Searching for a Lifeline: Transcriptome Profiling Studies of Influenza Susceptibility and Resistance

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Abstract

Excess or dysregulated host inflammatory responses cause much of the morbidity and mortality caused by severe influenza. Given the limitations of vaccines and antiviral drugs, novel therapeutics to modulate host responses and improve outcomes in severe influenza are needed. One strategy is to learn from the direct comparison of high-survivor versus high-mortality animal models. This review surveys the results of lung transcriptome profiling studies in murine models that directly compare susceptible versus resistant hosts challenged with identical influenza infections. The potential contributions and limitations of these studies are discussed. To amplify their power, the studies are subjected to a meta-analysis, which helps identify frequently dysregulated pathways and potentially novel areas for investigation. Using connectivity map-based tools (LINCS), transcriptome signatures linked to susceptibility can identify candidate drugs that merit testing for in vivo efficacy.

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Introduction

Seasonal influenza remains a major cause of mortality. A related concern is that a new virulent influenza strain might mimic the 1918 pandemic's notoriously high death rate [1]. Public health interventions and current vaccines have their limitations, as seen in the 2009 pandemic [2]. Moreover, current antiviral drugs (e.g., oseltamivir) have a narrow window of efficacy and face increasing viral resistance. Hence, there is substantial need for new insights into the mechanisms of the disease that might lead to new therapeutics.

The pathobiology of influenza has been extensively studied, and recent reviews highlight tremendous progress [3–5]. There exists a wealth of data about the cells and molecules involved in the host inflammatory responses to influenza, but unfortunately there is little clarity about which are critical and useful targets for drugs to improve outcomes. Even the relative importance of the virus strain or load compared to host response is the subject of controversy and discordant findings [6, 7]. At the cellular level, important roles have been reported for almost every cell type found in the lungs [3–5].

Transcriptome analysis and “systems biology” approaches have been eagerly embraced [8, 9]. However, the

hope that an omics-based, pathway-centered approach might identify key mediators and focus attention on the most promising therapeutic targets has not yet been realized. One promising strategy uses gene expression profiling of mouse models that show quite distinct outcomes – high survival versus high mortality – after infection with identical inocula of the virus.

The rationale for this approach begins with the well-documented spectrum of disease severity in people. Experimental studies of infected human volunteers also show a remarkable diversity in symptom severity and inflammatory responses, even in mild self-limited infections [10]. During the 1918 pandemic, case-fatality rates in children aged 5–14 years were substantially lower than those in the 18- to 40-year age range, even though overall infection rates were similar [11, 12]. Other host factors linked to the severity of disease include sex [13] and pregnancy [14]. More recently, infection with the H5N1 avian flu has been linked to nearly 60% fatality rate [15], although fortunately the total number of infections has been relatively limited. Understanding the basis for the difference in survival versus mortality has been a goal of numerous clinical and experimental research efforts, including many that take a systems biology approach to the question.

In severe influenza, death from hypoxemic respiratory failure is a major mechanism of mortality [16]. The pathophysiologic basis is the influenza-mediated damage to lung epithelium and endothelium, resulting in exudative inflammation and the development of acute respiratory distress syndrome [5]. However, which factors determine the magnitude and extent of the host inflammatory response remain incompletely characterized. It is worth noting that in a significant number of cases a further complication of secondary bacterial pneumonia contributes to respiratory compromise and death [17].

Many experimental studies have profiled responses to influenza strains known to differ in pathogenicity or to a range of inocula of the same virus to compare mild versus severe disease. These efforts have helped to establish the importance of variations in both the virus strain and host innate immune responses to the severity and outcome of influenza, which are concepts that have previously been thoroughly reviewed [6, 7, 18–20]. However, a more direct way to identify determinants of survival or death is to compare transcriptome responses to the same virus in hosts known to differ in the fatality rate. Indeed, a small, but fascinating, subset of studies directed to this question have been performed and are the focus of this review.

Gene Set Identification

A search of the GEO Database (www.ncbi.nlm.nih.gov/geo/browse/) in July 2016 using the keyword “influenza” yielded 278 data series, of which 50 studied whole lung RNA transcriptomes in mice (49 by microarray, 1 by RNASeq). To focus on host differences, studies in which different mouse strains exhibited differences in mortality after infection with the same dose of influenza were identified. The data series fulfilling these criteria are all whole lung transcriptome profiles and are tabulated in Table 1. They are first reviewed individually, and then together using a meta-analysis approach.

