Reciprocal Congenic Lines of Mice Capture the *Aliq1* Effect on Acute Lung Injury Survival Time

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Acute lung injury (ALI) is a devastating condition resulting from diverse causes. Genetic studies of human populations indicate that ALI is a complex disease with substantial phenotypic variance, incomplete penetrance, and gene-environment interactions. To identify genes controlling ALI mortality, we previously investigated mean survival time (MST) differences between sensitive A/J (A) and resistant C57BL/6J (B) mice in ozone using quantitative trait locus (QTL) analysis. MST was significantly linked to QTLs (*Aliq1-3*) on chromosomes 11, 13, and 17, respectively. Additional QTL analyses of separate and combined backcross and F₂ populations supported linkage to *Aliq1* and *Aliq2*, and established significance for previously suggestive QTLs on chromosomes 7 and 12 (named *Aliq5* and *Aliq6*, respectively). Decreased MSTs of corresponding chromosome substitution strains (CSSs) verified the contribution of most QTL-containing chromosomes to ALI survival. Multilocus models demonstrated that three QTLs could explain the MST difference between progenitor strains, agreeing with calculated estimates for number of genes involved. Based on results of QTL genotype analysis, a double CSS (B.A-6,11) was generated that contained *Aliq1* and *Aliq4* chromosomes. Surprisingly, MST and pulmonary edema after exposure of B.A-6,11 mice were comparable to B mice, revealing an unpredicted loss of sensitivity compared with separate CSSs. Reciprocal congenic lines for *Aliq1* captured the corresponding phenotype in both background strains and further refined the QTL interval. Together, these findings support most of the previously identified QTLs linked to ALI survival and established lines of mice to further resolve *Aliq1*.

Keywords: acute respiratory distress syndrome; chromosome substitution strain; congenic; mean survival; pulmonary edema

Acute lung injury (ALI), resulting from both pulmonary and extrapulmonary insults (1-3), is a common outcome of diverse injuries. Although painstaking clinical research has investigated an ever-increasing number of viable candidate genes as potential therapeutic targets, little headway has been made to impact the survival rates of patients with ALI or acute respiratory distress syndrome (ARDS), the most severe manifestation of the disease (4-6). A recent report estimated an annual U.S. mortality rate of 74,500/year and 59,000/year for ALI and ARDS, respectively (6).

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CLINICAL RELEVANCE

This work verifies previous regions linked to acute lung injury (ALI) survival. Results have refined the Chr 11 region that contains *Aliq1*, a significant susceptibility locus for ALI survival, and has established separate lines of congenic mice to further resolve *Aliq1*.

To complement recent studies of ALI/ARDS candidate genes in human populations (7-12), we initiated studies in inbred mice aimed at identifying the genetic factors controlling ALI. Because our long-term goal is to improve mortality rates, survival time from oxidant-induced ALI has been a common theme used as the quantitative trait (phenotype). Ozone, one of the most potent oxidants known (13), quickly produces a pulmonary response in inbred mice that resembles the exudative phase of ARDS; at doses greater than or equal to 4 ppm, ozone causes death within 2 days (14). In contrast to the rapid time course of ozone, nickel (15) and hyperoxia (16, 17) can induce ALI mortality within 3 to 4 days in sensitive strains, but resistant strains of mice can survive as long as 10 to 12 days. Importantly, at a time just before their respective mean survival times (MSTs), certain pathologies of ozone-, nickel-, and hyperoxia-induced ALIs are similar in various inbred mouse strains (18, 19), suggesting a possible overlap in the injury response to the different oxidants. Similarities and differences between time course and pathologies support the potential benefits of all these agents to investigate the genetics of ALI progression and its associated morbidity and mortality.

For ozone- and nickel-induced ALI, we previously performed genetic studies with offspring generated from sensitive A/J (A) and resistant C57BL/6J (B) inbred mouse strains (18-20). Separate quantitative trait locus (QTL) and recombinant inbred (RI) analyses identified loci significantly linked to ozone survival time on mouse chromosomes 11 (*Aliq1*, for Acute lung injury-1), 13 (*Aliq2*), and 17 (*Aliq3*), and suggestive QTLs on chromosomes 1, 6, 7, and 12. In addition, QTL analysis of A- and B-strain-derived backcross mice exposed to nickel identified significant linkage to a region on chromosome 6 (*Aliq4*), and suggestive QTLs on chromosomes 1 and 12. Of note, similar regions on chromosomes 1, 6, and 12 were identified at different significance levels for both ozone- and nickel-induced lung injuries, suggesting possible overlap of the genes and pathologic mechanisms induced by the two insults.

The objectives of these studies were to investigate the importance and contribution of the chromosomes that contain each of the significant and suggestive QTLs linked to ozone-induced ALI survival and to capture the phenotypic effect of *Aliq1* in reciprocal congenic lines; four general strategies were used to accomplish this. First, the separate A- and B-strain-derived

backcross and F_2 datasets (18, 20) were recombined and reanalyzed for linkage using Map Manager QTX (21). Results were verified with R/QTL (22) and QTL Express (23) computer programs when appropriate. Reciprocal backcrosses, which were previously analyzed for linkage as separate backcrosses, were combined and reanalyzed as a single backcross dataset. The total F_2 dataset (18) was reanalyzed for linkage to take advantage of the newer capabilities of these programs. Permutation testing (24) was performed on each dataset to identify empirical threshold LOD (log of odds ratio for linkage) scores; previously, theoretical threshold values (25) were used for linkage criteria. Next, QTL analysis and permutation testing of the combined backcross plus F_2 data (i.e., all data as a single dataset) were performed using QTL Express. Second, QTL genotype analysis was carried out on the combined backcross plus F_2 dataset to determine likely combinations of QTL alleles contributing to the overall survival time phenotype (i.e., allelic effects). Third, B.A-derived chromosome substitution strains (CSSs) for seven purported suggestive and significant ALI QTLs were individually tested in ozone to determine whether chromosomes that contain a linked QTL affected survival time. Because QTL genotype analysis predicted a combination of *Aliq1* and *Aliq4* would further increase sensitivity in ozone, we generated a B.A-6,11 double CSS and assessed MST and lung edema. Fourth, to verify *Aliq1* as a susceptibility QTL, we constructed and tested reciprocal congenic lines. Results from these studies confirmed the importance of several QTLs in ALI survival, narrowed the *Aliq1* interval, and established congenic mice to further resolve this major ALI susceptibility locus.

