A Functional Variation in CD55 Increases the Severity of 2009 Pandemic H1N1 Influenza A Virus Infection

Jie Zhou,1,2 Kelvin Kai-Wang To,1,2,3,4 Hui Dong,6 Zhong-Shan Cheng,1 Candy Choi-Yi Lau,1 Vincent K. M. Poon,1 Yan-Hui Fan,1 You-Qiang Song,5 Herman Tse,1,2,3,4 Kwok-Hung Chan,1,2,3 Bo-Jian Zheng,1,2,3 Guo-Ping Zhao,6 and Kwok-Yung Yuen1,2,3,4

1Department of Microbiology, 2Research Center of Infection and Immunology, 3State Key Laboratory of Emerging Infectious Diseases, 4Carol Yu Center for Infection, 5Department of Biochemistry, The University of Hong Kong, and 6Chinese National Human Genome Center at Shanghai, China

Infection due to 2009 pandemic H1N1 influenza A virus (A[H1N1]pdm09) is commonly manifested as mild infection but occasionally as severe pneumonia. We hypothesized that host genetic variations may contribute to disease severity. An initially small-scale genome-wide association study guided the selection of CD55 single-nucleotide polymorphisms in 425 Chinese patients with severe (n = 177) or mild (n = 248) disease. Carriers of rs2564978 genotype T/T were significantly associated with severe infection (odds ratio, 1.75; \(P = .011\)) under a recessive model, after adjustment for clinical confounders. An allele-specific effect on CD55 expression was revealed and ascribed to a promoter indel variation, which was in complete linkage disequilibrium with rs2564978. The promoter variant with deletion exhibited significantly lower transcriptional activity. We further demonstrated that CD55 can protect respiratory epithelial cells from complement attack. Additionally, A(H1N1)pdm09 infection promoted CD55 expression. In conclusion, CD55 polymorphisms are associated with severe A(H1N1)pdm09 infection. CD55 may exert a substantial impact on the disease severity of A(H1N1)pdm09 infection.

Influenza A virus is a major threat to human health because of its ability to cause recurrent epidemics and global pandemics. Annually, the global disease burden associated with influenza epidemics is around 3–5 million cases of severe illness and 300 000 to 500 000 deaths. During the 2009 influenza pandemic, a novel influenza virus, influenza A virus subtype H1N1 (A[H1N1]pdm09), rapidly spread around the world. Unlike seasonal influenza, people <65 years of age were infected preferentially [1], with a high rate of severe infection and mortality among younger patients globally and in Hong Kong [2–5]. Among these patients, about one-third with severe and fatal cases did not have comorbid conditions, suggesting that interindividual genetic variation may have accounted for the distinct disease severity of A(H1N1)pdm09 infection.

In experimental mice, a common animal model for influenza virus infection in humans, it is well acknowledged that genetic background profoundly influences disease presentation [6]. In humans, extremes of age, obesity, pregnancy, and comorbid conditions, such as immunosuppressive illness, chronic heart or pulmonary diseases and metabolic disorders, are risk factors for severe disease [7]. Nevertheless, inheritable host factors have been unraveled as important determinants of severe influenza. An earlier study of influenza-associated death records over the past 100 years in a community in Utah provided evidence for an increased risk in close and distant relatives, indicating a genetic contribution to the risk of influenza-associated death [8]. It was recently documented that single-nucleotide mutations in the gene encoding carnitine palmitoyltransferase II (CPT2) can cause influenza-associated...

Received 6 October 2011; accepted 9 February 2012; electronically published 12 June 2012.

Correspondence: Kwok-Yung Yuen, MD, Centre for Infection, Department of Microbiology, The University of Hong Kong, Queen Mary Hospital, Pokfulam Rd, Pokfulam, Hong Kong Special Administrative Region, China (kyyuen@hkucc.hku.hk).

The Journal of Infectious Diseases 2012;206:495–503
© The Author 2012. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.
DOI: 10.1093/infdis/jis378
encephalopathy in Japanese and Chinese patients [9, 10]. These findings, together with the relatively increased mortality in young people, prompted us to investigate whether host genetic variations may play a substantial role in the severity of A(H1N1)pdm09 infection.

