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Short Communication

Susceptibility to Anthrax Lethal Toxin Is Controlled by Three Linked Quantitative Trait Loci

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Anthrax lethal toxin (LT) is the principal virulence factor associated with lethal pathologies following infection with *Bacillus anthracis*. Macrophages are the primary effector cells mediating lethality since macrophage-depleted mice are resistant to LT challenge. Recently, *Ltxs1*, the gene controlling differential susceptibility of murine macrophages to cytolysis following *in vitro* exposure to LT, was identified as *Kif1c*. To directly assess the *in vivo* role of *Kif1c* alleles in mortality, we studied a panel of interval-specific recombinant congenic lines carrying various segments of central chromosome 11 derived from LT-resistant DBA/2 mice on the LT-susceptible BALB/c background. The results of this study reveal that mortality is controlled by three linked quantitative trait loci (QTL): *Ltxs1/Kif1c* (42–43 cM), *Ltxs2* (35–37 cM), and *Ltxs3* (45–47 cM). The *Ltxs3* interval encompasses *Nos2*, which is an attractive candidate gene for *Ltxs3*. In this regard, we demonstrate that selective, pharmacologically based inhibition of Nos2 activity *in vivo* partially overrides genetic resistance to LT and that *Nos2* expression as determined by reverse transcription-polymerase chain reaction differs significantly between DBA/2 and BALB/c macrophages. Additionally, to recapitulate dominant resistance to mortality as seen in (BALB/c × DBA/2) F_1 hybrids, DBA/2 alleles are required at all three QTL. *(Am J Pathol 2003, 163:1735–1741)*

The gram-positive bacterium *Bacillus anthracis* is the causative agent of anthrax. It infects humans and animal livestock alike, producing illness and death in those infected. While the relatively benign cutaneous anthrax is the most common form of the disease, *B. anthracis* also causes inhalational anthrax, and even gastrointestinal anthrax, both of which generally result in death of the host. The *in vivo* lethal effects of infection with *B. anthracis* are due, in part, to an exotoxin composed of three separate proteins called protective antigen (PA), edema factor (EF), and lethal factor (LF). Both LF and EF combine with PA to form distinct variants of the A-B model of bacterial exotoxins: lethal toxin (LT) and edema toxin (ET), respectively.[1] On its own, PA is nontoxic, and current research suggests PA binds a specific cell-surface receptor and translocates both EF and LF to the cytosol of host cells.[2] EF is an adenylate cyclase that acts to increase intracellular levels of cAMP.[3] LF, the principle virulence factor, is a zinc metalloprotease,[4] which, when translocated to the cytoplasm, is capable of cleaving mitogen-activated protein kinase kinases.[5–7] Systemic *B. anthracis* infection leads to lethal pathologies that can be mimicked in animal models by the delivery of anthrax LT, which is produced at high levels during infection.[8] However, the mechanisms leading to LT induced mortality are poorly defined.

Inbred strains of mice vary in their susceptibility to LT.[8–11] Watters and Dietrich[12] took advantage of the genetically controlled differential susceptibility of murine macrophages to cytolysis *in vitro* to map *Ltxs1* on murine chromosome 11. Recently, *Ltxs1* was identified as *Kif1c*.[13] In C57BL/6J and C3H/HeJ mice the *Kif1c* alleles are distinguished by a single amino acid substitution ($L^{578} \rightarrow P^{578}$), whereas in BALB/cJax and DBA/2J mice

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the amino acid differences are $L^{578}\rightarrow P^{578}$, $S^{1027}\rightarrow P^{1027}$, and $S^{1066}\rightarrow Y^{1066}$. To assess the role of the BALB/c and DBA/2 *Kif1c* alleles in mortality we studied a panel of interval-specific, recombinant-congenic (ISRC) lines carrying various segments of central mouse chromosome 11 derived from LT-resistant DBA/2 mice on the LT-susceptible BALB/c background. The results indicate that mortality is controlled by three linked quantitative trait loci (QTL) on chromosome 11: *Ltxs1*/*Kif1c*, *Ltxs2*, and *Ltxs3*. Additionally, we found that (BALB/c × DBA/2) F_1 hybrid (CD2) mice are resistant, demonstrating that resistance is dominant over susceptibility. Individually, DBA/2 alleles at each QTL have a limited protective effect. The presence of resistance alleles at all three QTL is required for complete protection.