MATERIALS AND METHODS

Mice

Male and female A/J (A) and C57BL/6J (B) mice were originally received from The Jackson Laboratory (Bar Harbor, ME). Breeder pairs for seven chromosome substitution strains (CSSs) were obtained and tested. The specific CSSs tested were based on our previous reports demonstrating significant and suggestive linkages. CSSs are designated as B.A-x, where x is the A-strain-derived chromosome that substitutes for the original B-strain chromosome through a series of backcrosses, marker screening, and selection (26). B.A-1 (contains a suggestive linkage in nickel-induced ALI), B.A-6 (contains *Aliq4*), B.A-11 (contains *Aliq1*), B.A-12 (contains a suggestive linkage in both ozone- and nickel-induced ALI; now called *Aliq6*), B.A-13 (contains *Aliq2*), and B.A-17 (contains *Aliq3*) CSSs were obtained as a generous gift from Dr. Joseph Nadeau (Case Western Reserve University, Cleveland, OH). B.A-7 (suggestive linkage in ozone-induced ALI; now called *Aliq5*), additional B.A-13, and B.A-17 CSSs were purchased from The Jackson Laboratory. The B.A-6,11 double CSS was constructed in our facility at the University of Cincinnati. Separate lines for B.A-1, B.A-6, B.A-11, and B.A-12 CSS lines were bred and maintained in our AALAC-approved facilities at the University of Cincinnati. Small colonies for B.A-7, B.A-13, and B.A-17 CSS lines were bred and maintained at Children's Hospital Medical Center (Cincinnati, OH). Specifics for the B.A-6,11 double consomic line and the B.A- and A.B-derived reciprocal congenic lines are detailed separately below. Housing was located in viral and pathogen-free, HEPA-filtered environments with a 14-hour/10-hour artificial light/dark cycle to help optimize breeding. The IACUC Committees at the University of Cincinnati and Children's Hospital have approved all mouse procedures.

Ozone Generation, Analysis, and Exposures

The generation of ozone differed at the two institutions. At the University of Cincinnati, ozone was generated from 100% extra-dry O_2 (Wright Brothers, Cincinnati, OH) using a Model V1-0 ultraviolet ozone generator (OREC, Phoenix, AZ) and monitored continually with a Dasibi Model 1008-PC direct reading instrument (Dasibi, Glendale, CA). This instrument has an internal calibration system, which was routinely checked and calibrated against a USEPA transfer

standard (Hamilton County Environmental Services, Cincinnati, OH). At Cincinnati Children's Hospital, ozone was generated from in-house feed O_2 passing through a corona discharge VM-120 ozone generator system (Ven-Mar Scientific, Hempstead, TX) and monitored continually with an ultraviolet absorption ozone analyzer (Model 400E; Teledyne Instruments, San Diego, CA). Animals were placed in identical 0.32 m³ inhalation chambers (Bertke Manufacturing, Cincinnati, OH) capable of complete air exchange every 2 minutes, and exposed continuously to HEPA-filtered room air containing 10 ppm ozone. Up to 48 animals were exposed in each trial (maximum of 12 cages; up to 4 mice per cage) and exposures included control mice, when appropriate. Status of mice and concentration of ozone were checked hourly throughout the entire exposure periods. Parental A and B strains, CSSs containing a significant or suggestive QTL for ALI survival time in published reports (i.e., chromosomes 1, 6, 7, 11, 12, 13, and 17), and the newly generated B.A-6,11 double CSS (see below) were exposed continuously to 10 ppm ozone and the survival times recorded to the nearest next hour for each animal. Exposures were performed throughout the year with no significant seasonal variations. MSTs of control A and B strains differed only slightly between the two institutions, so the appropriate group-matched comparisons were made. Statistical analyses were performed with SigmaStat 3.1 (Systat, Point Richmond, CA). MSTs of CSSs exposed at each institution were compared with those of their respective controls using one-way repeated measures ANOVA, with the Holm-Sidak method of multiple comparisons among groups. Statistical significance was accepted at $P \leq 0.05$.

Estimation of Heritability and the Minimum Number of Genes Involved in the Survival Trait

Narrow sense heritability (h^2) of the survival trait was estimated using the following formula: $h^2 = V_A/V_P$; where V_P (total phenotypic variance) is the variance of the total B×A-derived F_2 population, and V_A (variance of the additive effects) is estimated by $2V_{F_2} - (V_{BC1} + V_{BC2})$, where V_{F_2} , V_{BC1} , and V_{BC2} represent the variances of the total F_2 and reciprocal backcross populations, respectively. To estimate the minimal number of genes that segregate with the survival time difference in ozone, we used the following formula for F_2 mice: $n = (P_2 - F_1)^2/4 \cdot (V_{F_2} - V_{F1})$, where n is an estimate of the number of independent loci; P_2 and F_1 are the MSTs of A and (B×A) F_1 mice, respectively; and V_{F_2} and V_{F1} are computed variances of the average F_2 and F_1 cohorts, respectively (27). A modified formula was used to estimate the minimal number of genes segregating in the backcross population, in which the variance of the N_2 population was substituted for that of the F_2 population (28). These estimates assume that the genes are unlinked, semi-dominant, and contribute equally to the phenotype. Because of these assumptions, the calculations tend to underestimate the true number of genes involved.

QTL Analysis

To genotype mice, DNA was isolated from tail biopsies (3–5 mm) taken from each mouse (stored at -20°C). Genomic DNA was isolated using DNeasy columns (Qiagen, Valencia, CA) and the accompanying instructions. Samples were analyzed for purity (A_{260}/A_{280}) and DNA concentrations (A_{260}) were quantified using a Beckman DU-64 spectrophotometer. Samples were diluted to approximately 20 ng/ μl for subsequent PCR analysis. To further evaluate putative significant and suggestive QTLs identified in initial QTL analyses for the separate backcross and F_2 populations, we performed additional QTL analyses for all B- and A-strain-derived backcross and F_2 mice using different features available within three freely available QTL analysis packages: R/QTL (22), Map Manager QTX (21), and QTL Express (23). First, total backcrosses ($A \times F_1$ and $F_1 \times A$), previously analyzed separately (20), were evaluated as a single backcross dataset ($n = 174$). Next, the combined backcross and F_2 ($n = 240$) populations were analyzed as a total dataset ($n = 414$) using QTL Express. Before QTL Express, no freely available QTL analysis program could take into account these mixed breeding crosses.

