Next-Generation Computational Genetic Analysis: Multiple Complement Alleles Control Survival after Candida albicans Infection†

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Candida albicans is a fungal pathogen that causes severe disseminated infections that can be lethal in immunocompromised patients. Genetic factors are known to alter the initial susceptibility to and severity of C. albicans infection. We developed a next-generation computational genetic mapping program with advanced features to identify genetic factors affecting survival in a murine genetic model of hematogenous C. albicans infection. This computational tool was used to analyze the median survival data after inbred mouse strains were infected with C. albicans, which provides a useful experimental model for identification of host susceptibility factors. The computational analysis indicated that genetic variation within early classical complement pathway components (C1q, C1r, and C1s) could affect survival. Consistent with the computational results, serum C1 binding to this pathogen was strongly affected by C1r/s alleles, as was survival of chromosome substitution strains. These results led to a combinatorial, conditional genetic model, involving an interaction between C5 and C1r/s alleles, which accurately predicted survival after infection. Beyond applicability to infectious disease, this information could increase our understanding of the genetic factors affecting susceptibility to autoimmune and neurodegenerative diseases.

Genetic factors are known to alter susceptibility to and severity of Candida albicans infection in mice (1, 3, 22) and humans (42). Therefore, characterizing genetic factors affecting host susceptibility to C. albicans infection is of great importance. Since systemic candidiasis in mice closely resembles the human disease, inbred mouse strains provide a useful experimental model for identification of host susceptibility factors. Although virtually all organs are infected, the kidney is the major target, and the histopathology of infected lesions is similar in mice and humans. Mutations in several immune response genes have been associated with susceptibility to chronic mucocutaneous candidiasis in human families (14, 17, 36, 48), and several have been verified in murine models. Differences in survival after hematogenous C. albicans infection among inbred mouse strains have been associated with complement factor 5 (Hc or C5) alleles (1, 2, 4, 34). A 2-bp deletion polymorphism at the 5′ end of the C5 transcript shifts its reading frame and causes ~50% of inbred strains to be C5 protein deficient (54). Disseminated candidiasis is rapidly fatal in C5-deficient strains because of uncontrolled fungal proliferation in most organs (34). Although C5 alleles make an important contribution, several previous analyses indicated that there are other genetic factors that affect the severity of tissue damage or survival after C. albicans infection (2, 38). However, no one has yet been able to identify these other genetic factors.

Since its inception in 2004, haplotype-based computational genetic mapping (HBCGM) (30) has been used to identify the genetic basis for many biomedical trait differences among inbred mouse strains, including differences in gene expression (30), pharmacogenetic factors (19, 20, 58), susceptibility to invasive aspergillosis (56) and respiratory syncytial virus infections (47), analgesic medication (43) and inflammatory pain responses (26, 27), incisional wound biology (23, 24), and narcotic drug responses (12, 28, 29, 43). In a mapping experiment, a property of interest is measured in ≥10 inbred mouse strains; genetic factors are then predicted computationally by identifying genomic regions where the pattern of genetic variation correlates with the distribution of trait values among the inbred strains (30). Despite multiple successes, this genetic mapping method has been unable to identify the underlying genetic differences in other, more complex biologic systems (59). The paucity of genomic regions covered by the genetic map was a significant contributor to these failures. The previous haplotype map covered only ~15% of the genes in the mouse genome (30), and gene families were selected to enable analyses of specific phenotypes (i.e., drug metabolism). Also, the existing haplotype block construction algorithm (30) rewarded the inclusion of more single-nucleotide polymorphisms (SNPs), penalized the generation of more haplotypes in a block, and did not allow for overlapping blocks within a region. As a consequence, a causative block could easily be missed (pro-
duting false-negative results) if another block in a region with fewer haplotypes and fewer SNPs was selected. A new HBCGM method with whole-genome coverage and an improved method for haplotype block construction were needed to enable a wider range of biomedical phenotypes (including infectious disease) to be evaluated. Therefore, we produced a next-generation version of the HBCGM method and used it to analyze survival after hematogenous C. albicans infection in a panel of inbred mouse strains. The results led us to produce a novel combinatorial, conditional genetic model, involving an interaction between C5 and C1s alleles, that accurately predicted survival after infection.