Materials and Methods

Animals

BALB/cAnNCr and DBA/2NCr mice were purchased from the National Cancer Institute (Frederick, MD), whereas BALB/cByJ, BALB/cJax, DBA/2J, and (BALB/cByJ × DBA/2J) F_1 hybrid (CD2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). ISRC F_1 hybrids were generated at the University of Vermont. ISRC lines were generated using (BALB/cAnPt × DBA/2NCr) × BALB/cAnPt backcross mice. Third generation backcross mice carrying the DBA/2 *Evi2* allele were identified and subsequent progeny selected using *D11Mit67* for six backcross generations to BALB/cAnPt mice. The C.D2.*Evi2* mice were intercrossed and additional recombinants identified using informative chromosome 11 markers. The recombinant mice were analyzed for background contamination and C.D2 ISRC mice carrying BALB/c alleles at all marker loci were backcrossed an additional two generations. As a control for BALB/c substrain variability the C.D2 ISRC lines were also backcrossed to BALB/cByJ and BALB/cAnNCr mice before fixing.

Microsatellite Genotyping

Genomic DNA was isolated from 2 to 3 mm of mouse tail tissue[14] and genotyping performed by PCR using polymorphic microsatellite markers distinguishing BALB/c and DBA/2 mice.[15,16] Microsatellite primers were either purchased from Research Genetics, Inc. (Huntsville, AL) or synthesized according to primer sequences obtained from MGI (www.informatics.jax.org). Microsatellite size variants were resolved by electrophoresis on either agarose gels visualized by ethidium bromide or on large format denaturing polyacrylamide gels visualized by autoradiography on Kodak film.

Kif1c Genotyping

DNA samples from BALB/c and DBA/2 mice were prepared from tissues on hand as described above. Oligonucleotide primers flanking the C→T transition at nucleotide 1733 leading to the $P^{578}\rightarrow LP^{578}$ amino acid polymorphism distinguishing the DBA/2 and BALB/c Kif1c alleles were designed.[13] *Kif1c* allele specific oligonucleotide forward primer: 5′-GTCTAACCACAGTACAGCGTT-3′ and reverse 5′-CCTGCTTCCTCTGGTGAG-3′. The 489-bp fragment when cleaved with NlaIV results in a 406-bp BALB/c fragment and a 358-bp DBA/2 fragment. PCR and cleavage products were detected and resolved by electrophoresis in agarose gels and visualized by staining with ethidium bromide.

LT Challenge

Mice received standard mouse chow and water *ad libitum*. All mice were maintained in a conventional vivarium. Cohorts of 6- to 12-week-old male and female mice received intravenous injections of LT (μg PA:μg LF) as previously described[8] using recombinant PA and LF purified in the laboratory of Y.S.[17] or purchased from List Biological Laboratories, Inc. (Campbell, CA). No significant difference in *in vivo* bioactivity was detected between the two toxin sources.

Selective Inhibition of Nos2 Activity in Vivo

N-[3-(Aminomethyl)benzyl]acetamidine, dihydrochloride (1400W) was purchased from Calbiochem (San Diego, CA). Mice were injected with 14 mg/kg 1400W in saline 2X daily at 12-hour intervals by i.p. injection starting 2 days before the i.v. injection of 125:25 LT and daily thereafter. Control mice received saline.

