Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER 3

Use of Functional Genomics to Understand Influenza–Host Interactions

Jamie L. Fornek,* **Marcus J. Korth,*** and **Michael G. Katze*,†**

Contents

Abstract

Infection with influenza typically results in mild-to-moderate illness in healthy individuals; however, it is responsible for 30,000–40,000 deaths each year in the United States. In extreme cases, such as the influenza pandemic of 1918, tens of millions of people have died from the infection. To prepare for future influenza outbreaks, it is necessary to understand how the virus interacts with the host and to determine what makes certain strains of influenza highly pathogenic. Functional genomics provides a unique approach to this effort by allowing researchers to examine the effect of influenza infection on global host mRNA levels. Researchers are making

* Department of Microbiology, University of Washington, Seattle, Washington 98195
† Washington National Primate Research Center, University of Washington, Seattle, Washington 98195

Advances in Virus Research, Volume 70
ISSN 0065-3527, DOI: 10.1016/S0065-3527(07)70003-9

increasing use of this approach to study virus–host interactions using a variety of model systems. For example, data obtained using microarray technology, in combination with mouse and macaque infection models, is providing exciting new insights into the pathogenicity of the 1918 virus. These studies suggest that the lethality associated with this virus is in part due to an aberrant and unchecked immune response. Progress is also being made toward using functional genomics in the diagnosis and prognosis of acute lung infections and in the development of more effective influenza vaccines and antivirals.

I. INTRODUCTION

For centuries, influenza virus has plagued humankind. While influenza infection typically causes mild-to-moderate illness in healthy individuals, it still results in 30,000–40,000 deaths per year in the United States. Those most susceptible to influenza infection are infants, the elderly, and those individuals that are immunocompromised due to HIV/AIDS infection or organ/tissue transplant (CDC, 2006). In extreme cases, such as the 1918 pandemic, it is estimated that 50 million people died as a result of influenza infection (Taubenberger and Morens, 2006). What was unique about this pandemic is that the most susceptible to this disease were young, otherwise healthy, individuals. Since 1918, multiple influenza pandemics have occurred, although none nearly as deadly. Another influenza pandemic is inevitable and much effort is being placed on disease surveillance and monitoring of transmission across species (Pandemic Flu, 2007; Subbarao and Joseph, 2007).

Of particular concern is the H5N1 family of avian influenza viruses (Horimoto and Kawaoka, 2005). While the transmission rate of H5N1 viruses from birds to humans is extremely low, the case mortality rate in humans is greater than 50% (WHO, 2007). Fortunately, human-to-human transmission is extremely rare (WHO, 2005). It is difficult to predict for how long this will be the case and there is increasing concern that H5N1 viruses will recombine with human viruses. This could result in an H5N1 virus with the capacity for human-to-human transmission and perhaps generate a catastrophic pandemic (Subbarao and Joseph, 2007).

Understanding the ways in which influenza interacts with the host is an important component of preparing for the next pandemic. It is necessary to understand these interactions in order to improve existing vaccines, to develop new and more efficacious vaccines that will provide protection against multiple strains and subtypes, and to develop new antiviral therapeutics (Subbarao and Joseph, 2007). Because of its ability to provide a global view, functional genomics is one of the most useful approaches for studying virus–host interactions. Our laboratory is using

functional genomics to study a variety of viruses, including HCV, SIV/HIV, Ebola virus, HSV, SARS coronavirus, West Nile virus, and influenza virus (Baas *et al.*, 2006a,b; Baskin *et al.*, 2004; Fredericksen *et al.*, 2004; Geiss *et al.*, 2000, 2001, 2002, 2003; Kash *et al.*, 2004, 2006a,b; Kobasa *et al.*, 2007; Lederer *et al.*, 2006; Pasieka *et al.*, 2006; Smith *et al.*, 2003a,b, 2006; Thomas *et al.*, 2006; Walters *et al.*, 2006, 2006a,b). This chapter will focus on how microarray technology is being utilized to uncover the mysteries of influenza pathogenesis. We will explore increasingly complex models for studying influenza–host interactions using functional genomics, including cell culture systems, murine models of infection, and nonhuman primates (Fig. 1). Finally, we will discuss the promise of using genomics to define molecular signatures of the disease that could lead to the evolution of the microarray as a diagnostic tool.

II. MODEL SYSTEMS OF INFLUENZA A INFECTION USED IN FUNCTIONAL GENOMICS

A. Cell culture models

Initial functional genomic endeavors in our laboratory utilized established cell lines to understand the ways in which influenza virus disrupts cellular processes. We first performed a series of experiments to determine replication-dependent and -independent events during influenza infection. HeLa cells were mock infected or infected with either active or heat-inactivated A/WSN/33 (H1N1). Using the dual-labeling technique, cDNA arrays were hybridized with RNA from mock versus active or heat-inactivated virus or with RNA from heat-inactivated versus active virus, allowing us to determine which genes were regulated by actively replicating virus (Geiss *et al.*, 2001).

We found that while there are distinct subsets of genes whose regulation is replication dependent or independent, more gene expression changes were observed in the presence of replicating virus. Further analysis revealed that these genes could be classified in five major categories: protein synthesis, cytokine and growth factor signaling, transcription factors and DNA-binding proteins, processing and export of mRNA, and the ubiquitin pathway. In contrast, genes whose regulation was replication independent were grouped representative of the following categories: metallothioneins, cell cycle related, transcriptional regulators, part of the ubiquitin pathway, or cellular kinases (Geiss *et al.*, 2001). Although specific aspects of influenza replication-independent and -dependent events could have been assessed using conventional laboratory techniques, our gene expression studies allowed us to examine numerous gene expression changes at the same time. From this data, it was possible to