QTL Genotype Analysis

To predict the potential individual and collective effects of these QTLs on the overall survival time in ozone, QTL genotype analysis was also performed on the combined dataset for B×A-derived backcross and F_2

mice. For this analysis, each QTL effect was assumed to be located at the microsatellite marker nearest to the peak LOD score for the purported QTL regions (i.e., *D1Mit213*, *D3Mit203*, *D6Mit183*, *D7Mit310*, *D11Mit263*, *D12Mit5*, *D13Mit59*, and *D17Mit38*). To determine the contributions of these QTLs to the overall survival phenotype, MSTs for groups of mice with the same genotype (i.e., all sensitive A alleles) at each QTL or combination of QTLs (referred herein as the QTL genotypes) were calculated and then compared to the MSTs of mice with the opposing genotype (i.e., all resistant B alleles) and to mice that were heterozygous at all loci. QTL genotypes for each marker or set of markers were analyzed for differences using an unpaired Student's *t* test. Statistical significance was accepted at $P \leq 0.05$.

Lung Wet-to-Dry Weight Ratios

Lung wet-to-dry (W:D) weight ratios were determined for control mice and selected CSSs immediately after 3, 6, 8, 12, or 14 hours of ozone exposure. The exposure times tested for each mouse line were based on their MSTs. The methods used to determine W:D weight ratios were as described previously (29) and were analyzed for differences using an unpaired Student's *t* test. Statistical significance was accepted at $P \leq 0.05$.

Generation of the Double CSS and Genotype Analysis

B.A-6 and B.A-11 CSSs were intercrossed to generate mice heterozygous for chromosomes 6 and 11. Male and female heterozygotes were crossed and offspring screened by PCR with microsatellite markers on chromosomes 6 and 11 to identify breeders for the subsequent crosses. Primers were chosen based on polymorphisms between the A and B strains and purchased from Research Genetics/Invitrogen (Frederick, MD). Mice heterozygous and/or homozygous for A alleles at all markers tested on chromosome 6 (nine microsatellite markers) and chromosome 11 (fourteen microsatellite markers) were identified and crossed to fix both full chromosomes for homozygous A-alleles. Barring any extremely rare double crossover events, these mice are consomic for the two chromosomes. PCR was performed in 15- μ l reactions in 96-well plates (MJ Research; BioRad Laboratories, Hercules, CA) using a 2-block thermocycler (Dyad, Model PTC-220; MJ Research; BioRad). PCR and electrophoresis was completed as described previously (29).

Construction of Reciprocal Congenic Lines for Aliq1

For chromosome 11 congenic lines on the B-strain background (B.A lines), the B.A-11 CSS was used as the starting point. B.A-11 males were bred to B females to generate mice heterozygous for all of chromosome 11 (i.e., F_1), but with B alleles everywhere else in the genome. These chromosome 11 F_1 mice were repetitively bred back to B females and screened for the desired recombinations. A male and

female with a recombination between the same two markers were intercrossed to fix the region of interest as homozygous-A alleles. A total of 28 polymorphic microsatellite markers on chromosome 11 were screened for the B.A-derived lines.

For the congenics on the A-strain background (A.B lines), a speed congenics approach was attempted (30, 31), beginning with the A (female) and B (male) parental strains. However, the poor breeding performance of the A-strain females did not yield enough males for effective use of this protocol. Therefore, the approach was closer to the traditional backcross strategy (32), with genome-wide screening to assist selection of males when possible. At each generation, males were bred back to parental A females. In total, 9 backcross generations were required for the A.B-congenic lines, before fixing the desired regions in homozygous A alleles. All markers screened throughout the A.B genome ($n = 74$ markers outside of chromosome 11) typed as homozygous A. In addition, 21 polymorphic microsatellite markers were screened on chromosome 11 to identify specific recombinants for the desired *Aliq1*-containing intervals.

RESULTS

QTL Analysis

The datasets of the two separate backcrosses and the total F_2 population were reanalyzed using Map Manager QTX, R/QTL, and QTL Express software programs to take advantage of the additional features within these newer QTL analysis packages that appeared after the original analyses were performed with Mapmaker EXP/QTL software (33–35). Separate dataset analyses essentially confirmed the original QTLs, with no important differences noted from previous F_2 or separate backcross analyses. The two separate backcrosses ($A \times F_1$ and $F_1 \times A$) were combined into a single backcross dataset and reassessed for linkage disequilibrium. Ten thousand permutations were performed on this total backcross dataset using Map Manager QTX, which established an empirical threshold LOD score for significance at 2.6, rather than the more stringent theoretical threshold LOD score of 3.3 (25) used in the original report (20). Results of these analyses are presented in Table 1. Using the empirical threshold LOD score of 2.6, the previously suggestive linkages on chromosomes 7 and 12 (i.e., *D7Mit310* + 5 Mb and *D12Mit36* + 6 Mb) reached significance, and thus were named *Aliq5* and *Aliq6*, respectively. The loci had additive effects, but no significant interactions were identified with any of the three analyses. A previously unlinked QTL on chromosome 3

TABLE 1. SIGNIFICANT QTLs FOR OZONE-INDUCED ACUTE LUNG INJURY SURVIVAL IN SEPARATE AND COMBINED B $\times$ A DERIVED BACKCROSS AND F_2 DATASETS

Population	Size	QTL Marker	Position (Mb)	LOD Score	Linkage *	QTL Name
Total Backcross ($A \times BAF_1 + BAF_1 \times A$)	174	<i>D11Mit263</i>	95.38	4.1	Significant	<i>Aliq1</i>
		<i>D13Mit179</i>	44.78	3.1	Significant	<i>Aliq2</i>
		<i>D7Mit310</i> + 5 Mb	44.80 est.	2.8	Significant	<i>Aliq5</i> [†]
		<i>D12Mit36</i> + 6 Mb	68.77	2.9	Significant	<i>Aliq6</i> [†]
		<i>D3Mit203</i>	27.13	3.0	Significant	pending
Total F_2	240	<i>D11Mit67</i>	96.82	6.8	Highly significant	<i>Aliq1</i>
Combined	414	<i>D11Mit54</i>	96.10	10.1	Highly significant	<i>Aliq1</i>
		<i>D13Mit59</i> + 4 Mb	39.88	3.4	Significant	<i>Aliq2</i>
		<i>D7Mit310</i> + 4 Mb	43.81	3.6	Significant	<i>Aliq5</i> [†]

Definition of abbreviations: LOD, log of odds ratio for linkage; QTL, quantitative trait locus.

* QTL analyses were performed on survival time using Map Manager QTX (21) and checked when appropriate with R/QTL (22) and QTL Express (23). Threshold values for linkage in separate F_2 and total backcross populations were determined by 10,000 permutations of the respective population datasets, as described by Doerge and Churchill (24), using Map Manager QTX. QTL analysis for the combined populations (total backcrosses plus F_2) was determined with QTL Express, and linkage threshold values were based on 1,000 permutations. Highly significant and significant threshold values were set at 4.2 and 2.6, respectively, for the total backcross analysis ($A \times BAF_1 + BAF_1 \times A$), at 5.1 and 3.4, respectively, for the total F_2 analysis, and at 4.3 and 3.4, respectively, for the combined analysis of total backcross plus F_2 populations. *D3Mit203* was not named at this time, due to a lack of additional support to substantiate significant linkage.