GSE64750

Boon et al. [21] compared responses to a highly pathogenic H5N1 influenza (HK213) and found that infection with 10^2 or 10^3 EID50 resulted in 80–100% mortality in DBA/2 mice, while all of the C57Bl6 mice survived. The more susceptible DBA/2 mice showed a greater viral load in the lungs at 48 h and higher amounts of inflammatory cytokines (e.g., TNF, IFN- α , - β , CCL2). Transcriptome profiling showed some basal differences in RNA expression between the lungs of DBA/2 mice and BL6 mice. At 72 h after infection, 1,833 genes were significantly up- or downregulated, of which subsets (521 genes) were statistically different between the infected BL6 mice and DBA/2 mice. Many of the genes, including *Ifna4*, *Tnf*, *Cxcl11*, *Cxcl2*, and *Irf1* (interferon regulatory factor 1), were associated with inflammation and antiviral immunity. They noted that a link between disease severity and high viral loads and increased production of proinflammatory cytokines was previously found in humans infected with H5N1 viruses [22]. This study also identified 5 quantitative trait loci linked to influenza resistance. Their data further implicated a defective *Hc* gene in DBA/2 mice, which encodes the complement component C5 previously shown to be vital for resistance to influenza [23]. Some strain-dependent differences in pathways were identified using the KEGG database (<http://www.kegg.jp>). Especially noteworthy was the frequency of overrepresentation of metabolic pathways in DBA/2 versus BL6, for example (with rank): valine, leucine, and isoleucine degradation (1), propanoate metabolism (2), metabolism of xenobiotics (6), fatty acid metabolism (8), oxidative phosphorylation (9), and glutathione metabolism (10).

E-MTAB-835

In this study (available only at the European counterpart to GEO, ArrayExpress, www.ebi.ac.uk/arrayex),

Table 1. Transcriptome profiling studies reviewed

Study	Susceptible strain	Resistant strain	Virus	Dose	Sex	Age, weeks	Infected sample, days	Mock sample, days	Platform code	Platform name	Comparisons, <i>n</i>	Ref.
GSE64750	DBA/2	C57BL/6J	H5N1	10 ⁴ EID50	F	6–11	3	3	GPL1261	Affymetrix 430 2.0	1	19
EMTAB-835	DBA/2	C57BL/6J	H1N1	2 × 10 ⁴ FFU	F	10–12	1, 2, 3, 4	2	GPL1261	Affymetrix 430 2.0	4	22
GSE64798	DBA/2 129/SvImJ A/J	SM/J C57BL/6J Balb/cJ	H5N1	10 ⁴ EID50	F	6–8	1, 7	0	GPL8833	Illumina V6.1.1	17	6
GSE55403	129/SvImJ	C57BL/6J	H3N2	8 × 10 ³ TCID50	M	6–12	5	5	GPL6887	Illumina MouseWG-6 v2.0	2	24
EUK-207	Balb/cJ	Balb/cJ + EUK-207	H1N1	100 PFU	F	8–9	8	8	GPL11202	Agilent 4x44K v2	1	25
GSE66040	DBA/2	C57BL/6J	H1N1	2 × 10 ⁴ FFU	F	10–12	3, 5	1, 3	GPL16790	AB 5500	2	26
GSE74074	CAST/EiJ	PWK/PhJ C57BL/6J 129S1/SvImJ	H3N2	1 FFU	F	7–13	3, 5	3	GPL11202	Agilent 4x44K v2	6	27
GSE51526	RIPk3 KO	B6	H1N1	10 ⁵ PFU	F	6–8	2, 4	2, 4	GPL7202	Agilent 4x44K G4122F	2	28
GSE40792	B6	IDO KO	H5N1	10 ⁴	F	6	2	2	GPL7202	Agilent 4x44K G4122F	2	30
GSE35933	Balb/cJ	Balb/cJ-A2G-Mx1+	H1N1 (1918)	3.2 × 10 ⁵ PFU	NA	NA	0.5, 1, 3	0.5, 1, 3	GPL7202	Agilent 4x44K G4122F	1	59