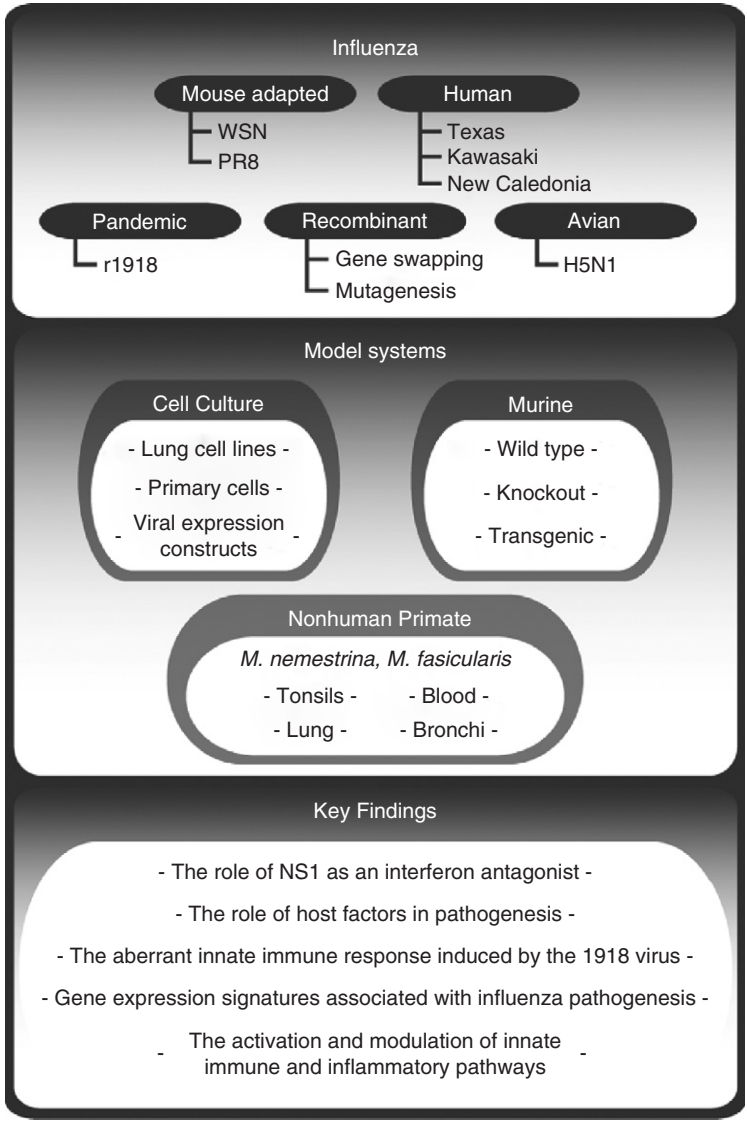

FIGURE 1 Representation of the range of viruses and experimental systems we have used to evaluate influenza virus–host interactions using genomic technologies. Highlights of experiments related to the use of these experimental systems are summarized in this chapter. WSN: A/WSN/33; PR8: A/PR/8/34; Texas: A/Texas/36/91; Kawasaki: A/Kawasaki/173/01; New Caledonia: A/New Caledonia/99; r1918: reconstructed 1918 virus.

identify specific processes that are related to influenza replication-independent and -dependent events and speculate on how these events work together in influenza pathogenesis.

Further studies using a cell culture system were aimed at discerning viral determinants of virulence. Of particular interest was the viral NS_1 protein. NS_1 appears to play a role in subverting the host response to the virus. It has been suggested that NS_1 attenuates the interferon response to the virus through its double-stranded (ds) RNA-binding domain (García-Sastre *et al.*, 1999). It has also been suggested that the dsRNA-binding domain of NS_1 functions to inhibit the 2′5′-OAS/RNaseL antiviral response (Min and Krug, 2006). This may indicate that NS_1 from different influenza viruses plays distinct roles in subverting the host response to the virus.

To better understand the effect of NS_1 on virus–host interactions, we infected an established human lung epithelial cell line, A549, with A/PR/8/34 (H1N1), A/PR/8/34 in which NS_1 was deleted, or with A/PR/8/34 in which NS_1 contained a deletion in the C-terminus (Geiss *et al.*, 2002). The latter two viruses were reconstructed using plasmid-based reverse genetics (Fodor *et al.*, 1999). These studies allowed us to examine the global host response to influenza infection in the absence of NS_1 or in response to infection with a virus exhibiting attenuated NS_1 function.

Numerous genes were preferentially upregulated in response to infection with the mutant viruses compared to the parental strain. Many of these genes were related to the antiviral and interferon responses. These data suggest a role for the NS_1 of A/PR/8/34 as an antagonist of the interferon response to the virus (Geiss *et al.*, 2002). Antagonism of this crucial defense response to influenza most likely contributes to the lethality of this virus in mice. Therefore, these initial studies from our laboratory were crucial in understanding the importance of the interferon response in the host response to influenza.

This study was also the first to use functional genomics to examine the role of specific genes from the 1918 strain. In addition to the viruses mentioned above, A549 cells were infected with A/WSN/33 or a recombinant in which the NS_1 of A/WSN/33 was replaced with the NS_1 from the 1918 virus. We noted that there was greater suppression of interferon-stimulated genes in cells infected with the 1918 NS_1 recombinant virus than in cells infected with the parental strain. The host response to A/WSN/33 virus containing the 1918 NS_1 was also compared with the response to wild-type A/PR/8/34 and with the A/PR/8/34 NS_1 mutant viruses. From these analyses, we determined that the expression of numerous interferon-stimulated genes was anti-correlated between these viruses and A/WSN/33 containing the 1918 NS_1. For example, NMI and STAT1 expression were upregulated in cells infected with the A/PR/8/34 viruses but downregulated in cells infected with A/WSN/33

containing the NS_1 from the 1918 virus. These studies suggest that the NS_1 from the 1918 virus is more adept at suppressing key interferon responses. It will be interesting to use functional genomics to compare the effect of the NS_1 from the 1918 virus on host–virus interactions to that of the NS_1s from modern day low pathogenicity human influenza viruses and both low and high pathogenicity avian H5N1 viruses. Such comparisons will lend a global view into how different influenza NS_1s affect the host response and lead to important observations as to the role of NS_1 in influenza pathogenicity (Geiss *et al.*, 2002).

Interestingly, mice infected with a virus containing the NS_1 of 1918 and the other seven genes from A/WSN/33 did not succumb to the infection. In contrast, all mice infected with A/WSN/33 died by 10 days postinfection (Basler *et al.*, 2001). These data, in conjunction with the array studies described above, suggest that the NS_1 of the 1918 virus is an important virulence factor, but it is not solely responsible for the high lethality of the 1918 virus. Therefore, it was imperative to study the effect of other 1918 genes on mortality and examine the critical interplay of all of the 1918 genes. Such studies will be discussed in the following section covering murine models of influenza infection.

Using cell culture systems in the application of functional genomics is crucial to the understanding of how influenza infection affects antiviral responses on the cellular level. However, these systems are limited in that the data obtained from them can only lead to inferences as to what is occurring in the host as a whole. For this reason, it is necessary to study influenza infection in the context of the whole organism. The use of functional genomics in conjunction with various mammalian models of infection, and in humans, will be discussed in the next sections.

B. Murine models

In order to study the effects of influenza in the context of the whole organism, many scientists have utilized mouse models of infection. Although mice are not a natural host for influenza virus, their accessibility and the vast repertoire of genetically altered species makes them a useful tool in many areas of research, including functional genomics. Since laboratory strains of mice are inbred, this reduces host variation, making it easier to clarify how influenza is affecting the host. Of particular interest to our laboratory is how the host response induced by highly pathogenic influenza infection differs from that induced by viruses with lower pathogenicity.

