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Mapping of Clinical and Expression Quantitative Trait Loci in a Sex-Dependent Effect of Host Susceptibility to Mouse-Adapted Influenza H3N2/HK/1/68

Gregory A. Boivin,*† Julien Pothlichet,*† Emil Skamene,‡ Earl G. Brown,*§ J. Concepción Loredo-Osti,*, Robert Sladek,*‖,# and Silvia M. Vidal*†,**

Seasonal influenza outbreaks and recurrent influenza pandemics present major challenges to public health. By studying immunological responses to influenza in different host species, it may be possible to discover common mechanisms of susceptibility in response to various influenza strains. This could lead to novel therapeutic targets with wide clinical application. Using a mouse-adapted strain of influenza (A/HK/1/68-MA20 [H3N2]), we produced a mouse model of severe influenza that reproduces the hallmark high viral load and overexpression of cytokines associated with susceptibility to severe influenza in humans. We mapped genetic determinants of the host response using a panel of 29 closely related mouse strains (AcB/BcA panel of recombinant congenic strains) created from influenza-susceptible A/J and influenza-resistant C57BL/6J (B6) mice. Combined clinical quantitative trait loci (QTL) and lung expression QTL mapping identified candidate genes for two sex-specific QTL on chromosomes 2 and 17. The former includes the previously described Hc gene, a deficit of which is associated with the susceptibility phenotype in females. The latter includes the phospholipase gene Pla2g7 and Tnfrsf21, a member of the TNFR superfamily. Confirmation of the gene underlying the chromosome 17 QTL may reveal new strategies for influenza treatment. The Journal of Immunology, 2012, 188: 3949–3960.

Influenza pandemics are the leading viral cause of mortality in the industrialized world (1, 2). Because of the broad host range and segmented genome of the influenza virus, concurrent infection of the same host with different influenza strains can result in reassortment and adaptation, with the production of new, highly pathogenic viruses (such as avian H5N1, swine-origin 2009 H1N1) from which host populations are not protected by conventional vaccination or pre-existing Abs. Such reassortment

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*Department of Human Genetics, McGill University, Montreal, Quebec H3A 1B1, Canada; †Centre for the Study of Host Resistance, McGill University, Montreal, Quebec H3G 1A4, Canada; ‡Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada; †Emerging Pathogens Research Centre, Faculty of Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada; ‡Department of Mathematics and Statistics, Memorial University of Newfoundland, St. Johns, Newfoundland and Labrador A1C 5S7, Canada; ‡McGill University and Genome Quebec Innovation Centre, Montreal, Quebec H3A 0G1, Canada; ‡Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, Quebec H3A 1A2, Canada; §McGill Life Sciences Complex, 3649 Promenade Sir William Osler, Room 367, Montreal, QC H3G 0B1, Canada. E-mail address: silvia.vidal@mcgill.ca

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Expression datasets presented in this article have been submitted to the National Center for Biotechnology Information/Gene Expression Omnibus (http://www.ncbi. nlm.nih.gov/geo/) under accession number GSE35888.

Address correspondence and reprint requests to Dr. Silvia M. Vidal, McGill Life Sciences Complex, 3649 Promenade Sir William Osler, Room 367, Montreal, QC H3G 0B1, Canada. E-mail address: silvia.vidal@mcgill.ca

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BG, background; eQTL, expression quantitative trait loci; LDH, lactate dehydrogenase; qPCR, quantitative PCR; QTL, quantitative trait loci; RNF, recombinant congeneric strain; SNP, single nucleotide polymorphism.

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a genetic-mapping study in female mice revealed five quantitative trait loci (QTL) associated with resistance to a highly pathogenic H5N1 virus (33). Hc, located in one of these five QTL, was shown to decrease viral load and increase survival time after influenza infection (33).

Identifying the causative genes underlying mouse QTL for complex phenotypes remains challenging. A major reason is the difficulty of resolving QTL into sufficiently small genomic intervals to make gene identification possible. In the current study, we aimed to overcome this problem by using a unique set of closely related mouse strains in which the genetic composition and genetic expression are well characterized (34).

The AcB/BcA reciprocal series of recombinant congenic strains (RCS) was derived by inbreeding the second backcross generation of A/J and B6 progenitor inbred mice (35). In this breeding scheme, each strain inherits only a limited portion (one eighth) of the genes from either A/J or B6. The relatively small size of the congenic segments fixed in individual RCS facilitates the search and testing of candidate genes. To identify primary candidates within genetic regions of linkage, we used lung microarray data to identify expression QTL (eQTL) for transcripts that are differentially cis-regulated across the AcB/BcA set. The colocalization of clinical QTL (cQTL) and eQTL in the RCS provides a strong tool for the identification of new genes involved in the host response to influenza infection.

In the current study, we examined host genetic control of the mouse-adapted influenza strain A/HK/1/68-MA20 (H3N2) (36), which induces an overwhelming inflammatory response and death in A/J mice. Combined qCt and lung eQTL mapping identified candidate genes for two sex-specific QTL on chromosomes 2 and 17.

