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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

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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 3^3NaJ (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 3^3NaJ) 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, H28, and Tnni3k as likely candidates for Vms1.

Viral myocarditis and its long-term sequela, 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 cytolytic damage of cardiomyocytes; 2) a phase of intense myocardial infiltration by immune effectors, which may resolve infection or persist indefinitely in susceptible animals; 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 IFNAR (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 PS54S) were found in excess among patients presenting entro viral 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 [Vms1]), 3 (Vms1), and 4 (Vms3). 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 diplod 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: Fptg, H28, and Tnmi3k.

Materials and Methods

Mice and virus

Inbred A/J (000469), B10.A-H2* H2-T18*/SgSnAl (B10.A, 000646), C57BL/6-J-Chr 3+v/Naj (CSS3, 004381), and 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 used for time-course and microarray experiments was the CVB3-CG strain, as used previously (16). All other experiments including CSS3/B6/F1 as well as [CSS3 × B6]F2, were infected with the plaque purified CVB3-H3 (18), generously provided by the mouse genome project group at the Wellcome Trust Sanger Institute. In this model, CVB3-H3 behaves comparatively to CVB3-CG and has the added benefit of being a known and widely used genotype allowing for more direct comparisons by other researchers.

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. Uninfected controls in microarray experiments were mock infected with 100,000 plaque forming units (PFU) CVB3-H3 diluted in sterile 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 d post-infection (PI). Moribund mice were humanely euthanized at an acceptable clinical endpoint. At necropsy, mouse hearts were removed aseptically, cut in half sagittally, 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 10,000 × 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 3.1 × 10⁶ cells per well. Homogenized organs were serially diluted 10-fold in non supplemented 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 proteinase 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, and D3Mcg1). 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 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 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 linkages were performed with package “Rqtl.” The scanone function of the “R/qtl” library was used to perform maximum likelihood interval mapping of phenotype on genetic markers. Significance values were evaluated with 1000 permutations. In joint analysis between [A/J × B10.A]F2 and [CSS3 × B6]F2, 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 “R/qtl.” 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/genomes/. 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 solution (Ambion). Hearts were homogenized 1 h before freezing at −30°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 MO100K microarray (Mouse-430 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 RMA configuration of Lumi. 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
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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., A/J 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: [(μA/J − μA/J infected) − (μB10.A − μB10.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 strain was significantly different (e.g., A/J responds to infection by increased expression of a gene, and B10.A does not; this is an A/J-specific gene). See Supplemental Fig. 1 for example and distribution of this parameter. Highly differentially expressed genes were identified with an arbitrary cutoff of 0.2 ≥ FC ≥ 5 (p < 0.05). Quantitative PCR (qPCR) primers were designed to span exon junctions with the help of primer3plus (27).

List of primers used

CVB3-H3 forward: 5′-ATGCGACAAAGCTCCCAACGG-3′; CVB3-H3 reverse: 5′-AGTTCTGCCCCAACTGCTC-3′; Gbp1 forward: 5′-ACTGAGAAGATGGGACAGGAAC-3′; Gbp1 reverse: 5′-GTTCAGCCCTCTTGCTG-3′; H28 forward: 5′-ACAGAAGCTGGATTCTGGT-3′; H28 reverse: 5′-GACATGCGACTTTCAACCCAGAG-3′; Ifnb1 forward: 5′-TGACGGAGAAGATGCAGAAG-3′; Tnni3k (as described in Ref. 28) forward: 5′-AAGACATCACGCTTGTGGTG-3′; Eif4e reverse: 5′-TACCATAATCCCCCACTCG-3′; Eif4e forward: 5′-GGTGTGCTGGCAAGTTTTT-3′; Lrc40 forward: 5′-CCACAAAAAGACCCCTCTG-3′; Lrc40 reverse: 5′-CCAACACCATCTTCCCTAGC-3′; Gapdh forward: 5′-AAAGGCTGTATGACCACTGC-3′; Gapdh reverse: 5′-GATGCAGGGATGTTGTGG-3′; Adh1 forward: 5′-TGTGCCGCTTACATGCTG-3′; Adh1 reverse: 5′-TGATGGCTCGAATTCCCTG-3′; D3Mcg1 forward: 5′-GGGATCTGAGGGAG-3′; D3Mcg1 reverse: 5′-CATGGGCTAAGGAAAACAAA-3′.

Cytokine measurement

IFN-β concentration was measured using an ELISA kit from PBL InterferonSource.

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 < 0.001) (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 Vms1S susceptibility to viral replication and intensity of peak inflammation; they also suggest Vms1S is recessive to Vms1F.