Of all the influenza viruses that have surfaced in the last century, very few have caused as much intrigue as the 1918 pandemic strain. Among the most perplexing questions surrounding the influenza pandemic of 1918 is what made this virus so deadly. Environmental, biological, or

demographic factors could have contributed to its virulence; however, the most pertinent factors may be related to how this virus interacts with the host innate immune response. As mentioned in the previous section, we used functional genomics to study the effect of the 1918 NS_1 on global gene expression using a cell culture system. While this study provided an important first step in understanding this deadly virus, it only hints at what might be occurring in the whole host.

With the sequencing and reconstruction of the 1918 virus using reverse genetics (Tumpey *et al*., 2005a), our laboratory and others have been able to study the effects of various genes from this virus and the fully reconstructed virus on the host (Basler *et al*., 2001; Reid *et al*., 1999, 2000, 2002, 2004; Taubenberger, 1998; Taubenberger *et al*., 1997). In initial studies, we infected mice with the lethal, A/WSN/33 stain or with a recombinant of this virus containing the HA and NA from the 1918 virus. A recombinant A/WSN/33 virus containing the HA and NA of a contemporary human strain (A/New Caledonia/99) was also included in these studies. Both HA and NA are major virulence factors and HA is the major viral factor against which host antibodies are produced (Lamb and Krug, 1996) and evidence suggests that the HA of the 1918 virus is necessary for transmission (Tumpey *et al*., 2007). Gene expression profiling was then performed on lungs isolated from these mice. Increased gene expression in the lungs of mice infected with either A/WSN/33 or the recombinant virus containing the HA and NA from the 1918 virus was observed at 24 h postinfection. In contrast, relatively few gene expression changes were observed in the lungs of mice infected with the A/WSN/33 recombinant strain containing the HA and NA from A/New Caledonia/33. By 72 h postinfection, gene expression changes were similar between the two infection groups, indicating that the HA and NA of the 1918 virus were sufficient to accelerate the host response to the virus (Kash *et al*., 2004).

A subset of genes was preferentially upregulated in mice infected with the A/WSN/33 recombinant virus containing the 1918 HA and NA. Among this group were genes that are indicative of T cell activation, macrophage activation, and cell death (Kash *et al*., 2004). In support of these findings, Tumpey *et al*. demonstrated that mice infected with A/Texas/36/91 containing the HA and NA from the 1918 virus or with A/WSN/33 containing these genes developed severe lung pathology, including varying degrees of necrotizing bronchitis, alveolitis, and pulmonary edema. Strikingly, there was also an increase in neutrophils and alveolar macrophages in the lungs of these animals. To analyze the importance of these immune cells in the context of A/Texas/36/91 recombinant virus, animals in which neutrophils and/or alveolar macrophages had been depleted were infected with a sublethal dose of the virus. Infected neutrophil-depleted mice had a 60% survival rate.

In contrast, all animals in which alveolar macrophages or both alveolar macrophages and neutrophils were depleted died by 9 days postinfection with the recombinant virus (Tumpey *et al.*, 2005b). Taken together, these data emphasize the importance of certain immune mediators in combating infection with a recombinant virus containing the HA and NA from the 1918 virus. However, as discussed below, these findings also suggest that an inappropriate activation of the host response to the virus may contribute to its pathogenicity.

We have also used functional genomics to analyze the host response of mice infected with the fully reconstructed 1918 virus. These studies, led by John Kash, revealed that genes related to various immune cells, notably NK cells, neutrophils, macrophages, and T helper 1 (Th1) cells, were upregulated in mice infected with the fully reconstructed 1918 virus as early as 1 day postinfection. These genes were persistently activated in the lungs of r1918-infected mice throughout the course of the experiment (5 days) (Kash *et al.*, 2006b). Key mediators of the immune response to influenza virus are cytokines and chemokines that are responsible for the activation of and recruitment of immune cells into the infected tissue (Julkunen *et al.*, 2001). In agreement with early and persistent activation of immune cells in r1918-infected mice, this same gene expression pattern was observed for genes related to pro-inflammatory cytokines and chemokines such as Tnf, Il6, and Ccl5 (Kash *et al.*, 2006b). It is crucial that a delicate balance of immune responses is maintained during infection in order to limit excessive damage to the host. If these responses go unchecked, or are insufficient, it can result in dire consequences for the host (La Gruta *et al.*, 2007). Our data suggest that a hyperactive and persistent host response is associated with the 1918 virus and that this is a key contributor to the high mortality associated with this virus.

Another important aspect of our study was an examination of what effect the full constellation of genes from the 1918 virus had on gene expression and virus-induced morbidity and mortality. In order to accomplish this, mice were infected with the fully reconstructed 1918 virus (r1918), with A/Texas/91/36 containing the HA and NA from 1918 (2:6 1918), or with A/Texas/91/36 containing the HA, NA, M, NP, and NS_1 genes from the 1918 virus (5:3 1918) (Fig. 2). Compared with the response of mice infected with the r1918 virus, which exhibited early and persistent upregulation of genes related to NK cells, neutrophils, macrophages, and T helper 1 (Th1) cells, mice infected with either the 5:3 1918 or 2:6 1918 virus exhibited a delay in the upregulation of these genes. However, expression levels of genes related to these immune cells was similar in all three 1918 recombinant viruses by day 5 postinfection.

Interestingly, animals infected with either the 5:3 1918 virus or the 2:6 1918 virus exhibited lung pathology intermediate to mice infected with A/Texas/91/36 and those infected with r1918 at day 3 postinfection.