press/), Alberts et al. [24] also studied the marked difference in survival between DBA/2 and C57BL/6 mice. They analyzed lung transcriptome profiles over the first 4 days of infection to identify shared and distinct genes in the 2 strains. On days 1–4, respectively, 9, 26, 114, and 153 genes were exclusively regulated in C57BL/6J, whereas 75, 528, 993, and 929 genes, respectively, were only regulated in DBA/2J, supporting the concept that expression profiling can reflect and illuminate differences in survival from severe influenza. Gene ontology analysis found increased immune response terms in the susceptible (DBA/2) mice, while abundant cell cycle and division terms in BL6 profiles were interpreted as reflecting the onset of repair processes linked to a better survival. Groups of inflammatory genes (e.g., interferon related, cytokine and chemokine) were increased in the susceptible DBA/2 mice to a greater degree than in the BL6 strain. Finally, pathway analysis using the Ingenuity IPA tool found 3 pathways that were only active in DBA/2J-infected mice: “eicosanoid signaling,” “apoptosis signaling,” and “coagulation system.”

GSE64798

Boon et al. [6] conducted a large study in which lung tissue from 3 susceptible (DBA/2, 129/SvIm, and A/J) and

3 resistant (SM, C57BL/6, and BALB/c) strains of mice was used for transcriptome profiling after infection with the highly pathogenic H5N1 HK213 influenza virus. They identified changes in gene expression in samples from day 1 and day 7 of infection. The key findings included: (1) most of the differences were attributable to altered or increased gene expression in the susceptible mouse strains; (2) while inflammatory and cytokine pathways were elevated in both groups of mice, the total number of genes identified within a particular pathway showed many more upregulated genes in the susceptible (e.g., DBA/2) strains compared to the resistant mice. By day 7, the susceptible mice continued to exhibit an increased expression of cytokine-cytokine receptor pathway members, while the resistant strains manifest pathways, cell adhesion molecules, antigen processing and presentation, and the graft-versus-host disease. The latter was interpreted as reflecting adequate virus-specific T and B cell responses in the resistant mice. The authors attributed the overrepresentation of proinflammatory genes in the susceptible strains to higher viral replication rates and larger viral loads. An additional valuable experiment in this paper is also informative. Using bone marrow chimeras, they found that the transfer of hematopoietic cells from resistant strains does not rescue the pathogenic phe-

notype of susceptible strains. This supports a critical role for lung structural cells (e.g., epithelium) although a small proportion of surviving host-resident lung macrophages in radiated recipient mice could also theoretically contribute to their findings [25].

GSE55403

Davidson et al. [26] found an important pathogenic role for type I interferons in acute influenza. IFN- $\alpha\beta$ receptor deficiency decreased morbidity and mortality, lung damage, proinflammatory cytokines and infiltrating inflammatory cells when compared to infection in wild-type mice with normal receptor levels. Transcriptome profiling on day 5 after infection showed that ISGs (interferon-stimulated genes) with antiviral function were similarly upregulated in receptor-deficient and normal mice, possibly reflecting the effects of IFN λ in triggering this upregulation in the absence of IFN- $\alpha\beta$ signaling. Nevertheless, the receptor-deficient mice showed much lower amounts of proinflammatory cytokines, indicating a secondary and likely harmful effect of excess type I IFN signaling.

EUK-207 Study Dataset

Kash et al. [27] treated 1918 influenza virus-infected mice with a superoxide dismutase mimetic (EUK-207) beginning 3 days postinfection. The postexposure treatment of mice infected with a lethal dose of the 1918 influenza virus resulted in significantly increased survival and reduced lung pathology without a reduction in viral titers. Moreover, EUK-207 treatment did not affect 1918 influenza viral replication. These findings add to the mix of discordant findings regarding the importance of viral load versus host inflammatory responses in determining the severity of influenza disease [6, 7]. This is likely a false dichotomy, and a reasonable position is that both factor into outcome depending on the experimental or clinical situation. Importantly, Kash et al. [27] conducted transcriptome profiling of the lungs of susceptible (control) or resistant (EUK-207-treated) mice on day 8 of infection (authors Kash and Walters generously provided access to this dataset, see below). Using both high-throughput sequencing and microarray analysis, they found that treatment resulted in a decreased expression of inflammatory response genes and increased lung metabolic and repair responses. Gene ontology analysis revealed that EUK-207 treatment (higher survival) increased biologic processes associated with ribosomal biogenesis, metabolism, cell proliferation and differentiation, tricarboxylic acid cycle, and macromolecule biosynthesis. In contrast, genes ex-

pressed in the vehicle-treated lungs were associated predominantly with immune responses and inflammation.