[†] Previously suggestive QTLs (20).

(*D3Mit203*) also reached significant linkage, although no other subsequent analyses strongly supported this QTL.

To gain further support for the published QTLs, the combined genotype and phenotype data from all backcross and F_2 mice ($n = 414$) were analyzed as a single dataset using QTL Express (Table 1). Permutation testing of this combined dataset established threshold values at 4.3 and 3.4 for highly significant and significant linkage, respectively. QTL analysis of this combined dataset identified *Aliq1* on chromosome 11 as highly significant, reaching an LOD score of 10.1, near the cumulative LOD scores of the separate backcross and F_2 analyses (4.1 and 6.8, respectively). Because the peak LOD scores mapped to a similar region in the separate crosses, we anticipated that the LOD score of the combined dataset would approach the added LOD scores. *Aliq2* (chromosome 13) and *Aliq5* (chromosome 7) were significantly linked, and *Aliq6* (chromosome 12) reached suggestive linkage to ALI survival time in this combined analysis. Suggestive linkage of the chromosome 12 QTL in the total combined analysis again supported earlier analysis results in both ozone- and nickel-induced ALI, suggesting possible overlap of one or more genes and pathologic mechanisms induced by these two insults. Additional analyses reported herein further supported the existence of *Aliq5* and *Aliq6*. The chromosome 3 QTL was not further tested in these studies, as QTL analyses results for this locus were equivocal and QTL genotype analysis did not support its role.

CSS Phenotype Analysis

The availability of CSSs constructed from the A and B strains (26) afforded us the opportunity to initially evaluate each chromosome carrying a putative significant or suggestive QTL. Once the most important QTLs are identified by phenotyping, the CSSs also represent an advanced starting point to more quickly determine the region(s) of interest and generate the desired congenic strains. The MSTs for B.A-1, B.A-6, B.A-7, B.A-11, B.A-12, B.A-13, and B.A-17 CSS lines were determined in 10 ppm ozone (Figure 1).

As predicted by QTL and QTL genotype analyses, B.A-11 mice (carrying *Aliq1* and the rest of chromosome 11 from the sensitive A strain) had a significant decrease in MST, succumbing at 16.0 hours on average compared with a MST of 19.7 hours

for concurrently exposed B-strain controls (Figure 1). Based on previous QTL results, B.A-13 mice, which house the A-strain-derived *Aliq2*, were also predicted to be sensitive. Interestingly, with an MST of 13.1 hours, the B.A-13 mice were by far the most sensitive CSS tested (Figure 1). Similarly, B.A-1 (17.2 h) and B.A-6 (17.5 h) mice, which house suggestive and significant QTLs in nickel-induced ALI, respectively (36), displayed significant decreases in MSTs in ozone. B.A-17 mice carry the sensitive A-alleles for *Aliq3*, a significant linkage identified in RI analysis (20), but did not demonstrate an MST difference from control B mice in ozone.

Mice with substituted chromosomes carrying the significant QTLs *Aliq5* and *Aliq6* were also sensitive when compared to the B strain mice. *Aliq5* on chromosome 7 was suggestive for linkage in previous backcross and RI analyses (20, 29), but was significantly linked in the total backcross and the combined backcross plus F_2 datasets. In agreement, the B.A-7 mice had an increased sensitivity (MST = 17.9 h). B.A-12 mice (MST = 16.2 h), which carry *Aliq6*, a significant linkage for ozone-induced ALI (Ref. 29 and Table 1) and suggestive linkage for nickel-induced ALI (36), were also significantly more sensitive than control B mice.

To assess lung injury in select CSSs carrying significant QTLs (e.g., *Aliq2* and *Aliq4*), lung W:D weight ratios were determined for B.A-13 and B.A-6 mice (Figure 2). All unexposed lines had a lung W:D weight ratio of about 4.0. The overall pattern of the lung W:D weight ratios for each line over time directly correlated with MSTs, and MSTs closely approximated the time to reach a W:D weight ratio of about 7.0. A-strain mice reached a W:D ratio near 7.0 by 6 hours of ozone, while this ratio was projected to be about 13 hours for B.A-13 mice and 17 hours for B.A-6 mice. In contrast, B-strain mice had a mean W:D weight ratio of 5.8 by 14 hours of exposure.

QTL Genotype Analysis

To estimate the contribution of individual QTLs and QTL combinations to the overall survival time, MSTs of mice from the total dataset (backcross plus F_2 mice) with the same genotype (i.e., all sensitive alleles, all heterozygous, or all resistance alleles) at each QTL marker at or near the peak of the QTL were calculated and then compared to the MSTs of mice with the opposing QTL genotype. In this combined dataset, only the F_2 crosses could generate homozygous B genotypes, compared to the backcross and F_2 crosses generating homozygous A or heterozygous genotypes at each locus. Thus, the number of mice

Figure 1. Phenotype analysis of chromosome substitution strains. To determine the importance of each linked quantitative trait locus (QTL) to the overall survival phenotype, chromosome substitution strains (shaded bars) carrying a significant or suggestive QTL (as determined in previous analyses; see Refs. 20, 29, and 36) were exposed to continuous 10 ppm ozone. The number at the base of each bar represents sample size. Results are presented as means $\pm$ SE. Because the mean survival times (MSTs) of control B-mice did not differ between institutions, they were combined in the analysis. MSTs of chromosome substitution strains (CSSs) exposed at each institution were compared against control B-mice using one-way repeated measures ANOVA, with the Holm-Sidak method of multiple comparisons among groups. $^{\#}P < 0.05$ from all other strains listed. $^*P < 0.05$ from B-strain.

Figure 2. Lung wet-to-dry (W:D) weight ratios for representative CSSs. Lung W:D weight ratios for B.A-6 and B.A-13 CSSs were compared to A (A/J) and B (C57BL/6J) progenitor strains exposed to 0, 3, 6, 8, 12, or 14 hours of continuous 10 ppm ozone. Plotted values represent the mean $\pm$ SE for each strain, with 5–7 mice per exposure time. Before exposure, all lines had a lung W:D weight ratio of about 4.0.

with homozygous B genotypes is considerably fewer. When the MSTs were determined for the groups of mice containing homozygous A, heterozygous, or homozygous B genotypes at each of the QTL peak markers, the largest difference in MSTs was found for *D11Mit263*, the marker representing the *Aliq1* peak (Figure 3). This finding was anticipated given the previous QTL analyses results.