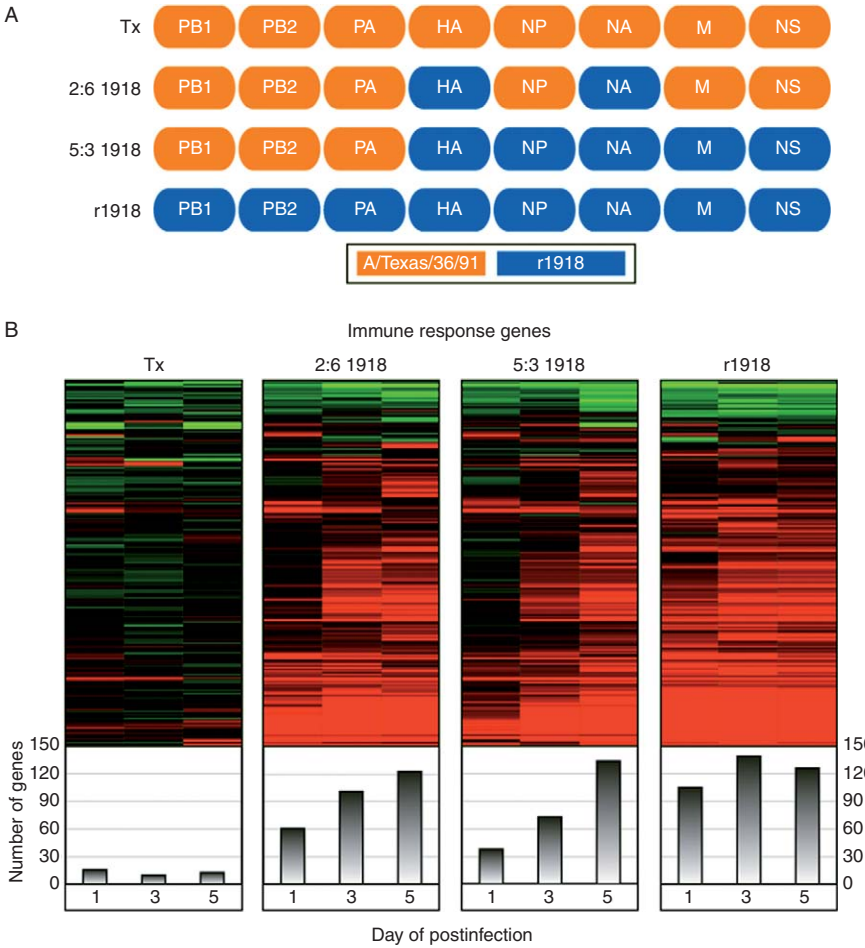

FIGURE 2 Effect of the full constellation of genes from the 1918 virus on gene expression. (A) Mice were infected with A/Texas/91/36, with A/Texas/91/36 containing the HA and NA from 1918 (2:6 1918), with A/Texas/91/36 containing the HA, NA, M, NP, and NS_1 genes from the 1918 virus (5:3 1918), or with the fully reconstructed 1918 virus (r1918). (B) Global gene expression profiles and number of differentially regulated genes for mice infected with each virus at days 1, 3, and 5 postinfection.

The differences in gene expression not only correlated with lung pathology, but also with viral titers and morbidity, demonstrating the usefulness of gene expression profiling in understanding molecular mechanisms of disease and disease outcome (Kash *et al.*, 2006b).

Studies in our laboratory are now focusing on combining genomics with the use of knockout or transgenic mice to further understand the complex host–virus interactions that occur in response to infection with the 1918 virus. We are also interested in using genomics to examine the effects of H5N1 infection on mice and to determine if there are molecular signatures of disease that are present as a consequence of infection with highly pathogenic strains of influenza. In addition, we are taking advantage of the vast repertoire of transgenic and knockout mice available to gain further insight into key regulators of the innate and/or adaptive immune response to influenza infection in general. For example, in collaboration with Michael Gale, we are working to understand the role of the pattern recognition receptor, retinoic acid inducible gene I (RIG-I), during influenza infection. RIG-I plays an important role in the interferon response to many viruses, including influenza (Foy *et al.*, 2005; Fredericksen and Gale, 2006; Kato *et al.*, 2005, 2006; Liu *et al.*, 2007; Sumpter *et al.*, 2005; Yoneyama *et al.*, 2005, 2004). We recently examined the gene expression profiles in RIG-I deficient mouse embryonic fibroblasts (MEFs) infected with A/PR/8/34. Global gene expression profiles revealed significant differences in gene expression between wildtype and RIG-I deficient MEFs. We are in the process of furthering analyzing data, but preliminary analyses have revealed an important role for RIG-I in the host response to the virus (Loo *et al.*, submitted).

C. Nonhuman primate models

Even though mouse models have provided critical insights into the pathogenesis of influenza, the information gained from these studies is limited since mice are not natural hosts for the virus. For this reason, data obtained from mouse studies can be difficult to translate to human infection. Numerous studies have utilized nonhuman primate models to study influenza pathogenesis (Berendt, 1974; Grizzard *et al.*, 1978; Liu *et al.*, 1997; Rimmelzwaan *et al.*, 2001; van Riel *et al.*, 2006). Unlike mouse models of infection, influenza infection in nonhuman primate models mimics human infection. For example, nonhuman primates can be infected with human influenza strains without prior adaptation and the virus is transmissible between animals. In addition, nonhuman primates and humans are close evolutionary relatives. As a consequence, nonhuman primates are increasingly being utilized to examine influenza pathogenesis, and with the sequencing of the rhesus macaque genome (Rhesus macaque genome sequencing and analysis consortium, 2007), genomic and proteomic resources for working with these animals are becoming progressively more available (Magness *et al.*, 2005; Spindel *et al.*, 2005; Wallace *et al.*, 2007).

Although there are numerous advantages to using nonhuman primates in influenza research, certain considerations must be taken before working with them. Nonhuman primates exhibit host variation similar to that in humans, a factor that needs to be taken into account when analyzing genomics data. Additionally, the numbers of nonhuman primates available for research are limited. Therefore, most nonhuman primate studies are restricted in their sample size. Lastly, considerable ethical concerns must be taken into account when using nonhuman primates in research studies. Our laboratory, along with others, has diligently worked to address these concerns and yet still obtain the insights into influenza–host interactions that only studies in nonhuman primates can provide.

In a seminal study led by Carole Baskin, pigtailed macaques (*Macaca nemestrina*) were infected with the reconstructed H1N1 human influenza strain, A/Texas/36/91. Physical symptoms, such as throat inflammation, loss of appetite, and weight loss, correlated with the upregulation of interferon-stimulated genes at days 4 and 7 postinfection. Gene expression profiling also revealed the upregulation of genes related to neutrophil and monocyte/macrophage function. Accordingly, an influx of neutrophils and macrophages into the lungs of infected monkeys was observed. Although not the first to use nonhuman primates as a model of influenza infection, this study was unique for two reasons. It was the first to use pigtailed macaques and it was the first in which functional genomics was used to examine influenza infection in nonhuman primates (Baskin *et al.*, 2004).

To expand upon the above study, we have also employed functional genomics to assess the effect of influenza infection on the early innate immune response in the lungs of pigtailed macaques, how genes related to this response were regulated over time, and whether gene expression signatures of infection could also be detected in the blood. Finally, we examined the correlation between genomic and proteomic data collected for both lung and PBMC samples. Significantly, this study was also the first to use macaque-specific oligonucleotide arrays, which were developed in our laboratory (Wallace *et al.*, 2007).