Real-Time PCR Analysis of Nos2 Expression

Real-time quantitative RT-PCR was performed using the Taqman system.[18] PCRs were performed using the Taqman Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System. The probes and primer set sequences were as previously described.[19] *Nos2*: 5′-TGACGGCAAACATGACTTCAG-3′ (forward), 5′-GCCATCGGGCATCTGGTA-3′ (reverse), 5′-FAM-(AATTCACAGCTCATCCGGTACGCTGG)-BHQ-1–3′ (probe). *Hprt*: 5′-TTTGCCGCGAGCCG-3′ (forward), 5′-TAACCTGGTTCATCATCGCTAATC-3′ (reverse), 5′-FAM-(CGACCCGCAGTCCCAGCGTC)-BHQ-1–3′ (probe). Total RNA was extracted from BALB/cByJ and DBA/2J peritoneal macrophages cultured for 24 hours in the presence of 100 ng/ml *Escherichia coli* 0111:B4 LPS (Sigma Chemical Company, St. Louis, MO) + 25 U/ml mouse IFNγ (Biosource, Camarillo, CA). RNA was isolated, DNase-treated, and 1.0 μg of total RNA used as a template to synthesize first-strand cDNA using random primers. *Nos2* levels were analyzed using the standard curve method. A non-RT control was also prepared for each sample and no genomic DNA was detected. As a positive control for *Nos2* expression, cDNA was prepared from *Nos2*-positive cell lines. Varying amounts of the positive control cDNA were used in the PCR reaction to determine the linear range of the PCR reactions. 1.0 μl of the sample cDNA was used in the PCR reaction and the comparative

threshold cycle (C_T) value obtained used to calculate the amount of sample cDNA required to reach the linear range established by the standard. *Nos2* levels were normalized to *Hrpt* and the data are presented as "Fold Induction" following stimulation with LPS + IFNγ normalized to the levels observed in DBA/2.

Statistical Analyses

The binomial test was used to test for significant differences in mortality between the C.D2 ISRC lines or the BALB/c and DBA/2 lines and the CD2 F_1 hybrids. analysis of variance with LSD comparisons was used to test for significant differences among post hoc groupings. The mortality rates were first transformed using the arcsine transformation (2 × arcsine $\sqrt{p}$). Student's *t*-test was used to assess the significance of the differences in *Nos2* mRNA expression between DBA/2 and BALB/c macrophages.

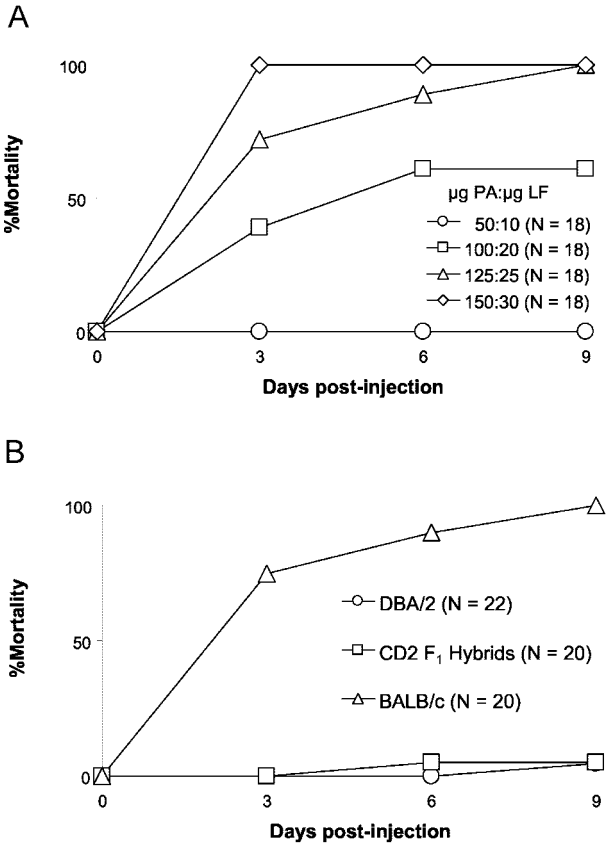

Figure 1. A: Anthrax LT dose-response curves for BALB/c mice. All mice received intravenous injections of LT on day 0. The data are expressed as % mortality (number of animals dead on a given day/number of animals studied × 100) and are the cumulative combined results obtained with male and female BALB/cByJ, BALB/cAnN and BALB/cJax mice. **B:** Mortality in BALB/c, DBA/2, and CD2 F_1 hybrid mice following the intravenous injection of 125:25 LT (125 µg PA:25 µg LF) on day 0. The data are expressed as % mortality (number of animals dead on a given day/number of animals studied × 100) and are the cumulative combined results for male and female mice by strain and/or substrain.

