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Quantitative Trait Locus Analysis, Pathway Analysis, and Consomic Mapping Show Genetic Variants of Tnni3k, Fpgt, or H28 Control Susceptibility to Viral Myocarditis

Sean A. Wiltshire, Gabriel André Leiva-Torres, and Silvia M. Vidal

Coxsackievirus B3 (CVB3) infection is the most common cause of viral myocarditis. The pathogenesis of viral myocarditis is strongly controlled by host genetic factors. Although certain indispensable components of immunity have been identified, the genes and pathways underlying natural variation between individuals remain unclear. Previously, we isolated the viral myocarditis susceptibility 1 (Vms1) locus on chromosome 3, which influences pathogenesis. We hypothesized that confirmation and further study of Vms1 controlling CVB3-mediated pathology, combined with pathway analysis and consomic mapping approaches, would elucidate both pathological and protective mechanisms accounting for natural variation in response to CVB3 infection. Vms1 was originally mapped to chromosome 3 using a segregating cross between susceptible A/J and resistant B10.A mice. To validate Vms1, C57BL/6J-Chr 3/NaJ (a chromosome substitution strain that carries a diploid A/J chromosome 3) were used to replicate susceptibility compared with resistant C57BL/6J (B6). A second segregating F2 cross was generated between these, confirming both the localization and effects of Vms1. Microarray analysis of the four strains (A/J, B10.A, C57BL/6J, and C57BL/6J-Chr 3/NaJ) illuminated a core program of response to CVB3 in all strains that is comprised mainly of IFN-stimulated genes. Microarray analysis also revealed strain-specific differential expression programs and genes that may be prognostic or diagnostic of susceptibility to CVB3 infection. A combination of analyses revealed very strong evidence for the existence and location of Vms1. Differentially expressed pathways were identified by microarray, and candidate gene analysis revealed Fpgt and Tnni3k as likely candidates for Vms1. The Journal of Immunology, 2011, 186: 6398–6405.

Virial myocarditis and its long-term sequelae, dilated cardiomyopathy (DC), are a common cause of morbidity and mortality (1). Adenoviruses, influenza, and herpesviruses have been implicated; however, enteroviruses, particularly coxsackievirus B3 (CVB3) strains, are the most prominent etiology (2). In mice, experimental infection with CVB3 has served to elucidate three distinct clinical aspects of human disease progression: 1) an initial phase of viral replication and cytolysis of cardiomycocytes; 2) a phase of intense myocardial infiltration by immune effectors, which may resolve infection or persist indefinitely in susceptible animals (3); and 3) a delayed phase in which initial infiltration and response may transition into autoimmune or inflammatory cardiomyopathy (4). Furthermore, different individuals or inbred mouse strains display variable degrees of cardiomyopathy, likely influenced by their genetic background. Myocarditis and DC can also be induced experimentally in the absence of virus by administration of cardiac myosin with adjuvant. As with viral myocarditis, inbred strains of mice also differ in their inherent susceptibility to experimental autoimmune myocarditis, implicating genetic controls of myocardial inflammation (5). Therefore, two outstanding questions in the field are: 1) does uncontrolled viral replication drive inflammation during myocarditis, or is virus merely an equivalent trigger to unequal inflammation; and 2) which host genetic variants are associated with the outcome of virus-induced disease?

Phenotype characterization of CVB3 infection in mutant mouse stocks has so far delimited three major systems essential in predicting disease severity: integrity of myocardial structure, protection by innate immunity, and the balance between curative immunity and chronic inflammation or autoimmunity. The cytoskeletal protein dystrophin is cleaved by CVB3 protease 2A, a protein essential for viral life cycle; this cleavage affects cardiac function and morphology (6). Type I IFN is produced by immune and nonimmune cells in response to pathogen sensing through innate receptors. High levels of type I IFN activate antiviral response systems (7) and enhance early cytotoxic effectors such as NK cells (8). Thus, mice deficient in TLR3 suffer from severe myocarditis and a high viral load in the heart and liver (9). Similarly, type I IFNR (10) and IFN-β knockout mice (11) show increased viral burden in the liver and heart, respectively. These data strongly suggest a protective role for type I IFN, particularly IFN-β, during coxsackieviral infection. Recently, the constitutive expression of CXCL10 in the heart has been shown to attract NK cells to the heart, reducing overall viral replication (12). Though much progress has been made, there is still a need to account for differential susceptibility to viral myocarditis among otherwise normal individuals due to natural variation.

