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ORIGINAL ARTICLE

Genetic analysis of susceptibility to Chlamydia trachomatis in mouse

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Chlamydia trachomatis is a bacterial pathogen that is a major cause of blindness and infertility in diverse populations across the world. In an effort to model genetic complexities that are observed in human populations and to identify novel genes involved in susceptibility to C. trachomatis, we have adapted a murine model of systemic infection for use in genetic analysis. In this model, chlamydial colonization and replication is measured in the spleens of mice shortly after intravenous delivery of C. trachomatis L2. Here, we show that C57BL/6J and C3H/HeJ inbred mice are differentially susceptible to this systemic infection. Additionally, fibroblasts cultured from C57BL/6J and C3H/HeJ embryos are differentially permissive for chlamydial replication. We have taken advantage of this natural variation to map quantitative trait loci on Chromosomes 2, 3, and 11 that segregate with the bacterial load in F2 cross progeny during the acute phase of C. trachomatis infection in vivo. To validate our mapping results, we also generated mice that are congenic for a portion of Chromosome 11 from the susceptible parent. This congenic interval confers increased susceptibility to C. trachomatis, both in vivo and in vitro, suggesting that our screen identified at least one gene that is involved in cellular resistance to C. trachomatis replication.

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Introduction

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that causes a wide range of diseases in humans. Depending upon the serovar that infects the host, *C. trachomatis* can cause infection of either the ocular or genital mucosa. Serovars A–C are responsible for the ocular infection, trachoma, which is the leading cause of preventable blindness in the world. In contrast, serovars D–K are associated with inflammation and pathology in the genital tract that leads to urethritis, cervicitis, pelvic inflammatory disease, and infertility. In developing countries, genital infection can also be caused by the lymphogranuloma venereum serovars L1–L3. These highly-invasive serovars quickly reach the draining lymph nodes of the genital tract and spread systemically throughout the host.[1]

A defining characteristic of chlamydiae is their unique biphasic developmental cycle. Outside of the host cell, *C. trachomatis* exists as infectious, but metabolically quiescent elementary bodies (EBs). Once EBs enter the host cell, they convert within hours to noninfectious reticulate bodies that are metabolically active and replicate within a specialized vacuole known as an inclusion. Approximately 16–20 h following infection, the reticulate bodies begin to convert back to EBs. By 36–48 h postinfection, the host cell lyses and EBs are released into the extracellular space to begin further rounds of infection and replication.[2]

Exposure to chlamydiae can be common, especially in endemic regions, although it is clear that exposure does not necessarily lead to disease in every individual. Similarly, even among those that develop disease, the duration and severity of pathology may vary substantially from one individual to the next.[3,4] Host genetic factors likely play an important role in this variability, and indeed, human genetic studies have shown associations between increased prevalence or severity of *Chlamydia* infection and certain human leukocyte antigen haplotypes[5–8] or polymorphisms in CD-14, tumor necrosis factor-α, or interleukin-10.[8–10] Although these reports show associations that are likely to be relevant, it is difficult to confirm their biological significance using human studies.

Genetic manipulation of *Chlamydia* is not currently possible; therefore, genetic studies of chlamydial pathogenesis must focus instead on host models of infection. Indeed, murine infection models provide an important set of tools to identify host factors that function in response to *Chlamydia* spp. and have revealed interesting differences in susceptibility among inbred mouse strains across a range of infection models. For example, there is variability among several inbred strains in susceptibility to genital tract infection with serovars of *C. trachomatis*.[11–16] Recent experiments with the mouse pneumonitis (MoPn) biovar of *C. trachomatis* have demonstrated that this variability is partly related to the cytokine profile in the genital tract during infection. C57BL/6 mice, which

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exhibit relatively mild pathological outcomes when compared to BALB/c or C3H/HeN mice, seem to have a more vigorous Th1 response with higher levels of tumor necrosis factor-α, interferon-γ, and macrophage inflammatory protein-1α.[15–17]

