

1 Lung epithelial cells have virus-specific and shared gene expression responses 2 to infection by diverse respiratory viruses

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9 **Abstract:** The severity and outcome of respiratory viral infections is partially determined by the cellular
10 response mounted by infected lung epithelial cells. Disease prevention and treatment is dependent on our
11 understanding of the shared and unique responses elicited by diverse viruses, yet few studies compare host
12 responses to different viruses while controlling other experimental parameters. We used microarray analysis
13 to compare changes in gene expression of murine lung epithelial cells infected individually by three respiratory
14 viruses causing mild (rhinovirus, RV1B), moderate (coronavirus, MHV-1), and severe (influenza A virus,
15 PR8) disease in mice. RV1B infection caused numerous gene expression changes, but the differential effect
16 peaked at 12 hours post-infection. PR8 altered an intermediate number of genes whose expression continued
17 to change through 24 hours. MHV-1 had comparatively few effects on host gene expression. The viruses
18 elicited highly overlapping responses in antiviral genes, though MHV-1 induced a lower type I interferon
19 response than the other two viruses. Signature genes were identified for each virus and included host defense
20 genes for PR8, tissue remodeling genes for RV1B, and transcription factors for MHV-1. Our comparative
21 approach identified universal and specific transcriptional signatures of virus infection that can be used to
22 discover mechanisms of pathogenesis in the respiratory tract.

23 1. Introduction

24 Viruses from several different families, including *Picornaviridae*, *Orthomyxoviridae*, *Paramyxoviridae*,
25 *Coronaviridae*, and *Adenoviridae*, infect and cause diseases in the respiratory tract. These diseases range from
26 mild infections of the upper respiratory tract to severe diseases when infecting the lungs. Respiratory viruses
27 commonly target epithelial cells of the airways and lungs. These epithelial cells are responsible for detecting
28 viral pathogens and initiating antiviral responses at the level of infected cells and the immune system, and
29 therefore their response to infection has an important role in determining disease outcomes. Knowledge of the
30 shared and virus-specific responses of respiratory epithelial cells to infection by diverse viruses is fundamental
31 to understanding viral pathogenesis and developing therapies to treat severe respiratory infections.

32 Murine models of respiratory viral infections have been widely used to identify the mechanisms that
33 determine disease severity in the respiratory tract. While these models are invaluable for evaluating pathology
34 and host responses to infection, parallel *in vitro* studies can be used to identify gene expression and signaling
35 pathway changes that occur in infected cells to mediate pathogenesis. In this study, we compare the gene
36 expression response of murine lung epithelial cells to infection by three respiratory viruses used in murine
37 models: rhinovirus (serotype RV1B) in the family *Picornaviridae*, mouse hepatitis virus (MHV strain 1) in the
38 family *Coronaviridae*, and influenza A virus (strain PR8) in the family *Orthomyxoviridae*. Viruses from
39 different families and with different replication strategies were chosen to identify which responses to infection
40 in respiratory epithelial cells are shared and which are virus-specific. In the following paragraphs, we describe
41 some of the key features of these three viruses in murine models.

42 Minor group rhinoviruses, including RV1B, use low-density lipoprotein receptor to enter either human or
43 murine cells. Because RV1B can replicate in mouse cells, it has been used to study immune responses and/or
44 mechanisms of asthma exacerbation in infected mice [1-5]. Intranasal inoculation of mice with a high dose of
45 RV1B results in viral replication and an early inflammatory response in the respiratory tract without clinical
46 signs of disease [1, 3-5]. RV1B antigens have been detected in cells of the epithelia and lamina propria of the
47 upper respiratory tract of infected mice [2, 4].

48 MHV-1 is a strain of mouse hepatitis virus that preferentially replicates and causes disease in the
49 respiratory tract of specific mouse strains and thus serves as a model for respiratory coronaviral infections [6,
50 7]. MHV-1 causes severe disease in A/J-strain mice and milder pathology in other mouse strains [6, 8]. Mouse
51 strain-dependent disease severity corresponds to inflammatory responses, fibrin deposition, and reduced type I
52 interferon (IFN) production [6]. Further, type I IFN-mediated signaling is required for protection from severe
53 disease during MHV-1 infection of resistant mice [8]. MHV-1 has been detected in pulmonary macrophages of
54 infected mice, but has not been reported to infect respiratory epithelial cells *in vivo* [6]. Although primary
55 murine alveolar epithelial cells are susceptible to MHV-1 infection *in vitro*, their role during *in vivo* infection
56 is not clear [9].

57 Mice have been used for decades to study the pathogenesis of influenza viral disease. One of the most
58 commonly used strains, PR8, has been serially passaged in mice to produce a model for pulmonary infection.
59 PR8 infection results in a range of disease severities that is mouse strain-dependent [10]. Although susceptible
60 mice mount a type I IFN response to PR8 infection, lethal infection is associated with spread of virus to the
61 alveoli and an excessive inflammatory response [10-13]. PR8 replicates in bronchiolar and alveolar epithelial
62 cells of the lower respiratory tract *in vivo* and in primary murine respiratory epithelial cells *in vitro* (Blazewicka
63 et al., 2011; [9, 14, 15].