The MHC genes have been linked to susceptibility to myocarditis in both humans and experimental mouse models (13, 14).
Non-MHC genes may also have an important impact on disease susceptibility. Recently, functional TLR3 variants (homozygous 412F and heterozygous 554S) were found in excess among patient presenting enteroviral myocarditis or DC (15). To identify novel genetic factors associated with disease, we have applied quantitative trait locus (QTL) mapping in an experimental mouse model. Previously, using an F2 cross between susceptible A/J and resistant B10.A-H2" (B10.A) mice, we identified three QTL on chromosomes 1 (viral myocarditis susceptibility 2 [Vms2]), 3 (Vms3), and 4 (Vms4). Only Vms1 was linked to the three phenotypes under study [i.e., myocarditis score (p = 0.005), extent of sarcolemmal damage (p = 0.001) (16), and heart viral load (p = 0.010)]. This suggested that Vms1 is a central determinant of disease severity; in the current study, we seek to determine the precise contribution of Vms1 in viral myocarditis. To achieve this, we validated the existence of Vms1 through phenotyping of a chromosome substitution strain that carries a diploid A/J chromosome 3 (CSS3) on a C57BL/6 (B6) background (17). We then used a second F2 cross, this time between B6 and CSS3 mice, to refine the Vms1 genetic interval. Investigation of the mechanistic basis of Vms1 function using genomewide expression and pathway analysis in the four parental strains (susceptible A/J and CSS3 mice, as well as resistant B10.A and B6 mice) indicated that Vms1 susceptibility alleles impair antiviral mechanisms downstream of viral sensing or type I IFN pathways. This combined effort demonstrated that viral load and myocarditis severity are likely controlled by a more resolved locus, Vms1.1, for which the critical interval contains three candidate genes: Fgpt, H2b, and Tnml3k.

Materials and Methods

Mice and virus

Inbred A/J (000469), B10.A-H2" H2-T18/SgSnJ (B10.A, 000646), C57BL/6-J (B6), and C57BL/6J-C57BL/6J (B6; 000664) mice were purchased from The Jackson Laboratory. The mice were maintained in the McGill University animal facility in compliance with the Canada Council on Animal Care as approved by the McGill University Animal Care Committee. Coxsackievirus B3 was used for mouse and microarray experiments was the CVB3-CG strain, as used previously (16). All other experiments, including CSS3/B6F1 as well as [CSS3 × B6F1], were infected with the plaque purified CVB3-H3 (18), generously provided as a plasmid by the laboratory of Dr. Kirk Knowlton (University of California, San Diego, CA). Like CVB3-CG, CVB3-H3 was also as a plasmid by the laboratory of Dr. Kirk Knowlton (University of California, San Diego, CA). Like CVB3-CG, CVB3-H3 was also provided by the laboratory of Dr. Kirk Knowlton (University of California, San Diego, CA). Like CVB3-CG, CVB3-H3 was also provided by the mouse genome project group at the Wellcome Trust Sanger Institute, and can be obtained at http://www.sanger.ac.uk/resources/mouse/geneses/. SNPs were analyzed using the sorts intolerant from tolerant algorithm (21) for potentially deleterious effects. IFN-stimulated genes (ISG) were annotated using the Interferon Stimulated Gene and Interferome databases (22, 23), the Database for Annotation, Visualization and Integrated Discovery (24), and by Ingenuity Pathways Analysis (Ingenuity Systems, http://ingenuity.com). Pathways identified through Ingenuity are characterized with a Network Score, which is defined as the negative exponent of the right-tailed Fisher’s exact test. The p values shown in figures are a result of a two-tailed test, with *p < 0.05, **p < 0.01, and ***p < 0.001.

Mouse infection and phenotype determination

Mice were inoculated i.p. with 400 PFU CVB3-CG/g of body weight (or 10 PFU/g CVB3-H3) diluted in sterile PBS at 7 to 8 wk of age. Experiments shown in Fig. 1 were replicated with both viruses with equivalent results. Uninoculated controls in microarray experiments were mock infected with PBS in parallel. Survival, weight, and signs of infection were evaluated on a daily basis, and the mice were humanely sacrificed 1, 2, 4, or 8 post-infection (PI). Mortibund mice were humanely euthanized at an acceptable clinical endpoint. At necropsy, mouse hearts were removed aseptically, cut in half sagitally, and fixed in buffered formalin; the remainder was stored at ~80˚C. The apex of hearts were subsequently homogenized and submitted to three freeze-thaw cycles to release infectious virus. After centrifugation at 1000 x g for 5 min to remove debris, the supernatant was isolated for quantification by plaque assay. Samples to be used for microarray were taken from a transverse section above the apex of ~30 mg.

Plaque assay

HeLa cells (American Type Culture Collection: CCL-2) were grown and maintained in DMEM supplemented with 10% FBS and 100 µg/ml penicillin/streptomycin (1× media). One day prior to infection, the cells were harvested at 70–80% confluence and plated on 12-well plates at 3.1 x 10^5 cells per well. Homogenized organs were serially diluted 10-fold in nonsupplemented DMEM, and the dilutions were used to infect HeLa cells in triplicate for 60 min. After initial infection by homogenate, HeLa cells are covered in a layer of media with 0.25% agarose and incubated for 3 d. Cells were fixed in formaldehyde and stained with crystal violet to count plaques.