MATERIALS AND METHODS

Survival after Candida albicans infection. All mouse experiments were approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee and were performed according to the Guide for the Care and Use of Laboratory Animals (35a). Male mice were obtained from Jackson Laboratories and were used in survival studies at approximately 6 weeks of age. C. albicans strain SC5314 was grown in yeast extract-peptone-dextrose (YPD) broth at 30°C. The yeast-phase organisms were washed twice in phosphate-buffered saline (PBS) and enumerated with a hemacytometer. To induce disseminated candidiasis, the kidney homogenates was measured to assess renal phagocyte accumulation from these mice were harvested and homogenized in ice-cold PBS containing one strain.

Computational genetic mapping. The methods for producing the genetic map and the new mapping method and the characterization of the method are described in the supplemental material. Using the new genomewide haplotype map, genetic factors were identified computationally using our previously described methods (30, 51). In brief, the pattern of genetic variation within each block was correlated with the distribution of trait values among the strains analyzed by using analysis of variance (ANOVA)-based statistical modeling. P values from the ANOVA were used as a corresponding block size, and blocks were calculated for each block (30, 51). The blocks were then ranked by their P values, and those below an input threshold were used as candidate predictions. The SNPs within the blocks were annotated using Ensembl mouse genome annotation information (http://www.ensembl.org; NCBI mouse genome build 37), and our software automatically identified SNPs causing nonsynonymous coding changes. The haplotype pattern, chromosomal location, presence of nonsynonymous SNPs, and calculated P values and genetic effect size for each block meeting the input criteria were also outputs of this program. For analysis of C. albicans survival, the median survival was used as the phenotypic input, since the median is more resistant to occasional outliers than the average. NZW mice had an extremely long survival time (median, 13 days) relative to the other strains. This complicated the computational analysis, since all blocks with NZW-specific haplotypes were automatically identified SNPs causing nonsynonymous coding changes. The haplotype blocks were annotated using Ensembl mouse genome annotation information on chromosome 6 (124,489,460 bp), CTGCCTGCTTATTCCTTCTG and AGTTG GTTCTTTCTCTAGGT for amplification and GCTCAAGGTACACCAGCCA and GAAATCAG for sequencing; for C1qa on chromosome 6 (124,490,473 bp), CAGTTGGCCAAAGCTGGTGTC and AGACAGAAGGAGGAGGAGG for amplification and GCACAAAA GCTGAGGCTTGGAG and AGGGGAGAGAATGGGGAGG for sequencing; for C1qb on chromosome 4 (136,446,076 bp), CTGCCTTCATGGAGCCCA and AGGTGTGAGGCGCAT ACAA for amplification and AAATGGCCACAGGAATACCA and GCCGATACAAAAAGAACAC for sequencing; for C1qc on chromosome 4 (136,446,490 bp), TGCCGGCTATGCGGTTAGTAGT and TCTCCGGAAAGG GAAACTGGA and TG CTTGCTTGTGGTAC for amplification and sequencing; and for C1s on chromosome 6 (136,453,706 bp), GAGAGAAGGGAGGGAGGGAGG and GG GTTGTGTGCTTGGAGTAAAG for amplification and GGAAGAGAAACTGGACAAA and AGGGAGGGAGAAACAGCAAC for sequencing; for C1s on chromosome 6 (136,484,450 bp), AGACAACCTGTCCCAGCCTG and GAA AACCTGAAAATGGACAGTGC and CTCAGGTTACCTCCTGAAACCA for sequencing; for C1qa on chromosome 4 (15).