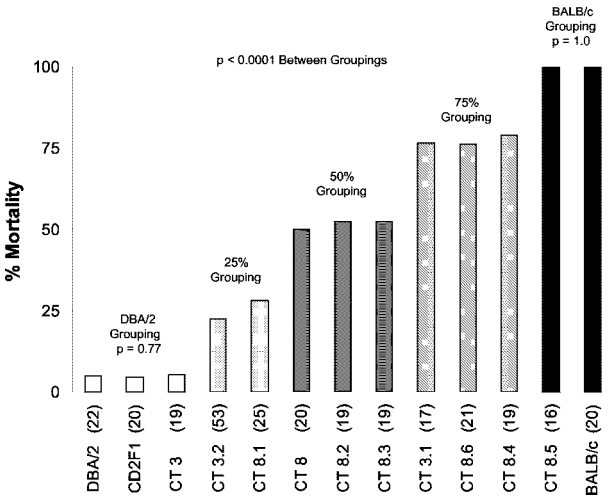

Figure 2. Mortality in C.D2 ISRC lines following the intravenous injection of 125:25 LT on day 0. The data are expressed as % mortality and are the cumulative combined results for both male and female mice for each ISRC line.

Results

Since BALB/c sublines are known to differ in susceptibility to a number of immunopathologic phenotypes including infectious agents,[20–24] the initial studies were carried out using BALB/cByJ, BALB/cAnNCr, and BALB/cJax mice to assess potential substrain differences in susceptibility to LT. Cohorts of male and female mice were challenged with 50:10, 100:20, 125:25, and 150:30 (µg PA:µg LF) LT by intravenous injection. No significant sex or substrain differences in mortality were observed at any of the doses studied. Therefore, the data were pooled to generate the dose response curves presented in Figure 1A. The 50:10 dose of LT failed to elicit any mortality. In contrast, BALB/c mice challenged with 100:20, 125:25, and 150:30 LT exhibited increasing mortality rates, with the 125:25 and 150:30 doses both eliciting 100% mortality by day 9 postinjection. Because the 125:25 dose of LT was the minimal effective dose that elicited 100% mortality in susceptible BALB/c mice, this dose was used to assess the *in vivo* susceptibility status of male and female DBA/2J, DBA/2NCr, and CD2 F_1 hybrid mice. At this dose, DBA/2 mice were essentially resistant to LT challenge with no significant sex or substrain difference being observed. Importantly, CD2 F_1 hybrid mice also exhibited resistance, indicating that resistance to LT is dominant (Figure 1B).

Susceptibility of the C.D2 ISRC lines to LT was also assessed by the intravenous injection of 125:25 LT (Figure 2). All lines (CT 3.2, $P = 0.0001$; all others, $P < 0.0001$) except CT 3 ($P = 0.77$) exhibited significantly greater mortality compared to DBA/2 and CD2 F_1 hybrid mice. In contrast, all lines ($P < 0.0001$) except CT 8.5 ($P = 1.0$) were significantly more resistant to LT compared to BALB/c mice. The remaining eight C.D2 ISRC lines clustered into three groups, resulting in five quantitatively distinct phenotypes based on mortality rates ($P < 0.0001$).

Table 1. Genotypes of ISRC Lines Indicate that Mortality is Controlled by Three QTL