Analysis of genetically modified mice has also demonstrated the importance of certain immune factors in the host response to *Chlamydia* infection. For example, mice that are deficient for cytokines like interferon-γ or interleukin-12, or key immune molecules like major histocompatibility complex class II or CD-8, show increased susceptibility to various types of *Chlamydia* infections (reviewed in Rottenberg *et al*. [18]). Studies using congenic mice have also demonstrated the effect of major histocompatibility complex haplotype on susceptibility to *Chlamydia* spp. (our unpublished observations).[19]

Although the mouse studies described above provide important clues about the roles played by specific cytokines and immune molecules under certain infection conditions, they supply only limited information about the wide range of factors that might be involved in the host response to *Chlamydia*. In contrast, genetic analysis represents an unbiased approach to identifying genes important for resistance to infection, from conventional immune sensors and effectors to non-immune proteins that may nonetheless influence the ability of *Chlamydia* to cause disease. Indeed, for many pathogens, classical mouse genetics has served as a valuable tool for the dissection of host factors involved in the response to infection.[20,21]

In an effort to apply the host genetics approach to *Chlamydia* pathogenesis, and to identify novel genes involved in resistance or susceptibility to *Chlamydia*, we have adapted a systemic model of *C. trachomatis* infection for use in genetic analysis.[22] In this model, mice are injected intravenously with *C. trachomatis* L2, and the ensuing infection is monitored in the spleen during the acute phase of the infection. This model system is particularly well suited for genetic analysis of the host response to infection because *C. trachomatis* reliably and predictably colonizes the spleen within hours of intravenous delivery. C3H mice support more chlamydial replication than C57BL/6 mice, and this difference emerges very early in the course of infection. We have exploited this early strain difference to map three quantitative trait loci (QTL) that appear to affect the ability of the host to restrict chlamydial replication during the acute phase of the infection. Therefore, this difference is unlikely to involve differences in the function of acquired immunity. Furthermore, we validated the use of this genetic approach by showing that one of our loci seems to impact resistance to *C. trachomatis* in fibroblasts isolated from embryos congenic for one of our QTL. Together, our data provide an important first step towards identifying novel genes involved in susceptibility to *C. trachomatis* and demonstrate the utility of host genetics in identifying loci that function in resistance to pathogens.

Results

The course of systemic C. trachomatis *infection differs in the spleens of* C57BL/6J *and* C3H/HeJ *mice*

We were interested in developing a genetically tractable model of susceptibility to *C. trachomatis* that would allow us to identify genes involved in resistance to this pathogen. To that end, we infected C57BL/6J and C3H/HeJ inbred mice via the tail vein with *C. trachomatis* L2, and monitored the infection in the spleens of these mice. To assay chlamydial colonization and replication during the acute phase of the infection, we devised a multiplex, real-time quantitative PCR assay that allowed us to simultaneously determine the amount of *Chlamydia* and host DNA as a measure of bacterial load in the spleens of infected animals. In preliminary experiments involving known amounts of *C. trachomatis* and host tissue, we found this assay to be a sensitive and reliable measure of chlamydial load (not shown). When we applied the PCR assay to infected mice within hours postinfection, we found that the amount of *C. trachomatis* present in the spleens of C57BL/6J and C3H/HeJ mice is similar at 6, 10 and 12 h postinfection (Figure 1). By 14 h postinfection, however, there is roughly fourfold more *C. trachomatis* in the spleens of C3H/HeJ mice relative to C57BL/6J mice; this difference increases to more than 10-fold by 20 h postinfection.