Next-generation computational genetic mapping method. A 2.7-million-SNP database was generated from analysis of data obtained from 2 different sources and was used to provide genomewide coverage (≥95% of the genes in the murine genome) for 16 inbred strains: 129S1/SvImJ, A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, C57BL/6N, CBA/J, DBA/2J, LP/J, NOD/ShiLtJ, NZO/HILJ, BALB/cByJ, FVB/NJ, BTBR T+tf/J, KK/HJ, and NZW/LacJ. We also developed a new haplotype block...
A construction method that reduced the possibility that a computational genetic mapping experiment would miss a true causative haplotype block (i.e., produce false-negative results). The new “maximal” haplotype block construction method identifies all patterns of genetic variation within a region by allowing the haplotype blocks to overlap (see Fig. S1 in the supplemental material). When the allelic data from all 16 strains were evaluated, the maximal method generated 6-fold more blocks ($n_{H11005} 580,565$) than the prior method ($n_{H11005} 92,109$) (see Table S1).

Our previous method generated a single haplotype map by using all available allelic data for all strains (30), and this map was used for all analyses. However, phenotypic data are usually available only for a subset of the strains in a typical mapping experiment, and inclusion of irrelevant alleles can disrupt haplotypic patterns that are uniform among the strains of interest. The 30,000-fold improvement in the computational efficiency of this implementation enabled customized haplotype blocks to be produced dynamically in real time for the strains with available phenotypic data. We also found that the use of whole-genome sequencing data enabled the haplotype map to provide a more complete representation of the pattern of genetic variation for new strains than could be obtained using genotyping arrays that characterize only previously known SNPs (see Tables S2 and S3). As described in the supplemental material, this new genetic map and mapping method exhibit superior performances over those of our prior genetic mapping method (see Fig. S4) and other available methods for association mapping (see Tables S4 and S5).

Survival after fungal infection. We examined the survival of 15 inbred mouse strains after hematogenous C. albicans infection. There was substantial variation in strain survival; the median survival for 7 strains was $\leq 3$ days, while other strains survived for 10 to 13 days (Fig. 1). One day after infection, we also measured the kidney fungal burden and kidney myeloperoxidase activity (as a measure of phagocyte accumulation). Kidney fungal burden ($P$ value, 0.007) and myeloperoxidase activity ($P$ value, 0.03) were both inversely correlated with survival, while the normalized myeloperoxidase activity (relative to fungal burden) was directly correlated with survival ($P$ value, 0.01). Since both measurements were made at a single time after infection, they provide an imperfect assessment of a dynamic and evolving response to infection. However, these correlations verify the expected result that the host’s abilities to recruit phagocytes and limit fungal growth in the kidney are determinants of survival. C5 alleles could explain some, but not all, of the observed interstrain differences. All C5-deficient strains had a median survival of $\leq 3$ days after infection, while the median survival among the C5-sufficient strains ranged from 3 to 13 days (Fig. 2). The large variation in survival among the C5-sufficient strains indicates that genetic factors other than C5 could affect survival.