Among the backcross and F₂ mice, those homozygous B at *D11Mit263* survived an average of 7.3 hours longer than those mice homozygous for the A allele (i.e., 21.0 h versus 13.7 h, respectively). Mice heterozygous for *D11Mit263* survived 4.0 h longer (17.7 h versus 13.7 h, respectively) than A-A mice (data not shown). The MSTs of backcross and F₂ mice that were homozygous B or heterozygous at *D11Mit263* were not significantly different than control B and (B×A)F₁ mice (21.0 h versus 20.9 h and 18.6 h versus 17.7 h, respectively). However, the group of mice homozygous A for *D11Mit263* differed from A-strain mice (13.7 h versus 6.7 h), strongly supporting the existence of other resistance genes contributing to the overall phenotype. Interestingly, the group of mice homozygous A for *D6Mit183* (the peak marker representing *Aliq4* on chromosome 6, identified in nickel-induced ALI) ranked second in increased sensitivity, with an average survival time of 14.7 hours. Mice A-A at *Aliq2* had an MST of 14.8 hours. By comparison, the group of mice that were homozygous A at an unlinked marker (i.e., *D5Mit139*) had an MST of 16.8 hours (data not shown).

Comparing different allelic combinations at two peak QTL markers identified an additive survival effect in ozone for chromosomes 7 and 11, with mice homozygous B for both markers surviving an average of 11.9 hours longer than mice homozygous A at both loci (Figure 3). Of note, the best two-QTL model was identified for the combination of QTLs on chromosomes 6 and 11 (i.e., *Aliq4* and *Aliq1*, respectively). This combination of QTLs was unexpected, but of considerable in-

terest, because *Aliq4* was identified in both ozone- and nickel-induced ALI (suggestive and significant linkages, respectively). To assess additivity or a potential QTL interaction, we generated a double CSS containing the A-strain chromosomes for these two QTLs.

Examination of different combinations of three QTLs revealed that mice homozygous B for QTL peak markers on chromosomes 7, 11, and 13 (*Aliq5*, *Aliq1*, and *Aliq2*) had an MST 12 hours longer than mice homozygous A at these markers. This difference in MSTs approached the difference between the total means for parental A (6.7 h) and B strains (20.9 h). Similarly, the combination of three QTLs on chromosomes 7, 11, and 12 (*Aliq5*, *Aliq1*, and *Aliq6*) and chromosomes 6, 11, and 12 (*Aliq4*, *Aliq1*, and *Aliq6*) had MST differences of 12.6 hours and 12.9 hours, respectively, between homozygous B and homozygous A mice, further supporting the importance of *Aliq5* and *Aliq6*.

In a four-QTL model, a group of 16 mice were homozygous A for the combination of markers representing QTLs on chromosomes 6, 7, 11, and 13 (*Aliq4*, *Aliq5*, *Aliq1*, and *Aliq2*) had an MST of 10.0 hours and only 2/16 mice died outside the normal A-strain range of 4 to 12 hours (both died at 14 h). Only one mouse was homozygous B for all four of these markers, and it died at 21 hours, but 104 mice had BB or H at all four markers and an MST of 20.5 hours. A second four-QTL model, which included 18 mice that were homozygous A for chromosomes 6, 11, 12, and 13 (*Aliq4*, *Aliq1*, *Aliq6*, and *Aliq2*), had an MST of 10.2 hours, and only 2/18 mice died outside the A-strain range (both died at 14 h). Unfortunately, none of the 414 mice in the total dataset were homozygous B at all four of these markers; however, the MST of the 103 mice that were BB or H at all four of these markers was 20.4 hours. Adding additional QTL markers to the four-QTL model yielded group sizes too small to adequately assess. MSTs of mice heterozygous for different QTL combinations (two or more QTLs) were similar to those for each single QTL, with MSTs ranging from 17.3 to 18.8 hours. These results were statistically the same as (B×A)F₁ mice, which had a MST of 18.1 hours. Overall, the QTL genotype analysis demonstrated that ALI survival time is a multigenic trait, for which different combinations of the linked QTLs can yield a significantly increased sensitivity.

Heritability and Number of Genes

Descriptive statistics for control, reciprocal backcrosses, F₂, and combined populations are presented in Table 2 and were used to calculate heritability and the minimum number of genes segregating with ALI survival. Survival time for ozone-induced ALI had a narrow sense heritability of 53%, suggesting that about half of the phenotypic variance is due to additive genetic effects. Separate calculations for backcross and F₂ populations both estimated a minimum of three genes segregating with ALI

Figure 3. QTL genotype analysis. Survival time differences were determined for combined backcross and F₂ mice that were homozygous for sensitivity alleles versus mice with resistance alleles at microsatellite markers representing the putative QTL peaks for ozone- and/or nickel-induced ALI. The group on the far right represents the MSTs of A/J (A) and C57BL/6J (B) control strains. Open bars represent mice with an AA genotype and solid bars denote mice with a BB genotype for the indicated representative QTL markers on chromosomes 11, 7, 13, and 6. Values are the mean ± SE. All MST differences between AA (open bars) versus BB (solid bars) QTL genotypes were significant (*P* < 0.05, *t* test).

TABLE 2. SURVIVAL TIME STATISTICS FOR A AND B INBRED STRAINS AND ALL DERIVED CROSSES

	A	B	(B×A)F ₁	(A×B)F ₁	Total F ₁	BC1	BC2	Total BC	Total F ₂
MST, h	7.8	19.7	19.2	17.0	18.1	11.6	14.4	13.8	19.1
SD	2.7	2.6	3.6	3.1	3.5	3.7	4.9	4.8	5.1
Variance	7.3	6.8	12.6	9.4	12.1	13.8	24.4	23.3	25.7
Size	84	46	59	60	119	38	136	174	240

Definition of abbreviations: BC, backcross; BC1, (B×A)F₁ × A; BC2, A × (B×A)F₁; MST, mean survival time.

Data are the compiled results from previous reports (20, 29), with inclusion of additional control and F₁ data for use in estimates of heritability and number of genes segregating with the survival time phenotype.

survival time. A minimum of three genes is consistent with the results from QTL genotype analysis, which demonstrated that QTLs on Chrs 7, 11, and 13 could explain most of the genetic variation. QTL analysis of the total backcross dataset identified four significant linkages (*Aliq1*, *Aliq2*, *Aliq5*, and *Aliq6*); a fifth significant linkage (Chr 3) was not well supported by other analyses. The three-gene estimate also agrees with the combined backcross plus F₂ dataset, which identified three highly significant (*Aliq1*) or significant (*Aliq2*, and *Aliq5*) QTLs (Table 1).