Materials and Methods

**Animals and ethics**

Inbred A/J, B6, 129X1/SvJ, and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Recombinant congenic mice (n = 398) of the AcB/BcA set were derived from two successive backcrosses (N_{0}) to either A/J (AcB) or B6 (BcA) parental mice, as previously reported (35). Briefly, in this breeding scheme, each AcB strain inherits a different set of discrete congenic segments, including ~12.5% genes from the B6 genome and ~87.5% genes from A/J, and reciprocally for each BcA strain. Progeny (n = 177) from reciprocal (BcA70 x B6) F1 crosses were generated in-house. Experimental protocols were in accordance with the institutional guidelines of the Canadian Council on Animal Care. Mice were maintained at McGill University animal facilities in compliance with the Canada Council on Animal Care, as regulated by the McGill University Animal Care Committee.

**Cell lines and virus strains**

MDCK (ATCC CCL-34) and mouse fibroblast L-929 (ATCC CCL-1) cell lines were maintained, respectively, in DMEM and RPMI 1640 (Wiset) medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and FBS (10%). Influenza viruses A/PRI/34 (H1N1) and A/HK/1/68-MA20 (H3N2) were grown in 10-d-old embryonated hen’s eggs. A/HK/1/68-MA20 is a mouse-adapted influenza virus strain derived from a H3N2 strain that was clinically isolated in Hong Kong during the 1968 pandemic, as previously reported (36). Briefly, A/HK/1/68-MA20 was prepared by serially passaging lung homogenates from mice intranasally infected with the wild-type clinical isolate for 20 rounds of infection. The experimental adaptation led to an increase in virulence representing a 10^{1.5} change in the LD_{50} and the selection of 11 mutations, several of which are in common with the virulent human H5N1 isolate A/HK/1/5697 (36). Titration of infectious virus was determined by a plaque assay, as described previously (37).

**Infection of mice, definition of phenotype, and tissue collection**

For each RCS, at least six male mice and six female mice were infected. A/J and B6 mice were included as controls in each infection. The F_{1} mice were infected in groups of 46–65 mice, together with control mice from the parental strains. All RCS and F_{1} mice were 84 ± 15 d old at the time of infection. For the susceptibility screen, mice were monitored daily for 2 wk following intranasal inoculation with a weight-adjusted dose of 10^{5} PFU influenza virus/22 g body weight. At the experimental endpoint, we measured the semiquantitative lung-consolidation score, which was defined as: 0, no signs of hemorrhagic consolidation; 1, small or sporadic loci involving <10% of lung tissue; 2, 10–20% lung involvement; 3, 20–50% lung involvement; 4, 50–70% lung involvement; and 5, 70–100% lung involvement. Clinical signs (weight loss, labored breathing, lack of grooming, and low motility) were recorded daily. Mice presenting respiratory distress were humanely sacrificed. Human intervention at this decision point may minimally influence our definition of susceptibility and survival. For gene-expression analysis, male mice were inoculated with a dose of 10^{5} PFU influenza virus/22 g body weight at 1, 3, or 7 d prior to sacrifice and tissue harvest.

For further phenotype analysis, male mice were euthanized at different time points postinfection by CO_{2} in preparation for three different experiments. In the first experiment, the trachea was exposed and cannulated immediately after death. The lung lobes were inflated under constant pressure (25 cm H_{2}O) with 10% neutral buffered formalin (Sigma-Aldrich), excised, and embedded in paraffin for histopathology. In the second experiment, mouse tracheas were cannulated, and four volumes of 0.5-ml cold sterile saline were instilled through the tracheal cannula, withdrawn, and pooled to recover bronchoalveolar lavage fluids (BALF), as previously described (38). In the third experiment, lungs were perfused with cold saline, flash frozen, and stored at −80°C until processing for viral titr determination or RNA isolation.

**Histopathology, immunohistochemistry, and image analysis**

Paraffin-embedded lung samples were sent to the Morphology Unit in the Department of Pathology and Laboratory Medicine, University of Ottawa. Tissue blocks were excised in 5-μm-thick sections and stained with H&E for light microscopic examination. A board-certified pathologist blindly categorized each lung section according to a histopathological score based on the number and distribution of inflammatory cells within the lung parenchyma, as well as on noninflammatory changes, such as evidence of bronchial epithelial injury and repair. Sections were also analyzed using custom color-recognition software designed to identify white space (open airspaces); hematoyxin-associated nuclei and eosin-associated collagen, muscle fiber, and interspersed inflammatory infiltrates. Briefly, each pixel in the histological image was filtered into one of three preset binary conditions (white, blue, and red) based on 8-bit color space. Other sections were stained with primary Ab, rabbit serum against A/HK/1/68-MA20 virus, and secondary Ab conjugated to Alexa Fluor 555 (Molecular Probes) to detect viral Ag, as described previously (39).

**BALF cell content and determination of lactate dehydrogenase activity**

BALF was harvested from each mouse were centrifuged at 1000 rpm for 10 min. Supernatants were frozen and stored for protein analyses, whereas the cell pellets were resuspended in sterile PBS. The total number of leukocytes in BALF was counted with a hemocytometer, and the cells were spun onto frosted microscope slides (Fisher) at an approximate concentration of 5 × 10^{3} cells/ml. The percentage of total leukocytes, consisting of eosinophils, lymphocytes, macrophages, and neutrophils, was blindly determined from counts of 200 cells in a cytopsin sample stained with Diff-Quick (Dade Behring). The level of lactate dehydrogenase (LDH) activity in the BALF supernatant was determined by commercial colorimetric assay (Roche). The absorbance was read at 490 nm and reported as OD in BALF.