The median survival data for the 14 strains with available
allelic information were analyzed using the next-generation computational genetic mapping method. Two haplotype blocks encoding C1q components (C1qa and C1qc) had the highest correlation with median survival (P = 10^-10). C1rb and C1s (P = 0.0002) were also among the 13 most highly correlated genes (Fig. 2). All three C1q component genes (C1qa, -b, and -c) are adjacent to each other on chromosome 4 (136.4 Mb), while C1rb and C1s are carried by opposite strands of the same genomic region on chromosome 6 (124.5 Mb) (see Fig. S2 in the supplemental material). There was high linkage disequilibrium among SNP alleles within each of these two genomic regions. SNPs within the four C1 genes gave the following alterations: Glu293Gln, Asn122Arg, Arg68Lys, and Asp82Gly substitutions within C1s; an Asn59His substitution within C1r; a Thr16Ile substitution in C1qa; and Val208Ala, Arg70Gln, and Pro10Gln substitutions in C1qc (see Fig. S2B). C1q binding to an antigen-antibody complex induces the Ca^{2+}-dependent assembly of a C1s-C1r-C1s-C1r tetramer (5), which activates the classical complement pathway. C1s is a serine protease with a modular structure; all 4 nonsynonymous C1s SNPs are located within the NH2-terminal bone morphogenic protein and epidermal growth factor (EGF)-like modules (CUB1-EGF-CUB2) (reviewed in reference 5). This Ca^{2+} binding region of C1s mediates the interaction with C1r and C1q, which is essential for C1s activation (18). The haplotypic groupings created by the C1rs and C1q alleles are very similar (see Fig. S2). However, for the reasons discussed below, the C1s haplotype (Fig. 2) was used in the subsequent analyses. Nevertheless, it is possible that C1q haplotypes (which have a very similar pattern to those for C1rs) have an independent

![Fig. 2. (Top) Median survival after hematogenous *C. albicans* infection for the 14 indicated strains. The C5-deficient strains are shown in red, and the C1s haplotypes of the C5-sufficient strains are indicated. The amino acids at the indicated positions for the two C1s haplotypes are also shown. All C5-deficient strains survived for ≥3 days after infection, while the C1s haplotype influenced the survival of the C5-sufficient strains. BALB/cByJ mice have a different C1s haplotype (due to a C allele at SNP NES16184810) from the other C5-sufficient strains, which is why the data for this strain are shown with a bar with a unique color. (Bottom) The survival data were analyzed by computational haplotype-based genetic mapping, and the 15 genes with the most correlation are shown. The columns show the gene symbol, calculated P value and genetic effect size, chromosome, and locations of the starting and ending positions for each correlated haplotype block. The haplotype pattern within each haplotypic block is also shown; each rectangle represents a single strain that appears in the same order as in the bar graph above. Each haplotype within a block is represented by a rectangle of a different color; strains with rectangles of the same color have the same haplotype.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Position</th>
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<td>0.72</td>
<td>1</td>
<td>4</td>
<td>350,000</td>
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FIG. 2. (Top) Median survival after hematogenous *C. albicans* infection for the 14 indicated strains. The C5-deficient strains are shown in red, and the C1s haplotypes of the C5-sufficient strains are indicated. The amino acids at the indicated positions for the two C1s haplotypes are also shown. All C5-deficient strains survived for ≥3 days after infection, while the C1s haplotype influenced the survival of the C5-sufficient strains. BALB/cByJ mice have a different C1s haplotype (due to a C allele at SNP NES16184810) from the other C5-sufficient strains, which is why the data for this strain are shown with a bar with a unique color. (Bottom) The survival data were analyzed by computational haplotype-based genetic mapping, and the 15 genes with the most correlation are shown. The columns show the gene symbol, calculated P value and genetic effect size, chromosome, and locations of the starting and ending positions for each correlated haplotype block. The haplotype pattern within each haplotypic block is also shown; each rectangle represents a single strain that appears in the same order as in the bar graph above. Each haplotype within a block is represented by a rectangle of a different color; strains with rectangles of the same color have the same haplotype.
effect on survival, but analyzing this would require a much larger strain panel.

**C1 allelic differences have functional consequences.** The complement system can be activated by three distinct mechanisms: C1 binding to the Fc region of an antigen-antibody complex activates the classical pathway, while the alternative and mannose-binding lectin pathways are activated by the direct binding of other complement proteins to the pathogen surface (49, 50). Of relevance here, the classical pathway plays an important role in host defense against many types of pathogens (55); it is the dominant pathway for protecting mice against *Streptococcus pneumoniae* infection (10). Bacteria can activate the classical pathway through direct binding of C1q to the bacterial surface (11) or via naturally occurring IgM antibodies bound to the bacterial surface (10). Similarly, naturally occurring mannan-specific human IgG antibodies can activate the classical complement pathway and induce C3 deposition on *Candida albicans* (57).