As in the previous study, animals were infected with A/Texas/36/91. Subsequent analysis focused on gene expression changes present at day 2 postinfection in order to determine molecular correlates of early influenza infection. In lesions where viral mRNA was present, there was increased expression of interferon-stimulated genes and antiviral-related genes. Notably, the majority of these genes were significantly upregulated, suggesting a robust host response against the virus. Differential expression of cytokine, chemokine, and immune-related genes was also present in samples isolated at 7 days postinfection (Baas *et al.*, 2006a). These data indicate a robust and sustained host response in the lungs of influenza-infected pigtail macaques.

We also compared the signatures of infection in the lung with those found in whole blood. This analysis identified numerous genes whose expression was upregulated in the lung and in the blood throughout the time course of the infection. There was an upregulation in interferon-stimulated genes and antiviral-related genes such as IRF7, IFIT2, OAS1, and OAS3. Our findings suggest that there are common signatures of influenza infection between the lung and whole blood, indicating that gene expression profiling of blood may eventually prove useful for diagnostic or prognostic applications. This subject is further discussed in the following section.

We also worked with Richard Smith's group at Pacific Northwest National Laboratory to perform the first ever global proteomic analyses on macaque lung samples. Side-by-side comparison of genomic and proteomic data from infected macaque lung samples revealed that there were many correlations between the two sets of data. Of particular interest, were the correlations observed for interferon-stimulated genes and antiviral-related genes. Members of these families, such as IFIT1, IFIT2, STAT1, and MX1, were identified by both genomics and proteomics. In further support for the use of whole blood as a surrogate marker of influenza pathogenesis in the lungs, gene expression data for the above markers and others correlated with the lung genomics and proteomics data (Baas *et al*., 2006a). We would like to further these studies by determining if similar proteomic results are observed in whole blood.

From these analyses, we also identified an increase in the abundance of certain proteins in influenza-infected lung that would not have been predicted by our genomics data. This observation points to the need for the integration of genomics and proteomics data to gain a more complete understanding of influenza pathogenesis. Furthermore, integration of genomic and proteomic data will enhance our understanding of the differences between mRNA levels and protein abundance.

We have also recently used functional genomics and a macaque infection model to study the pathogenesis of the 1918 virus. For these studies, cynomolgus macaques (*Macaca fasicularis*) were infected with the human H1N1 virus, A/Kawasaki/173/01, or with the reconstructed 1918 virus (Kobasa *et al*., 2007). Microarray analysis on bronchi from infected animals revealed a robust activation of numerous pro-inflammatory chemokine and cytokine genes in both A/Kawasaki/173/01 and 1918-infected animals at day 3 postinfection. Additionally, there was an increased activation of genes related to the interferon-α response in response to infection with either of these viruses at this time-point. Strikingly, many of the genes related to these responses exhibited a more robust upregulation in the A/Kawasaki/173/01-infected animals at day 3 postinfection, but returned to baseline levels or were downregulated later in infection compared with r1918-infected animals. In contrast, animals infected with

the r1918 virus exhibited an increased and robust upregulation of expression of interferon-stimulated genes and chemokines and cytokines through the study endpoint.

In support of our genomics observations, CCL2, CCL5, IL-8, and IL-6 levels were increased in the serum of r1918-infected macaques compared with the levels present in A/Kawasaki/173/01-infected animals at days 3 and 6 postinfection. Viral titers were substantially greater in both the upper and lower respiratory tracts of macaques infected with r1918 at all three time-points postinfection. r1918-infected animals also exhibited severe lung pathology at 8 days postinfection (Kobasa *et al*., 2007). Taken together, these data agree with data obtained using mouse models, suggesting that the pathogenesis induced by infection with the 1918 virus is associated with, and potentially caused by, an aberrant and unchecked immune response to the virus. As a consequence, this response turns from one that is beneficial to the host to one that is extremely detrimental.

The studies described above demonstrate how nonhuman primate models can be used in combination with functional genomics to understand influenza–host interactions. Our studies, in conjunction with those of others, firmly demonstrate that nonhuman primate models of influenza provide crucial information into disease progression and pathogenesis. Currently, we are focused on using functional genomics to assess the effectiveness of influenza vaccination in nonhuman primates (Baskin *et al.*, submitted). These studies illustrate a novel use for functional genomics in influenza vaccine development. Genomic analyses during vaccine trials may reveal gene expression markers of protective immunity or gene expression changes that are indicative of a predisposition to a particular response to immunization and subsequent challenge.

D. New diagnostic approaches

As mentioned previously, a major promise of genomics is the capacity to use this technology in the more precise and efficient diagnosis of disease. Of major interest, is the use of functional genomics to ascertain molecular signatures of infection that permit the distinction among diseases. Discussed below is how this technology is being tested for influenza diagnosis.

To identify the gene expression signatures induced by various pathogens, Chaussabel *et al*. examined peripheral blood mononuclear cells (PBMCs) obtained from pediatric patients presenting with various illnesses (2005). Specifically, they examined diseases with distinct immunological components such as systemic lupus erythematosus (SLE), influenza A, *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pneumoniae*. They also examined adult patients who received liver transplants with immunosuppressive therapy or patients who received bone marrow transplants

and experienced graft versus host disease. These samples were compared with PBMCS from healthy volunteers. The authors were able to identify unique gene expression patterns for patients presenting with influenza and SLE. They then determined expression profiles common to all of the diseases using genes that were either up- or downregulated in patients infected with influenza or SLE. Analyses also demonstrated that the genes whose expression was regulated in a similar manner in both influenza and SLE patients fell into distinct categories such as defense response, interferon induction, and heavy metal binding. Furthermore, the authors were able to determine how many genes related to these processes were expressed in individual patients (Chaussabel *et al.*, 2005).

In an extension of the above studies, the authors examined the gene expression profiles of PBMCs from young patients presenting with acute infections including influenza A, *S. aureus*, *S. pneumoniae*, and *E. coli* (Ramilo *et al.*, 2007). Analysis of these samples was performed in a methodical manner using statistical comparison, sample classification, validation of classifier genes using a test set, and validation of microarray platforms and chips. The authors were able to identify subsets of genes that distinguished patients with influenza (viral infection) from those that presented with either *E. coli* or *S. pneumoniae* (Gram-negative and Gram-positive bacterial infections, respectively). The same was found for patients infected with influenza compared to those infected with *S. aureus* (Gram-positive bacterial infection). Distinct expression patterns were also present in PBMCs from patients infected with *E. coli* or *S. aureus*.

Using sets of classifier genes obtained from the above analyses, the authors examined the gene expression profiles of PBMCs isolated from patients presenting with lower respiratory infections the same as those listed above or from healthy volunteers. From these analyses, the authors were able to classify the samples from these new patients into the correct disease categories. In addition, the authors tested a separate set of samples using a different array platform. These studies also demonstrated that patients presenting with these illnesses could be accurately classified into distinct groups based on gene expression profiles (Ramilo *et al.*, 2007). Through these painstaking efforts, the authors convincingly used functional genomics to discriminate between patients with a variety of acute infections, including influenza.