Congenic line		CT 3	CT 3.2	CT 8.1	CT 8	CT 8.2	CT 8.3	CT 3.1	CT 8.6	CT 8.4	CT 8.5	
	% Mortality[1]*	5.3	22.6	28.0	50.0	52.6	52.6	76.5	76.2	78.9	100	
	N	19	53	25	20	19	19	17	21	19	16	
Marker	cM†											
D11Mit339	34.0	C	C	C	C	C	C	C	C	C	C	
D11Mit318	34.0	D	C	C	C	C	C	C	C	C	C	
D11Mit156	34.0	D	C	C	C	C	C	C	C	C	C	
D11Mit275	34.0	D	C	C	C	C	C	C	C	C	C	
D11Mit261	34.2	D	C	C	C	C	C	C	C	C	C	
D11Mit157	34.3	D	C	C	C	C	C	C	C	C	C	
D11Mit88	34.5	D	C	C	C	C	C	C	C	C	C	
		D	C	D	C	C	C	D	D	D	C	*Ltxs2*
D11Mit4	37.0	D	C	D	D	C	C	D	D	D	C	
D11Mit278	40.0	D	C	D	D	D	C	C	D	C	C	
D11Mit298	40.0	D	D	D	D	D	D	C	C	C	C	
D11Mit30	40.0	D	D	D	D	D	D	C	C	C	C	
D11Mit31	40.0	D	D	D	D	D	D	C	C	C	C	
D11Mit15	40.0	D	D	D	D	D	D	C	C	C	C	
D11Mit29	40.0	D	D	D	D	D	D	C	C	C	C	
D11Mit90	42.0	D	D	D	D	D	D	C	C	C	C	
D11Die30		D	D	D	D	D	D	C	C	C	C	
Kif1c		D	D	D	D	D	D	C	C	C	C	*Ltxs1*
D11Die31		D	D	D	D	D	D	C	C	C	C	
D11Die33		D	D	D	D	D	D	C	C	C	C	
D11Mit320	43.0	D	D	D	D	D	D	C	C	C	C	
D11Die34		D	D	D	D	D	D	C	C	C	C	
D11Die35		D	D	D	D	D	D	C	C	C	C	
D11Die36		D	D	D	D	D	D	C	C	C	C	
D11Die24		D	D	D	D	D	D	C	C	C	C	
D11Mit243	43.0	D	D	D	D	D	D	C	C	C	C	
D11Die37		D	D	D	D	D	D	C	C	C	D	
D11Die38		D	D	D	D	D	D	C	C	C	D	
D11Mit279	43.0	D	D	D	D	D	D	C	C	C	D	
D11Mit219	43.0	D	D	D	D	D	D	C	C	C	D	
D11Bhm163	45.6	D	D	D	D	D	D	C	C	C	D	
D11Mit364	44.0	D	D	C	D	D	D	C	C	C	D	
D11Bhm149	44.7	D	D	C	C	C	C	C	C	C	C	
D11Bhm154	45.1	D	D	C	C	C	C	C	C	C	C	
Nos2	45.6	D	D	C	C	C	C	C	C	C	C	*Ltxs3*
D11Mit262	46.0	D	D	C	C	C	C	C	C	C	C	
D11Mit40	46.0	D	D	C	C	C	C	C	C	C	C	
D11Mit94	46.1	D	D	C	C	C	C	C	C	C	C	
D11Mit34	46.1	D	D	C	C	C	C	C	C	C	C	
D11Mit8	46.2	D	D	C	C	C	C	C	C	C	C	
D11Mit118	46.4	D	D	C	C	C	C	C	C	C	C	
D11Bhm169	46.5	D	D	C	C	C	C	C	C	C	C	
D11Mit165	46.0	D	C	C	C	C	C	C	C	C	C	
D11Mit36	47.0	C	C	C	C	C	C	C	C	C	C	
Ltxs QTL per line		1, 2, 3	1, 3	1, 2	1	1	1	2	2	2	0	

*The mortality values are the combined cumulative totals observed at day 9 post-LT injection for all C.D2 ISRC mice studied and correspond to the values in Figure 2.

†Map locations for marker loci are as reported by the MGI (www.informatics.jax.org) and are ordered form the centromere to the telomeric end of mouse chromosome 11. However, order and placement of marker loci are ultimately based on minimizing recombinational events. This occasionally results in marker orders that are discordant with their reported locations. For example, *D11Bhm163* is reported to be at 45.6 cM; however, the best placement for this marker is between *D11Mit219* at 43.0 cM and *D11Mit364* at 44.0 cM. Similarly, *D11Mit165* and *Tcf2,* which are reported to be at 46.0 and 44.0 cM, respectively, fit best between *D11Bhm169* at 46.5 cM and *D11Mit195* at 47.0 cM. The most significant discrepancy, however, is the location for *Ltxs1/Kif1c.* MGI places *Kif1c* at 37.0 cM while Watters and Dietrich[12] place it between *D11Mit90* and *D11Mit320* at 42.0 and 43.0 cM, respectively. In order to orient and integrate their data with ours, we genotyped the ISRC lines with all of the informative markers between *D11Mit90* and *D11Mit279* including an allele-specific marker for *Kif1c.* Our data are consistent with the placement of *Ltxs1/Kif1c* within this interval.