GSE66040

Wilk et al. [28] used RNASeq to obtain transcriptome profiles of lungs from susceptible DBA/2 and resistant BL6 mice on days 1, 3, and 5 after infection with the PR8 H1N1 virus. Their comparison of strains revealed differentially expressed genes (DEGs) in mock-treated controls (82 and 478 on days 1 and 3, respectively) as well as in infected samples (105, 198, 512 on days 1–5, respectively). These authors focused their efforts on a search for genes that were exclusively upregulated in C57BL/6J but not in DBA/2J mice after infection. They identified 5 DEGs that were significantly regulated on day 3 and 5 postinfection in C57BL/6J mice: *Lhx2*, *2210415F13Rik*, *Trim15*, *Reg3g*, and *Cd72*. Eliminating the first 3 due to low absolute expression levels, they infected *Reg3g*–/– mice and observed no significant effect on survival. They concluded that *CD72* merited further attention since it is an important receptor that negatively regulates BCR signaling and also has inhibitory functions in murine NK cells. The 2-fold higher upregulation of *Cd72* in BL6 compared to DBA/2J mice could result in a relative deficit in the inhibitory effect on NK cells and might contribute to the exaggerated immune response observed in DBA/2J mice.

GSE74074

Leist et al. [29] compared lung transcriptomes from days 3 and 5 after infection with an H3N2 virus (A/HK/01/68) in a susceptible strain (CAST/EiJ), 2 strains with intermediate resistance (C57BL/6J, 129S1/SvImJ) and 1 highly resistant strain (PWK/PhJ). Initial analysis showed increased expression of a panel of inflammatory genes in CAST/EiJ mice compared to 129S1/SvImJ and C57BL/6J, whereas PWK/PhJ mice showed minimal upregulation or even downregulation of these genes.

To identify genes that linked to the high susceptibility of CAST/EiJ mice, they searched for genes that were upregulated in the lungs of C57BL/6J and 129S1/SvImJ at days 3 and 5 postinfection (compared to their controls) but not in CAST/EiJ. The filtering revealed 8 genes that failed to be upregulated in CAST/EiJ mice: *Prm1* (protamine 1), *Gdf3* (growth differentiation factor 3), *Nts* (neurotensin), *Plekhs1* (pleckstrin homology domain containing, family S member 1), *Hpse* (heparanase), *F830016B08Rik* (RIKEN cDNA F830016B08 gene), *Insrr* (insulin receptor-related receptor), and *Wfdc17* (WAP 4-disulfide core domain 17). Based on other findings, they concluded that the susceptible CAST/EiJ mice did

not exhibit a general deficiency in the overall activation of an inflammatory response, but rather a specific defect in the recruitment of cells to the lung, reflecting gene networks that play a crucial role in the migration and recruiting of leukocytes. The specific role of the genes they identified remains to be directly tested.

GSE51526 and GSE40792

Two additional public datasets not described in specific publications are available from the lungs of mouse strains that differ in survival after influenza. In GSE51526, Katze and colleagues [unpubl. data] provide data from mice deficient in RIPK3 (receptor interacting protein kinase 3) and wild-type mice for comparison. The RIPK3^{-/-} mice were protected from mortality in other studies [30, 31], and serve as the resistant group in this comparison. In GSE40792, Katze and colleagues [unpubl. data] again shared data from lungs infected with H5N1 influenza, using both wild-type and IDO1-deficient mice. Mice deficient in IDO1 (indoleamine 2,3 dioxygenase) were found to be protected against influenza by another group of investigators [32]. For the interested reader, these researchers provide additional information and important meta-data regarding their experiments at the Influenza Research Database (www.fludb.org).