Generation and Testing of a B.A-6,11 Double CSS

The significant ALI QTLs *Aliq1* and *Aliq4* were identified in at least two separate studies. The *Aliq1* interval on chromosome 11 was determined in backcross (20) and F₂ (29) mice, and a similar region was also identified in ozone-induced pulmonary inflammation (37). The *Aliq4* region on chromosome 6 was identified for ozone- (29) and nickel- (36) induced ALI. QTL genotype analysis supported an additive effect for the QTLs on

chromosomes 6 and 11 and, although not the two highest LOD scores, these loci represented the best two-QTL model predicted by QTL genotype analysis. But, because other susceptibility QTLs and the rest of the genome differ for the individual mice within the groups used in QTL genotype analysis, the combined effects of only the chromosome 6 and 11 QTLs were still uncertain. Therefore, to test the hypothesis that having both susceptibility QTLs (*Aliq1* and *Aliq4*) rendered mice even more sensitive to ozone-induced ALI than either QTL alone, a double CSS was generated that contained the entire chromosomes for 6 and 11 from the A-strain introgressed onto the B-strain background. Although the B.A-6 and B.A-11 CSSs demonstrated increased sensitivities in ozone (Figure 1), the MST for the B.A-6,11 double CSS was 22.9 ± 2.2 hours (SD, *n* = 19), not different than the resistant B parental strain (Figure 4A). Therefore, the *in vivo* results of the B.A-6,11 double CSS differed from QTL genotype analysis, which predicted an MST of 12.8 hours for mice with both sensitivity

QTLs. To compare the B.A-6,11 double CSS survival time results with lung pathology, we obtained lung W:D weight ratios. After 8 hours and 14 hours of 10 ppm ozone, B.A-6,11 mice had equivalent W:D weight ratios to B-strain mice. However, the more sensitive B.A-11 CSS significantly differed from B strain mice, demonstrating increases in the W:D weight ratios of 22% (8 h) and 59% (14 h) over controls, compared to 10% and 43% at the same times for B strain mice (Figure 4B). These results disagreed with our hypothesis of an additive effect by *Aliq1* and *Aliq4* and demonstrated a reversal of the individual susceptibility phenotypes of the B.A-6 and B.A-11 CSSs, when both A-derived chromosomes are present in the same strain (B.A-6,11).

To further investigate the discrepancy of results between QTL genotype analysis and testing the B.A-6,11 double CSS, additional QTL analysis was performed. For this analysis, the separate backcross and F₂ datasets were used, because QTL Express (which can analyze a dataset of mixed crosses for QTLs) cannot accommodate fixing a locus. Fixing a locus removes its effect (variance explained) from the analysis and allows a search for other QTLs that can explain more or less of the variance between parental strains. Using Mapmaker/QTL, loci on chromosomes 6 and 11 were fixed one-by-one and assessed for a possible reduction of QTL effect. Agreeing with QTL genotype analysis (two-QTL model), but contrary to our hypothesis for *in vivo* studies, results did not implicate opposing interactions between loci on chromosomes 6 and 11.

Reciprocal Congenic Lines for *Aliq1*

To verify the contribution of *Aliq1* to survival time, reciprocal congenic lines were constructed for different segments of the purported *Aliq1* interval. A summary of these congenic lines is displayed in Figure 5. Two congenic lines were generated on the B-strain background (designated B.A11-5 and -6) and two congenic lines were generated on the A-strain background (A.B11-1 and -2). Segments marked with vertical lines in the figure represent regions of unknown genotype (i.e., AA, AB, or

BB), located between two typed markers. The B.A11-derived congenic lines locate the QTL effect distal to *D11Mit67* at 96.82 Mb, because B.A11-5 demonstrates a susceptibility phenotype (MST = 15.9 h) when compared with B.A11-6 (MST = 21.4 h) and B strain controls (MST = 20.0 h). In fact, the difference between B.A11-5 and the B strain was the same as the difference between the CSS B.A-11 and the B strain, demonstrating that the overall chromosome 11 effect was captured in the B.A11-5 congenic line. Both of the A.B congenic lines had an increased resistance over the A-strain control. Comparing A.B congenic lines with the B.A congenic lines revealed a minimal region of overlap between 96.82 Mb (*D11Mit67*) and 110.44 Mb (*D11Mit336*). Mining this region for genes with biological relevance to inflammation, fluid permeability, and homeostasis, and lung injury or wound repair identified many candidate genes. Further *in silico* analysis identified that several of these positional candidate genes also contain genetic variation (e.g., nonsynonymous SNPs) between the A and B parental strains, adding strength to their potential role in the overall phenotype (Table 3).

DISCUSSION

Current supportive therapy has positively impacted survival outcome; however, mortality from ALI and ARDS is still unacceptably high (4, 6, 38–40). Using different agents to induce ALI mortality, we have carried out genetic studies with a long-term objective of identifying major gene(s) affecting survival. Results from these studies, obtained by recombinant inbred (RI) analysis and separate QTL analyses of backcross and F₂ mice derived from A- and B-strains of inbred mice, identified multiple QTLs (i.e., *Aliq1-4*) significantly linked to ALI survival, with several additional QTLs suggestive of linkage (20, 29, 36). The present studies were initiated to further assess the genetic factors involved in ALI survival, to delineate the contributions of each of these significant and suggestive QTLs linked to ALI survival time, and to establish refined mouse models to identify the gene(s) comprising the major effect QTL *Aliq1*.

Figure 5. Chromosome 11 reciprocal congenic lines derived from A and B strains of mice. Reciprocal congenic lines of mice were constructed to contain different segments around the *Aliq1* interval (*D11Mit245*–*D11Mit146*). Control A and B strains, and the B.A-11 CSS are displayed as controls. B.A11-x (where x is 5 and 6) are two congenic lines on the B-strain background and A.B11-x (where x is 1 and 2) are two congenic lines on the A-strain background. Solid regions represent alleles that derive from the B strain, and open segments are alleles that derive from the A strain. Vertical lined regions denote segments between typed markers that are unknown (i.e., AA, AB, or BB). A schematic of chromosome 11 is presented on the left, listing MIT microsatellite markers and mapping locations of the crossover points in the congenic lines. On the far right is an overview of the QTL interval. The *Aliq1* interval from the original (orig) data is identified by the hatched region (*D11Mit245*–*D11Mit146*), from the B.A11-congenic (B.A) is the open bar (*D11Mit67*–*ter*), from the A.B11-congenics (A.B) is the solid bar (*D11Mit119*–*D11Mit336*), and the refined *Aliq1* interval is depicted as the black and white checkerboard bar (*D11Mit67*–*D11Mit336*).