**Quantitative PCR**

Total RNA was extracted from whole lung using RNeasy columns (QIA-GEN) and transcribed into cDNA using M-MLV with random hexamers (Invitrogen), according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed using Platinum SYBR Green SuperMix-UDG (Invitrogen) together with experimental or control primers designed for the Ct of the experimental primers targeting a panel of cytokines and a housekeeping gene and designed to span exon junctions with the help of primer3 (Table 1). Target transcripts were normalized to the control housekeeping genes Gapdh or Hprt. Samples were run in duplicate, with five mice/condition. Reactions were performed using the PTC200 Thermal Cycler with Chromo4 Continuous Fluorescence Detector (MJ Research), and expression was analyzed using Opticon Monitor 3 software (MJ Research). Relative mRNA expression was calculated by subtraction of the mean ΔCt of the control samples from the ΔCt of the infected samples (ΔΔCt). The amount of target mRNA, normalized to the endogenous reference, was calculated as 2^{−ΔΔCt}.
Ex vivo macrophage preparation, infection, and analysis

Bone marrow-derived macrophages were isolated from male mice, as previously described (40). Briefly, marrow was extracted by flushing with 2 ml RPMI 1640. RBCs were lysed, and the remaining cells were resuspended in complete media (RPMI 1640, 10% FBS, and 30% L-929 supernatant). Cells were maintained in culture for 5 d and supplemented with additional L-929 supernatant at days 2 and 4 prior to plating. Cells were seeded at 4 × 10^5 cells in 500 μl Opti-MEM (Invitrogen) medium/well in 24-well plates and incubated overnight. After two washes with 200 μl Opti-MEM, cells were infected with different amounts of influenza virus. Cell supernatants were harvested 18 h postinfection, and TNF-α, MCP-1, G-CSF, MIP-1β, IL-10, and keratinocyte chemoattractant (KC) were quantified by ELISA assays, using the McGill University and Genome Quebec Innovation Centre (13BcA, 12AcB, B6 and A/J mice in duplicate) using Custom CDFv12 to model association (http://mouse.cs.ucla.edu/emma/). Because flexible association was assessed via Bonferroni correction controlling the genome-wide type 1 error.

Genotyping DNA was prepared from tail biopsies using Proteinase K and serial phenol/chlorform extractions, followed by ethanol precipitation, as previously described (35). The RCS of mice were genotyped using 110,567 polymorphic markers from the Mouse Diversity Genotyping Array (Affymetrix), 1,449 polymorphic markers from the MD linkage panel (Illumina), and 625 microsatellite markers (35). Polymorphic markers with 95% call rate and two or more informative strains (i.e., AcB strains with a B genotype or BcA strains with an A genotype) were used to identify haplotype blocks. Information markers (the first and last markers of each haplotype block) were included in the final marker map. We selected a set of 1215 markers that spanned the entire mouse genome (Build 37) and covered the relevant break points in the AcB/BcA panel of mice. The complete AcB/BcA genotypes are presented in Supplemental Table IA. The reciprocal (BcA70 × B6) F2 crosses (n = 177) were genotyped with a selection of 68 markers spanning the informative A/J segments of BcA70 mice using Sequenom iPLEX Gold technology. Genotyping of the F2 was performed at the McGill University and Genome Quebec Innovation Centre.

Statistical and bioinformatics analyses

All of the statistical analyses were performed using the freely available package R (41). Because of the large background effect identified in the AcB/BcA panel of mice, we used a mixed-model statistical test designed to correct for genetic relatedness in mouse models, known as Efficient Mixed Model Association (http://mouse.cs.ucsd.edu/emma/). Because flexible time-to-event models are not implemented in Efficient Mixed Model Association, we transformed the continuous variable “survival,” representing time of sacrifice, into the binary variable “susceptibility” that indicated whether an animal was sacrificed during the experimental period. One locus mapping for the time-to-sacrifice phenotype for F2 mice was analyzed using a parametric survival regression at the markers. The significance was assessed via Bonferroni correction controlling the genome-wide type 1 error.

To identify eQTL, we reanalyzed lung-expression data previously obtained by Lee et al. (34) on MGI74Av2 microarrays (Affymetrix) for 54 mice (13BcA, 12AcB, B6 and A/J mice in duplicate) using Custom CDFv12 to reorganize oligonucleotide probes based on the latest genome and transcriptome information. Probe sets that were deemed problematic (e.g., strain single nucleotide polymorphism [SNP] differences) were removed. The remaining probe sets were mapped to genomic locations based on the mouse genome (Build 37) and positioned with respect to individual RCS genotypes. Probes mapping to multiple locations were removed from the analysis. As a result, 11,294 of the initial 12,488 probes were mapped to sections of the genome for each AcB and BcA strain. We inferred an A/J or B6 strain of origin for each gene based on the genotype of surrounding markers with a call rate of 96.7%. If a gene was in between A/J and B6 markers for a given RCS, it was coded as NA. Expression values were normalized using the Robust Multiarray Analysis for Affymetrix gene chips.

To define the association between differentially expressed genes and genotypes, ANOVA was conducted on a per-gene basis using the linear model Expression ~ DSO + BG + DSO*BG, where background (BG) and donor strain of origin (DSO) were coded as binary phenotypes corresponding to A/J or B6. The cutoff for genome-wide significance was computed using the Benjamini–Hochberg correction (42). Expression datasets have been deposited at National Center for Biotechnology Information (Accession number GSE35888).