64 We used a murine lung epithelial cell line (LA4) to compare the gene expression response to these three
65 unrelated viruses. LA4 cells were derived from neoplastic lung epithelia from strain A (A/He) mice and have
66 some properties of alveolar type II cells [16]. Strain A (A/J) mice are highly susceptible to respiratory viral
67 infections, including MHV-1 and influenza A viruses [6, 10]. Other studies have demonstrated that LA4 cells
68 are susceptible to infection by PR8 and RV1B [15, 17]. In this study, we show that LA4 cells are also susceptible
69 to infection by MHV-1 (hereafter referred to as MHV). The gene expression response of LA4 cells to infection
70 by MHV, PR8, and RV1B (hereafter referred to as RV) differed in timing and magnitude of the changes. While
71 we expected to see highly divergent transcription responses to these three viruses, they induced expression of a
72 large overlapping set of shared genes, including genes involved in antiviral responses. Each virus also altered
73 expression of unique genes, which highlight their different replication strategies and mechanisms of
74 pathogenesis in murine hosts.

75 2. Materials and Methods

76 2.1. Virus stocks and cell lines

77 PR8 (A/Puerto Rico/8/1934 (H1N1)), obtained from BEI Resources (NR-3169), was grown and titrated
78 by plaque assay in MDCK (ATCC: CCL-34) cells. MHV, obtained from ATCC (VR-261), was grown and
79 titrated by plaque assay in 17Cl.1 cells [18] (provided by Dr. Kathryn Holmes, University of Colorado Denver
80 School of Medicine). RV, obtained from ATCC (VR-1645), was grown and titrated by tissue culture infectious
81 dose 50% (TCID₅₀) assay in HeLa cells (ATCC: CCL-2). LA4 (ATCC: CCL-196), a murine lung epithelial cell
82 line, was cultured in Ham's F12K medium (Mediatech, Manassas, VA).
83

84 2.2. Epithelial cell infection and microarray sample

85 Our experimental approach was to infect LA4 cells with the three viruses at times t=0 h and t=12 h and
86 harvest RNA for microarray analysis at t=24 h. Controls were mock-inoculated at both time points. Preliminary
87 experiments were done to establish a multiplicity of infection (MOI) for each virus that resulted in comparable
88 numbers of cells positive for viral antigen at 24 h post-infection (Figure S1). Based on this, LA4 cells were
89 inoculated with 3 TCID₅₀/cell RV, 1 PFU/cell PR8, or 3 PFU/cell MHV. Triplicate wells of LA4 cells in 6-well
90 plates were inoculated with each virus diluted in serum-free medium or were mock-inoculated with serum-free
91 medium for 1 h at 37°C. Viral inocula were removed and the cells were rinsed twice with serum-free medium.
92 The cells were incubated in Ham's F12K medium with 2% FBS for 12 or 24 h, at which time RNA was isolated
93 from cell cultures using an RNAsasy Plus kit (QIAGEN) and transcript levels were measured by microarray
94 (NimbleGen *Mus musculus* MM9 Expression Array). For the 24 h samples, the media were removed and
95 replaced with fresh media 12 h after inoculation. Raw and processed data are available from NCBI Gene
96 Expression Omnibus under accession number GSE89190.
97

98 2.3. Data analysis

99 NimbleScan v2.5 software (NimbleGen, Madison, WI) was used to extract raw intensity data for each probe
100 on each array. Intensity data were read into the R statistical computing environment and checked for quality
101 [19]. Data were prepared for processing using the pdInfoBuilder package and then normalized using the robust
102 multichip average (RMA) function in the oligo package [20].

103 Statistical tests for differences in expression between treatments were conducted on the normalized
104 expression data using a linear mixed-effect model followed by linear contrasts corrected for multiple
105 comparisons. More specifically, expression was modeled as a function of treatment while probes for a particular
106 gene were treated as a random effects using the nlme::lme function in R. The data contained seven treatments:
107 three viruses tested at two time points (12 h and 24 h) each plus the mock treatment (RV₁₂, RV₂₄, MHV₁₂,
108 MHV₂₄, PR8₁₂, PR8₂₄, and mock). The following nine post hoc, two-sided contrasts were then performed on
109 the fitted models using the multcomp::glht function in R: each virus-time combination vs. mock (RV₁₂ vs. mock,
110 RV₂₄ vs. mock, MHV₁₂ vs. mock, MHV₂₄ vs. mock, PR8₁₂ vs. mock, PR8₂₄ vs. mock) and each pairwise
111 combination of viruses at the 24 h time point (RV₂₄ vs. MHV₂₄, RV₂₄ vs. PR8₂₄, MHV₂₄ vs. PR8₂₄). These 9
112 tests have their *p*-values adjusted by the multcomp::summary.glht function according to their joint distribution.
113 Any factors detected to be significant at the family (gene) level were then subsequently corrected using the
114 Benjamini-Hochberg algorithm [21] with a false discovery rate set at 1%.

115 Genes associated with type I IFN responses were identified among the sets of genes with differential
116 expression for each virus compared to mock at 12 h and 24 h using the Interferome v.2.01 database
117 (<http://interferome.its.monash.edu.au>; [22]). This database was queried using the search criteria: Type I IFN
118 (all), *in vitro*, *Mus musculus*, 2.0 fold change (up or down). Interferome genes were manually sorted into
119 functional categories: antiviral, IFN signaling, viral detection, immune response, MHC class I, inhibitory,
120 apoptosis, ubiquitination, miscellaneous, and unknown. The significance of each virus having genes in the
121 specific categories was tested using a chi-squared test.

122 Gene expression responses to RV1B were compared between our data from mouse cells and published
123 data using human cells [23] using the MGI vertebrate homology database provided by The Jackson Laboratory
124 [24] as well as the annotate package in R.