Complement activation has been implicated in the pathogenesis of lung injury and disease [11, 12]. Notably, a recent study demonstrated that aberrant complement deposition in the respiratory tract caused severe A(H1N1)pdm09 infection in middle-aged patients without previous comorbidities [13]. CD55, an important complement regulatory protein, inhibits C3 and C5 activation by preventing the formation of new C3 and C5 convertase and accelerating their decay [14, 15]. Both C3 and C5 convertase can mediate the downstream effect of all 3 complement-activation pathways. Therefore, CD55 functions to protect host cells from complement attack and inhibits amplification of the complement cascade. There are 2 major isoforms of CD55 in human, a glycosyl phosphatidylinositol (GPI)–anchored membrane form (mCD55) and a soluble form (sCD55) [16]. mCD55 is widely expressed on almost all cell types in immediate contact with plasma components, such as peripheral blood cells, epithelial cells, and endothelial cells [14], whereas sCD55, devoid of the C-terminal GPI anchor, is generated by alternative use of optional exon 10. Present in body fluids and extracellular matrix, it is a more potent inhibitor for complement activation in the fluid phase [17]. The high expression of CD55 in human lung [18] may, to some extent, reflect its indispensable role to prevent this organ from complement-mediated tissue damage as a result of long-term evolutionary pressure.

This study was initiated in May 2010, when cases of severe A(H1N1)pdm09 infection, especially in individuals without preexisting comorbidities, started to accumulate. A genome-wide association study (GWAS) was conducted in 25 patients with severe A(H1N1)pdm09 infection and in 26 controls with mild symptoms, when the number of severe cases was very limited in Hong Kong. From the GWAS, 34 single-nucleotide polymorphisms (SNPs) in CD55 (GenBank accession number NG_007465) emerged. The allelic distributions of these SNPs were highly significantly different between severe cases and mild controls. On the basis of this preliminary result, we proceeded to corroborate whether genetic variations in CD55 are associated with severe A(H1N1)pdm09 infection and whether CD55 may play an important role in the pathogenesis of this infection.

STUDY PARTICIPANTS AND METHODS

Study Participants
Adult patients who were infected with A(H1N1)pdm09 and had clinical specimens sent to Queen Mary Hospital were recruited. Patients who were <18 years of age, were not Chinese, or did not have sufficient archived specimens were excluded. A(H1N1)pdm09 infection was confirmed by either positive results of reverse transcription–polymerase chain reaction (RT-PCR) or virus culture of respiratory tract specimens [3, 4]. Patients with severe disease were defined as those who required oxygen supplementation, were admitted to the intensive care unit, or died, whereas patients with mild disease were defined as those who did not satisfy these criteria. Risk factors for severe disease were classified according to World Health Organization guidelines [19]. The clinical characteristics of all patients are listed in Table 1. Additionally,buffy coat from 50 healthy blood donors was obtained from the Hong Kong Red Cross Blood Transfusion Service with authorized approval. This study was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority of Hong Kong.

Genotyping Methods
The initial GWAS study involved 51 patients and used the Genome Wide Human SNP Array 6.0 (Affymetrix). Genomic DNA samples from 205 patients were analyzed by the SEQUENOM MassArray Genotyping platform. Additional 169 DNA samples were genotyped for rs2564978 by restriction fragment–length polymorphism (RFLP) analysis. Detailed information about experimental methods and data analysis is available in the Supplementary Materials.