While these studies provide evidence that genomics can be used to define molecular signatures of disease associated with certain pathogens, they also have significant limitations. For example, these studies were performed on samples that had been taken from patients that had already been diagnosed with a particular illness and genomic analyses only had to distinguish between a relatively few possibilities. However, in order to be effective in a clinical setting, gene expression profiling will need to provide a high degree of accuracy and overcome numerous confounding

factors such as age, race, gender, immune status, and co-infection with more than one pathogen. All of these issues must be addressed before functional genomics can function in disease diagnosis. However, once these challenges have been met, genomic diagnosis may decrease the amount of elapsed time between sample collection and disease diagnosis thereby allowing doctors to treat patients more quickly. This is particularly important for patients presenting with acute infections. Additionally, the use of microarrays in this manner may eliminate the need for patients to undergo certain painful and potentially dangerous diagnostic procedures, such as tissue biopsies.

III. CONCLUSIONS

Functional genomics has clearly provided critical information regarding virus–host interactions and has made significant contributions to influenza research. As we have described, functional genomics has been utilized to study influenza infection in a variety of model systems including cell culture, mice, and macaques. Researchers are also utilizing functional genomics to study influenza infections in chickens, but these endeavors are still in their infancy (Degen *et al.*, 2006). It will also be desirable to use functional genomics to examine influenza infection in ferrets. Unlike mice, ferrets can be productively infected with human influenza viruses and ferret-to-ferret transmission occurs. Due to these characteristics, ferrets provide a useful and unique model for influenza infection studies. Unfortunately, genomic studies using ferrets are currently limited due to the lack of ferret nucleotide sequence information. We therefore strongly recommend that the ferret genome be sequenced and that ferret-specific microarrays be developed.

From the functional genomics experiments published so far, we have been able to gain invaluable insight into influenza pathogenesis. Perhaps the most critical use of this technology has been in the study of the virus responsible for the deadly 1918 influenza pandemic. In regards to highly pathogenic influenza, future experiments should also focus on the effect of avian H5N1 infection on global gene expression, using multiple model systems such as those that are being used to study the 1918 virus.

Functional genomics has provided us with numerous insights into influenza–host interactions. In particular, we have utilized this technology to discern how low and high pathogenicity viruses affect host responses. However, there are many challenges facing our laboratory and others that use functional genomics. Of utmost concern, is the integration of the vast amounts of genomics data that is available and has yet to be generated. Among the major obstacles are microarray platform differences, species differences, cell type differences, and annotation differences

(Wallace *et al.*, 2006). Data from microarray studies also needs to be integrated with conventional biological approaches and with data that will be obtained from the burgeoning field of proteomics. As demonstrated throughout this manuscript, our laboratory has worked tirelessly to achieve these goals. We firmly believe that functional genomics will be crucial to the development of novel therapies necessary for the prevention of influenza infection and spread.

ACKNOWLEDGMENTS

We thank Tracey Baas, Carole Baskin, Gary Geiss, John Kash, and our many other colleagues past and present that contributed to the studies discussed in this review. We apologize for not being able to list them all by name. We also thank Sean Proll for figure preparation. Funding for genomic and proteomic studies in our laboratory is provided by Public Health Service grants R01AI022646, R01HL080621, R21AI017892, R21AI063436, R24RR016354, P01AI052106, P01AI058113, P30DA015625, and P51RR000166 from the National Institutes of Health.

REFERENCES

Baas, T., Baskin, C. R., Diamond, D. L., Garcia-Sastre, A., Bielefeldt-Ohmann, H., Tumpey, T. M., Thomas, M. J., Carter, V. S., Teal, T. H., Van Hoeven, N., Proll, S., Jacobs, J. M., *et al.* (2006a). Integrated molecular signature of disease: Analysis of influenza virus-infected macaques through functional genomics and proteomics. *J. Virol.* **80:**10813–10828.

Baas, T., Taubenberger, J. K., Chong, P. Y., Chui, P., and Katze, M. G. (2006b). SARS-CoV virus-host interactions and comparative etiologies of acute respiratory distress syndrome as determined by transcriptional and cytokine profiling of formalin-fixed paraffin-embedded tissues. *J. Interferon Cytokine Res.* **26:**309–317.

Baskin, C. R., Garcia-Sastre, A., Tumpey, T. M., Bielefeldt-Ohmann, H., Carter, V. S., Nistal-Villan, E., and Katze, M. G. (2004). Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). *J. Virol.* **78:**10420–10432.

Basler, C. F., Reid, A. H., Dybing, J. K., Janczewski, T. A., Fanning, T. G., Zheng, H., Salvatore, M., Perdue, M. L., Swayne, D. E., García-Sastre, A., Palese, P., Taubenberger, J. K., *et al.* (2001). Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. *Proc. Natl. Acad. Sci. USA* **98:**2746–2751.

Berendt, R. F. (1974). Simian model for the evaluation of immunity to influenza. *Infect. Immun.* **9:**101–105.

CDC (2006). CDC Flu Shot. www.cdc.gov/flu/about/qa/flushot.htm

Chaussabel, D., Allman, W., Mejias, A., Chung, W., Bennett, L., Ramilo, O., Pascual, V., Palucka, A. K., and Banchereau, J. (2005). Analysis of significance patterns identifies ubiquitous and disease-specific gene-expression signatures in patient peripheral blood leukocytes. *Ann. N. Y. Acad. Sci.* **1062:**146–154.

Degen, W. G. J., Smith, J., Simmelink, B., Glass, E. J., Burt, D. W., and Schijns, V. E. J. C. (2006). Molecular immunophenotyping of lungs and spleens in naive and vaccinated

chickens early after pulmonary avian influenza A (H9N2) virus infection. *Vaccine* **24:**6096–6109.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G., and García-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73:**9679–9682.

Foy, E., Li, K., Sumpter, R., Jr., Loo, Y. M., Johnson, C. L., Wang, C., Fish, P. M., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M., Jr. (2005). Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc. Natl. Acad. Sci. USA* **102:**2986–2991.

Fredericksen, B. L., and Gale, M., Jr. (2006). West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. *J. Virol.* **80:**2913–2923.

Fredericksen, B. L., Smith, M., Katze, M. G., Shi, P. Y., and Gale, M., Jr. (2004). The host response to West Nile virus infection limits viral spread through the activation of the interferon regulatory factor 3 pathway. *J. Virol.* **78:**7737–7747.

García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P., and Muster, T. (1999). Influenza A virus lacking the NS_1 gene replicates in interferon-deficient systems. *Virology* **252:**324–330.

