in countries with few reported MERS cases in humans which may represent another potential zoonotic reservoir with the potential to launch another epidemic (Peiris and Perlman 2022). The identification of biomarkers or host signatures that indicate disease severity would improve response time for novel respiratory pathogens which in turn would reduce the likelihood of another epidemic.

Systems biology approaches provide global snapshots of infection induced changes in host cells/tissues and provide extremely rich datasets for understanding host pathogen interactions. Identification of transcripts and proteins with increased differential expression provide clues to the cellular pathways that are being activated post infection but do not capture all pathogen induced changes. Levels of metabolites, especially lipids, are critical for viral replication but less is understood about infection induced changes to lipids due to limits in lipid species detection and identification. Human coronavirus infection causes rearrangement of host intracellular membranes (comprised of a variety of lipid species) early in infection to establish viral replication complexes to protect nascent viral RNA species and later in infection host cell membranes in the intermediate compartment between the endoplasmic reticulum and Golgi apparatus are constantly turning over as these membranes form the outer membrane of progeny viral particles (Doyle et al. 2021; Sims et al. 2021). In addition, lipids are key signaling molecules and are likely to play a variety of roles during virus infection.

To characterize how individual lipid species and proteins with lipid associated functions contribute to highly pathogenic human coronavirus replication and cytopathic effect existing datasets were probed to determine cell type specific lipid responses to MERS-CoV infection and verification studies were performed to determine if modification of the host lipid signature (by inhibiting enzymatic functions that produce specific lipid species) can perturb CoV replication human lung cells. All lipid host signatures identified will strengthen the BioRisk Beyond the List Directorate Objective host response database and support for the use of host response to unidentified pathogens to predict disease severity.

2.0 Results and Discussion

2.1 MERS-CoV Induces Apoptosis in Primary Human Lung Endothelial Cells

MERS-CoV infection of epithelial, endothelial and fibroblast cells within the human lung produces large amounts of virus (titers great than 10e7) by 24 hours post infection, however only endothelial cells die within 48 hours. To identify cellular pathways leading to MERS-CoV induced endothelial cell death, experiments in mock-infected and MERS-CoV infected primary human lung cells were designed to capture transcriptomic, proteomic, metabolomic and lipidomic samples over time to identify host pathways that are critical for MERS-CoV replication. Duplicate samples were harvested from mock-infected and MERS-CoV infected primary human lung epithelial, endothelial and lung fibroblasts every 12 hours over a 48-hour time course from a total of three donors. The first set of samples were harvested to collect total RNA for microarray analysis while the second set of samples were harvested in a 2:1 mixture of chloroform methanol to collect proteins, metabolites, and lipids for further analysis. The findings discussed below were published and all results can be found here (Sims et al. 2021). Despite similar viral replication kinetics, numbers of infected cells and rates of infection, MERS-CoV infected endothelial cells demonstrated significant virus induced cell death, while infected primary human lung fibroblast monolayers were viable for the 48 hour infection time course. Functional enrichment analysis of the transcriptomic, proteomic and lipidomic datasets suggested that MERS-CoV infected endothelial cells were dying via an unfolded protein response (UPR) driven apoptotic mechanism. Verification studies confirmed activation of death caspases supporting endothelial cell death via apoptotic pathway(s). Treatment of MERS-CoV infected endothelial cells and fibroblasts with an UPR specific inhibitor reduced levels of viral replication (in vitro) and prophylactic treatment of MERS-CoV infected mice reduced disease severity in a mouse model of MERS-CoV induced lung disease supporting the importance of the UPR for MERS-CoV replication and disease outcomes. The death of MERS-CoV infected endothelial cells may represent the first breach of the epithelial-endothelial barrier and allow for disease progression to acute respiratory distress syndrome (ARDS) in infected individuals. Defining the differentially expressed host signatures for individual primary host cell types following infection with highly pathogenic human coronaviruses will provide critical clues for how each cell type responds to infection and likely identify novel host targets for countermeasure development and evaluation.