Results

Interstrain phenotypic differences in host response to mouse-adapted H3N2 clinical isolate

In a preliminary screen to identify differential host responses to influenza virus infection, we used two well-characterized influenza strains: the prototype A/PR/8/34 (PR8) strain and A/HK/1/68-MA20 (in this article HK/68), a mouse-adapted influenza virus derived from a pandemic H3N2 clinical isolate from Hong Kong. HK/68 has 11 mutations that were selected through mouse adaptation, including several mutations in common with a highly pathogenic human H5N1 isolate (36). We monitored survival in male mice over 14 d following infection with intranasal inoculation of serial dilutions of PR8 and HK/68 viruses of four inbred mouse strains, 129X1, A/J, B6, and BALB/c, which are known to present marked interstrain differences in response to HK/68; A/J and 129X1 were relatively susceptible compared with B6 mice, represented by severe hemorrhagic consolidation. 

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>Pla2g7</td>
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<td>5′-TCA TCA TCT CTT TCC ACC AGC-3′</td>
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<td>5′-ACC CAG TGC TGA AGA AAT TG-3′</td>
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<tr>
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<td>5′-TTC TGG AAG TGC ATC ACG G-3′</td>
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<tr>
<td>KC</td>
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<td>5′-CTG GAG TGG GCT CTA TTG G-3′</td>
</tr>
<tr>
<td>Fas</td>
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<td>5′-TGG GAG TAG AAG CGA AGC A-3′</td>
</tr>
<tr>
<td>Tnfα</td>
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<td>5′-TTG GAG TAC AAG ACC CC-3′</td>
</tr>
<tr>
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<td>5′-CTT GGA CGG AGA GAC ACA AG-3′</td>
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<tr>
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<td>5′-CAT CTT GGT GCA ATC TCC TC-3′</td>
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<tr>
<td>Tral</td>
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<td>Hprt</td>
<td>5′-CAG GCC ACA GGT TGT TGG AT-3′</td>
<td>5′-TGC CTC TCA TCT TAG GCT TT-3′</td>
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</table>
and increased wet lung weight (Supplemental Fig. 1B, \( p < 0.0001 \)) and increased bronchial epithelial necrosis and perivascular lymphocytic infiltrates in B6 mice, whereas A/J mice presented more extensive, disseminated interstitial inflammation and luminal protein infiltration in H&E-stained lung sections (D). Distribution into one of three preset binary conditions (white, blue, and red) of each pixel in digitized H&E slides identified a significant increase in eosin-associated collagen, muscle fiber, and proteinaceous infiltrates in infected A/J mice (E) \( (p < 0.001, \text{ANOVA}) \). Original magnification \( \times10 \) (D, E).

Increased lung neutrophilia, cytotoxicity, and inflammation, but not viral load, correlate with susceptibility

To further characterize the differential susceptibility observed between A/J and B6 mice, we examined molecular, cellular, viral, and inflammatory phenotypes in BALF and lungs at days 1, 3, and 7 postinfection. LDH levels in BALF are a measure of cytotoxicity and cell lysis of damaged cells and serve as a marker of lung pathology (43), particularly during infection with highly pathogenic influenza strains (Ref. 44 and references therein). By day 3 postinfection, both mouse strains showed evidence of LDH activity in BALF. However, by day 7, LDH levels were significantly higher in A/J mice (Fig. 2A, left panel, \( p < 0.001 \)). The two
strains had a comparable total number of infiltrating cells (Fig. 2A, middle panel, $p = 0.3317$). LDH activity correlated with recruitment of neutrophils in BALF, which were significantly more numerous in A/J mice by day 7 (Fig. 2A, right panel, $p < 0.001$). Additionally, A/J mice showed significantly fewer macrophages on day 3 ($p < 0.01$) and day 7 ($p < 0.05$) compared with B6 mice (Fig. 2A, right panel). No differences were found for eosinophil or lymphocyte populations (Fig. 2A, right panel). These results seem to indicate that the immune response in A/J animals is primarily neutrophil oriented by day 7 postinfection, as opposed to the predominantly macrophage-oriented immune response in resistant B6 mice.