Geiss, G. K., Bumgarner, R. E., An, M., Agy, M. B., van't Wout, A., Hammersmark, E., Carter, V. S., Upchurch, D., Mullins, J. I., and Katze, M. G. (2000). Large-scale monitoring of host cell gene expression during HIV-1 infection using cDNA microarrays. *Virology* **266:**8–16.

Geiss, G. K., An, M. C., Bumgarner, R. E., Hammersmark, E., Cunningham, D., and Katze, M. G. (2001). Global impact of influenza virus on cellular pathways is mediated by both replication-dependent and -independent events. *J. Virol.* **75:**4321–4331.

Geiss, G. K., Salvatore, M., Tumpey, T. M., Carter, V. S., Wang, X., Basler, C. F., Taubenberger, J. K., Bumgarner, R. E., Palese, P., Katze, M. G., and García-Sastre, A. (2002). Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: The role of the nonstructural NS_1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc. Natl. Acad. Sci. USA* **99:**10736–10741.

Geiss, G. K., Carter, V. S., He, Y., Kwieciszewski, B. K., Holzman, T., Korth, M. J., Lazaro, C. A., Fausto, N., Bumgarner, R. E., and Katze, M. G. (2003). Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. *J. Virol.* **77:**6367–6375.

Grizzard, M. B., London, W. T., Sly, D. L., Murphy, B. R., James, W. D., Parnell, W. P., and Chanock, R. M. (1978). Experimental production of respiratory tract disease in cebus monkeys after intratracheal or intranasal infection with influenza A/Victoria/3/75 or influenza A/New Jersey/76 virus. *Infect. Immun.* **21:**201–205.

Horimoto, T., and Kawaoka, Y. (2005). Influenza: Lessons from past pamdemics, warnings from current incidents. *Nat. Rev. Microbiol.* **3:**591–600.

Julkunen, I., Sareneva, T., Pirhonen, J., Ronni, T., Melen, K., and Matikainen, S. (2001). Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. *Cytokine Growth Factor Rev.* **12:**171–180.

Kash, J. C., Basler, C. F., García-Sastre, A., Carter, V., Billharz, R., Swayne, D. E., Przygodzki, R. M., Taubenberger, J. K., Palese, P., Katze, M. G., and Tumpey, T. M. (2004). Global host immune response: Pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. *J. Virol.* **78:**9499–9511.

Kash, J. C., Muhlberger, E., Carter, V., Grosch, M., Perwitasari, O., Proll, S. C., Thomas, M. J., Weber, F., Klenk, H. D., and Katze, M. G. (2006a). Global suppression of the host

antiviral response by Ebola- and Marburgviruses: Increased antagonism of the type I interferon response is associated with enhanced virulence. *J. Virol.* **80:**3009–3020.

Kash, J. C., Tumpey, T. M., Proll, S. C., Carter, V., Perwitasari, O., Thomas, M. J., Basler, C. F., Palese, P., Taubenberger, J. K., Garcia-Sastre, A., Swayne, D. E., Katze, M. G., *et al.* (2006b). Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* **443:**578–581.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O., and Akira, S. (2005). Cell type-specific involvement of RIG-I in antiviral response. *Immunity* **23:**19–28.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K. J., Yamaguchi, O., Otsu, K., *et al.* (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441:**101–105.

Kobasa, D., Jones, S. M., Shinya, K., Kash, J. C., Copps, J., Ebihara, H., Hatta, Y., Hyun Kim, J., Halfmann, P., Hatta, M., Feldmann, F., Alimonti, J. B., *et al.* (2007). Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature* **445:**319–323.

La Gruta, N. L., Kedzierska, K., Stambas, J., and Doherty, P. C. (2007). A question of self-preservation: Immunopathology in influenza virus infection. *Immunol. Cell Biol.* **85:**85–92.

Lamb, R. A., and Krug, R. M. (1996). Orthomyxoviridae: The viruses and their replication. *In* ''Fields Virology'' (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), pp. 1353–1395. Lippincott-Raven, Philadelphia.

Lederer, S., Walters, K. A., Proll, S., Paeper, B., Robinzon, S., Boix, L., Fausto, N., Bruix, J., and Katze, M. (2006). Distinct cellular responses differentiating alcohol- and hepatitis C virus-induced liver cirrhosis. *Virol. J.* **3:**98.

Liu, M. A., McClements, W., Ulmer, J. B., Shiver, J., and Donnelly, J. (1997). Immunization of non-human primates with DNA vaccines. *Vaccine* **15:**909–912.

Liu, P., Jamaluddin, M., Li, K., Garofalo, R. P., Casola, A., and Brasier, A. R. (2007). Retinoic acid-inducible gene I mediates early antiviral response and toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J. Virol.* **81:**1401–1411.

Magness, C., Fellin, P. C., Thomas, M., Korth, M., Agy, M., Proll, S., Fitzgibbon, M., Scherer, C., Miner, D., Katze, M., and Iadonato, S. (2005). Analysis of the macaca mulatta transcriptome and the sequence divergence between macaca and human. *Genome Biol.* **6:**R60.

Min, J. Y., and Krug, R. M. (2006). The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2′-5′ oligo (A) synthetase/RNase L pathway. *Proc. Natl. Acad. Sci. USA* **103:**7100–7105.

Pandemic Flu (2007). www.pandemicflu.gov/.

Pasieka, T. J., Baas, T., Carter, V. S., Proll, S. C., Katze, M. G., and Leib, D. A. (2006). Functional genomic analysis of herpes simplex virus type 1 counteraction of the host innate response. *J. Virol.* **80:**7600–7612.

Ramilo, O., Allman, W., Chung, W., Mejias, A., Ardura, M., Glaser, C., Wittkowski, K. M., Piqueras, B., Banchereau, J., Palucka, A. K., and Chaussabel, D. (2007). Gene expression patterns in blood leukocytes discriminate patients with acute infections. *Blood* **109:** 2066–2077.

Reid, A. H., Fanning, T. G., Hultin, J. V., and Taubenberger, J. K. (1999). Origin and evolution of the 1918 ''Spanish'' influenza virus hemagglutinin gene. *Proc. Natl. Acad. Sci. USA* **96:** 1651–1656.

Reid, A. H., Fanning, T. G., Janczewski, T. A., and Taubenberger, J. K. (2000). Characterization of the 1918 ''Spanish'' influenza virus neuraminidase gene. *Proc. Natl. Acad. Sci. USA* **97:**6785–6790.

Reid, A. H., Fanning, T. G., Janczewski, T. A., McCall, S., and Taubenberger, J. K. (2002). Characterization of the 1918 ''Spanish'' influenza virus matrix gene segment. *J. Virol.* **76:**10717–10723.