2.2 Key Lipid Modifying Enzymes for MERS-CoV Infection

MERS-CoV infection alters the detectable quantities of three predominant species of lipids: ceramides, sphingomyelins, and triacylglycerol. To determine if increases in these lipid species were critical for MERS-CoV replication, inhibitors targeting enzymatic functions that produce these lipid species were used to treat MERS-CoV infected cells and levels of viral replication assessed. As described above, samples to determine differentially expressed lipids, proteins, metabolites, and transcripts were collected from MERS-CoV and mock-infected primary lung epithelial cells, endothelial cells and fibroblasts derived from three human donor lungs. Following sample and statistical analysis, functional enrichment and bioinformatic analysis was performed to identify differentially expressed lipids and proteins with lipid associated functions. Two lipid species were found to be significantly upregulated following MERS-CoV infection, ceramides and triacylglycerol (predominantly in MERS-CoV infected microvascular endothelial cells for all three donors) both of which are indicative of cells undergoing apoptotic cell death. In contrast, sphingomyelins, lipid molecules that can serve as a precursor for ceramide synthesis,

had significantly decreased expression in MERS-CoV infected microvascular endothelial cells in all donors but decreased expression was also observed in the other two cell types. Ceramide synthesis can occur via conversion of sphingomyelin to ceramide (and phosphorylcholine (Kornhuber et al. 2010)) or can be synthesized by a number of reactions de novo in the endoplasmic reticulum starting with the activity of serine palmitoyltransferase to combine serine and palmitoyl-CoA (Kurek et al. 2014). To verify the importance of ceramide production during MERS-CoV and to determine if ceramide production occurred predominantly in the lysosome (acid sphingomyelinase functional inhibitors of acid sphingomyelinase FIASMA drugs (Kornhuber et al. 2010)) or in the endoplasmic reticulum (serine palmitoyltransferase myriocin (Kurek et al. 2014)) dose response curves of inhibitors specific for each ceramide production pathway were added to MERS-CoV infected human lung cells and levels of nanoluciferase expression monitored as a surrogate for viral replication using published methods (Table 1 (Sims et al. 2021)). MERS-CoV induced expression of nanoluciferase was reduced following treatment with the FIASMA inhibitor, Desipramine, but not the inhibitor of de novo synthesis, suggesting that acid sphingomyelinases in the lysosome mediate the increased synthesis of ceramide following infection (Table 1). Interestingly, SARS-CoV 2 induced nanoluciferase expression was reduced following treatment with two of the FIASMA inhibitors, Amitriptyline and Desipramine (Table 1) suggesting that the increase levels of ceramide produced in SARS-CoV 2 infected cells (Farley et al. 2022) may also be occurring in lysosomes and mediated by acid sphingomyelinases. For both MERS-CoV and SARS-CoV 2 preventing ceramide production blocks a key pathway required for viral replication.

Table 1. Ceramide Production Inhibitors. Cells were infected with MERS-CoV (Calu3 cells) or SARS-CoV 2 (Vero E6 cells) expressing nanoluciferase and serial dilutions of the inhibitors listed below were added at the time of infection. Drug induced cytotoxicity was measured in uninfected cells using Promega's CellTiterGlo kit according to manufacturer's recommendations at 24 and 48 hours post treatment. Nanoluciferase expression was monitored as a surrogate for infection and was assayed using Promega's NanoGlo kit according to manufacturer's recommendations at 24 and 48 hours post infection. Data below is representative of at least two experiments. ND not done

Inhibitor	*Class*	*Inhibits MERS-CoV*	*Inhibits SARS-CoV 2*	*Cytotoxic*
Amitriptyline	FIASMA	No	15 to 25 μM	>25 μM
Imipramine	FIASMA	No	ND	50 μM
Desipramine	FIASMA	15 to 25 μM	15 to 25 μM	>25 μM
Myriocin	De novo	No	ND	>25 μM

Analysis of proteomics data collected from the MERS-CoV infected primary lung cells revealed that only one lipid associated protein had increased differential expression in more than one cell type and donor. The protein, acyl-coenzyme A synthase long chain fatty acid family member 3 (ACSL3), is the first enzyme in the pathway to convert long chain fatty acids to triacylglycerol by fatty acid oxidation. This was particularly interesting as triacylglycerol was the second lipid species with increased abundance following MERS-CoV and SARS-CoV 2 infection (Farley et al. 2022; Sims et al. 2021). ACSL3 converts long chain fatty acids to acyl-CoA thioesters and its activity is blocked by the inhibitor Triacsin C. The next step in the fatty acid oxidation pathway is conversion of acyl-CoA thioesters to acyl carnitines by carnitine palmitoyltransferase-1 is