Genotype analysis

Genomic DNA was prepared from tail biopsies of individual F2 mice by overnight incubation at 55˚C in 700 µl buffer (100 mM Tris-HCl [pH 8], 0.05 mM EDTA, 200 mM NaCl, and 0.2% SDS) containing 0.5 mg/ml protease K and followed by RNase treatment (0.3 mg/ml 2 h at 37˚C). DNA was purified by serial phenol-chloroform extractions and ethanol precipitation. In this genome scan, nine MIT markers spaced ~15 cm apart from each other, with increased density toward distal chromosome 3 to distinguish between A/J and B6 alleles (D3Mit164, D3Mit224, D3Mit346, D3Mit291, D3Mit147, D3Mit128, D3Mit116, D3Mit19, and D3Mit46). The markers in the peak of Vms1 were chosen to match those previously used to facilitate direct comparison between the two datasets. Reactions performed manually used a 10 µl total reaction volume, 200 µM d-2'-deoxy-nucleoside 5'-triphosphate, 1.5 mM MgCl2, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen, Burlington, ON, Canada). Reactions were performed as follows: 96˚C for 2 min; 30 cycles of 94˚C for 45 s, 56˚C for 45 s, 72˚C for 60 s; and a final extension step at 72˚C for 7 min. PCR products were then separated on 2 to 3% high-resolution agarose (USB, Cleveland, OH) gels containing ethidium bromide and visualized under UV light. Identification of likely genotyping errors was performed using R/qtl; all genotypes with an error logarithm of odds (LOD) scores >3 were repeated and verified (19).

Statistical and bioinformatics analysis

Statistical analyses were conducted with the freely available program R, and linkage was performed with package “Rqtl.” The scannone function of the “Rqtl” library was used to perform maximum likelihood interval mapping of phenotype on genetic markers. Significance values were evaluated with 10,000 permutations. In joint analysis between [A/J × B6F1] and [CSS3 × B6F1], combined cross-analysis was performed with experimental cross used as a covariate term in all calculations as previously described (20). LOD support interval was calculated using a 1.5 LOD drop by “Rqtl.” Single nucleotide polymorphisms (SNPs) were identified using the mouse phenome database (http://phenome.jax.org/SNP), were also provided by the mouse genome project group at the Wellcome Trust Sanger Institute, and can be obtained at http://www.sanger.ac.uk/resources/mouse/geneses/. SNPs were analyzed using the sorts intolerant from tolerant algorithm (21) for potentially deleterious effects. IFN-stimulated genes (ISG) were annotated using the Interferon Stimulated Gene and Interferome databases (22, 23), the Database for Annotation, Visualization and Integrated Discovery (24), and by Ingenuity Pathways Analysis (Ingenuity Systems, http://ingenuity.com). Pathways identified through Ingenuity are characterized with a Network Score, which is defined as the negative exponent of the right-tailed Fisher’s exact test. The p values shown in figures are a result of a two-tailed t test, with *p < 0.05, **p < 0.01, and ***p < 0.001.

Microarray and quantitative PCR

Hearts used for microarrays were first perfused with cold sterile buffer (110 mM NaCl, 10 mM NaHCO3, 16 mM KCl, 16 mM MgCl2, 1.2 mM CaCl2, and 5 U/l heparin [pH 7.9]), and tissues were preserved in RNA-later stabilization reagent (Ambion) before being frozen at ~80˚C. Total RNA was extracted from ~30 mg heart tissue from a transverse section using the Qiagen RNeasy extraction kit (Qiagen). Quality control by Agilent bioanalyzer (Agilent Technologies), cDNA synthesis, and microarray hybridization was performed at the McGill University and Genome Quebec Innovation Centre. Three infected and three uninfected female mice of each genotype were independently prepared and analyzed on an Affymetrix MouseWG-6 version 2.0 array (Illumina) (one genotype and six mice per array, four arrays total). A/J versus B10.A and CSS3 versus B6 experiments were performed and analyzed separately. Raw data are available through the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE19496. Normalization analysis was performed using FlexArray (25), and expression values were normalized using the log2 and RNA configuration of Lumini. Principal component analysis figures (Supplemental Fig. 3) were also generated within FlexArray. Genes were filtered for consideration as follows: the 40th percentile was determined across all normalized probes as an arbitrary cutoff value. If no probes from any experimental group were greater than.
the 40th percentile, the gene was considered absent in all samples and not considered further. A starting list was used in which at least one genotype either infected or uninfected met this criterion for 20,261 targets. Paired experimental groups (e.g., AJ-infected versus uninfected) were compared by CyberT (26) with default parameters and considered to be differentially expressed if the fold change (FC) was <-0.5 or >2 and unadjusted p value was <0.05. Interaction between strain and genotype was defined as: at least one strain is differentially expressed (0.5 < FC < 2, p < 0.05), ANOVA (strain/infection) significant (p < 0.05); as well as the absolute difference of difference between strain means: [(\mu_{AA} - \mu_{AI} infected) - (\mu_{B10.A} - \mu_{B10.A} infected)] > 1.25. This final step was determined as an effective filter in removing parallel changes between genotypes following infection. This much-abbreviated list was then annotated to identify which significantly higher levels of inflammation in the heart than B6 mice (2.61 ± 0.23 versus 4.04 ± 0.11). Furthermore, CSS3 mice have significantly higher levels of inflammation in the heart than B6 mice (2.85 ± 0.26 versus 1.4 ± 0.23; p < 0.001) (Fig. 1B). No significant differences were observed between the sexes, and [CSS3 × B6]F1 mice were not significantly different from B6 mice in terms of viral replication or inflammation. These results replicate Vms1 susceptibility to viral replication and intensity of peak inflammation; they also suggest Vms1r is recessive to Vms1s.