125 3. Results and Discussion

126 3.1. MHV, PR8, and RV alter cellular gene expression by different magnitudes and with different timing

127 In order to compare how unrelated respiratory viruses (MHV, PR8, and RV) alter gene expression of
128 murine epithelial cells, we infected LA4 cells with each of the three viruses and evaluated cellular gene
129 expression by microarray analysis at 12 and 24 h after infection compared to mock-inoculated controls. Figure
130 1 shows the \log_2 -fold change in expression level of genes that were differentially expressed in virus-infected,
131 compared to mock-inoculated, cells. By plotting the changes in gene expression at 12 vs. 24 hours, we
132 observed differences in magnitude and timing of gene expression changes mediated by the three viruses. The
133 genes with significantly different expression in MHV-infected cells had low fold change values (Figure 1A).
134 At 24 h, when gene expression changes were the highest, genes that were up-regulated by MHV infection had
135 \log_2 -fold change values of less than five. In contrast, PR8 and RV induced expression of many genes by
136 greater than five \log_2 -fold at 24 h, and genes were spread consistently across the full range of values. By 24 h,
137 the genes most strongly up-regulated by PR8 and RV induced changes of 7 - 9.5 \log_2 -fold and 6 - 7.5 \log_2 -
138 fold compared to mock, respectively. This same pattern was observed with the down-regulated genes (Figure
139 1).

140 The three viruses also differed in the timing of gene expression changes. MHV altered expression of
141 relatively few host genes, most of which were only significantly different from mock at 24 h (Figure 1A).
142 While both PR8 and RV induced expression of large subsets of host genes, they did so with different timing.
143 PR8-induced changes in gene expression occurred at a constant rate: the expression level of most genes at 24
144 h was approximately twice the expression value at 12 h (Figure 1B). In contrast, RV infection altered
145 expression of a large number of genes by 12 h and the expression levels were maintained at approximately the
146 same levels at the 24 h time point (Figure 1C).

147 Taken together, we observed differences in magnitude and timing of gene expression changes mediated
148 by the three viruses: MHV changes were low and slow, PR8 induced gene expression to high levels at a
149 steady rate, and RV altered gene expression more quickly to peak levels by 12 h. The limited response to
150 MHV infection is in agreement with other coronaviruses, such as MHV-A59 [25] and SARS-CoV [26, 27]. In
151 addition to inducing minor transcriptional up-regulation of host genes, MHV-A59 shuts down host gene
152 expression by enhancing mRNA degradation [25]. A related coronavirus, SARS-CoV, also induces
153 degradation of host mRNAs [28]. The low numbers of host mRNAs that were altered in response to MHV
154 infection in our study could be due to one or both of these mechanisms. While rhinoviruses are also known to
155 down-regulate host gene expression by inhibiting transcription, we saw a robust increase in host RNAs early
156 upon RV infection. This is in agreement with other transcriptome studies of major and minor serogroup
157 rhinoviruses in human respiratory epithelial cells and experimental infections of humans [23, 29-32]. The
158 plateau in gene expression changes in RV-infected cells at 24 h may be due to transcriptional inhibition later
159 in infection. PR8 infection induced a strong transcriptional response in LA4 cells, which has also been seen
160 with multiple strains of influenza A viruses in primary human and mouse airway or lung epithelial cells [33-
161 36].

162 3.2. Host genes have shared and unique responses to RV, PR8, and MHV infection

163 We identified which genes were altered by each virus at 24 h compared to mock and the degree of
164 overlap among the differentially expressed genes. As was also observed in Figure 1, at 24 h RV infection
165 resulted in up-regulation of the largest number of genes, followed by PR8 then MHV (Figure 2A). A similar
166 pattern was seen with down-regulated genes (Figure 2B). While one might worry that the small number of
167 significant genes that were altered by MHV could be false positives, the majority of these genes (65% of up-
168 regulated and 86% of down-regulated genes) were also significantly altered by at least one other virus
169 suggesting that most of these genes are true positives. For both up- and down-regulated gene sets, RV had the
170 largest proportion of unique genes, while the majority of genes affected by both PR8 and MHV were shared
171 by at least one other virus.

173 Supplemental table 1 contains the list of genes whose expression was significantly up-regulated by all
174 three viruses compared to mock-inoculated cells. These genes may reflect a global response of epithelial cells
175 to viral infection. Several of the genes with the highest fold change values are involved in antiviral defense at
176 the level of infected cells (eg., *Mx1*, *Bst2*, *Oas2*, *Gbp10*) or recruitment of immune cells (eg., *Cxcl10*, *Cxcl11*,
177 *Cxcl1*). These genes are upregulated by type I IFNs, suggesting that induction of a type I IFN response is
178 shared by these viruses. In contrast to the shared up-regulated genes, genes that were significantly down-
179 regulated by all three viruses have diverse functions (Table S2). Some examples of genes that were down-
180 regulated by all three viruses included genes that encode transmembrane proteins (*Tmem 119*, *231*, *19*, *50a*,
181 and *14c*), extracellular matrix proteins (*Spon2*, *Ogn*, *Aspn*), and apoptotic signaling proteins (*Sdpr*, *Bmf*,
182 *Bnip3l*).

183

184 3.3. Identification of signature genes that were uniquely altered by each virus

185 Comparing the number of genes altered by each virus provides insight into shared and unique cellular
186 responses elicited by the viruses, but it does not provide information on the relative magnitudes of gene
187 expression changes between viruses. To compare gene expression changes between viruses, we plotted the
188 log₂-fold change of each gene at 24 h for MHV vs. RV vs. PR8 (Figure 3A). We only included genes that
189 were differentially expressed in at least one viral infection compared to mock. Like Figure 1, this 3D plot
190 illustrates that PR8 and RV not only caused a larger number of genes to be up-regulated compared to MHV,
191 but they also induced higher fold change values (Figure 3A).