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, p = 0.54; p < 0.0001). Unlike the previous [A/J × B10.A]F2 sex differences in susceptibility within the F2 mice, both sexes were susceptible with CVB3-H3 infections ([CSS3 × B6]F2 controls) and were replicated with CVB3-CG. *p < 0.05, ***p < 0.001.
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*I mice were protected from high viral load in the heart (2.87 ± 0.29 log [PFU/mg]) when compared with Vms1* (3.75 ± 0.13 log [PFU/mg]) (Fig. 3B). Vms1 linkage to infection was also significant (LOD = 3.57; p = 0.0032); Vms1*I (1.07 ± 0.23) mice were protected compared with Vms1* 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]F2 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*I resistance or Vms1* 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-β than resistant B10.A mice during infection (Fig. 4C). CSS3 and B6 mice did not produce significantly different amounts of IFN-β 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-β 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.
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 ($Jr7$), 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 increase 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 $Vms1$ ($Adh1$, $Ghp1$, 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 allelespecific expression, differential response to infection, or coding sequence variation. Of these, only Tnni3k, Lrcr-40, H28, and Fpgtr were 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 nonsynonymous SNP (cSNPs) within five genes (Twn3, Tnni3k, Fpgtr, Sfrs11, and Rpe65) have been identified within Vms1.1, but only two are predicted to be nonconservative and damaging to function: Fpgtr<sup>350203847-C</sup> (C517R) and Tnni3k<sup>35071223-T</sup> (T659I). After alignment, the Fpgtr<sup>350203847-C</sup> allele possessed by B6, AKR, and 129S1 mice is predicted to be damaging (score 0.03) because the Fpgtr<sup>350203847-R</sup> allele (A/J, BALB/c, C3H) is shared with most vertebrates. In fact, the cSNP 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 Fpgtr.

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). There remains, however, a need to account for variation of 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.

CSS3 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<sup>α</sup>), CSS3 and B6 mice are H2<sup>β</sup>; H2 is already 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 recapitulated. Variability in mean viral titer was similar in [C57BL/6]F<sub>2</sub> (3.22 ± 0.09 log [PFU/mg]) compared with the larger [A/J × B10.A]F<sub>2</sub> 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<sup>5</sup> PFU/mg. This provides an example of increased resolution and power by isolating genetic variation to a single chromosome despite considerable environ-

Refined QTL and candidate gene prioritization

A common set of genetic markers, as well as the high degree of similarity between B10.A and B6 on chromosome 3, permitted a joint analysis with the aim of increasing linkage resolution using both the previous [A/J × B10.A]F<sub>2</sub> and the present [CSS3 × B6] F<sub>2</sub> crosses. An increased number of meioses in the combined cross has considerably shortened the interval under consideration. The interval controlling viral replication was narrowed between D3Mit116 and D3Mcg1 (78.7–83.2 cM), which we will refer to as Vms1.1a. Using the same technique, a region between D3Mit116 and D3Mcg1 (78.7–83.2 cM), which we will refer to as Vms1.1b. The overlapping region of Vms1.1a and Vms1.1b is Vms1.1. Candidate genes identified by differential expression (Adh1, Gbp1, H28), by basal expression difference (Tnni3k), and containing nonsynonymous mutations (Fpgtr) are shown in approximate position. B, Box plots of microarray data for candidate genes with expression differences. Horizontal lines represent the 40th and 60th percentile of expression of all genes. Adh1, Gbp1, and H28 are expressed at significantly higher levels in A/J and CSS3 mice following infection, but not in B10.A and B6 mice. Gbp1, Eif4e, and Tnni3k were expressed at different levels even in the absence of infection. C and D, Quantitative RT-PCR confirmation that H28 is induced preferentially in Vms1’ following infection and that Tnni3k transcript is less present in Vms1’ at basal levels.
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 Ifi1 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 C3SS3 mice (1.3–3-fold). Amical is a costimulatory molecule that becomes strongly induced on ITAC Ts upon ligation to the CVB3 receptor (Cxadr) on injured cells (44). ITAC Ts are known to play a pathogenic role during viral myocarditis (45). Conversely, all mice of the B6/B10 background, including susceptible C3SS3 mice, expressed several markers (Cd8b1, Nkg7, Cd244/B24, Klrα7) of antiviral CD8/NK immune responses. Lack of CD8/NK cells and overabundance of inflammatory ITAC Ts 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 (Tyw3, 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 (Tyw3, 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 P19c6 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 subcongeneric mice, as well as reverse genetic approaches, to assess the contribution of individual genes before progressing further.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Differential gene expression filter
A Simple comparison of difference of difference was used to exclude as many equivalent changes in gene expression as possible.
A) Most genes do not change expression following infection, and when they do, changes between the 2 genotypes are roughly equivalent. A histogram of distribution of strain mean difference differences shows that a cutoff of 1.25 excludes ~97% of all genes.
B,C) Two genes within Vms1 H28 and Ifi44 were expressed qualitatively differently. Ifi44 was roughly equivalently expressed following infection whereas H28 (both results were confirmed by qPCR) was only expressed by A/J genotype following infection.