Since the allelic differences induced significant amino acid changes in C1 components, we compared C1 functional activities in sera obtained from 5 strains by measuring C1q binding to *Candida albicans*. In our initial experiment, the amount of C1q bound was 10-fold higher in SJL/J serum than in CBA/J serum (Fig. 3A and B). Furthermore, the sera of all 3 strains with C1s haplotype 1 (C57BL/6, 129S1/SvImJ, and CBA/J) had low levels of C1q binding activity, while both strains (SJL/J and LG/J) with C1s haplotype 2 had high levels of C1q binding (Fig. 3B and C), and this strain-specific difference was reproducible in multiple independent experiments. Although LG/J and SJL/J sera had higher C1q binding activities, they had lower levels of anti-*Candida* antibody than CBA/J sera (Fig. 3D). Thus, the interstrain differences in the level of C1q binding to *C. albicans* were independent of the amount of naturally occurring anti-*Candida* antibody but were correlated with the C1 haplotype and with survival after *C. albicans* infection.

C57BL/6 and A/J mice have different C1q and C1r/s alleles. Therefore, C1q binding to *C. albicans* was measured using sera obtained from two chromosome substitution strains (CSS) that were derived from these 2 strains and had selectively altered C1q (chromosome 4) or C1rs (chromosome 6) alleles. Each CSS strain is homosomic for a single specified A/J chromosome on an otherwise C57BL/6 (C5-sufficient) genetic background (35). At all serum dilutions tested, A/J and B6.CSS4 (both C1q haplotype 2) sera had higher levels of C1q binding than C57BL/6 (C1q haplotype 1) sera (Fig. 4A). The level of C1q binding in B6.CSS6 (C1rs haplotype 2 and C1q haplotype 1) sera was substantially lower than that in C57BL/6 sera. The C1qa-C1qb-C1qc and C1r-C1s proteins have different haplotypes in B6.CSS6 mice, and all 4 nonsynonymous C1s SNPs are located within the coding sequence for the Ca^2+^ binding region that mediates the interaction of C1s with C1r and C1q (5, 18). Thus, the interaction between C1 components with different haplotypes likely reduces the formation or stability of the
obtained from C57BL/6, A/J, or the indicated CSS mice. The C. albicans tometry as described in the legend to Fig. 3, using dilutions of sera strain mice (10 mice per group).

C. albicans togenous infection, which appears to also depend upon the C1q binding to the fungal pathogen and on survival after infection. These data and the other data indicate that C1rs alleles have a major effect on C1q binding to the fungal pathogen and on survival after C. albicans infection, which appears to also depend upon the C1q and C5 alleles.

A combinatorial genetic model predicts survival. These results led us to propose a conditional, multiallelic model (involving 2 complement genes) for predicting inbred strain survival after hematogenous C. albicans infection. Four different genetic backgrounds or with different phenotypes, could also impact the outcome after infection. These data indicate that C1 alleles are at least some of the genetic factors that were postulated to affect survival but could not be identified using the available genetic discovery methods (2).

Although we have shown the combinatorial effect that C1 and C5 alleles have on survival in this model, their effect on the response to other infectious agents remains to be determined. It is also possible that other genetic factors, especially in different genetic backgrounds or with different phenotypes, could also impact the outcome after C. albicans infection. For example, a genetic analysis was performed using kidney fungal burden as an outcome after C. albicans infection, utilizing C5-deficient mouse strains, and this revealed that other genetic loci could affect the outcome (38). However, given the important role of the complement pathway in the response to multiple infectious agents, these findings should stimulate others to investigate whether this genetic mechanism impacts the response to other infectious agents. We do not yet know if a