192 For each of the three viruses, we defined a signature gene as a gene that is both differentially regulated at
193 24 h compared to the mock treatment and has an effect size significantly larger than the other two viruses (i.e.
194 fold change on the X axis is significantly different from Y-axis, Z-axis, and mock). These genes are colored in
195 Figure 3A and appear along the diagonal in Figure 3B. As expected, RV had the largest number of signature
196 genes, followed by PR8, then MHV (Figure 3B). Interestingly, the genes with the highest fold change values
197 compared to mock were not signature genes, but were up-regulated by both PR8 and RV infection. A pairwise
198 analysis was performed to identify the number of genes with altered expression compared with mock in two
199 viruses compared with the third. This analysis, shown in Figure 3B, reveals that RV and PR8 had the most
200 similarities in both up- and down- regulated genes (Figure 3B, purple blocks). The pattern of up-regulated
201 gene expression changes during MHV infection was more similar to PR8 (24 genes) than RV (6 genes).

202 Several host defense genes were identified as signature genes uniquely up-regulated by PR8 infection
203 (Table S3). These genes included cytokines and chemokines (*Cxcl9*, *Ccl5*, *IL12b*, *Ccl8*), IFN response genes
204 (*Ifitm6*, *Ifi2712a*, *Ifna2*, *Ifit2*, *Ifitm5*, *Ifna11*), and genes involved in processing MHC class I antigens (*Psmb10*,
205 *Tap2*, *H2-Q2*, *H2-K1*, *Psmc9*, *Psmc2*, *Psmc1*). The significant up-regulation of host defense genes in response
206 to PR8 in the LA4 cell line corresponds with the expression profile of murine type II alveolar epithelial cells
207 in response to PR8 infection in mice [37]. Furthermore, strong up-regulation of immune response-related
208 genes upon PR8 infection of mice correlates with disease severity [11]. Several genes that were uniquely
209 down-regulated by PR8 are involved in cellular metabolic pathways (*Cdo1*, *Aldh1a7*, *Acad11*, *Hsd17b4*) or
210 intracellular transport (*Myl6b*, *Ifi88*, *Anxa8*).

211 Although RV induced expression of several genes involved in host defense, these were largely shared by
212 PR8 so were not identified as signature genes. The signature genes up-regulated by RV included kallikrein-1
213 and 10 kallikrein-1-related peptidases and additional proteins involved in tissue remodeling (Table S4).
214 Rhinovirus infections are a significant cause of asthma exacerbations, which correspond with inflammatory
215 responses in the airways. Kallikreins generate kinins and contribute to many disease processes, including
216 inflammation. Kinins are induced by rhinovirus infections and kallikrein-1 is up-regulated by rhinovirus
217 infection in humans, especially those with asthma [38, 39]. Up-regulation of these genes in mouse cells upon
218 RV infection would provide a tractable animal model in which to study the roles of kallikreins in rhinovirus-
219 induced asthma exacerbations. Rhinoviruses are also known to up-regulate expression of mucins by airway
220 epithelial cells *in vitro* and *in vivo*, which may contribute to mucus hypersecretion [1, 40]. *Muc2* was the only
221 mucin gene up-regulated by RV in our study, and was unique to RV infection (Table S4).

222 MHV infection resulted in regulation of a small set of signature genes (Figure 3B, Table S5). Signature
223 genes that were uniquely up-regulated by MHV infection included multiple transcription factors from the
224 double homeobox (*Duxf3*, *Dux*, *Dux4*) and zinc finger and SCAN domain (*Zscan4d*, *Zscan4c*, *Zscan4-ps1*, *2*
225 *and 3*) families. Despite up-regulating expression of transcription factors, MHV infection had a minor impact
226 on the host cell transcriptome. This may be due to enhanced degradation of mRNAs as discussed above,
227 which has been shown to occur during other coronaviral infections [25, 28]. Therefore, LA4 cells may be up-
228 regulating transcription in response to MHV infection through expression of various transcription factors
229 while MHV causes degradation of these transcripts, which would reflect the small number of up-regulated
230 transcripts in MHV infected samples. In contrast to MHV-A59, MHV-1 infection did not cause down-
231 regulation of a substantial number of host genes. Differences may be due to virus strain, host cell type, and
232 timing differences between the studies.

233

234 3.4. Type I IFN-related genes had increased expression in LA4 cells infected by PR8, RV, and MHV

235 As described above, several of the genes with up-regulated expression in response to all three viruses
236 and those that were unique to PR8 are induced by type I IFNs. To specifically evaluate how IFN response
237 genes were altered by the three viruses, genes that were significantly up-regulated by each virus at the 24 h
238 time point were used to query the Interferome v2.01 database (see Materials and Methods). A Venn diagram
239 was generated to visualize the degree of overlap in IFN-related genes whose expression was induced by at
240 least one of the three viruses (Figure 4). PR8 induced expression of the greatest number of IFN-related genes,
241 a majority of which were shared by at least one other virus. RV up-regulated slightly fewer IFN-induced
242 genes compared to PR8 and MHV infection resulted in up-regulation of the fewest IFN-induced genes. It was
243 somewhat surprising that PR8 induced a higher type I IFN response than RV, given that RV induced
244 expression of nearly twice as many genes than PR8 (Figure 2).