GSE35933

Cilloniz et al. [33] evaluated the host response to the 1918 virus in wild-type BALB/c and resistant Mx1^{+/+} mice. Mx1 is a dynamin-like GTPase that prevents transcription by viral RNA polymerase, effectively blocking both transcription and replication of influenza. Mx1 is a 72-kDa IFN-inducible protein that is genetically deficient in most inbred laboratory mouse strains and linked to their profound susceptibility to influenza virus. In contrast, the human counterpart MxA is normally expressed and contributes to innate defenses against viral infection [34]. In their experiments, the Mx1^{+/+} mice were partially protected from lethal 1918 virus infection; however, complete protection was achieved only upon interferon treatment before infection. In contrast, untreated and interferon-treated BALB/c mice succumbed to 1918 virus infection.

To further assess the basis for differential susceptibility, the researchers used transcriptome data from early time points (12, 24, and 72 h) and identified 547 genes that were differentially expressed. A large proportion of these genes were downregulated in BALB/c mice, whereas the same genes were robustly upregulated in the Mx1^{+/+} mice. Network analysis identified apoptosis, cell

migration, connective tissue disorders, and the production of reactive oxygen species as being linked to resistance. Genes from these networks were generally upregulated in the Mx1^{+/+} mice but were downregulated in BALB/c mice, suggesting that increased survival of Mx1^{+/+} mice is associated with the upregulation of these pathways.

Synthesis

The good news is that these studies provide clear support for the now well-established concept that susceptibility to mortality from influenza is associated with excess or greater inflammation than seen in matching resistant, high-survivor groups. As evident from the brief summaries above, the bad news is that there is little additional concordance that might provide more detailed insight and lead to new therapeutic targets. For example, using GSE64750, Boon et al. [21] found increased expression of metabolism-related pathways in susceptible DBA/2 mice early after infection, but Kash et al. [27] found metabolic pathways upregulated later (day 8) in resistant (EUK-207) treated mice. Additional differences in mouse and virus strains, sex, age, and protocols across these studies (Table 1) may also contribute to the different results and conclusions reached. It is worth noting that most studies did not attempt direct validation of potential leads from their DEG lists. In some cases, this reflects the ancillary role of the transcriptome data in the context of a given paper. In a minority of cases, PCR validation of microarray findings was obtained, or the correlation of DEGs or pathways to other inflammatory parameters were analyzed. Finally, even less frequently, specific leads were tested functionally (e.g., the testing of Reg3g^{-/-} mice by Wilk et al. [28]). This reflects in part the large effort needed to pursue leads from transcriptome profiling, and in part the uncertainties of how to prioritize subsequent studies.

Can Meta Be Better?

The problem of discordant transcriptome profiling results when the same biologic process is analyzed by different laboratories is well recognized [35]. One potential solution has been the application of meta-analysis approaches which seek to combine multiple datasets to increase analytical power [35–37]. The pros and cons of the multiple methodologies (16 or more!) for a meta-analysis

of transcriptome data have been extensively reviewed [38–40]. To explore whether a meta-analysis of the panel of studies reviewed above could be informative, a variation of the “vote-counting” method [40] was used (see below). This method essentially counts the frequency of genes or pathways identified as significant (up- or down-regulated) in a group of studies. The rationale is that the more often genes/pathways are identified in multiple studies, the more likely they are to represent robust, true findings rather than quirks of a single experimental study. Vote-counting is recognized as one of the least powerful but perhaps the easiest method to apply among the many meta-analysis tools [40]. The corollary is that the method may fail to find “hits” that would be identified with more complex methods, but those positives identified are likely to represent strong signals that can be detected by this less powerful methodology.

Identification of DEGs

The original data and annotation files for the panel of studies in Table 1 were downloaded from GEO, Array Express or the NCBI Sequence Read Archive (SRA, www.ncbi.nlm.nih.gov/sra). The sole exception is the study of a potential therapeutic (EUK-207), data files from which were generously provided by authors Kash and Walters. The microarray data were analyzed with Partek Genomic Suite version 6.6 (Partek, St. Louis, MO, USA) using standard workflows. After normalization, ANOVA-based analysis was conducted to identify DEGs with the criteria of a false-discovery rate ≤ 0.05 and fold-change of 1.5. DEGs from infected versus mock samples from high- and low-mortality groups in each study were compared using Venn diagram tools to identify DEGs unique to the high-survivor or high-mortality group within each study. For the single RNASeq study, the Partek Flow software package was used for analysis, with a workflow that subjected the downloaded FASTQ files to alignment using the STAR program [41], and quantitated the data using the Gencode mm10 reference mouse genome, normalized by total count, and analysis of differential gene expressions using the Partek Gene-Specific Analysis algorithm. The DEGs were further divided by those increased or decreased versus mock-infected samples, and the lists were tabulated for each study to group DEGs in resistant or susceptible strains. As discussed above, the frequency of DEGs in each group were counted to allow the ranking of frequency across the studies (vote counting).