blocked by the inhibitor Etomoxir. Inhibitor assays as described above were employed to determine if these pathways were critical for MERS-CoV replication. Inhibitors that directly block ACSL3 (Triacsin C) but not steps later in the pathway (Etomoxir) inhibit MERS-CoV replication suggesting that triacylglycerol production and/or ACSL3 activity is also key for viral replication (Table 2). ACSL3 expression is also upregulated in lung cancer and is predicted to ensure the availability of sufficient energy sources in cells to promote continued cell viability which would also enhance viral replication. The differentially expressed lipid and lipid-associated protein species identified in our studies suggest that MERS-CoV infection is activating cellular death pathways to limit the number of cells that become infected but also stimulating the production of lipid-associated enzymes that can prolong the time progeny virions can be produced and released. As the inhibitors that worked against MERS-CoV infection were also efficacious against SARS-CoV 2 infection, countermeasures that target these host pathways may provide novel ways to block highly pathogenic human coronavirus infection and/or prevent severe disease outcomes.

Table 2. **ACSL3 Inhibitors.** Cells were infected with MERS-CoV (Calu3 cells) or SARS-CoV 2 (Vero E6 cells) expressing nanoluciferase and serial dilutions of the inhibitors listed below were added at the time of infection. Drug induced cytotoxicity was measured in uninfected cells using Promega's CellTiterGlo kit according to manufacturer's recommendations at 24 and 48 hours post treatment. Nanoluciferase expression was monitored as a surrogate for infection and was assayed using Promega's NanoGlo kit according to manufacturer's recommendations at 24 and 48 hours post infection. Data below is representative of at least two experiments. ND not done

Inhibitor	*Class*	*Inhibits MERS-CoV*	*Inhibits SARS-CoV 2*	*Cytotoxic*
Triacsin C	ACSL3 Pathway	5 to 25 μM	10 to 25 μM	>25 μM
Etomoxir	ACSL3 Pathway	No	No	>25 μM

2.3 PALLID (Protein and Lipid Linkage for Integration and Directionality) Improving Software to Facilitate Systems Biology Analysis of Lipidomics Datasets

An analysis tool designed to link differentially expressed lipid species with lipid associated/modifying proteins was evaluated and improved as a part of our project. New analysis tools for lipidomics are required for a variety of reasons. The number of lipid species and isomers is very large making database only searches impractical. Lipid modifying enzymes can act on a variety of lipid species making it difficult to use only a substrate or product-based database. PALLID (**P**rotein **a**nd **L**ipid **L**inkage for **I**ntegration with **D**irectionality) links individual differentially expressed lipid species to co-expressed lipid modifying enzymes. Linkage is only identified where directionality is consistent with enzymatic activity, e.g., both lipid product(s) and enzyme expression must be upregulated in their respective datasets.

PALLID consists of 1) a database of linkages between enzymes and specific traits of individual lipid molecules, and 2) a software tool that matches observed lipid traits and enzyme identifiers in data from matched protein/lipid samples. PALLID software scans the protein data from each individual sample for lipid-related identifiers and compares all lipid species within the same

sample with activity specifications for matched enzymes for that sample. Importantly, matches are not considered valid unless they are directionally consistent. For example, if enzyme X is known to recognize lipid A as substrate and convert it to lipid B, then matches of both lipids to enzyme X are only made when expression of enzyme X is up-regulated, lipid A down-regulated, and lipid B up-regulated. Conversely, if enzyme X expression is decreased in comparison to non-perturbed samples, then its activity would be assumed to be decreased and linkage would require expression of lipid A to increase and lipid B to decrease.

2.4 Analysis of SARS-CoV 2 Patient Samples from an Early Pandemic Patient Cohort

In collaboration with the University of Wisconsin patient plasma samples from 151 donors were analyzed to identify changes in immune pathology specific to mild and severe cases of COVID-19. 77 samples were collected from mild cases with just positive COVID19 tests, 34 samples were collected from patients with severe cases that required hospitalization and 40 samples were collected from age matched healthy controls. Plasma was immunodepleted to remove abundant proteins and improve levels of detection for less abundance proteins. Distinct sets of differentially expressed proteins and metabolites were identified in samples derived from mild and severe cases in comparison to the samples from healthy donors which is consistent with previously published studies. Differentially expressed proteins and metabolites from patients with mild cases (COVID19 positive test but did not require hospitalization) suggested that responses to infection dampening oxidative stress and limiting the formation of neutrophil extracellular traps could lessen or prevent severe COVID19 disease outcomes. These results are described in the draft manuscript in the final stage of revision which has been sent back to the reviewers for final approval (Bramer et. al. “Multi-omics of NET Formation and Correlations with CNDP1, PSPB, and L-Cystine Levels in Severe and Mild COVID-19 Infections”).

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