TABLE 3. CANDIDATE GENES MAPPING TO THE REFINED *Aliq1* INTERVAL

Gene Name	Abbreviation	Potential Role	*Total A versus B SNPs	Nonsynonymous A versus B SNPs	SNP Position (bp, Bld 36)	nt Change	Amino Acid Change
Angiotensin I-converting enzyme	<i>Ace</i>	Effects on pulmonary vascular tone, permeability, epithelial cell survival	14	rs8279472	105,785,071	T/G	I/M
ATP-binding cassette, subfamily A, member 6	<i>Abca6</i>	Macrophage lipid homeostasis; lipid transport	170	NT_165773.1_21589121	110,004,734	C/T	R/K
Granulin Integrin alpha 2b	<i>Grrn</i> <i>Itga2b</i>	Wound repair Inflammation, cell recruitment	16	rs29405296	110,028,407	G/A	A/V
				rs3690064	110,046,591	T/C	K/E
				rs27019312	110,066,564	T/C	H/R
				rs29400625	102,252,540	C/T	P/L
				rs29455575	102,272,086	G/A	P/L
Mitogen-activated protein kinase kinase 6	<i>Map2k6</i>	p38 activation	266	NT_165773.1_13856735	102,272,348	A/G	V/A
				rs27040377	102,273,580	T/G	R/S
				NT_165773.1_21891333	110,306,946	G/A	G/E
Mitogen-activated protein kinase kinase kinase 14	<i>Map3k14</i>	NF- κ B signaling	128	rs27050588	103,047,223	A/G	M/T
Sodium channel, voltage-gated, type IV, alpha	<i>Scn4a</i>	Water imbalance	55	rs27048182	106,142,929	T/C	P/L
				NT_165773.1_17730685	106,146,298	G/A	Q/R

Definition of abbreviations: A, A/J; B, C57BL/6J; nt, nucleotide; SNP, single-nucleotide polymorphism.

* From GeneNetwork SNP Browser at <http://www.genenetwork.org/cgi-bin/snpBrowser.py>.

As an initial strategy, additional QTL analyses were performed on total backcross and F₂ datasets. The backcross population had not been analyzed previously as a single population and prior threshold values were established based on theoretical values (25). Permutations of the total backcross population set an empirical threshold LOD score at 2.6, which was less than the proposed theoretical value for a backcross (3.3). This analysis supported previous QTLs and added *Aliq5* and *Aliq6*, previously identified as suggestive QTLs on chromosomes 7 and 12, respectively. These QTLs were further supported by results from QTL genotype analysis and by *in vivo* results with corresponding CSSs.

Using QTL genotype analysis, MSTs of mice carrying the same sensitive or resistant genotypes at each single and combination of QTLs predicted that a three-QTL model could explain nearly all of the phenotypic variance between the A and B progenitor strains. In addition, a four-QTL model yielded a group of mice for which up to 89% (16/18) died within the sensitive A-strain range. This analysis identified *Aliq1* as the best single QTL model; mice A-A for *D11Mit263* ($n = 133$) had a MST of 13.7 hours. Including the markers for chromosomes 7 and 12 (and either chromosome 6 or 13) into the QTL genotype analysis identified mice with a MST of about 10 hours. Groups of mice for the two-, three-, and four-QTL models averaged a survival time that fell within the normal range of the sensitive A-strain (i.e., 4–12 h). QTL genotype analysis results generally supported QTL analysis results, providing additional strength to the earlier QTLs.

QTL genotype analysis is a method to quantify the genotype to phenotype relationship. However, the method has an inherent level of uncertainty. For example, when assessing all mice that are A-A at one specific marker, this total group will include mice with differing genotypes at other important QTL markers, and at markers throughout the rest of the genome. Adding additional markers to the analysis decreases the number of mice with the desired genotype (e.g., A-A or B-B for all markers), but also decreases the genetic variability of the mice

within the group. Therefore, the method can detect general trends of phenotype–genotype associations and can estimate the number of interacting QTLs contributing to the overall phenotypic difference between the parental strains. The three-QTL model parsimoniously agrees with separate estimates for the number of genes segregating with the survival time phenotype and also agrees with the three highly significant or significant linkages identified in the QTL analysis of the combined backcross plus F₂ dataset.

Because a complete line of CSSs derived from the A and B strains (all 19 autosomes and the X- and Y-chromosomes) are available, we examined whether resistant B-strain mice that contained the corresponding individual chromosomes from the sensitive A-strain (each linked to a decrease in ALI survival time) would have increased sensitivity in ozone. Results of QTL analysis and QTL genotype analysis predicted that B.A-11 mice (carrying *Aliq1*, the QTL with the largest LOD score and percent variance explained) would have the greatest increase in susceptibility (i.e., decreased MST). The decreased MST of about 4 hours for B.A-11 mice suggests that *Aliq1* controls around a third of the phenotypic difference between B and A parental strains. This *in vivo* result compares favorably to QTL analysis, which determined that *Aliq1* explained 42% of the variance in backcrosses derived from these strains (20). B.A-13 mice (carrying the sensitive *Aliq2* alleles) demonstrated the most sensitivity in ozone, with a MST of 13.1 hours. Thus, chromosome 13 controls at least half (53%) of the phenotypic variance between progenitor A and B strains. Neither QTL nor QTL genotype analysis forecasted this, and results suggest that one or more additional QTLs may occur on chromosome 13. Other putative susceptibility QTLs (chromosomes 1, 6, 7, and 12) were also confirmed using the appropriate CSSs. However, *Aliq3* on chromosome 17—identified only by RI analysis—may not, on its own, contribute to overall phenotype.

Lung W:D weight ratios were also determined in B.A-6 and B.A-13 CSSs to correlate survival time to lung injury. Results were consistent with MSTs for these strains. The initial 3-hour

ratio showed the slowest gain in lung edema for all strains, suggesting a certain innate ability to initially withstand the insult. After 3 hours, the slopes of all curves increased due to an increase in lung water; however, as predicted by its MST, the A-strain slope was by far the steepest. Thus, these data support the premise that the A, B.A-6 and B.A-13 strains lack one or more resistance genes that initially slow the progression of lung edema in the B strain. Alternatively, compared to the A strain, the B, B.A-6 and B.A-13 strains lack one or more sensitivity genes that cause or allow lung edema to progress. Extending these W:D weight ratio curves beyond the last exposure times recorded, identified that a W:D weight ratio of about 7.0 (Figure 2) correlated well with the MST of each line. From the projected intersections of W:D weight ratios and MSTs, it is evident that the different lines of mice succumb with similar lung W:D weight ratios, but the time to reach this threshold differs among the lines. This finding gives hope to the possibility that identifying a gene or set of genes that allow one to withstand such alveolar flooding may yield insight into pathologic mechanisms that could ultimately direct pharmacologic intervention.