This reanalysis effort generated a total of 38 gene lists comprised of DEGs corresponding to the various days of infection represented in the studies in Table 1. GSE35933

was excluded from further study because, in contrast to the other datasets, only a very small number of DEGs were identified that passed the FDR.05, 1.5-fold-change criteria (<20 per comparison). Subsequent analysis focused on days 3–5, a time window of biologic interest as it precedes the terminal events that begin on around day 7. This grouping also offered a relatively large number of different studies contributing datasets, consistent with the goal of benefitting from pooling multiple experiments (6 studies with 14 comparisons).

Analysis of Gene Lists

Initial comparisons were based on up- versus down-regulated genes (compared to mock-infected controls) in the susceptible or resistant strains on days 3–5 after the onset of infection. Ranking of frequency in the resistant mice revealed that very few genes were found in $>50\%$ of the studies (e.g., 3 up- and 0 downregulated genes). In contrast, frequency evaluation of datasets from susceptible mice identified 248 upregulated genes shared by 8 or more of the 14 available comparisons. There were also 450 downregulated genes shared by 8 or more studies in susceptible mice. Results of pathway and drug identification efforts were similar to those obtained using a more comprehensive analysis of all of the up- and downregulated genes from each individual study in susceptible mice, as detailed below.

Pathway-Based Analysis

To investigate pathways and other features of resistant or susceptible mice, we used each of the unique DEG gene lists from samples obtained on days 3–5 after infection (14 each, susceptible and resistant groups) to query the online tool Enrichr (amp.pharm.mssm.edu/Enrichr/) [42, 43], which allows users to submit gene lists for rapid analysis by a large collection of relevant bioinformatics tools. Enrichr currently contains 102 gene set libraries belonging to 8 categories and populated by approximately 180,000 annotated gene sets. The main task for which Enrichr is used here is to evaluate the enrichment of pathways or drug-related gene signatures. Enrichr offers 3 approaches to compute enrichment. The first is the standard Fisher exact test. This is a proportion test that assumes a binomial distribution and independence for probability of any gene belonging to any set. The second test is a correction to the Fisher exact test developed by the Ma’ayan laboratory (source for Enrichr). They computed enrichment using the Fisher exact test for many random input gene lists in order to compute a mean rank and standard deviation from the expected rank for each

term in each gene set library. Then, using a lookup table of expected ranks with their variances, they compute a *z*-score for deviation from this expected rank, which serves as a new corrected score for ranking terms. Finally, they offer a combined score, which multiplies the Fisher exact test outcome ($\log p$) by the *z*-score of the deviation from the expected rank. They report optimal results using the combined score metric [42, 43] and, when ranking pathways etc., scores ≥ 2 were used to identify enrichment.

The frequency of pathways in each group were counted to allow the ranking of frequency across the studies. Using upregulated genes in the resistant group, an Enrichr-based query of the Reactome database identified 10 pathways shared by more than half the studies, 9 related to cell cycle and division and 1 broadly linked to the immune system (R-HAS-168256). KEGG database analysis also found the most common pathways to be cell cycle and cytokine-cytokine receptor interactions, but these were found in only 7 and 5 studies, respectively.

This type of analysis produced more shared results when applied to the upregulated genes in the susceptible group. Table 2 shows the 12 KEGG pathways that were shared by more than half of this group, and a subset (25) of the 88 Reactome pathways that met this criterion. Innate and immune response signaling pathways were abundantly represented, as were cell cycle and apoptosis. It is noteworthy that both lists contain pathways related to proteasomal and ubiquitin activity, since ubiquitination was recently reported to upregulate influenza virus polymerase function [44] and earlier work found that inhibition of the ubiquitin proteasome system can inhibit influenza A infectivity [45]. Moreover, proteasome inhibitors showed improved survival in mice infected with influenza [46] and in a murine model of SARS-like pneumonia [47]. No further *in vivo* studies using proteasome inhibitors in preclinical models of fatal influenza are available, so the potential benefit of targeting this pathway remains an intriguing possibility in need of more definitive testing. A similar analysis of the downregulated genes produced much smaller lists of shared pathways, for example for the susceptible group only 7 KEGG and 2 Reactome pathways were identified (e.g., axon guidance, cGMP-PKG signaling, valine, leucine and isoleucine degradation, Hippo signaling, propanoate metabolism, ECM-receptor interaction, and Rap1 signaling KEGG pathways). The lower yield in downregulated pathways may reflect a generally lower number of downregulated DEGs compared to those upregulated in the gene signatures, the lower sensitivity of the vote-counting method used, or other factors. For example, all but 1 of the studies