<table>
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<th>Strain</th>
<th>C1s haplotype</th>
<th>Predicted Median (±SD) survival (days)</th>
<th>Measured Median (±SD) survival (days)</th>
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</thead>
<tbody>
<tr>
<td>SM/J</td>
<td>1</td>
<td>4 ± 1</td>
<td>3</td>
</tr>
<tr>
<td>MRL</td>
<td>2</td>
<td>9 ± 3</td>
<td>8</td>
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<tr>
<td>DBA/1</td>
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<td>LG/J</td>
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<td>9 ± 3</td>
<td>12</td>
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</table>

* All strains were positive for the C5 allele.
similar genetic mechanism involving the complement pathway will be found in humans. However, if an allelic effect is identified, genotyping at-risk individuals could identify those that would best benefit from increased monitoring or preventative therapy.

Beyond its potential applicability to other infectious diseases, this combinatorial genetic model could provide insight into the genetic architecture of susceptibility to autoimmunity and neurodegenerative diseases. The relationship between complement alleles and autoimmunity disease susceptibility in humans and mice has been puzzling. While kidney inflammation in human systemic lupus erythematosus and related murine models is driven by immune complex deposition and complement activation, paradoxically, C1q-deficient humans have the highest risk for lupus susceptibility (7, 31). Similarly, a C1q knockout can accelerate the development of renal disease in mice with certain genetic backgrounds, but its effect on autoimmunity disease expression is highly strain dependent (6, 33). Investigators have partially explained these paradoxical observations by proposing that early complement proteins play multiple roles in autoimmune disease pathogenesis (reviewed in reference 31). Besides driving complement-dependent tissue inflammation, C1q is also a pattern recognition protein that facilitates the clearance of apoptotic cells (6, 15, 16, 33), and a deficiency in this activity could facilitate the development of autoimmunity.

The combinatorial allelic model described here provides a new mechanism for modulating complement pathway activity, which could explain the significant effect that genetic background has on the autoimmune phenotype in a C1q knockout mouse (6, 33). Also, the MRL and NZW strains provide prototypic models for human lupus, and both have C5 and C1 alleles that favor efficient classical complement pathway activation, which may partially explain why they spontaneously develop autoimmune disease (39, 39a). Despite intensive investigation, we do not fully understand the genetic basis for the renal disease that spontaneously develops in F1(NZB × NZW) mice but not in either parent (60). We previously demonstrated that the NZB Ifi202 allele promotes autoantibody production. However, congenic C57BL/6 mice expressing the NZB Ifi202 allele (B6.NZBIfi202) produce multiple autoantibodies but do not develop renal disease, while NZW mice expressing the NZB Ifi202 allele develop renal pathology at the same rate as F1(NZB × NZW) mice (41). Similarly, the lpr mutation promotes autoimmunity in MRL/lpr mice, and C57BL/6 mice expressing the lpr mutation develop high autoantibody titers but do not develop renal disease (53). This suggests that the complement alleles in NZW or MRL mice could be required for autoimmune disease expression. It was also demonstrated recently that early classical complement pathway components (C1q and C3) regulate synapse formation within the central nervous system and retina, that C1q binding “tags” selected synapses for elimination during development, and that C1q is an essential mediator of neurodegeneration in a murine glaucoma model mediated by retina-specific genetic factors (44). Polymorphisms in human C1q, C1r, and C1s are known to exist, and allelic associations with systemic lupus erythematosus and early Alzheimer’s disease have been investigated (25, 32, 37, 40). Whether these polymorphisms influence the susceptibility of hospitalized patients to disseminated candidiasis or the outcome of this disease remains to be determined. Nevertheless, characterizing the combinatorial interaction between allelic variants in different complement proteins and with other genetic factors required for autoimmune disease expression (major histocompatibility complex [MHC], Ifi202, and Fas/lpr) in mice could increase our understanding of susceptibility to autoimmunity and neurodegenerative diseases as well as disseminated candidiasis.

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