**Genomewide linkage replicates Vms1**

To characterize the inheritance of susceptibility to CVB3, we determined heart viral titer and myocarditis in 126 [CSS3 × B6]F2 mice. The mean of F2 viral titers (3.22 ± 0.09) and myocarditis scores (1.73 ± 0.11) were similar to that of the B6 parental strain, which is consistent with a major gene effect and recessive Vms1s susceptibility (Fig. 2A, 2B). As shown in Fig. 2C, correlation was noted between viral load and myocarditis severity (Spearman, ρ = 0.54; p < 0.0001). Unlike the previous [A/J × B10.A]F2 sex differences in susceptibility within the F2 mice were not evident in either myocarditis or viral replication (p = 0.470, p = 0.305, respectively).

**Results**

**Chromosome 3 controls viral myocarditis severity**

In mice, severity of viral myocarditis is controlled by genetic background (29). Strong linkage of chromosome 3 to myocarditis, sarcolemmal disruption (16), and viral titer (Supplemental Fig. 2) encouraged us to isolate the role of chromosome 3 in viral myocarditis. The CSS3 mice are ideally suited to assess the contribution of chromosome 3, as CSS3 mice carry the susceptible A/J allele of Vms1 (Vms1s) while controlling for background compared with B6. Differential control of viral replication was observed at day 8, with CSS3 mice being significantly more susceptible than B6 mice (2.61 ± 0.13 versus 4.04 ± 0.08 log [PFU/mg]; p < 0.0001) (Fig. 1A). Furthermore, CSS3 mice have significantly higher levels of inflammation in the heart than B6 mice (2.85 ± 0.26 versus 1.4 ± 0.23; p < 0.001) (Fig. 1B). No significant differences were observed between the sexes, and [CSS3 × B6]F1 mice were not significantly different from B6 mice in terms of viral replication or inflammation. These results replicate Vms1 susceptibility to viral replication and intensity of peak inflammation; they also suggest Vms1r is recessive to Vms1s.

**Figure 1.** Replication of Vms1 phenotypes in C57BL/6j-Chr3A/NaJ. A, Viral titer in CSS3, B6, and [CSS3 × B6]F1 mice 8 d postinfection, as measured by plaque assay. CSS3 mice of both sexes have significantly more virus in the heart than B6 or [CSS3 × B6]F1 (p < 0.001). B, Myocarditis is scored with 4 being most severe and 0 being no inflammation evident. CSS3 mice were more susceptible than B6 or [CSS3 × B6]F1 (p < 0.05). n ≥ 11 per group. Both A and B represent a composite of multiple CVB3-H3 infections ([CSS3 × B6]F2, controls) and were replicated with CVB3-CG. *p < 0.05, ***p < 0.001.

**Figure 2.** Phenotype distributions within the [CSS3 × B6]F2 population. A, Histogram of the distribution of viral titers in [CSS3 × B6]F2 mice, with a mean of 3.22 ± 0.09. B, Histogram of the distribution of myocarditis scores in [CSS3 × B6]F2 mice, with a mean of 1.73 ± 0.11. C, The distribution of [CSS3 × B6]F2 mice, showing significant correlation (r² = 0.29; p < 0.0001) between myocarditis score and viral titer. This reveals a trend in which higher myocarditis score is correlated to higher viral titer.
Linkage analysis revealed a locus on distal chromosome 3, overlapping the previously described Vms1 locus, controlling heart viral titer (LOD = 3.74; \( p = 0.018 \)) (Fig. 3A). Vms1 superscript I mice were protected from high viral load in the heart (2.87 ± 0.29 log [PFU/mg]) when compared with Vms1 superscript I (3.75 ± 0.13 log [PFU/mg]) (Fig. 3B). Vms1 linkage to inflammation was also significant (LOD = 3.57; \( p = 0.0032 \)); Vms1 superscript I (1.07 ± 0.23) mice were protected compared with Vms1 superscript I mice (2.28 ± 0.24). In a single QTL model, Vms1 accounted for 11.89% of the variance observed in viral titer and for 11.02% in myocarditis score, a notable increase from, respectively, 5.87 and 3.25% in the [A/J × B10.A]\(F_2\) cross. The Vms1 interval in both crosses is defined between D3Mit291 and D3Mcg1, spanning 16 cM and 16 Mb.