Table 2. Pathways enriched in upregulated gene signatures from susceptible mice

	Frequency
<i>Reactome pathway</i>	
Signaling by interleukins	13
Activated TLR4 signaling	13
Immune system	12
Cytokine signaling in immune system	12
TCR signaling	12
Toll-like receptor cascades	11
Signaling by VEGF	11
Activation of NF- κ B in B cells	11
Innate immune system	10
Apoptosis	10
Programmed cell death	10
p53-dependent G1 DNA damage response	10
Autodegradation of the E3 ubiquitin ligase COP1	10
Ubiquitin-mediated degradation of phosphorylated Cdc25A	10
Vpu-mediated degradation of CD4	10
Degradation of AXIN	10
Regulation of activated PAK-2p34 by proteasome-mediated degradation	10
Regulation of apoptosis	10
CDK-mediated phosphorylation and removal of Cdc6	10
Ubiquitin-dependent degradation of cyclin D	10
Ubiquitin-dependent degradation of cyclin D1	10
Cyclin A:Cdk2-associated events at S phase entry	10
Antigen processing cross-presentation	10
Regulation of ornithine decarboxylase	10
<i>KEGG pathway</i>	
NF- κ B signaling pathway	10
Osteoclast differentiation	10
Proteasome	10
Hepatitis B	9
Toxoplasmosis	9
Transcriptional misregulation in cancer	9
Toll-like receptor signaling pathway	8
Cytokine-cytokine receptor interaction	8
Apoptosis	8
Tuberculosis	8
Lysosome	8
Jak-STAT signaling pathway	8

here used a microarray methodology. RNA sequencing-based transcriptome profiling offers an increased dynamic range and, once more datasets using this approach become available, it may be possible to gain further insights.

The Enrichr tool also facilitates queries of the Library of Network-Based Cellular Signatures (LINCS, www.lincsproject.org) database [48, 49], which is based on the

Table 3. Candidate drugs and targets anticorrelated to the upregulated gene signature in susceptible mice

Drug	Frequency	Target
PD-325901	94	MEK
CGP-60474	61	CDK1.2
Geldanamycin	45	HSP90
Alvocidib	32	CDK9
GSK-2126458	28	PI3K mTOR
Trametinib	27	MEK
Selumetinib	26	MEK
AZD-8330	25	MEK
NVP-AUY922	24	HSP90
Palbociclib	23	CDK4, 6
BMS-387032	21	CDK2, 7, 9
PD-184352	21	MEK
Mitoxantrone	18	type II topoisomerase
Radicalcol	18	HSP90
Afatinib	17	EGFR
NVP-TAE684	17	ALK
AS-605240	14	PI3K
AT-7519	14	CDK1, 2, 4, 6, 9
Canertinib	14	EGFR Erb2
Dasatinib	14	Abl Src
PI-103	14	PI3K
GSK-1059615	13	PI3K mTOR
HG-6	12	Braf
NVP-BEZ235	12	PI3K mTOR
WZ-3105	12	CLK2, CNSK1E, FLT3, ULK1
WZ-4	12	c-Fes
A443654	11	Akt
AZD-5438	10	CDKx
Gefitinib	9	EGFR
PHA-793887	9	CDKx
Torin-1	9	mTOR
Torin-2	9	mTOR
Dovitinib	8	Multiple RTKs
GDC-980	8	PI3K