Given that *Aliq1* and *Aliq4* were both susceptibility QTLs for ALI survival, we expected that B.A-6,11 double CSS mice would be more sensitive than B.A-11 CSS mice. In addition, QTL genotype analysis also predicted an increased sensitivity by demonstrating that backcross and F₂ mice that were A-A for both QTL peak markers on chromosomes 6 and 11 had a MST of 12.8 hours, compared to 20.9 hours for B-strain control mice. Contrary to our hypothesis that the QTL effects would be additive, the B.A-6,11 double CSS mice totally lost the susceptibility phenotype seen in the separate CSSs, surviving ozone-induced ALI as long as the progenitor B-strain. This result was further supported by a reversal of the B.A-11 increase in W:D weight ratio by the double CCS mice, when compared to that of the resistant B-strain. Given that QTL genotype analysis only takes into account the genotypes at specific QTL markers, it is understandable how this method would not detect an interaction that eliminates the separate QTL effects, especially an effect that is due to other markers on chromosomes 6 and 11.

As with all *Aliq* loci, it is too soon to know whether the QTLs on chromosomes 6 and 11 represent individual genes or a combination of closely linked genes. Nor is it known whether the chromosome 6 and 11 QTLs act directly (i.e., causal) or indirectly (i.e., unmask suppressive loci), either alone or with other QTLs located on these or other chromosomes, to generate the CSS phenotypes. Therefore, numerous scenarios can be conceived to explain the loss of phenotype in the B.A-6,11 double consomic mice. QTL genotype analysis predicted an increased sensitivity in the combined B.A-6,11 line; thus, the loss of phenotype suggests that an epistatic effector(s) lies distant to the identified QTL peak marker(s). However, this effector could reside outside the QTLs on chromosomes 6 or 11 or elsewhere in the genome, depending on nature of the interaction. For example, the reversal of phenotype could be due to a combination of genes on chromosomes 6 and 11 interacting to suppress the susceptibility effects produced by genes on the individual chromosomes. Just as likely, the gene(s) on one chromosome may unmask the inhibitory effect of another gene, either within or outside of a QTL region, thereby blocking the sensitivity seen in the single consomic lines. Regardless, results clearly demonstrate that significantly different phenotypes can be generated from gene-gene interactions, which are directly or indirectly relevant to loci on these chromosomes. The exact mechanisms will ultimately depend on identification of the major genes involved. Generating and testing a panel of double congenic lines for *Aliq1* and *Aliq4* will help determine whether

the loss of sensitivity is due to loci within or outside the respective QTL intervals, and whether these loci act directly or indirectly.

To validate the *Aliq1* effect and to move towards identification of the quantitative trait gene(s) for *Aliq1*, we generated reciprocal congenic lines of mice (B.A11 and A.B11) with overlapping regions around the putative QTL interval. A significant change in survival time was successfully captured in both directions. B.A11-5 had an MST of 15.9 hours, accounting for 33% of the difference between A and B controls in these exposures (Figure 5), and similar to the difference seen in the B.A-11 CSS. A.B11-1 and A.B11-2 congenic lines demonstrated a significant increase in survival time (26% and 29%, respectively) over A-strain controls. Results from the B.A-derived congenic lines suggest that the QTL effect is distal to *D11Mit67* at 96.82 Mb (Build 36) and results from the A.B-derived congenic lines suggest that the QTL effect is proximal to *D11Mit336* at 110.44 Mb. This effectively reduces the *Aliq1* interval by 56%, from 30.6 Mb to 13.6 Mb. Interestingly, this region maps to, and extends beyond, the distal most region of the 1.5-LOD confidence interval identified by QTL analysis. Results from the reciprocal congenic lines give additional support for *Aliq1* containing a gene (or a set of closely linked genes) contributing to overall survival and provide further evidence that a positional cloning analysis for *Aliq1* should be possible.

Mining through the known genes mapping to the refined *Aliq1* interval identified several genes associated with inflammation, epithelial damage or repair, ALI, or water imbalance. Among these, many contain exonic SNPs between the A and B strains that result in amino acid changes (Table 3), as well as SNPs in 3', 5', and/or splice sites. One excellent candidate gene is Angiotensin converting enzyme (*Ace*), which maps near the center of the interval (105.81 Mb) and has been implicated in ALI/ARDS in humans and animals (11, 41, 42). Recent cohort studies demonstrated a significant association between an *ACE* insertion (I allele) or deletion (D allele) polymorphism and the susceptibility and outcome of patients with ARDS (11, 41). Specifically, the D/D genotype for the *ACE* gene was significantly associated with mortality in the ARDS group compared with a control cohort (41), and patients carrying the *ACE* I/I genotype had a significantly increased survival rate (11). It is important to note that the insertion/deletion polymorphism in *ACE* is a 287-bp noncoding intronic sequence; therefore, it likely cosegregates with the causative locus. One additional point of interest with *Ace* as a candidate gene for *Aliq1* is that the *Aliq2* two-LOD support interval on chromosome 13 contains the angiotensin II receptor, type 1a, further supporting a role for the renin-angiotensin system in ALI survival. Several additional positional candidate genes for *Aliq1* are listed in Table 3, along with their potential roles in differential ALI survival.

In summary, several methods have been utilized to further assess previously identified QTLs for their individual and collective contributions to ozone-induced ALI survival. QTL genotype analysis on the combined A- and B-strain-derived backcross and F₂ data demonstrated that the collective effects of three QTLs could explain much of the phenotypic difference seen between the A and B parental strains. These results were consistent with minimal gene estimates and results from QTL analysis of the combined dataset. B.A-derived CSSs for seven purported suggestive and significant ALI QTLs supported all but *Aliq3* on chromosome 17 as susceptibility QTLs. Chromosome 13 and, by extension *Aliq2*, had the most effect on ALI survival time. To examine whether the predicted QTL genotype interaction between the *Aliq1* and *Aliq4* leads to increased sensitivity, a double CSS line (B.A-6,11) was generated and tested in ozone. The double CSS lost its sensitivity phenotype,

demonstrating a similar MST to the resistant B-strain. The implications of this loss of phenotype await resolution of the individual QTLs. Reciprocal congenic lines for *Aliq1* captured the QTL effect and refined the QTL interval. Results from these studies confirm the importance of several QTLs in ALI survival and set the stage for further studies to resolve its complexity.

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