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FIGURE 2. Increased lung neutrophilia, cytotoxicity, and inflammation, but not viral load, correlate with susceptibility. Male mice (usually five/group) were infected with $10^4$ PFU (A–C) or $10^3$ PFU (D) of HK/68 virus. (A) A/J mice showed increased LDH levels (a measure of cell cytotoxicity) by day 7 postinfection (left panel, *$p < 0.001$, ANOVA) in BALF. After RBC lysis, the total number of cells in BALF was estimated using a hemocytometer. A/J and B6 mice showed a similar number of total cells in BALF across infection, with no significant differences occurring at day 0 (Control), day 3, or day 7 postinfection (middle panel). A small, but significant, increase in overall cell numbers was noted in A/J mice on day 1 (middle panel, *$p = 0.005$). The percentage of macrophages, neutrophils, eosinophils, and lymphocytes is shown (right panel). A/J mice showed significantly fewer macrophages compared with B6 mice on day 3 (*$p < 0.01$) and day 7 (*$p < 0.05$) postinfection (right panel). Similarly, A/J mice showed significantly increased neutrophils on day 7 postinfection (right panel, *$p < 0.001$). No significant differences were found within the eosinophil or lymphocyte populations counted (right panel). A/J and B6 mice had similar viral loads throughout infection, as determined by plaque assay. (B) A/J mice had slightly higher levels on day 3 postinfection (*$p < 0.001$, ANOVA). (C) Similar localization of the virus was observed in A/J and B6 mice at day 6 postinfection when slides were stained with primary Ab against HK/68. Original magnification $\times 20$. (D) A/J mice had increased proinflammatory gene expression in whole-lung tissue compared with B6 mice when measured by qPCR; genes with significant differences by ANOVA are marked by asterisks and the day of significance (i.e., day 1, 3, or 7). Il-9 was significant by ANOVA, but the day-specific post hoc tests were not significant. (E) Bone marrow-derived macrophages differentiated from A/J and B6 mice were incubated with various multiplicities of infection (MOI) of HK/68. TNF-$\alpha$, MCP-1, and KC levels in cell supernatant were quantified by ELISA at 16 h postinfection and normalized to LDH activity; all differences in protein levels between A/J and B6 mice were significant by ANOVA (*$p < 0.001$).
A/J mice showed a small, but significant, increase in lung virus load compared with B6 mice (6.8 \( \times 10^6 \) versus 6.2 \( \times 10^6 \) PFU/g, respectively) on day 3 postinfection (Fig. 2B, \( p < 0.001 \)). However, the kinetics of viral replication were otherwise comparable, reaching a maximum of 10^7 PFU/g on day 1 and decreasing to \( \sim 10^5 \) PFU/g by day 7 postinfection in both strains. This observation was confirmed using immunohistochemistry analysis with Abs against HK/68, which showed that the localization of the virus in the lung was similar in A/J and B6 mice (Fig. 2C).

As determined by qPCR using oligonucleotide primers (Table I), A/J mice had significantly higher lung cytokine and chemokine expression levels compared with B6 mice mostly at day 3 postinfection (Fig. 2D, Supplemental Table IIA; Ifn-β, \( p = 0.0394 \); Il-10, \( p = 0.0032 \); Il-6, \( p = 0.0009 \); Tnf-α, \( p = 0.0003 \); Mcp-1, \( p < 0.0001 \); Ifn-γ, \( p = 0.0025 \); Il-9, \( p = 0.0158 \)). The primers used for the qPCR reactions are shown in Table I. Because these differentially expressed molecules are mainly produced by macrophages, and to explore whether overreactive inflammation is a trait intrinsic to A/J macrophages, we derived bone marrow macrophages from A/J and B6 mice to confirm our results ex vivo. In agreement with the in vivo results, postinfection with influenza virus, macrophages from A/J mice had increased cytokine and chemokine expression compared with B6 mice at both the transcript (data not shown) and protein levels (Fig. 2E; MCP-1, \( p < 0.0001 \); Tnf-α, \( p < 0.001 \); KC, \( p < 0.001 \)), with no significant differences in viral replication (data not shown). Thus, susceptibility to HK/68 in A/J mice was characterized by increased expression of primarily macrophage-produced lung inflammatory mediators at day 3 postinfection, leading to neutrophilia and increased cytotoxic markers in BALF on day 7 postinfection.

**Complex sex-specific genetic control of host response**

Because the A/J and B6 mouse strains showed marked differences in their response to infection, we used the AcB/BcA panel of RCS to investigate the genetic control of susceptibility to the influenza HK/68 strain. Twenty-nine RCS were challenged with a dose of 10^5 PFU/22 g of influenza HK/68 and monitored for 14 d. Survival was a continuous variable identifying the day of sacrifice during the experimental period (days 1–14) (Fig. 3A). Susceptibility was a binary variable identifying whether the animal was sacrificed during the experimental period that minimally changed the strain distribution from survival (Fig. 3B) and was used for the statistical analysis.

The distribution of the AcB/BcA panels of strains is illustrated in Fig. 3. We observed a significant effect of strain background (AcB versus BcA, \( p < 0.0001 \)), as well as a significant effect of strain (\( p < 0.0001 \)) and a significant sex \( \times \) strain interaction for the BcA70 and BcA72 mouse strains (BcA70, \( p < 0.05 \); BcA72, \( p < 0.05 \)). The continuous distribution of both survival time and susceptibility across the panel of 29 strains suggests complex genetic control of the susceptibility to influenza virus in A/J and B6 mice.

**Increased susceptibility maps to sex-specific candidate cQTL**

We assigned a binary measure to each mouse according to its survival during the 14-d infection period (0 = sacrificed, 1 = survived). Using this simplified measure of survival, we performed genome-wide linkage analysis and identified two cQTL on chromosomes 2 (24–38 Mb, \( p < 0.01 \)) and 17 (37–48 Mb, \( p < 0.01 \)) associated with increased susceptibility to influenza HK/68 infection (Fig. 4A). Because of the significant sex \( \times \) strain interaction observed in our population, we also analyzed the females and males separately. Interestingly, the chromosome 2 locus reached genome-wide significance only in the female population (Fig. 4B), whereas the locus on chromosome 17 reached genome-wide significance only in males (Fig. 4C). Table II shows the cQTL identified while analyzing the full RCS cohort, the male RCS mice, and the female RCS mice.