A comparison of the alleles at each of the marker loci carried by the C.D2 ISRC lines is presented in Table 1. First, the DBA/2 *Ltxs1/Kif1c* allele does not fully protect mice from LT challenge. This is reflected by the fact that lines CT 8, CT 8.2, and CT 8.3 exhibit significantly greater mortality (50.0%, 52.6%, and 52.6%, respectively) than do either DBA/2 (5.0%) or CD2 F_1 hybrid mice (4.5%). However, the data show that DBA/2 alleles at marker loci between *D11Mit318* and *D11Mit165* result in mortality similar to that of DBA/2 (5.0%) and CD2 F_1 hybrids (4.5%), ie, line CT 3 mice (5.3%).

The most parsimonious interpretation of these results is that aside from *Ltxs1/Kif1c* there are at least two additional QTL within the interval between *D11Mit318* and *D11Mit165* controlling mortality. The first novel QTL is proximal of *Ltxs1/Kif1c* within the interval between

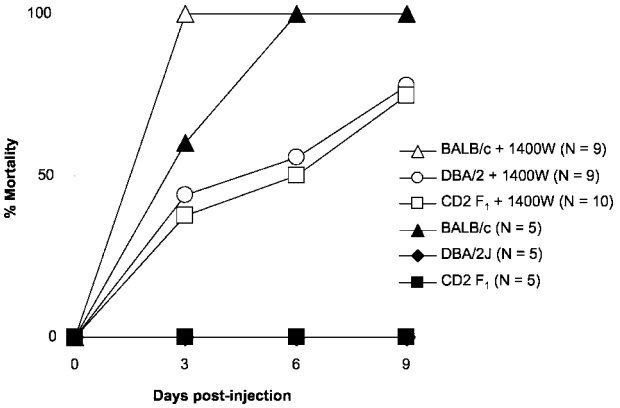

Figure 3. Selective, pharmacologically based inhibition of *Nos2* activity partially overrides genetic resistance to LT *in vivo*. The data are expressed as % mortality and are the combined results for both male and female mice.

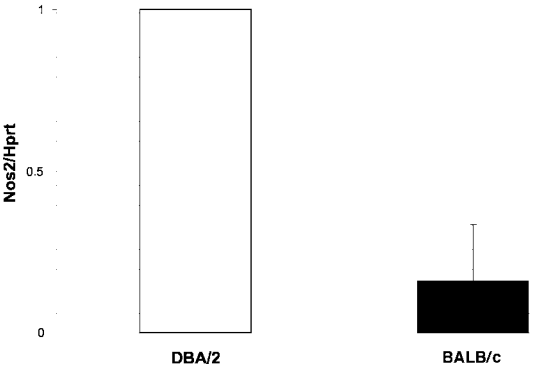

Figure 4. *Nos2* expression differs between DBA/2 and BALB/c peritoneal macrophages. Resident peritoneal macrophages were isolated by lavage from DBA/2 and BALB/c mice and *Nos2* expression levels assessed by Taqman PCR using cDNA generated by reverse transcription of mRNA isolated from equal numbers of cells stimulated for 24 hours with 100 ng/ml LPS and 25 U/ml mouse IFNγ. *Nos2* levels are normalized with respect to DBA/2 levels. Data are presented as the normalized means ± SD (P value <0.05).

D11Mit88 (34.5 cM) and *D11Mit4* (37.0 cM). We have designated this QTL as *Ltxs2*. The genetic effect at *Ltxs2* is manifest in lines CT 8.4, CT 8.6, and CT 3.1 which exhibit 78.9%, 76.2%, and 76.5% mortality compared to 100% mortality in BALB/c and CT 8.5 mice.