**Differential control of viral replication occurs between 2 and 4 d postinfection**

Differential gene expression in response to infection may be causally related to the observed phenotypes or may be a reaction secondary to the primary causal locus. Understanding differential gene expression globally and within Vms1 will aid in identifying potential candidates for Vms1. Presumably, the gene mediating Vms1 superscript I resistance or Vms1 superscript s susceptibility to viral myocarditis exerts some effect before the peak of myocarditis at day 8. As a prerequisite to transcriptional profiling by cDNA microarray, we first ascertained at which time point expressed genes might play a role. We assessed viral titer at multiple time points in A/J and B10.A mice (Fig. 4A), as well as in CSS3 and B6 mice (Fig. 4B), prior to the development of inflammation. After 96 h PI, A/J and CSS3 mice have significantly higher levels of viral replication than B10.A or B6 mice. Higher type I IFN levels have been reported in the B6 strain (30), suggesting a possible mechanism for resistance. We observed that susceptible A/J mice produce significantly more IFN-\(\beta\) than resistant B10.A mice during infection (Fig. 4C). CSS3 and B6 mice did not produce significantly different amounts of IFN-\(\beta\) at the times measured. These observations are consistent with a genetically determined lack of ability to control viral replication by 96 h PI, which is not accounted for by higher IFN-\(\beta\) in resistant mice.

**Microarray analysis of resistant and susceptible hearts reveals a core program of response to viral myocarditis**

Global gene expression 96 h PI was compared by microarray in A/J, B10.A, CSS3, and B6 hearts. Although it has not yet been demonstrated that Vms1 is a heart-specific effect, the lack of difference in viral control at 48 h PI suggests an equal seeding of the myocardium followed by an unequal response to infection toward 96 h PI (Fig. 4B). Therefore, the heart seemed the most likely tissue to interrogate to identify mechanisms or cells identifiable by gene expression. Principal component analysis indicates that infection accounted for the majority of variation, as expected (Supplemental Fig. 3), followed by variation due to genotype in A/J versus B10.A and CSS3 versus B6 mice.

Genes highly expressed or repressed PI (at least a 5-fold change in all strains) were considered to be part of a common pathway.

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**FIGURE 3.** Linkage of chromosome 3 to CVB3 replication in the heart and to myocarditis. Overlapping LOD plots of [A/J × B10.A]\(F_2\) (black) and [CSS3 × B6]\(F_2\) (gray) mice shows significant linkage on distal chromosome 3 to viral replication in the heart (A) and to myocarditis (C). Threshold values corresponding to \( \alpha = 0.03 \) are represented as horizontal lines of corresponding shade. Peak linkage to viral replication and myocarditis in [A/J × B10.A]\(F_2\) occurred at D3Mit19 (LOD = 3.74; \( p = 0.018 \)) and D3Mit16 (LOD = 3.57; \( p = 0.0032 \)). B. Viral titer versus genotype at D3Mit19 shows that the AA genotype leads to a higher viral titer than BB in both crosses: 3.5 ± 0.11 versus 2.84 ± 0.12 log [PFU/mg] in [A/J × B10.A]\(F_2\) and 3.75 ± 0.13 versus 2.87 ± 0.29 log [PFU/mg] in [CSS3 × B6]\(F_2\). D. D3Mit19 genotype of AA leads to higher myocarditis scores than BB in both crosses: 2.83 ± 0.11 versus 2.06 ± 0.12 in [A/J × B10.A]\(F_2\) and 2.28 ± 0.24 versus 1.07 ± 0.23 in [CSS3 × B6]\(F_2\).