To confirm our novel locus on chromosome 17, we generated an F2 mouse population from susceptible BcA70 and resistant B6 mice. BcA70 males were significantly more susceptible than BcA70 females in our initial RCS screen. The informative A/J genomic segments of BcA70 cover the linked region of chromosomes 2 (24–38 Mb, \( p < 0.01 \)) and 17 (37–48 Mb, \( p < 0.01 \)) associated with increased susceptibility to influenza HK/68 infection. To confirm whether overreactive inflammation is a trait intrinsic to A/J macrophages, we derived bone marrow macrophages from A/J and B6 mice to confirm our results ex vivo.
From the AcB/BcA set originally published by Lee et al. in 2006, we performed linkage analysis using the chromosome 17 cQTL, to identify primary candidate genes within our chromosome 2 and virus and more specifically confirms the novel male-specific locus on chromosome 17. Expression levels of genes were identified in whole-lung tissue of AcB/BcA mice using MOE430A microarrays. Co-regulated expression differences in the AcB/BcA set of mice were identified by ANOVA. (D) The negative log of the Benjamini–Hochberg corrected p value is shown for each gene. Different shades of gray indicate different chromosomes.

Increased susceptibility maps to sex-specific candidate cQTL. Genome-wide linkage analysis was done in the RCS population (n = 398) using a binary measure of susceptibility. The negative log genome-wide p values are shown for a pool of both sexes (A), females (B), and males (C), illustrating a female-specific locus on chromosome 2 and a male-specific locus on chromosome 17. Expression levels of genes were identified in whole-lung tissue of AcB/BcA mice using MOE430A microarrays. Co-regulated expression differences in the AcB/BcA set of mice were identified by ANOVA. (D) The negative log of the Benjamini–Hochberg corrected p value is shown for each gene. Different shades of gray indicate different chromosomes.

Primary candidate genes identified using eQTL

To identify primary candidate genes within our chromosome 2 and chromosome 17 cQTL, we performed linkage analysis using the microarray expression data from uninfected lung tissue of 54 mice from the AcB/BcA set originally published by Lee et al. in 2006 (34). Compared with Lee et al.’s analysis, the current analysis had increased resolution and accuracy in detecting eQTL, because of increased density of genotypes in the RCS and the exclusion of known problem microarray probes. We hypothesized that genes showing highly significant cis-regulated expression differences between A/J and B6 genotypes within cQTL would be primary candidates for further validation. Benjamini–Hochberg correction of ANOVA-derived p values identified a set of 458 probes representing 473 genes from the microarray analysis that showed genome-wide significance (Fig. 4D, Supplemental Table IB).

Significant cis-eQTL were colocalized with the cQTL identified above (Fig. 6A). Hc (34.8 Mb, p = 1.27 x 10^{-5}) was the most significant of five eQTL located within the female-specific cQTL on chromosome 2 (Fig. 6A, left panel). Hc was previously associated with influenza susceptibility, validating our candidate gene identification (33). A/J mice are known to have a mutation that causes a stop codon in Hc, resulting in A/J mice showing drastic reductions in the expression of transcripts for this gene compared with B6 mice. Tnfrsf21 and Pla2g7 were colocalized within the male-specific cQTL located on chromosome 17 (Fig. 6A, right panel). In both cis-eQTL on chromosome 17, the A/J allele had higher expression compared with the B6 allele. Tnfrsf21 (43.2 Mb, p = 1.18 x 10^{-5}) is a member of the TNFR superfamily and was shown to modulate Th cell activation (45). Pla2g7 (43.7 Mb, p = 4.65 x 10^{-5}) was the third most significant eQTL identified in our microarray analysis and the most significant eQTL within the male-specific locus. Pla2g7 is a phospholipase that was shown to modulate proinflammatory responses (46, 47). Fig. 6 has expanded views of the two loci, showing the colocalization of cQTL and eQTL (Fig. 6A), marker location (Fig. 6A), and SNP frequency in A/J and B6 mice (Fig. 6B). By identifying the most significant eQTL within our cQTL, we identified Hc, Pla2g7, and Tnfrsf21 as strong candidate genes underlying susceptibility to influenza in our model.

Table II. Significant cQTL in the total, male, and female RCS populations

<table>
<thead>
<tr>
<th>All</th>
<th>Male</th>
<th>Female</th>
<th>Position (Mb)</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>NS</td>
<td>2</td>
<td>24–38</td>
</tr>
<tr>
<td>NS</td>
<td>6</td>
<td>NS</td>
<td>127–135</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>NS</td>
<td>37–48</td>
</tr>
</tbody>
</table>

Susceptibility scores of RCS mice from a pool of both male and female mice, male mice only, or female mice only were used to identify cQTL associated with susceptibility or resistance to influenza in the RCS. Chromosomal positions of significant linkage are outlined for these three RCS populations.
Male-specific locus on chromosome 17 is associated with decreased survival and increased cytokine expression in RCS