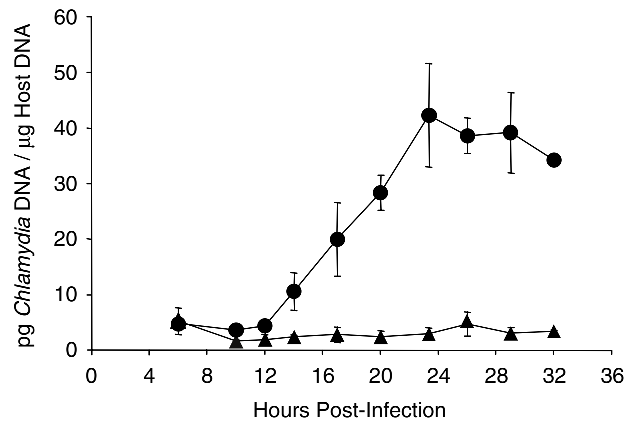

Figure 1 Time course showing the amount of *Chlamydia* DNA per unit weight of host DNA amplified from the spleens of C57BL/6J (triangles) and C3H/HeJ (circles) mice after intravenous delivery of *C. trachomatis* L2. Each point represents the mean of three animals; error bars represent $\pm$ one standard deviation.

In separate experiments, we carried the infection out to 17 days postinfection and assessed chlamydial load in the spleens of infected mice using standard titering methods. We found that more *C. trachomatis* can be titered from the spleens of C3H/HeJ mice relative to C57BL/6J mice at 3, 7 and 10 days postinfection (Figure 2). Furthermore, while C57BL/6J mice clear the splenic infection by 12 days postinfection, infectious particles can be titered from C3H/HeJ spleens beyond 17 days postinfection. Preliminary genetic mapping experiments involving day 17 splenic titers taken from a small number of backcross progeny revealed a complex trait that was only partly explained by suggestive linkages to the major histocompatibility complex on Chromosome 17 and to another locus on Chromosome 6 (data not shown).

The strain difference in susceptibility to C. trachomatis *can be recapitulated* in vitro *using primary cells*

We reasoned that certain elements of the *in vivo* susceptibility difference might be modeled *in vitro* using primary cells derived from the parental strains. To test this hypothesis, we prepared mouse embryonic fibro-

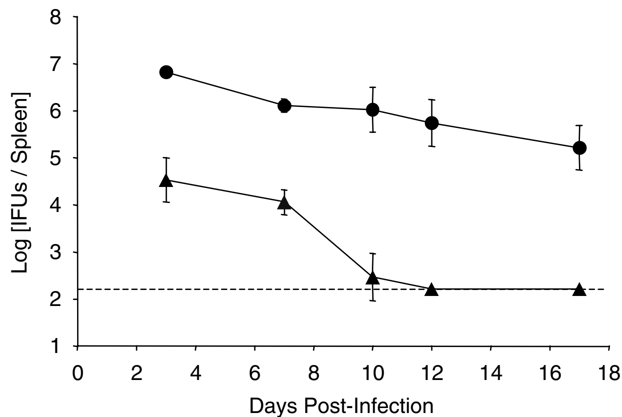

Figure 2 Time course showing the log of the number of inclusion-forming units (IFUs) titered from the spleens of C57BL/6J (triangles) and C3H/HeJ (circles) mice after intravenous delivery of *C. trachomatis* L2. Each point represents the mean of four animals; error bars represent ± one standard deviation. The limit of detection is 167 IFUs/spleen (dashed line).