**FIGURE 4.** Viral replication is higher in A/J and CSS3 than in B10.A and B6 96 h PI. Level of infectious CVB3 within the heart measured by plaque assay at 24, 48, and 96 h in A and B. Differential control is significant at 96 h postinfection in both comparisons (\( p < 0.05 \)). C and D. Concentrations of IFN-\(\beta\) in the serum were measured by ELISA in the same animals as in A and B. Significantly higher amounts of IFN-\(\beta\) were detected in A/J mice compared with B10.A at 48 and 96 h PI (\( p < 0.05 \)). No significant differences were observed between CSS3 and B6 mice (A and C, \( n = 4 \) per time point; B and D, \( n = 6 \) per time point, replicated with \( n = 8 \) per strain at 48 h).
This set of 129 genes was strongly indicative of an IFN response: 46 of the 129 (35%) genes were annotated as being IFN induced (23). The interferome database used for comparison contains 1925 mouse genes annotated as being IFN induced in various cell types, corresponding to 6.3% of the arrays used in this study. Of the 46 genes identified, 14 suggested a type I IFN response, 1 a type II IFN response, and the remaining 31 were inducible by either. Functional clustering of these by Database for Annotation, Visualization and Integrated Discovery revealed that the strongest enrichment terms were responses to virus and RIG-I–like receptor signaling ($p < 0.00005$). Highly expressed genes included effector ISG (Oas1g, Mx1), transcription factors (Irf7), and MHC class I (H2-d1, H2-k1). These results are summarized in Fig. 5 and detailed in Supplemental Table I. The observation that ISG pathways themselves are strongly upregulated in resistant (B6, B10.A) and susceptible (CSS3, A/J) strains is consistent with the hypothesis that although IFN is required to survive systemic CVB3 infection (10, 11), IFN may not be sufficient to control cardiac viral replication (31, 32).

**Differential microarray analysis reveals myocarditis susceptibility expression programs**

As a robust IFN response seems approximately equal between strains, differential expression of sets of genes within a pathway may represent unequal mechanisms of adaptation to infection or pathological processes. To determine expression programs differentially activated by infection, sets of genes were created that identify qualitatively different expression patterns (e.g., A/J differentially activated by infection, sets of genes were created that identify qualitatively different expression patterns (e.g., A/J increased expression of H28 PI, but B10.A do not). Using these priority ranked lists (Supplemental Table I), pathway analysis and functional grouping was performed.

A/J-specific programs (Fig. 6A) were involved in metabolism, cell structure, and cardiovascular development pathways. B10.A differed in that cell-mediated immunity markers, Ag presentation, and immune cell trafficking were identified as primary pathways for this strain (Fig. 6B). The CSS3-specific pathway (Fig. 6C) shared several component genes with A/J, most of which are not located on chromosome 3. CSS3-specific pathways were identified to be tissue and organ development related. Notably, cell-mediated immune response pathways not present in A/J were expressed in B6 and CSS3 hearts. No B6-specific pathways were identified, and only five genes are particular to a B6 response (Chad, Dkk3, Mybphl, Prss35, and Sln); of these, only Mybphl is expressed on chromosome 3, over 30 cM away from Vms1.

Differentially expressed genes following infection within Vms1 (Adhl, Ghp1, and H28) were identified between A/J and B10.A and confirmed in CSS3 and B6; no genes were found within Vms1 that were differentially expressed between CSS3 and B6 but not between A/J and B10.A (Fig. 7).

Similarly, genes not differentially expressed following infection, but with basal expression differences (e.g., B10.A express Tnni3k before and PI at equivalent levels, whereas A/J do not) represent possible candidate genes, as the presence or absence of one or more key genes may provide a mechanism of resistance (or susceptibility) for which activity does not require induction. Between CSS3 and B6, a total of 28 genes with basal expression differences were identified. Of these, 14 were located on chromosome 3, and 4 of those were located within Vms1: Ghp1, Eif4e, Tnni3k, and Lrrc40.

**FIGURE 5.** Core program of highly and not differentially expressed genes responding to CVB3 infection. Microarray analysis of A/J, B10.A, CSS3, and B6 hearts at 4 d PI. Genes highly expressed or repressed PI (at least 5-fold change in all strains) were considered to be part of a common pathway. Mice strains (A/J, B10.A, CSS3, and B6) are represented in rows stratified by infection status, where + indicates infected tissue and − indicates mock-infected control tissue. This set of 129 genes constitutes a shared program of response to infection and damage in both susceptible and resistant mice. Pathway analysis of these genes identifies inflammation and response to infection. Functional clustering suggested RIG-I–like signaling was involved, and Interferome annotation identified 46 of 129 genes as known ISG. Network score represents a statistical likelihood that the pathway identified is involved in the tissue analyzed given the genes involved.