Table 1. Demographic and Clinical Characteristics of Study Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Severe (n = 177)</th>
<th>Mild (n = 248)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic characteristic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years, median (range)</td>
<td>55 (18–97)</td>
<td>39 (18–94)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Female sex</td>
<td>75 (42.2)</td>
<td>142 (57.3)</td>
<td>.002</td>
</tr>
<tr>
<td><strong>Risk factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of patients, median (range)</td>
<td>1 (0–7)</td>
<td>0 (0–5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age ≥65 years</td>
<td>40 (22.6)</td>
<td>32 (12.9)</td>
<td>.009</td>
</tr>
<tr>
<td>Heart disease</td>
<td>36 (20.3)</td>
<td>21 (8.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>52 (29.4)</td>
<td>24 (9.7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Liver disease</td>
<td>8 (4.5)</td>
<td>10 (4.0)</td>
<td>.806</td>
</tr>
<tr>
<td>Renal disease</td>
<td>23 (13.0)</td>
<td>13 (5.0)</td>
<td>.005</td>
</tr>
<tr>
<td>Metabolic disorder</td>
<td>52 (29.4)</td>
<td>36 (14.5)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hemoglobinopathies</td>
<td>5 (2.8)</td>
<td>2 (0.8)</td>
<td>.133</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>25 (14.1)</td>
<td>37 (14.9)</td>
<td>.819</td>
</tr>
<tr>
<td>Neurological disease</td>
<td>24 (13.6)</td>
<td>17 (6.9)</td>
<td>.021</td>
</tr>
<tr>
<td>Obesity</td>
<td>30 (16.9)</td>
<td>4 (1.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>1 (0.6)</td>
<td>9 (3.6)</td>
<td>.051</td>
</tr>
</tbody>
</table>

Data are no. (%) of patients, unless otherwise indicated.

* By the Fisher exact test.

⁺ Data are limited to the risk factors cited in the table.
mCD55 Expression, Monocyte Isolation, and CD55 Messenger RNA (mRNA) Levels

Peripheral blood mononuclear cells (PBMCs) were obtained from 50 healthy blood donors. The mCD55 level in each PBMC sample was detected by flow cytometry and correlated to its genotype, which was determined by RFLP analysis. Monocytes isolated from the PBMC sample were examined for CD55 mRNA expression by quantitative RT-PCR (RT-qPCR).

Luciferase Assay of CD55 Promoter Variants

Luciferase vectors carrying either C/C or T/T genotype at rs2564978, with a corresponding insertion or deletion allele at rs3841376, were constructed. The vectors were then subjected to mutagenesis to generate 2 additional mutant constructs, to define the individual effect of rs2564978 and rs3841376. BEAS-2B cells (ATCC) were used for reporter plasmid transfection and subsequent luciferase assay.

Complement-Dependent Cytolysis (CDC) Assay in A549 and BEAS-2B Cells

A549 cells and BEAS-2B cells were assayed for CDC, using a standard calcein-release protocol.

Detection of CD55 Expression After A(H1N1)pdm09 Infection

A549 cells were inoculated with 5 multiplicities of infection (MOI) of A/HK/415742/09 virus, a locally isolated A(H1N1) pdm09 strain [20]. Cells and culture medium were harvested to detect CD55 mRNA and soluble CD55 protein at specified time points.

Statistical Analysis

The statistical programs PLINK [21] and SPSS 17.0 were used to analyze the genetic association. The linkage disequilibrium (LD) plot was generated using Haploview [22]. Additionally, stepwise multivariate analysis, the χ² test, the Fisher exact test, the Mann-Whitney U test, and the Student t test were used for data analysis.

RESULTS

CD55 Genetic Variants Associated With Severity of A(H1N1) pdm09 Infection

The initial GWAS study was performed in the first cohort of 25 patients with severe disease and 26 controls with mild disease. A total of 5166 SNPs in autosomes were identified as significantly different between the 2 groups, with P values <.01. Most of the SNPs with the lowest P values were tens or hundreds of kilobases upstream or downstream of genes, most of which are barely functionally relevant to viral infection on the basis of currently available knowledge. However, of the 5166 SNPs, 34 were identified as being proximally upstream from or within CD55, with P values ranging from $10^{-4}$ to $10^{-3}$, which were respectively ranked from thirty-second to three hundredth. Therefore, we believed that CD55 SNPs were appropriate candidates for further study. Although the P values of these CD55 SNPs were several magnitudes higher than those expected for GWAS, considering the small sample size of the study, as well as the proinflammatory role of complement in the pathogenesis of A(H1N1)pdm09, we proceeded to test the potential genetic association between CD55 SNPs and disease severity.