245 There was strong overlap between the IFN-induced genes up-regulated by each virus. The timing of
246 IFN-related gene expression followed the same trend as was seen in Figure 1, wherein all genes with
247 significantly altered expression were analyzed (data not shown). Most of the IFN-related genes up-regulated
248 by MHV were only increased at 24 h. PR8 induced expression of 110 IFN-related genes at 12 h and these
249 genes were a subset of the 179 genes up-regulated at 24 h. In contrast, RV infection induced expression of
250 more IFN-related genes at 12 h (148 genes) than at 24 h (123 genes). Relative to up-regulation, few IFN-
251 related genes were down-regulated at the 24 h time point (MHV=5, PR8=10, RV=26).

252 Type I IFNs induce expression of genes with different functions during an antiviral response. To
253 determine whether there were specific patterns in expression of IFN-induced genes that correspond with
254 function, the IFN-induced genes that had significantly increased expression by any of the three viruses were
255 separated into functional groups. Heatmaps that demonstrate differences in fold change (color scale) and
256 significant differences (outlined boxes) in expression compared to mock-inoculated controls were generated
257 (Figure 4 and S2). As shown in the Venn diagram, this analysis also demonstrates that PR8 infection resulted
258 in up-regulation of the most genes involved in type I IFN responses, followed by RV then MHV. The fold
259 change values induced by PR8 infection also were generally higher than the other two viruses. However, there
260 was not a significant correlation between virus identity and functional group. For most of the functional
261 groups, MHV up-regulated expression of a smaller subset of the same genes as PR8 and RV, with the
262 exception of the MHC class I pathway (Figure 4). MHV significantly up-regulated expression of only one
263 gene involved in the MHC class I pathway (*Blmh*), which was not significantly up-regulated by the other two
264 viruses. This observation suggests that cytotoxic T cell responses may differ in MHV infections compared to
265 PR8 and RV. T cell responses have complex roles in MHV-1 infections, as they contribute to protection in
266 resistant mouse strains but mediate pathology in susceptible strains [41]. However, mice with the CD8+ T cell
267 repertoire of a resistant strain in the background of a susceptible strain remain susceptible to severe MHV-1
268 infection [42]. The failure of MHV-1 to activate processing and presentation of MHC class I antigens could
269 explain the inability of a broadly reactive CD8+ T cell response to protect these mice.

270 The interferome analysis focuses on IFN-induced gene expression, but not expression of the type I IFNs
271 that induce these responses. Multiple type I IFNs exist, including IFN- β and 14 subtypes of IFN- α , all of
272 which signal through the type I IFN α/β receptor (IFNAR) [43]. Type I IFNs can induce autocrine and
273 paracrine signaling; thus the IFN-induced genes we detected could be from both infected and uninfected cells
274 in the cultures. To determine if differential expression of type I IFNs explains the differences in IFN-induced
275 gene expression upon infection by PR8, RV, and MHV, we analyzed the expression of type I IFN and
276 receptor genes for each virus compared to mock (Figure 5). Probes for IFN- β 1 and ten subtypes of IFN- α
277 were present on the arrays. In agreement with expression of IFN-induced genes, PR8 induced expression of
278 the largest set of type I IFNs, followed by RV. Both viruses induced expression of *Ifnb* and *Ifna4*, which
279 encode type I IFNs known to be expressed early during antiviral responses [44, 45]. Five subtypes of *Ifna*
280 were up-regulated by both PR8 and RV, while three *Ifna* subtypes were uniquely up-regulated by PR8 and
281 only *Ifnab* was uniquely up-regulated by RV. Only PR8 induced expression of *Ifnar2*, which encodes the high
282 affinity chain of the type I IFN α/β receptor [46]. This might allow for enhanced positive-feedback signaling
283 and account for the larger number of IFN-induced genes up-regulated by PR8 infection.

284 Rhinoviruses and influenza A viruses are known to induce type I IFN responses through recognition by
285 MDA-5 and RIG-I, respectively [47, 48]. Furthermore, both viruses are recognized by TLR3 in infected
286 epithelial cells [47, 48]. However, TLR3 predominantly induces expression of pro-inflammatory genes, rather
287 than type I IFN-dependent genes, during influenza A virus infection [47]. Differential signaling through
288 MDA-5 and RIG-I pathways may contribute to the differences in type I IFN responses by these two viruses.
289 Zaritsky et al. have demonstrated that the type I IFN response to Sendai virus differs when cells are infected
290 by different doses [49]. They further showed that these differences were mediated by differential signaling
291 through the IFN α/β receptor, with robust signaling in uninfected cells. This supports our findings that PR8
292 induces expression of *Ifnar2* and additional type I IFN genes that are not up-regulated by RV (Figure 5).

293 None of the type I IFNs or receptors had significantly altered expression upon MHV infection (Figure
294 5), despite up-regulation of a modest number of IFN-stimulated genes (Figure 4). This could be due to IFN-

295 independent expression of these genes, or induction by a type I IFN that was not represented on the
296 microarray. Coronaviruses are notorious for being able to replicate within cells without triggering type I IFN
297 responses, or delaying IFN induction until late in the replication cycle [34, 50-52]. Other studies have shown
298 that the IFN response to MHV-1 is a critical determinant of susceptibility. Severe disease in A/J mice
299 compared to C57Bl/6 mice correlates with lower type I IFNs detected in the lungs of A/J mice upon MHV-1
300 infection [6, 53]. Similarly, the expression of various type I IFNs in response to MHV-1 infection *in vitro* is
301 cell line-dependent [53]. Because the cell line we used, LA4, was derived from the lungs of A/He mice, we
302 would expect it to have a similar response as A/J mice. Thus the lack of type I IFNs induced by MHV-1 in
303 LA4 cells *in vitro* corresponds with pathogenesis observed in A/J mice *in vivo*.