**FIGURE 6.** Differentially expressed genes in A/J, B10.A, CSS3, and B6 mice identify common and distinct pathways. Mice strains (A/J, B10.A, CSS3, and B6) are represented in columns stratified by infection status, where + indicates infected tissue and − indicates mock-infected control tissue. A, A/J-specific genes (increased or decreased expression in A/J mice PI, but not in B10) cluster into pathways involving cardiovascular development, survival, inflammatory disease, and metabolism. B, B10.A-specific genes differentially expressed compared with A/J were primarily involved in cell-mediated immunity and strongly indicative of a cytotoxic response. C, CSS3-specific genes (increased or decreased expression in CSS3 mice PI, but not in B6) were less numerous than A/J, but fell into similar functional categories of development. There were no B6-specific pathways identified.
Candidate gene analysis for Vms1 (as defined by either F2 cross) identified seven candidate genes for Vms1 on the basis of allele-specific expression, differential response to infection, or coding sequence variation. Of these, only Tnni3k, Lrcr-40, H28, and Fpgt are contained within Vms1.1. Confirmation of significantly higher basal expression levels was found in Tnni3k by qPCR (Fig. 7D), but not in Lrcr-40 (data not shown). Tnni3k was found to be 2.7-fold less expressed in Vms1’ than Vms1’ mice. Increased expression of H28 following infection in Vms1’ and not Vms1’ mice was also confirmed by qPCR (Fig. 7C). Finally, five coding non-synonymous SNP (cisSNPs) within five genes (Twy3, Tnni3k, Fpgt, Sfrs11, and Rpe65) have been identified within Vms1.1, but only two are predicted to be nonconservative and damaging to function: Fpgt*_{rs30203847-C} (C517R) and Tnni3k*_{rs30712233-T} (T659I). After alignment, the Fpgt*_{rs30203847-C} allele possessed by B6, AKR, and 129S1 mice is predicted to be damaging (score 0.03) because the Fpgt*_{rs30203847-R} allele (A/J, BALB/c, C3H) is shared with most vertebrates. In fact, the cnSNP in Tnni3k predicted as damaging is secondary to a more recently identified SNP, rs49812611, which accounts for the lack of basal expression of Tnni3k in A/J or CSS3 mice due to aberrant splicing (28). This interval shortens the list of likely candidate genes for Vms1.1 to H28, Tnni3k, or Fpgt.

**Discussion**

The myocarditic potential of the coxsackieviruses was recognized shortly after their discovery (33). Despite considerable progress in the understanding of CVB3 pathogenesis, coxsackieviral infection is a persistent cause of human disease (34, 35). This remains, however, a need to account for variation in clinical presentation between any two otherwise healthy, normal, and immunocompetent individuals. Surveys of inbred mouse strains have long indicated a genetic component in susceptibility to CVB3-induced disease (14, 29). Our previous analysis identified a locus on chromosome 3, Vms1, which controlled several aspects of disease. This led us to isolate the role of chromosome 3 from other potentially segregating or confounding loci.

CSS represent a valuable model in which to study complex traits. As a prelude to further refinement and positional cloning of Vms1, it was necessary for us to at least replicate our initially observed phenotypes in the CSS3 mice. CSS3 mice control for other previously identified loci respective to B6 (Vms2, Vms3 and H2); however, it was not obvious that Vms1’ susceptibility would be recapTURED. Unlike our initial [B10.A × A/J]F2 cross (all H2”), CSS3 and B6 mice are H2” in known to be a strong modifier of disease (14, 29). CSS3 are also on a C57BL/6 and not C57BL/10 background; though B6 and B10 are closely related, we have found at least 7500 SNPs [S.A. Wiltshire, G.A. Leiva-Torres, and S.M. Vidal, unpublished observations and previous data (36)], some of which may cause interstrain differences in response to infection. Nevertheless, phenotypes of both myocardial inflammation and viral replication were recapTURED. Variability in mean viral titer was similar in [CSS3 × B6]F2 (3.22 ± 0.09 log [PFU/mg]) compared with the larger [A/J × B10.A]F2 cross (3.01 ± 0.06 log [PFU/mg]); however, Vms1 explained more variance in the present cross (11 to 12% versus 3–6%). Even in this genetically simplified context, a considerable amount of variation exists between mice, even of identical genotype (Fig. 1). As an enterovirus, CVB3 exists as a quasispecies with a high mutation rate (~1 aa substitution/ genome) (37, 38), providing ample opportunity for every possible mutation within an infection in which infected tissue can contain as many as 10^7 PFU/mg. This provides an example of increased resolution and power by isolating genetic variation to a single chromosome despite considerable environ-
mental variation. Replication of linkage between distal chromosome 3 and both inflammation and viral titer phenotypes strongly supports the strong role of Vms1 in infection.

Previous studies have successfully used gene expression to better understand myocarditis and dilated cardiomyopathy (5, 39–41). In addition to identifying likely candidate genes, global expression analyses give us key mechanistic insights that aid in the identification of Vms1. Because viral replication in the heart is controlled in Vms1+ mice as early as 4 d PI, this time point was chosen for expression analysis. It has previously been reported that A/J and B6 mice differ substantially in their production of type I IFN following infection (30, 42). Another QTL that has never been cloned but influences peak IFN levels called IIf1 colocalizes with Vms1 (43). These together suggested an initial hypothesis that a sensing or signaling defect in IFN might be present in susceptible mice. However, in this study, IFN response between susceptible and resistant mice was roughly equivalent, or in the case of A/J and B10.A mice, higher viral load was accompanied by higher IFN. Susceptible Vms1+ mice sense virus and produce antiviral IFN-β with similar kinetics and magnitude as compared with Vms1− mice. This makes it so that a defect in viral sensing, signaling, or response to IFN is unlikely to underlie Vms1−.