It was documented that complement-inhibition activity varied among CD55 variants generated by alternative mRNA splicing [18]. Therefore, from the 34 CD55 SNPs, we selected 5 SNPs from intragenic regions and 1 SNP, rs2564978, from the promoter region and examined them in the second cohort of 80 severe cases and 125 mild controls, using the Sequenom MassArray. As shown in Supplementary Table 1, 2 of the 6 candidate SNPs were significantly or marginally associated with disease severity, with risk-associated alleles overrepresented in patients with severe disease. Because this result was inconsistent with findings from the GWAS, we decided to proceed by recruiting more patients. LD analysis of CD55 in the second cohort revealed that all 6 SNPs were in a high LD block (Figure 1). When the 52-kilobase region covering CD55 in Chinese subjects in HapMap (data release 27) was applied to LD analysis, all of the SNPs in this region were in a tight LD block (data not shown). Since all 5 intragenic SNPs were located in the intron region, we performed in silico analysis to examine whether variations at these polymorphic sites can cause phenotypic variation. None of these SNPs seemed to be functional. However, it has been illustrated that more than one-third of genetic polymorphisms in the promoter were

![Figure 1. Linkage disequilibrium (LD) of 6 candidate single-nucleotide polymorphisms (SNPs) in study participants. The schematic illustration of CD55 is shown at the top. Vertical bars represent 14 exons of the gene, the gray bar represents the promoter region, and the gray triangles represent the 6 SNPs. The $R^2$ values for LD were calculated by Haploview software.](https://academic.oup.com/jid/article-abstract/206/4/495/864437)
Table 2 Meta-analysis of rs2564978 Risk Allele T in Study Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Subjects, No.</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cohort</td>
<td>51</td>
<td>4.32 (1.84–10.11)</td>
<td>.0005</td>
</tr>
<tr>
<td>Second cohort</td>
<td>374</td>
<td>1.37 (1.01–1.87)</td>
<td>.0445</td>
</tr>
<tr>
<td>Meta-analysis</td>
<td>425</td>
<td>1.57 (1.17–2.10)</td>
<td>.0024</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; OR, odds ratio.

Analysis of rs2564978 in Study Participants

Table 3 Genotypic Distribution and Logistic Regression Analysis of rs2564978 in Study Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Severe (n = 177)</th>
<th>Mild (n = 248)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype, patients, no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>94 (53.1)</td>
<td>94 (37.9)</td>
<td>1.85 (1.15–2.78)</td>
<td>.0019</td>
</tr>
<tr>
<td>CT</td>
<td>60 (33.9)</td>
<td>109 (44.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>23 (13.0)</td>
<td>45 (18.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Univariate regression analysisa

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.85 (1.15–2.78)</td>
<td>.0019</td>
</tr>
</tbody>
</table>

Multivariate regression analysisa

<table>
<thead>
<tr>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.75 (1.13–2.70)</td>
<td>.0110</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; OR, odds ratio; Ref, reference.

a Data are shown as genotype T/T under a recessive model.

mild controls (Table 1), we performed stepwise multivariate logistic regression analysis (Supplementary Table 2) to control for these confounders, using genotyping data from 177 severe cases and 248 mild controls. After adjustment for age, sex, and underlying risk conditions, rs2564978 remained significantly different between the 2 groups, with homozygous carriers of the rs2564978 risk allele (genotype T/T) significantly more susceptible to severe disease (OR, 1.75 [95% CI, 1.13–2.70]; P = .011).

Allele-Specific Effect on CD55 Expression

The disease association study identified rs2564978 as a risk marker of severe disease due to A(H1N1)pdm09 infection. Because rs2564978 resides in the CD55 minimal promoter region [24], we speculated that there is a correlation between rs2564978 genetic variation and gene expression level. We examined CD55 expression by flow cytometric analysis in 50 PBMC samples from healthy blood donors. In the first 20 PBMC samples, we found that, among 3 major components of PBMCs, monocytes expressed the highest level of mCD55, around 2 or 3 magnitudes higher than those on T and B lymphocytes (data not shown), which agreed with findings from an earlier study [25]. Additionally, mCD55 levels on monocytes were well correlated with rs2564978 genotypes, whereas little correlation was observed for T and B lymphocytes. Therefore, we focused on CD55 expression among monocytes in additional 30 PBMC samples. We found that rs2564978 T/T carriers (n = 14) exhibited significantly lower levels of mCD55 on monocytes, compared with C-allele carriers (C/C and C/T; n = 16), indicating a predominant effect of the C allele on CD55 expression. Despite the substantial interindividual variation, the allele-specific difference was dramatic: mCD55 levels defined by the molecule equivalent of fluorescence were highly significantly different between non-C carriers (mean [± SD], 2900 ± 3642) and C carriers (mean [± SD], 65006 ± 54176) (Figure 2A).