connectivity map concept [50]. Through an extraordinary effort, this resource has compiled gene signatures in 20 or more cell lines in response to over 20,000 drugs and compounds as well as numerous shRNA inhibitors and overexpression agents. Gene lists (like those generated here) can be compared to the database to identify in rank order agents that produce a similar (correlated) mRNA profile (signature) or those that produce an opposite (anticorrelated) signature. This allows rapid in silico identification of potential inhibitors (agents anticorrelated to the upregulated genes associated with an undesirable phenotype, e.g., high mortality from influenza) or agonists that might mimic desired phenotypes (e.g., upregulated gene signatures linked to high resistance to influ-

enza mortality). Using this tool inside the Enrichr system, the upregulated genes from each of the susceptible mouse groups was used to query the LINCS Chem Pert Down database, with the goal of identifying potential inhibitors that would downregulate the signature associated with high mortality in these susceptible mice. After collecting the drug list for each study, the frequency of agents was tabulated to identify the most common potential drugs and their targets. The results are shown in Table 3.

One of the prominent targets identified is MEK (mitogen-activated protein kinase kinase, aka MAP2K, MAPKK), a component of the Ras-Raf-MEK-ERK signaling pathway which is beneficial for the virus based on in vitro studies [51]. There is also 1 report of improved in vivo survival after influenza infection (70 vs. 0% in vehicle-treated controls) using a MEK inhibitor delivered by aerosol into the lungs [52]. The same paper also found that a single aerosol dose reduced viral lung loads at 24 h after infection (by 80–90%) by 4 different influenza strains. It is notable that a number of MEK inhibitors are in use or under investigation in human cancer patients [53].

Another frequent target in Table 3 is the cyclin-dependent kinases (CDKs), another drug class already in use in human patients [54]. The available evidence for the potential of CDK inhibitors is mixed. Influenza virus-infected p27^{-/-} mice (missing the CDK inhibitor 1B; Cdkn1b) accumulated significantly lower viral titers in the lung, suggesting at least that this inhibitor is harmful rather than helpful [55]. In contrast, CDK9 facilitates the interaction of viral and cellular polymerases which might lead to a benefit if inhibited [56]. Additional evidence suggests a potential benefit for CDK inhibition to allow resolution of inflammation by promoting inflammatory cell apoptosis [57]. Indeed, timing may be especially important when testing this category of agents as genes and pathways linked to cell division have been identified at different times in both susceptible and resistant mice.

A number of agents are available as inhibitors of the mammalian target of rapamycin (mTOR). For example, the mTOR inhibitor everolimus was used in a lethal mouse infection model of influenza A (H1N1 and H5N1) virus infection, where it significantly delayed death but did not prevent ultimate mortality [58]. No other in vivo data in animal models are available. Nevertheless, this hint of benefit, as well as the somewhat anecdotal clinical observations of improved outcomes in patients with severe H1N1 pneumonia and acute respiratory failure treated with an mTOR inhibitor (sirolimus) [59], indicates that further study of this class of agents is warranted.

A number of the other agents and targets identified have suggestive in vitro data to support their potential utility but the available data are relatively scant and in vivo testing for improved survival has not been reported. Heat shock protein (HSP90) associates with influenza polymerase proteins and HSP90 inhibitors geldanamycin or its derivative 17-AAG to delay the growth of influenza virus in cell culture resulting in a 1- to 2-log reduction in viral titer early in infection [60]. No in vivo studies have been reported to date. The identification of mitoxantrone, a type II topoisomerase inhibitor, is intriguing in view of the recent finding that type I topoisomerase inhibition suppresses inflammatory genes and protects from death by inflammation, including the improved survival of mice coinfecting with influenza and *Staphylococcus aureus* [61]. The PI3K component of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is another frequent target in Table 3, with some suggestive evidence in vitro that this molecule contributes to influenza propagation [62].

Conclusions

Excess or dysregulated host inflammatory responses play an important role in morbidity and mortality caused by severe influenza. It would be wonderful if efforts to develop a universal and more effective vaccine are successful. Until then, there remains a need for novel therapeutics to modulate host responses and improve outcomes in severe influenza. One strategy is to learn from the direct comparison of high-survivor versus high-mortality animal models, as reviewed here. Future meta-analyses using more sophisticated and potentially more powerful methods may allow further insights. Using transcriptome signatures linked to resistance or survival for in silico identification of therapeutic targets and agents is appealing based on the internal logic and the available data suggesting a benefit for many of the agents so identified. However, the ultimate test of this approach will be direct experimental testing.

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