To dissect male-specific susceptibility, we chose to focus on the BcA70 and BcA69 strains, which have the shortest survival time in males within the RCS panel. These two strains carry A/J alleles for Pla2g7 and Tnfrs21 at the chromosome 17 cQTL but also contain A/J segments for Hc, which was previously reported to control influenza susceptibility (33), and for the H2 cluster of immune genes, including Tnfr-α, known to be involved in the pathology of acute influenza infection (48) (Fig. 7A). H2 is located ~7 Mb proximal to the chromosome 17 cQTL mapped in this study. Therefore, to refine the localization of the chromosome 17 cQTL and its relative contribution to susceptibility, we examined the survival and expression profiles of a panel of cytokines and chemokines in a subset of informative RCS with ancestral recombination events segregating these three loci. Male mice from strain BcA74, which contains only the informative H2 segment, and strain BcA76, which contains only the informative Hc segment, both showed increased survival time compared with male mice from BcA70 and BcA69 strains (Fig. 7B). Thus, in this model, neither the Hc nor H2 regions affected male mouse vulnerability to influenza infection. Further, at day 3 postinfection with influenza virus, we examined the panel cytokines previously shown to distinguish A and B6 progenitor strains (Fig. 2D) and found significant interstrain differences by ANOVA. Pla2g7 expression segregated according to genotype and phenotype, with higher levels in susceptible mice. By contrast, Tnfrs21 expression was low in all RCS mice compared with the parental strain A/J. In addition, increased expression of Ifn-β, Il-6, Tnf-α, and Kc associated with susceptibility, as determined by the chromosome 17 cQTL (Fig. 7C, Supplemental Table IIB).

Discussion

We undertook this study to identify new candidate regions and genes involved in the host response to infection with a mouse-adapted H3N2 influenza strain. In our novel mouse model, influenza susceptibility was under complex genetic control, with sex significantly altering the severity of infection. We identified two sex-specific cQTL associated with influenza susceptibility. By comparing cQTL from expression profiles of uninfected lung tissue, we were able to identify primary candidate genes within these cQTL. A novel male-specific cQTL on chromosome 17 was confirmed by an F2 cross and was associated with increased cytokine and chemokine expression and decreased survival in male RCS mice.

Functional dissection of susceptibility in our mouse model

After identifying differential susceptibility to influenza infection in terms of clinical signs of disease and survival between A/J and B6 mice, we tested additional aspects of the host response and immune system function to further characterize the differences. Both A/J and B6 mice had similarly high viral loads in lung tissue in

FIGURE 5. Male-specific cQTL confirmed through a secondary F2 screen. F2 mice (177 total) bred from susceptible BcA70 and resistant B6 mice were infected with 10⁴ PFU of HK/68 virus and followed for 14 d. Survival of the F2 mice segregated by genotype at peak markers on chromosomes 4 (A) and 17 (B). Significant p values are indicated.

FIGURE 6. (A) Primary candidate genes identified by combining cQTL and eQTL. Colocalization of the female-specific cQTL and lung tissue-derived eQTL on chromosome 2 (left panel, Dpp7, Pla2g7, Notch1, St6galnac4, and Hc) and colocalization of the male-specific cQTL and lung tissue-derived eQTL on chromosome 17 (right panel, Tnfrs21 and Pla2g7) are shown. (B) SNP density per 25k bin is displayed for chromosomes 2 and 17 to indicate regions of genetic heterogeneity between A/J and B6 mice.
and increased apoptosis are all markers of severe disease in influenza (57). The differential pathogenicity in A/J and B6 mice mirrors some of the hallmarks of influenza in humans, including the overactive cytokine response. As such, our model provides a strong platform for genetic dissection of the inflammatory response. Further, host susceptibility genes to H3N2 identified in this study may be relevant for infection outcome with many influenza strains.

**Complex genetic control of influenza virus infection in mice**

To investigate host genetic factors contributing to infection outcome, we screened a panel of closely related mouse strains created from A/J and B6 progenitors. We found a continuous distribution of susceptibility across these mouse strains, suggesting that infection outcome was under complex genetic control. This is in line with the complex nature of susceptibility usually seen in human infectious diseases (17). Interestingly, we found a significant effect of sex in two of the RCS (male susceptibility in BcA70 and female susceptibility in BcA72) that was not present in progenitor strains. It is remarkable that the sexual dimorphism was only statistically significant in two strains, indicating a combined effect of background and sex in the susceptibility of the RCS mice to influenza. Sexual dimorphism with enhanced male susceptibility has been seen in model infections with many pathogens and are hypothesized to reflect endocrine-immune interactions (58, 59). For example, in the case of *Listeria monocytogenes* infection, increased female resistance was linked to estrogen-mediated inhibition of proinflammatory caspase-12 expression (60). On the contrary, in the case of both human and mouse influenza infection, females can appear more susceptible than males (61). Observational studies indicated that the incidence, severity, and fatality due to seasonal or pandemic influenza infections could be different between the sexes. However, they also often vary with age and geographic location, making the effect of sex alone difficult to interpret (62).

Further, it was shown that infection of 8-wk-old mice with H1N1 virus induced low estradiol levels, which had proinflammatory effects and rendered females more susceptible than males (61, 63). Notably, this sexual disparity was evident only when median infectious doses were used, whereas the effect of infections with low or high viral inoculi were sex independent (64). Thus, comparisons are not straightforward with our experiments that have used older (12-wk-old) mice and different virus strain and dose. Thus, to draw more precise conclusions about the impact of sex on the outcome of infection with influenza virus, it will be important to disaggregate not only sex, but also age and dose, in future studies. Regardless, to our knowledge, our study is the first to identify sexually dimorphic genetic control of influenza infection, but the precise mechanisms (hormonal or other) determining sex differences in this model remain to be defined.