The second novel QTL, *Ltxs3*, is distal of *Ltxs1*/*Kif1c* within the interval between *D11Bhm149* (44.7 cM) and *D11Bhm169* (46.5 cM). The location of *Ltxs3* is manifest by the fact that line CT 3.2 exhibits significantly reduced mortality compared to lines CT 8, CT 8.2, and CT 8.3 (22.6% *vs.* 50.0%, 52.6%, and 52.6%, respectively). *Ltxs3* is also present in line CT 3. The data suggest that *Ltxs1* and *Ltxs3* interact in controlling responsiveness. This also is the case for *Ltxs2* because line CT 8.1, which possesses DBA/2 alleles at both *Ltxs1*/*Kif1c* and *Ltxs2*, exhibits 28.0% mortality compared to 50.0%, 52.6%, and 52.6% in lines CT 8, CT 8.2, and CT 8.3, respectively. Given this interpretation, the data indicate that *Ltxs2* resides within the interval defined by the recombination break-point in lines CT 8 and CT 8.1 between *D11Mit88* (34.5 cM) and *D11Mit4* (37.0 cM).

The *Ltxs3* interval encompasses inducible nitric oxide synthase (*Nos2*), which is an attractive candidate gene for *Ltxs3*. LF inhibits NO production by macrophages[6] and *Nos2* knockout mice die sooner than wild-type mice when challenged with anthrax spores.[25] To assess the candidacy of *Nos2* for *Ltxs3* we examined the effects of N-[3-(Aminomethyl)benzyl]acetamidine, dihydrochloride (1400W) on susceptibility to LT. 1400W is a selective, irreversible *Nos2* inhibitor which is effective *in vivo*.[26] Inhibition of *Nos2* activity was capable of partially overriding genetically controlled resistance in both DBA/2 and CD2 F_1 hybrid mice (Figure 3). Additionally, DBA/2 macrophage produced significantly greater levels of *Nos2* mRNA following stimulation with LPS and IFNγ (Figure 4). Taken together these data strongly support *Nos2* as a candidate for *Ltxs3*.

To test the hypothesis that dominant interactive effects contribute to mortality we crossed several of the congenic lines and assessed the F_1 progeny for susceptibility and resistance (Figure 5). First, we crossed lines CT 8.1 and CT 3.2. Both parental strains exhibited 25% mortality whereas the (CT 8.1 × CT 3.2) F_1 hybrids were resistant. Since both *Ltxs2* and *Ltxs3* are heterozygous, this indicates that DBA/2 alleles at these two QTL are dominant. Dominance at *Ltxs3* is also supported by the data obtained with the (CT 8.2 × CT 3.2) F_1 hybrids. Finally, crossing line CT 8.6 with line CT 3.2 resulted in F_1 hybrids that are heterozygous at all three QTL. These mice were completely resistant to LT challenge, as were the CD2 F_1 hybrids in this particular experiment. Taken together these data suggest that the three QTL controlling mortality interact in a dominant fashion.

Discussion

Although *Ltxs1*/*Kif1c* was identified as the gene controlling LT-induced macrophage cytolysis *in vitro*, insufficient data existed to conclude whether or not *Ltxs1*/*Kif1c* plays a role in lethal pathologies *in vivo*.[13] Fortuitously, we were in a position to directly test this. We had previously established a panel of C.D2 ISRC lines covering the central portion of chromosome 11 as the first step in the positional cloning of *Orch3*, a gene controlling dominant resistance to autoimmune orchitis.[16] Since BALB/c and DBA/2 mice exhibit differential susceptibility to both macrophage cytolysis *in vitro* and lethality following infection,[8,9] and BALB/c and DBA/2 *Ltxs1*/*Kif1c* alleles are