On the basis of the aforementioned results, we proceeded to isolate CD14+ monocytes from PBMCs. After separation, the purity of the resultant monocytes was >95% (data not shown), as detected by flow cytometry. The isolated monocytes were then subjected to RNA extraction and RT-qPCR. As shown in Figure 2C, CD55 mRNA expression levels on monocytes from T/T carriers (n = 7) were about 3-fold lower than those on monocytes from C/C carriers (n = 4), intermediate in C/T carriers (n = 6). Taken together, we explicitly demonstrated the allele-specific effect of rs2564978 on CD55 expression in peripheral blood monocytes, in terms of both protein and mRNA levels. The risk genotype T/T, which has a significantly higher frequency in patients with severe disease, exhibited significantly lower levels of CD55 expression than the protective genotype C/C.
Luciferase Assay of Reporter Vectors With CD55 Promoter Variants and Mutational Analysis

To corroborate the allele-specific effect of rs2564978 on CD55 transcriptional regulation, we conducted the luciferase assay of reporter constructs carrying either the C/C or T/T genotype at this polymorphic site. Notably, by sequencing the CD55 promoter in DNA samples from 50 local healthy blood donors, we found that rs2564978 was in complete LD ($r^2 = 1$) with rs3841376, a 21–base pair indel variation 68 base pairs downstream in the CD55 promoter region. Specifically, the rs2564978 C/C genotype unequivocally coexists with the rs3841376 insertion, and the T/T genotype coexists with the rs3841376 deletion. This observation was replicated when we inspected these 2 markers in the genotype data for 30 Chinese individuals released from the 1000 Genomes Project (available at: http://www.1000genomes.org/home, last accessed on June 20, 2012), an international collaboration to produce genetic variation by deep sequencing. Therefore, there are only 2 naturally occurring variants (CI, TD) that were inserted in pGL3-Basic vector. Two mutagenized vectors (TI, CD) were generated to dissect the individual effect of 2 gene polymorphisms in complete LD, rs2564978, and rs3841376. The reporter vectors were cotransfected with pGL4.70RL into BEAS-2B cells in triplicate. Luciferase assay was performed 32 hours after transfection to compare the transcriptional activity of each construct. The normalized firefly luciferase (Relative Luc Activity) activities of 4 promoter reporter constructs were compared. The result represents one of the experiments performed at least 3 times independently. The Student t test was used for data analysis.

Luciferase Assay of Reporter Vectors With CD55 Promoter Variants and Mutational Analysis

To corroborate the allele-specific effect of rs2564978 on CD55 transcriptional regulation, we conducted the luciferase assay of reporter constructs carrying either the C/C or T/T genotype at this polymorphic site. Notably, by sequencing the CD55 promoter in DNA samples from 50 local healthy blood donors, we found that rs2564978 was in complete LD ($r^2 = 1$) with rs3841376, a 21–base pair indel variation 68 base pairs downstream in the CD55 promoter region. Specifically, the rs2564978 C/C genotype unequivocally coexists with the rs3841376 insertion, and the T/T genotype coexists with the rs3841376 deletion. This observation was replicated when we inspected these 2 markers in the genotype data for 30 Chinese individuals released from the 1000 Genomes Project (available at: http://www.1000genomes.org/home, last accessed on June 20, 2012), an international collaboration to produce genetic variation by deep sequencing. Therefore, there are only 2 naturally occurring haplotypes in Chinese individuals, namely, rs2564978C/rs3841376ins (CI) and rs2564978T/rs3841376del (TD). Two mutagenized vectors, rs2564978T/rs3841376ins (TI) and rs2564978C/rs3841376del (CD), were subsequently constructed to dissect the individual effect of the 2 linked variations. As shown in Figure 2D, the transcriptional activity of promoter variant TD was significantly lower than that of CI, which was consistent with differential expression levels on monocytes. Moreover, comparison of the activities of CI and CD, as well as of TD and TI, clearly indicated that the rs3841376 indel variation was the major determinant for transcriptional regulation of CD55.
CD55 Protects Respiratory Epithelial Cells From Complement-Mediated Cell Lysis