304 The finding that LA4 cells mount a stronger response to PR8 than RV or MHV infection may be due to
305 differences in the viral recognition and signaling pathways used to detect these different viruses and
306 amplification of the type I IFN response as discussed above. Alternatively, it could be due to differences in
307 replication kinetics of the viruses. RV-infected cells have started dying by the 24 h time point (not shown),
308 therefore host response genes may have been up-regulated at an earlier time point. In contrast, coronaviruses
309 are known to delay cellular responses to infection [54] so the 24 h time point may be too early to evaluate the
310 innate response to MHV infection. Alternatively, the cells may detect MHV and up-regulate transcription of
311 IFN response genes, but mRNA degradation would mask this process. By quantifying mRNA transcripts at
312 two time points after viral infection, our study cannot distinguish between these possibilities.

313
314 *3.5. RV1B induced a similar gene expression response in murine and human respiratory epithelial cells*

315 One limitation of our study is the analysis of three viruses that do not share a natural host. MHV is a
316 natural pathogen of mice and PR8 is a highly mouse-adapted strain of influenza A virus. However, RV1B is a
317 human rhinovirus whose receptor is conserved between mice and humans. RV1B is increasingly being used in
318 mouse studies [1-4, 55]. Despite the difference in host, we found similar changes to gene expression in murine
319 cells as studies with RV1B in human cells [23]. Of the 24,204 and 12,438 genes represented on our mouse
320 microarray and the human microarray chip used by Chen et al., respectively, 10,847 genes are shared. Using
321 the same 2-fold increase in expression cut-off and restricting our list only to homologous human genes studied
322 by Chen et al., we found that 196 mouse genes were upregulated by RV1B infection. Comparing this list of
323 196 genes to the 48 upregulated human genes identified by Chen et al., we found that 20 genes (Table S6)
324 were upregulated by RV1B infection in both human and mouse cells. A chi-squared test confirmed the
325 significance of this shared pattern of up-regulated genes ($\chi^2=431.7$, d.f.=1, $p<0.001$). Interestingly, all 20 of
326 the shared genes we identified are involved in type I IFN responses. While far from identical, the similarity of
327 the responses in the two cell types suggests conserved activation of type I IFN responses by these different
328 hosts and supports the validity of a murine model for studying rhinovirus infections in humans.

329 4. Conclusions

330 Alveolar epithelial cells have a key role in alerting the immune system to infection by respiratory viruses
331 and shaping immune responses [37, 56, 57]. As viruses from several different families all target respiratory
332 epithelial cells, it is important to understand the similarities and differences in how these cells respond to a
333 diverse set of viruses. A significant number of genes were up- or down-regulated in response to infection by
334 three distinct viruses from different families. Genes that were associated with a shared response to the three
335 viruses included those involved in defense against viruses, and particularly genes that are induced by type I
336 IFNs. However, there were differences in the timing, numbers of genes altered, and expression levels of these
337 genes. This may reflect differences in viral replication cycles and signaling pathways that are activated by
338 infection.

339
340 **Supplementary Materials:** Figure S1: Infection of LA4 cells visualized by immunofluorescent assay of viral
341 proteins and epifluorescent microscopy, Figure S2: Heatmap of differential expression values for interferome
342 genes, Table S1: Genes up-regulated in all virus infections, Table S2: Genes down-regulated in all virus
343 infections, Table S3: PR8 signature genes, Table S4: RV signature genes, Table S5: MHV signature genes,
344 Table S6: Up-regulated genes shared with Chen et al., 2006.

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353

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362

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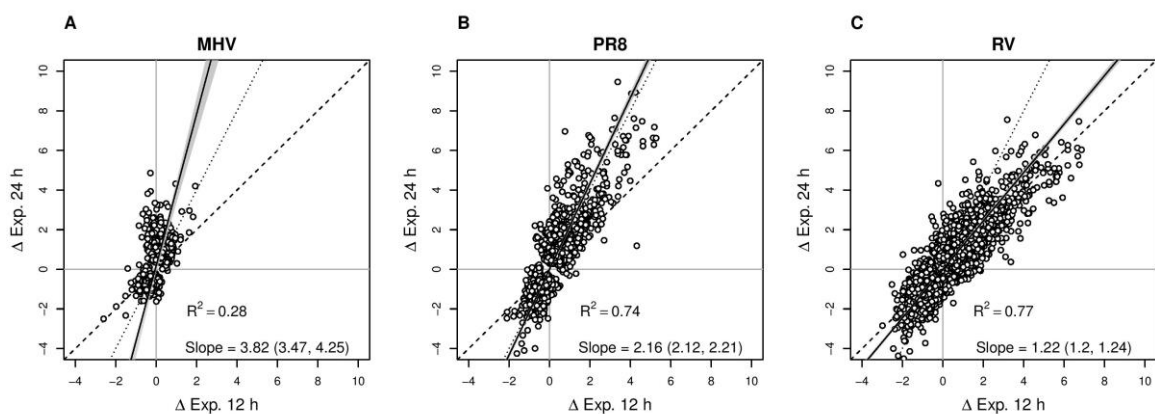
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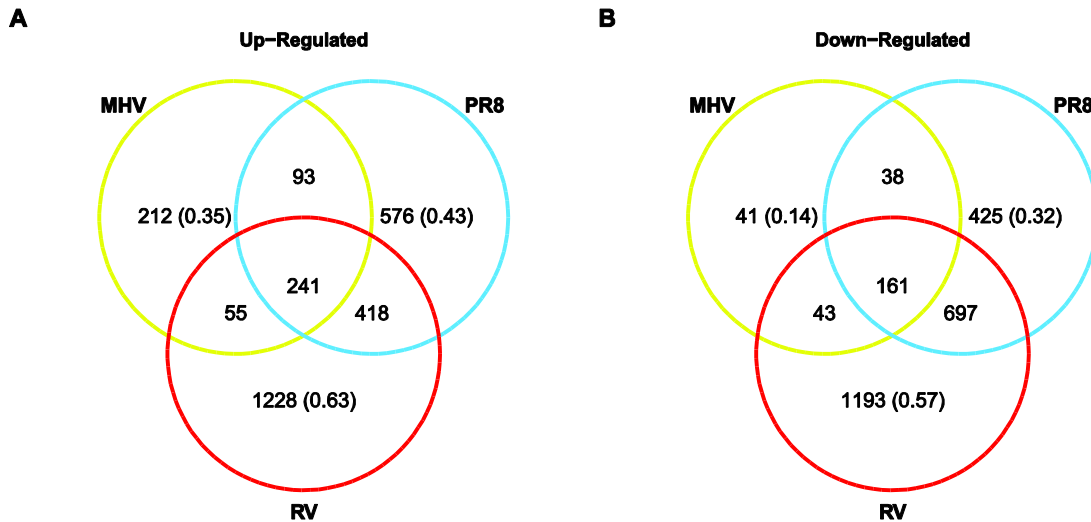
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529 5. Figures