Reid, A. H., Fanning, T. G., Janczewski, T. A., Lourens, R. M., and Taubenberger, J. K. (2004). Novel origin of the 1918 pandemic influenza virus nucleoprotein gene. *J. Virol.* **78:**12462–12470.

Rhesus macaque genome sequencing and analysis consortium (2007). The rhesus macaque genome sequence informs biomedical and evolutionary analyses. *Science.* **316:**222–234.

Rimmelzwaan, G. F., Kuiken, T., van Amerongen, G., Bestebroer, T. M., Fouchier, R. A. M., and Osterhaus, A. D. M. E. (2001). Pathogenesis of influenza A (H5N1) virus infection in a primate model. *J. Virol.* **75:**6687–6691.

Smith, M. W., Yue, Z. N., Geiss, G. K., Sadovnikova, N. Y., Carter, V. S., Boix, L., Lazaro, C. A., Rosenberg, G. B., Bumgarner, R. E., Fausto, N., Bruix, J., and Katze, M. G. (2003a). Identification of novel tumor markers in hepatitis C virus-associated hepatocellular carcinoma. *Cancer Research* **63:**859–864.

Smith, M. W., Yue, Z. N., Korth, M. J., Do, H. A., Boix, L., Fausto, N., Bruix, J., Carithers, R. L., Jr., and Katze, M. G. (2003b). Hepatitis C virus and liver disease: Global transcriptional profiling and identification of potential markers. *Hepatology* **38:**1458–1467.

Smith, M. W., Walters, K. A., Korth, M. J., Fitzgibbon, M., Proll, S., Thompson, J. C., Yeh, M. M., Shuhart, M. C., Furlong, J. C., Cox, P. P., Thomas, D. L., Phillips, J. D., *et al.* (2006). Gene expression patterns that correlate with Hepatitis C and early progression to fibrosis in liver transplant recipients. *Gastroenterology* **130:**179–187.

Spindel, E., Pauley, M., Jia, Y., Gravett, C., Thompson, S., Boyle, N., Ojeda, S., and Norgren, R. (2005). Leveraging human genomic information to identify nonhuman primate sequences for expression array development. *BMC Genomics* **6:**160.

Subbarao, K., and Joseph, T. (2007). Scientific barriers to developing vaccines against avian influenza viruses. *Nat. Rev. Immunol.* **7:**267–278.

Sumpter, R., Jr., Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M., and Gale, M., Jr. (2005). Regulating intracellular antiviral aefense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J. Virol.* **79:**2689–2699.

Taubenberger, J. K. (1998). Influenza virus hemagglutinin cleavage into HA1, HA2: No laughing matter. *Proc. Natl. Acad. Sci. USA* **95:**9713–9715.

Taubenberger, J. K., and Morens, D. M. (2006). 1918 Influenza: The mother of all pandemics. *Emerg. Infect. Dis.* **12:**15–22.

Taubenberger, J. K., Reid, A. H., Krafft, A. E., Bijwaard, K. E., and Fanning, T. G. (1997). Initial genetic characterization of the 1918 ''Spanish'' influenza virus. *Science* **275:**1793–1796.

Thomas, M. J., Agy, M. B., Proll, S. C., Paeper, B. W., Li, Y., Jensen, K. L., Korth, M. J., and Katze, M. G. (2006). Functional gene analysis of individual response to challenge of SIVmac239 in M. mulatta PBMC culture. *Virology* **348:**242–252.

Tumpey, T. M., Basler, C. F., Aguilar, P. V., Zeng, H., Solorzano, A., Swayne, D. E., Cox, N. J., Katz, J. M., Taubenberger, J. K., Palese, P., and Garcia-Sastre, A. (2005a). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* **310:**77–80.

Tumpey, T. M., Garcia-Sastre, A., Taubenberger, J. K., Palese, P., Swayne, D. E., Pantin-Jackwood, M. J., Schultz-Cherry, S., Solorzano, A., Van Rooijen, N., Katz, J. M., and Basler, C. F. (2005b). Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: Functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J. Virol.* **79:**14933–14944.

Tumpey, T. M., Maines, T. R., Van Hoeven, N., Glaser, L., Solorzano, A., Pappas, C., Cox, N. J., Swayne, D. E., Palese, P., Katz, J. M., and Garcia-Sastre, A. (2007). A

two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* **315:**655–659.

van Riel, D., Munster, V. J., de Wit, E., Rimmelzwaan, G. F., Fouchier, R. A. M., Osterhaus, A. D. M. E., and Kuiken, T. (2006). H5N1 virus attachment to lower respiratory tract. *Science* **312:**399.

Wallace, J. C., Korth, M. J., Diamond, D. L., Proll, S. C., and Katze, M. G. (2006). Virology in the 21st century: Finding function with functional genomics. *Future Virol.* **1:**47–53.

Wallace, J., Korth, M., Paeper, B., Proll, S., Thomas, M., Magness, C., Iadonato, S., Nelson, C., and Katze, M. (2007). High-density rhesus macaque oligonucleotide microarray design using early-stage rhesus genome sequence information and human genome annotations. *BMC Genomics* **8:**28.

Walters, K. A., Joyce, M. A., Thompson, J. C., Smith, M. W., Yeh, M. M., Proll, S., Zhu, L. F., Gao, T. J., Kneteman, N. M., Tyrell, D. L., and Katze, M. G. (2006). Host-specific response to HCV infection in the chimeric SCID-beige/Alb-uPA mouse model: Role of the innate antiviral immune response. *PLOS Pathog.* **2:**e59.

Walters, K. A., Joyce, M., Thompson, J., Proll, S., Wallace, J., Smith, M., Furlong, J., Tyrrell, D. L., and Katze, M. (2006a). Application of functional genomics to the chimeric mouse model of HCV infection: Optimization of microarray protocols and genomics analysis. *Virol. J.* **3:**37.

Walters, K. A., Smith, M. W., Pal, S., Thompson, J. C., Thomas, M. J., Yeh, M. M., Thomas, D. L., Fitzgibbon, M., Proll, S., Fausto, N., Gretch, D. R., Carithers, J., *et al.* (2006b). Identification of a specific gene expression pattern associated with HCV-induced pathogenesis in HCV- and HCV/HIV-infected individuals. *Virol.* **350:**453–464.

WHO (2005). Avian Flu Facts www.who.int/csr/disease/avian_influenza/avian_faqs/en/index.html#isit.

WHO (2007). Avian Flu Timeline www.who.int/csr/disease/avian_influenza/Timeline_2007_03_20.pdf.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5:**699–701.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr., Akira, S., Yonehara, S., Kato, A., *et al.* (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **175:**2851–2858.