To assess the potential role of CD55 in the pathophysiology of the respiratory tract, we chose the respiratory epithelial cell lines A549 (a lung adenocarcinoma cell line) and BEAS-2B (adenovirus 12-SV40 hybrid virus transformed human bronchial epithelial cells); both may physiologically mimic the influenza virus–infected epithelial cells in the airway in an authentic infection. By the CDC assay, we demonstrated that, in A549 cells, the mean percentage of complement-mediated cell lysis in anti-CD55, isotypic control antibody, and PBS-treated cells was 62.5%, 33.9%, and 28.1%, respectively (Figure 3A), indicating that CD55 can protect A549 cells from complement-mediated cell lysis. This observation was replicated in BEAS-2B cells (Figure 3B). Therefore, the protection provided by CD55 from complement attack was definitively operational in these 2 respiratory tract epithelial cell lines.

A(H1N1)pdm09 Infection Upregulates CD55 Expression

To evaluate the dynamics of CD55 expression in the context of A(H1N1)pdm09 infection, we inoculated A(H1N1)pdm09 with 5 MOI in A549 cells and detected mCD55 expression in infected cells and sCD55 in culture medium. As shown in Figure 4A, at 24 hours after infection, the normalized CD55 mRNA levels were >2-fold higher in A(H1N1)pdm09-infected cells than in mock-infected cells. Accordingly, the mCD55 levels on virus-infected A549 cells were significantly increased, as detected by CD55 flow cytometry (data not shown). Notably, sCD55 in the culture medium was also increased in virus-infected cells (Figure 4B). Taken together, A(H1N1)pdm09 infection can promote CD55 expression, which may represent a host-defense strategy against complement attack upon influenza virus infection.

**DISCUSSION**

Host genetic background underlying the susceptibility to viral infectious diseases has been studied intensively for several pathogens, including human immunodeficiency virus [26], hepatitis B virus [27], hepatitis C virus [28], and respiratory syncytial virus [29]. However, our understanding of the genetic susceptibility to influenza has been greatly hindered by variable background humoral or cell-mediated immunity in the population, the distinct pattern of morbidity and mortality caused by different influenza virus strains, and the high attack rate of influenza virus [30]. A unique feature of A(H1N1)pdm09 infection was the absence of background humoral immunity in individuals aged <70 years and the relatively higher mortality rate in younger people, compared with seasonal influenza. Instead of exploring the genetic susceptibility to the infection, we investigated the host genetic predisposition to severe infection, since the latter entails respiratory dysfunction, extrapulmonary complications, or even death [31]. Interestingly, when this manuscript was in preparation, a research group released their findings of a GWAS study on the susceptibility to severe A(H1N1)pdm09 infection in a small cohort of 91 patients with severe pneumonia and 98 asymptomatic controls in Mexico [32]. Two of 4 identified SNPs were mapped to genes involved in immune complex processing and complement activation. However, further replication was not performed in that study.

In this study, the initial GWAS was promptly followed by replication of candidate SNPs in the second cohort of patients, and functional studies were performed to accordingly validate the genetic association. Consistent with recent findings [7], patients with severe disease were older and more likely to have preexisting comorbidities (Table 1). We demonstrated that rs2564978 was significantly associated with severe A(H1N1)
pdm09 infection (Tables 2 and 3 and Supplementary Table 2), after adjustment for these confounding factors. The differences were significant at each step of the stepwise multivariate logistic regression analysis. When considered alone, the P value of the association between rs2564978 and severe A(H1N1)pdm09 infection may overstate the true type I error rate because of the choice of the regression model used. Hence, we proceeded to investigate the possible biological effect of rs2564978 that may influence the infection outcome. We uncovered the allele-specific effect of rs2564978 on CD55 expression in human monocytes (Figure 2A–C). The ex vivo observations in human cells were further validated by the in vitro assay for promoter activity. The promoter variant with rs2564978 genotype T/T exhibited significantly lower transcriptional activity than that with genotype C/C. Interestingly, an indel variation in the vicinity, rs3841376, was in complete LD with rs2564978. Subsequent mutational analysis showed that the rs3841376 indel dictated the functional variation (Figure 2D). Therefore, we identified a functional indel variation, rs3841376, as a genetic marker for severe A(H1N1)pdm09 infection.