Strain-specific expression analysis revealed similarities to previous work and several novel results. Comparison between A/J and B10.A mice showed A/J preferentially express genes and pathways involved in cardiovascular development and metabolism. This was consistent with previous findings (also A/J mice), which postulated that these changes reflect undamaged cardiomyocytes compensating for damaged neighbors (41). A notable addition to the A/J-specific response was Amical (also known as JAML), which was highly induced (19.6-fold) in A/J compared with B6, B10.A, or CSS3 mice (1.3–3-fold). Amical is a costimulatory molecule that becomes strongly induced on γδ T cells upon ligation to the CVB3 receptor (Cadr) on injured cells (44). γδ T cells are known to play a pathogenic role during viral myocarditis (45). Conversely, all mice of the B6/B10 background, including susceptible CSS3 mice, expressed several markers (Cd8b1, Nkg7, Cd44/2B4, Klrα7) of antiviral CD8/NK immune responses. Lack of CD8/NK cells and overabundance of inflammatory γδ T cells in A/J mice suggests that this is another possible (non-Vms1−) mechanism accounting for A/J-specific susceptibility to CVB3 infection. In contrast to A/J versus B10.A mice, expression profiling did not reveal any Vms1+ or Vms1−-specific cell type, suggesting that Vms1 does not control the recruitment of a cellular immune response.

Three candidate genes were identified within the refined Vms1.1 locus via expression and publicly available SNP data (150–159.6 Mb on chromosome 3): H28, Tnni3k, and Fpgt. This method of identifying candidate genes via associative data is limited in that if a modest (FC < 2-fold) change in expression underlies our phenotype, it would be missed. An alteration of protein function, but not expression level, in genes containing nonsynonymous SNPs (Twy3, Sfrs11, or Rpe65) is also possible even in the absence of predicted negative impact. We have also treated Vms1.1a and Vms1.1b controlling viral replication and myocarditis score, respectively, as a single entity Vms1.1, which controls both. In the absence of congenic and subcongenic mice that parse individual gene contributions, it is impossible at this stage to identify whether changes in expression are causal or consequential and of which phenotype. However, differential control of viral replication before myocarditis suggests protective mechanisms acting at day 4 or earlier in Vms1+ mice at which time H28, Tnni3k, and Fpgt remain the most attractive candidates.

H28 is expressed in the Vms1+ genotype following infection but not in Vms1−. H28 has no known function and has only been previously described as being presented during allograft rejection between BALB.B (H2b) and B6 mice. Of the five cnSNPs in Vms1.1 (Twy3, Tnni3k, Fpgt, Sfrs11, and Rpe65), only Tnni3k and Fpgt were both expressed in the heart and predicted to be deleterious. Fpgt catalyzes the formation of the fucosyl donor GDP-β-fucose as part of the fucose salvage pathway (46). L-fucose is a sugar present in glycoproteins involved in inflammation and immunity (e.g., sialyl-lewis X, the inflammatory ligand of selectins, contains L-fucose). A possible link between the ability of infected cells to effect immediate inflammatory signals and Fpgt may make this gene an attractive candidate.

Tnni3k was identified as having a basal expression difference: Vms1+ mice express significantly higher basal levels of the gene than Vms1− mice. Indeed, the SNP rs49812611 was recently identified as leading to nonsense mediated decay of Tnni3k transcripts (28). Tnni3k was identified as a cardiac specific tropinin-interacting kinase in 2003 (47). Although the normal function of Tnni3k is still obscure, some recent results have somewhat clarified the situation. Transgenic overexpression of Tnni3k in differentiated P19/crl cells led to increased contractile force and frequency, as well as increased adrenergic response and protection from ischemic injury (48). Tnni3k has also been implicated in dilated cardiomyopathy (39) and heart failure. Mice not expressing Tnni3k were resistant to CSQ heart failure (DBA/2, BALB, and C3H), whereas normal expressers (B6, 129/X1, and AKR) or mice transgenic for the human allele were highly susceptible to heart failure (28). This raises the possibility that Tnni3k may prevent myocardial injury during acute injury (48), but also become pathogenic and increasingly disregulated during chronic disease (28, 39).

This work was begun with the understanding that the outcome of CVB3 infection is largely influenced by three factors: integrity of the myocardium, IFN response, and progression to adaptive immunity. In this study, we present evidence that differential viral replication and myocardial inflammation at early time points may be controlled by Tnni3k (implicating myocardial integrity), Fpgt (implicating cellular immune response), or H28 (by unknown mechanisms). Future work will include the generation of congenic and subcongenic mice, as well as reverse genetic approaches, to assess the contribution of individual genes before progressing further.

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Disclosures

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References