Supplemental Figure 2: Previously unpublished data for [B10.A x A/J]F2 cross
A: Mice of B10.A-H2a background have low to moderate amounts of viral replication in the heart (3.43±0.90 Log[pfu/mg]) by day 8, whereas A/J mice fail to control viral replication in the heart (5.70±0.73 Log[pfu/mg]). F1 mice present predominantly susceptible phenotype (4.99±0.61 Log[pfu/mg]).
B: Histogram of distribution of viral load in the heart of all (A/J x B10.A-H2a)F2 mice with kernel density plotted as a line above. No significant effect of sex was detected on the phenotype.
C,D: Correlation between viral load in the heart with sarcolemmal damage (C) and Inflammation (D) within the (A/J x B10.A-H2a)F2 population. The distribution of F2 mice by viral load and sarcolemmal damage show a trend (Spearman ρ =0.61, p<0.0001) where high viral burden was correlated to higher sarcolemmal disruption, a similar trend of positive correlation with myocarditis score (Spearman, ρ=0.57, P<0.0001), was also observed. This correlation strongly suggests levels of virus in the heart are proportional to cardiomyocyte damage as well as inflammation, however variability within the correlation indicates further complexity.
Principal component analysis:
Principal component analysis (PCA) is divided into two separate analyses representing the two sets of arrays which were performed (A vs B10.A and CSS3 vs. B6). PCA represents a two dimensional simplification of sources of variance within microarray expression and a control that experimental groups cluster primarily according to experimental variables.
A) The first set shown in A includes an array with A/J infected and mock infect as well as B10.A infected and mock infected. Genotype accounts for 25.358% of variance observed and infection for 37.365%.
B) B6 and CSS3 are shown with genotype accounting for only 8.462% of variance, an expected but considerable decrease compared to A/J and B10.A. An increase in the variance explained by infection (from 37.365% up to 49.923%) may be explained by less background noise due to genes playing a role outside of chromosome 3 (ex: Amica1 see discussion).
### Supplemental Table 1

<table>
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<th>AI Groups</th>
<th>Fold Change</th>
<th>P-Value (Cyto-T)</th>
<th>P-Value (W/HNK) Str. Infections</th>
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<td>0.0002</td>
<td>0.813</td>
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<tr>
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<td>GM-CSF</td>
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<tr>
<td>IL-8</td>
<td>0.55</td>
<td>0.0014</td>
<td>0.445</td>
</tr>
</tbody>
</table>

**Notes:**
- SHH/MS: Sonic hedgehog/transforming growth factor-alpha
- FGF2: Fibroblast growth factor 2
- IL-6: Interleukin 6
- TNFα: Tumor necrosis factor alpha
- TGFβ: Transforming growth factor beta
- IL-1β: Interleukin 1 beta
- GM-CSF: Granulocyte-macrophage colony-stimulating factor
- IL-8: Interleukin 8

### Gene Expression Differences

- **Supplementary Table 1:**
  - **Gene Expression Changes:**
    - **ADAM10:**
      - Fold Change: 0.55
      - P-Value (Cyto-T): 0.0002
      - P-Value (W/HNK): 0.813
    - **FGF2:**
      - Fold Change: 0.73
      - P-Value (Cyto-T): 0.0004
      - P-Value (W/HNK): 0.939
    - **IL-6:**
      - Fold Change: 0.62
      - P-Value (Cyto-T): 0.0007
      - P-Value (W/HNK): 0.347
    - **TNFα:**
      - Fold Change: 0.72
      - P-Value (Cyto-T): 0.0008
      - P-Value (W/HNK): 0.259
    - **TGFβ:**
      - Fold Change: 0.55
      - P-Value (Cyto-T): 0.0011
      - P-Value (W/HNK): 0.903
    - **IL-1β:**
      - Fold Change: 0.62
      - P-Value (Cyto-T): 0.0012
      - P-Value (W/HNK): 0.800
    - **GM-CSF:**
      - Fold Change: 0.73
      - P-Value (Cyto-T): 0.0013
      - P-Value (W/HNK): 0.195
    - **IL-8:**
      - Fold Change: 0.55
      - P-Value (Cyto-T): 0.0014
      - P-Value (W/HNK): 0.445

**References:**