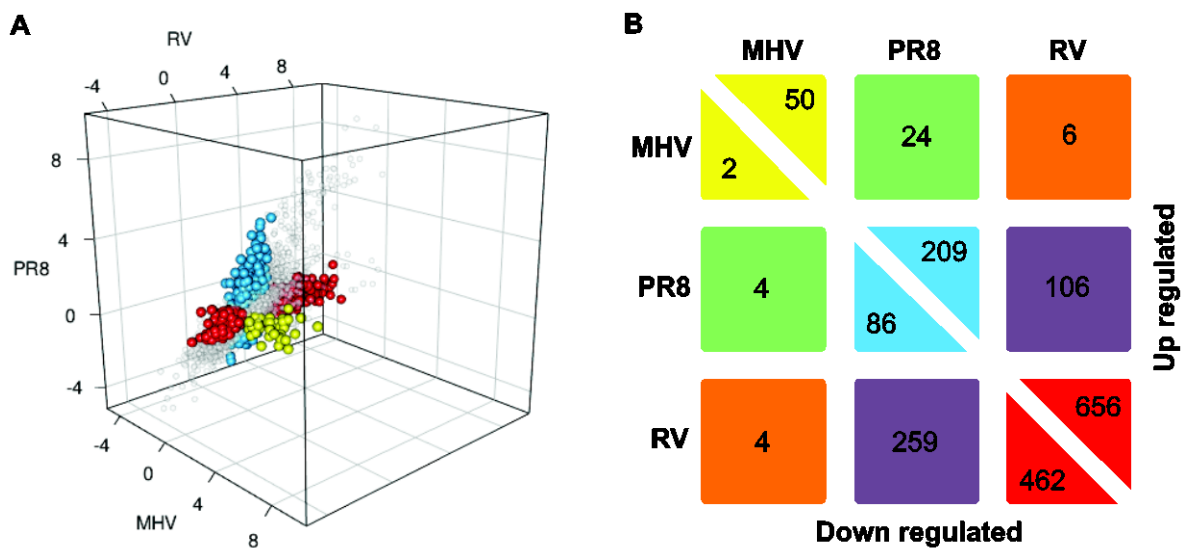


530
531 **Figure 1.** Magnitude and timing of gene expression changes mediated by MHV, PR8, and RV. The plots
532 show the estimated log₂ fold change in expression relative to mock at 12 h vs. 24 h for each of the three