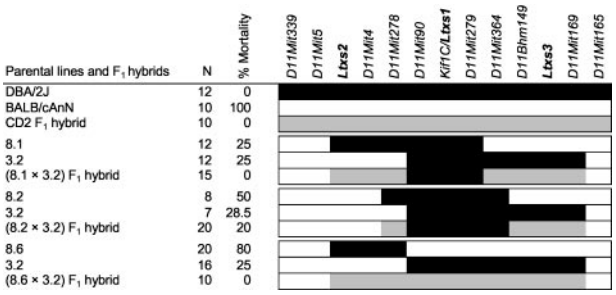

Figure 5. Mortality studies in C.D2 ISRC F_1 hybrid mice indicate that protection requires resistance alleles at *Ltxs1*, *Ltxs2*, and *Ltxs3* for complete protection from LT. **Solid bars** represent DBA/2 alleles, **open bars** represent BALB/c alleles and **gray bars** represent heterozygous regions. The mortality values are the cumulative totals observed at day 9 following the intravenous injection of 125:25 LT.

segregating among our lines, we undertook an experiment to assess the role of *Ltxs1/Kif1c* in the genetic control of mortality.

DBA/2 mice were reported to be susceptible to lethality following infection[8] while their macrophages are resistant to cytolysis.[11] In contrast, BALB/c mice were resistant to lethality following infection[8] but their macrophages are susceptible to cytolysis.[9,11] The susceptibility status of both strains following direct intravenous challenge with LT was unknown. In generating the C.D2 ISRC lines several different BALB/c sublines were intentionally used to control for subline differences in susceptibility to autoimmune orchitis.[23] Therefore, it was important to exclude potential substrain differences as a confounding factor in these studies. Similarly, DBA/2 substrain differences in susceptibility to infection with *Leishmania major* have been reported.[27] Our substrain survey clearly indicated that all BALB/c sublines were equally susceptible to mortality clearly establishing that BALB/c mice are susceptible to LT-challenge and that DBA/2 are resistant. Additionally, we showed that CD2 F_1 hybrid mice are also resistant, indicating that resistance is dominant over susceptibility. These results are consistent with the previously reported inverse relationships between mortality elicited by infection and LT challenge.[8]

The congenic mapping results confirm the role of the BALB/c and DBA/2 *Ltxs1/Kif1c* alleles in controlling mortality. However, the results of this study reveal that two additional QTL linked to *Ltxs1/Kif1c* control mortality: *Ltxs2* (34.5–37.0 cM) and *Ltxs3* (44.71–46.43 cM). Individually, DBA/2 alleles at each QTL contribute some protective effect. The presence of resistance alleles at all three QTL is, however, required for complete protection.

One QTL and one candidate gene within the *Ltxs2* interval is *Ity3*, controlling susceptibility to *Salmonella typhimurium* infection, and *Mgl*l, macrophage galactose N-acetyl-galactosamine specific lectin 1 mediating macrophage binding to galactose and N-acetyl-galactosamine residues on tumor and bacterial cells and bacterial products (www.informatics.jax.org). The interval encompassing *Ltxs3* encodes *Nos2*. LF inhibits NO production by macrophages[6] and *Nos2* knockout mice die sooner than wild-type mice when challenged with anthrax spores.[25] Our results demonstrating that *in vivo* inhibition of *Nos2* activity partially overrides genetic resistance to LT and that *Nos2* mRNA levels are higher in DBA/2 macrophages compared to BALB/c macrophages supports the hypothesis that either a structural or expression-level polymorphism in *Nos2* could underlie *Ltxs3*.[28] Additionally, *Tnfaip1* (tumor necrosis factor, α-induced protein 1) at 45.1 cM is also of interest given that TNFα treatment of LT-resistant macrophages converts them to LT-susceptible macrophages.[29] Tnfaip1 is specifically and transiently expressed by endothelial cells following exposure to TNFα.

Clearly, the identification of the genes underlying *Ltxs2* and *Ltxs3*, and their detailed characterization at the biochemical and molecular level, will provide significant additional insight into the mechanisms whereby LT elicits lethal anthrax pathologies. Importantly, since resistance is dominant at all three QTL, BAC and single gene transgenic mapping strategies can in theory be used to identify the respective genes and verify that *Kif1c* is the only gene within the DBA/2 *Ltxs1* interval controlling mortality and macrophage cytolysis.

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