**Identification of sexually dimorphic loci altering host response to influenza**

We used the AcB/BcA panel of RCS to identify genetic regions linked to the host response to influenza infection. Overall, two regions, on chromosomes 2 and 17, reached genome-wide significance and were significantly linked to influenza outcome in the full AcB/BcA population. The chromosome 2 cQTL was the only locus to reach significance in the female population, while the cQTL on chromosomes 6 and 17 reached significance in the male population. It is notable that the chromosome 6 cQTL, which was not highly evident in the full RCS screen, became very highly significant in the male population. This is most likely due to the loss
of power associated with halving the number of individuals in the analysis. With the loss of information that came with the decrease in sample size, it was impossible to differentiate between the chromosome 6 and chromosome 17 genetic regions in the male-only dataset. In this case, the chromosome 6 QTL is most likely a false positive that was inflated because it had a distribution of RCS genotypes similar to the chromosome 17 QTL. The chromosome 17 cQTL was confirmed in an additional F₂ male population generated from susceptible BcA70 and resistant B6 mice. The F₂ cross also detected a weaker chromosome 4 locus with A alleles associated with increased susceptibility in male and female mice. Perhaps due to a lower effect this locus was not detected in the RCS analysis. This result showcases the importance of secondary crosses to fully uncover the complexity of the genetic control of infectious traits.

Identifying primary candidate genes using eQTL

To identify primary candidates within the chromosome 2 and chromosome 17 loci, we looked for colocalization of our eQTL with that of lung eQTL identified in the same AcB/BcA mice. We hypothesized that highly significant cis-regulated expression differences between B6 and A/J mice may result from altered or absent gene function in mice containing the A/J or B6 genotype at a given locus. Integrating expression and clinical data previously identified candidate genes involved in complex disease (22). Within the AcB/BcA mice, the most significant differentially regulated gene in the female-specific chromosome 2 locus was Hc. Otherwise immunocompetent mice (35), A/J animals share a common natural 2-bp deletion in a 5' exon of this gene, leading to a primary stop codon and complement deficiency (65). Depletion of Hc was previously associated with increased susceptibility to influenza (33, 66) and infections by unrelated pathogens (67, 68), as well airway hyperresponsiveness (69). Intact complement function was shown to have an adjunctive effect on host recovery from primary influenza (66). The identification of this gene in our study supports our experimental approach, but it does not discount possible roles for other expression candidate genes in the chromosome 2 interval in influencing susceptibility in our model. Previous mouse work implicating Hc in influenza susceptibility was done only in female mice, which is in line with our mapping results that suggest the effect is stronger in females than in males. Interestingly, C5 (encoded by Hc) activity may be altered by female sex hormones (70) providing a potential explanation for the sexual dimorphism that we observed.

Similarly, of the significant eQTL within the male-specific chromosome 17 locus, Pla2g7 is the most compelling candidate. The protein encoded by Pla2g7 is a multifunctional enzyme that is involved in many disease processes, including cardiovascular disease, asthma, and macular degeneration (46, 47, 71, 72). Two contrasting effects on inflammation have been described. PLA2G7 can hydrolyze oxidized phospholipids to produce lysophosphatidylcholine and free oxidized fatty acids, leading to increased inflammation (73). In our AcB/BcA model, pharmacological inhibition of PLA2G7 reduced inflammation in the necrotic cores of atherosclerotic plaques (73). In our animal model, pharmacological inhibition of PLA2G7 reduced inflammation and disease severity by reducing levels of proinflammatory lysophosphatidylcholine (77). In our AcB/BcA model of influenza infection, the chromosome 17 locus and the A/J Pla2g7 genotype were associated with high Pla2g7 mRNA levels, increased inflammation (including increased KC expression), and decreased survival. Although our results provide an intriguing new candidate gene, more research is needed to dissect the exact genetic contributors of susceptibility within the sex-specific region of linkage on chromosome 17. As with Hc, previous work showed that female sex hormones have an effect on mRNA levels of Pla2g7 (78, 79), providing a potential explanation for the sexual dimorphism observed at this locus.

Another interesting candidate within the chromosome 17 cQTL stemming from eQTL analysis is Tnfrsf21 (also known as death receptor 6), a member of the TNF superfamily. TNFRSF21 activation is associated with NF-κB and JNK activation and apoptosis (80). The gene was associated with increased lung inflammation in a murine model of asthma (45). However, although we observed differential expression in the parental strains, we failed to observe differential regulation of the gene in RCS upon influenza infection, suggesting that, at least at the expression level, TNFRSF21 does not influence the course of infection.

We confirmed a previously identified cQTL and identified a novel sex-specific cQTL linked to aberrant host responses against influenza infection in mice. Expression mapping was used to identify primary candidates within these regions of linkage. Hc, located within the previously confirmed cQTL on chromosome 2, was depleted in susceptible mice, leading to reduced complement function. Pla2g7 and Tnfrsf21, located in the male-specific cQTL on chromosome 17, were overexpressed in susceptible mice and associated with increased pathogenic levels of proinflammatory cytokines. By using RCS mice, we could detect genes with small effects while maintaining high genetic resolution, thus anchoring expression results in small intervals. By searching for genes with highly significant cis-regulatory differences within regions of linkage to influenza susceptibility, we were able to more efficiently identify compelling candidate genes involved in the host response to this globally relevant pathogen. This study may be used as a reference for future studies attempting to incorporate eQTL with traditional forward genetics for efficient gene discovery within the context of infection.

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Disclosures

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