533 viruses. Each point represents one gene; only those genes that differ significantly from mock (at either time
 534 point) are included. The solid black line is the best fit regression line with the gray shading showing the 95%
 535 confidence interval. The slope with confidence interval and R^2 are given in the inset legend. The dashed line
 536 illustrates the hypothesis that all changes in expression occur in the first 12 h (slope = 1); the dotted line
 537 shows the constant rate of change hypothesis (slope = 2). (a) MHV has small effects and most of the
 538 expression changes occur between 12 and 24 h (slope \gg 2). (b) PR8 has larger effects than MHV and the
 539 changes approximate a constant rate of change across both 12 h intervals (slope \approx 2). (c) RV also has large
 540 effects on gene expression and changes occur in the first 12 h with very little further change in the next 12 h
 541 (slope \approx 1).
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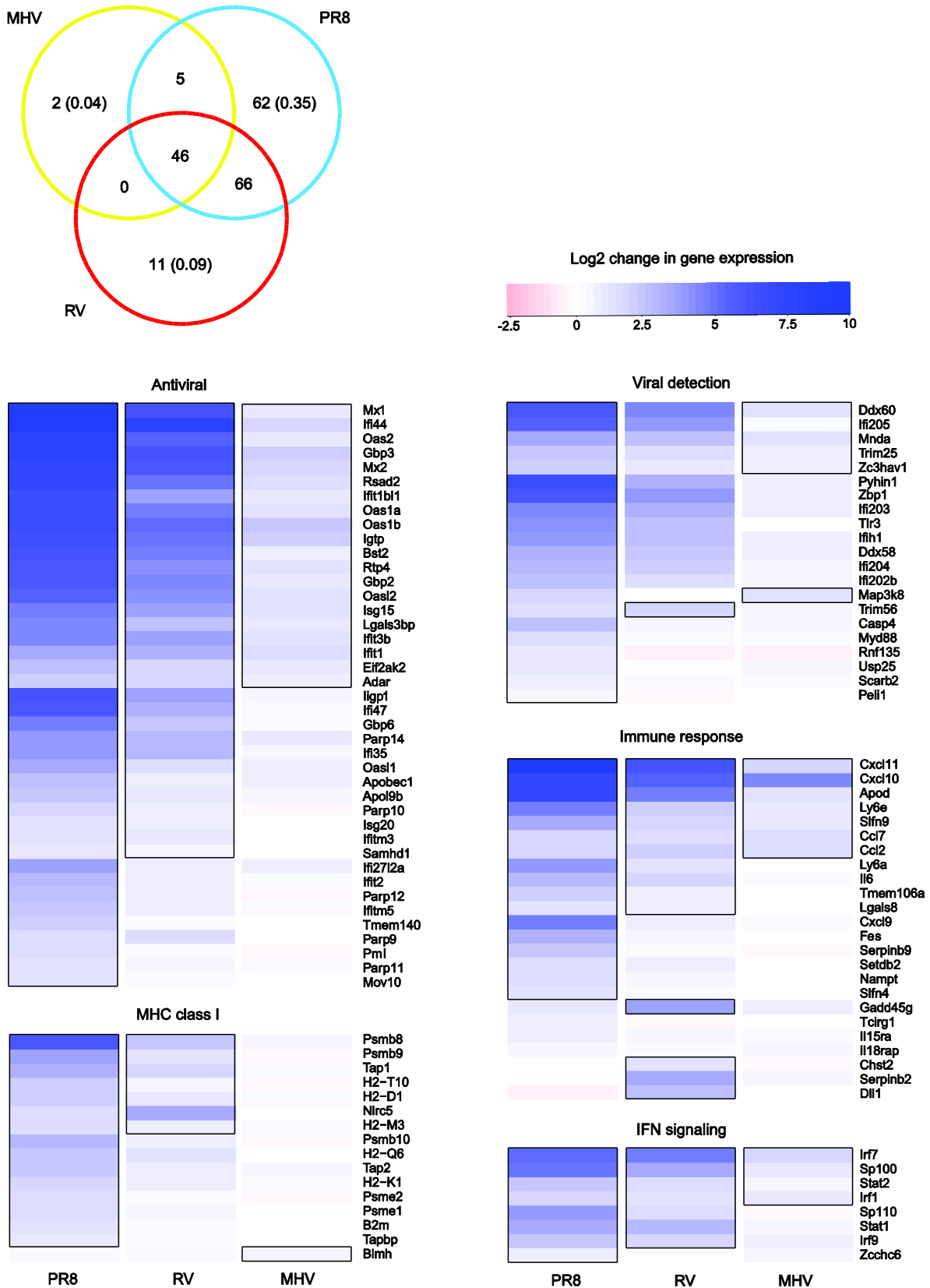


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 546 **Figure 2.** Numbers of genes with significantly altered expression upon viral infection. Venn diagrams show
 547 the number of significantly (a) up- and (b) down-regulated genes compared to mock 24 h after infection. The
 548 proportion of genes that are uniquely significant for each virus is indicated in parentheses.
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 555 **Figure 3.** Patterns of gene expression changes mediated by viral infection. (a) Genes differentially expressed
 556 in at least one viral infection at 24 h are plotted as log₂ fold change with each virus along a different axis.
 557 Signature genes, which have significantly larger effects in one virus compared to all other treatments, are
 558 colored: blue = PR8, red = RV, yellow = MHV. (b) The number of genes uniquely up- or down- regulated by

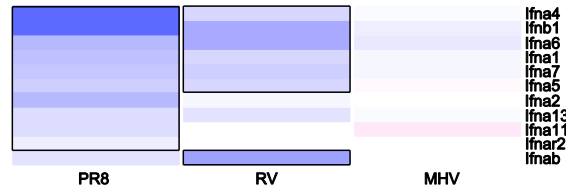
559 each virus or pairs of viruses. The numbers along the center diagonal are the signature genes with boxes
 560 colored as in (a). The off-diagonal numbers are genes that have differential expression in two viruses
 561 compared to mock and the third virus, but are not significantly different from each other.
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Figure 4. Differential expression of type I interferon-induced genes. Genes with significantly up-regulated expression compared to mock at 24 h (see Figure 2) were used to query the Interferome v.2.01 database. The

567 Venn diagram shows the number of shared and unique type I IFN-related genes that were up-regulated in each
568 viral infection. The proportion of genes up-regulated in only one virus treatment are shown in parentheses.
569 The genes represented in the Venn diagram were divided into functional groups and heat maps were generated
570 using \log_2 fold change values for each virus at 24 h compared to mock-inoculated controls. Heat maps of
571 additional functional groups can be found in Supplemental Figure 2. Gene names are indicated to the right of
572 each row and statistically significant values are outlined in black.
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578 **Figure 5.** Differential expression of type I interferons and receptors. The \log_2 fold change compared to mock
579 of up-regulated type I IFN cytokine and receptor genes. Gene names are shown to the right of each row and
580 the color scale is the same as in Figure 4.

581