More importantly, our genetic study implicated that CD55, a complement regulatory protein, may be an important determinant for the clinical presentation of A(H1N1)pdm09 infection. Upon natural influenza virus infection, a localized portion of respiratory epithelial cells were affected and underwent cytonecrosis eventually. If the infected epithelial cells can induce production of soluble CD55, as shown in Figure 4B, the protective effect of CD55 would cover the neighboring uninfected cells and greatly ameliorate the complement-mediated tissue pathology in influenza virus infection. A very recent study revealed that complement activation triggered by pulmonary deposition of immune complex represents an important pathogenetic mechanism for severe A(H1N1)pdm09 infection [13]. It is well-known that CD55 protects autologous cells from complement damage and disrupts the complement cascade [15]. Therefore, it is conceivable that distinct CD55 expression due to genetic variation may make a substantial difference in the manifestation of disease due to influenza virus infection.

Apart from the high level of CD55 expression in the lung, we and others [25] found that, among leukocytes in human peripheral blood, CD55 is highly expressed on monocytes. Influenza A virus infection is characterized by an early influx of a small amount of neutrophils, followed by the recruitment of a large amount of blood-derived monocytes to the site of infection. The accumulation of monocytes was ascribed to the selective induction of monocyte-attracting chemokines upon infection [33]. It was demonstrated that mice with diminished monocyte recruitment exhibited higher viral titer, enhanced inflammation, and delayed virus clearance after challenge with influenza virus [34, 35]. Therefore, monocyte recruitment to the respiratory tract may influence disease manifestation. Because of their important role in influenza virus infection, blood monocytes receive priority protection from CD55, an

Figure 4. Expression of CD55 in the context of 2009 pandemic influenza A virus subtype H1N1 (A[H1N1]pdm09) infection. A, A549 cells were inoculated with 5 multiplicities of infection of A[H1N1]pdm09 and harvested 7 hours or 24 hours after infection. The cells were subjected to RNA extraction and real-time quantitative polymerase chain reaction to measure the CD55 messenger RNA (mRNA) levels, which were normalized with human GAPDH mRNA. B, A549 cells were subjected to the same procedure as described above. Culture medium was collected at indicated time points for human scCD55 enzyme-linked immunosorbant assay.
inference based on the high level of CD55 expression on these cells. Therefore, the differential levels of CD55 expression derived from its genetic variations may influence monocyte viability and eventually dictate the disease outcome.

Recent years have witnessed major progress in disease-association studies, with GWAS evolving from a tool for discovering genetic markers to a tool for identifying novel genes that contribute to disease pathogenesis in an unbiased fashion [36, 37]. GWAS revolutionarily alters our understanding of diseases and paves a new path by which the mechanisms of complement-mediated tissue damage is involved. The role in the presentation of disease due to in associated injury has been intensively studied in the field of organ transplantation [38]. More detailed functional studies and related mice work are warranted for improving our understanding of the role of CD55 in influenza virus infection and may add a new dimension to the rationale of therapeutics for influenza and perhaps other respiratory diseases in which complement-mediated tissue damage is involved.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by the Providence Foundation Limited in memory of the late Lui Hac Minh, Research Fund for the Control of Infectious Diseases (Food and Health Bureau, Hong Kong Special Administrative Region, China), and Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Disease (Department of Health, Hong Kong Special Administrative Region, China).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

25. Christmas SE, de la Mata Espinosa CT, Halliday D, Buxton CA, Cummerson JA, Johnson PM. Levels of expression of complement