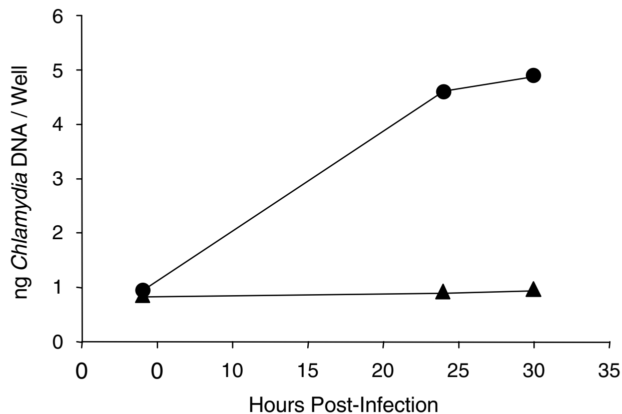

Figure 3 Time course showing the amount of *Chlamydia* DNA amplified from primary mouse embryonic fibroblasts isolated from C57BL6J (triangles) and C3H/HeJ (circles) and infected *in vitro* with *C. trachomatis* L2. Each point represents the mean of two wells of MEFs from a combined pool of at least 20 embryos; data are representative of five similar experiments. Cells were pretreated with interferon-γ as described in Materials and methods.

blasts (MEFs) from C57BL/6J and C3H/HeJ, infected them in culture with *C. trachomatis* L2, and assessed chlamydial replication by measuring the amount of *Chlamydia* DNA present in the cells using our quantitative PCR assay. Under standard culture conditions, we found that MEFs from both strains were equally permissive for chlamydial replication (not shown). Therefore, we elected to pretreat the cells with interferon-γ, a cytokine that is critical to the host response to *Chlamydia* infection,[18] and that might not otherwise be present in sufficient quantities in our fibroblast cultures. Exogenous interferon-γ markedly reduced the amount of chlamydial growth that took place in both MEF strains; however, the presence of interferon-γ rendered C57BL/6J MEFs comparatively more resistant to chlamydial replication than C3H/HeJ MEFs (Figure 3). Furthermore, when we looked at the cells by immunofluorescent microscopy at 29 h postinfection, we observed that this difference is related to a greater number of mature inclusions in C3H/HeJ MEFs relative to C57BL/6J MEFs and not to any discernable differences in inclusion size or morphology (not shown). Given that this *in vitro* effect is dependent upon the presence of interferon-γ, and that interferon-γ is not likely to be involved in uptake of *Chlamydia* into the cells, we hypothesize that the block in inclusion development in C57BL/6J MEFs is at the level of replication and not entry.

Susceptibility to C. trachomatis *segregates as a complex trait in the F2 generation*

To dissect the genetic origins of the *in vivo* susceptibility difference between C57BL/6J and C3H/HeJ, we infected 169 F2 progeny with *C. trachomatis* L2 and assessed chlamydial load in the spleens of these mice at 29 h postinfection, a time point that would allow us to capture host genetic differences operative during the acute phase of the infection. When assayed at 29 h postinfection, the amount of *C. trachomatis* in the spleens of these F2 mice exhibited a continuous distribution with a mean of 10.44 ± 5.69 pg *Chlamydia* DNA/μg host DNA. As the F2 animals were injected in groups of 30–40 animals, and because there was subtle variability in the average phenotype from batch to batch, we elected to normalize the data by subtracting the group-specific mean from each animal. The mean of this new distribution is zero, with a standard deviation of 5.30.

Each animal was initially genotyped at 93 simple sequence length polymorphism (SSLP) markers spaced an average of 16 centimorgans (cM) across the 19 autosomes; additional markers were subsequently added to the map to improve the resolution around linkage peaks. We then performed QTL analysis using MapManager QTX[23] to determine regions of the genome that segregated with the normalized phenotypes described above. A permutation test based on our data established a genome-wide significance level of $P = 0.01$ at a likelihood ratio statistic (LRS) of 19.4 (where LOD = LRS/4.6[23]). Regions of Chromosomes 2, 3, and 11 exceeded this significance level with respective LRS_{max} values of 20.5, 19.9, and 20.2 (Figure 4). Note that these linkages also exceed the conservative benchmark of LOD = 4.3 (LRS = 19.8) set forth by Lander and Kruglyak[25] for the reporting of significant QTL results. Accordingly, we have named the Chromosome 2, 3 and 11 loci, respectively: *Ctrq-1*, *Ctrq-2*, and *Ctrq-3* for C. trachomatis resistance QTL. All other regions of the genome were LRS <14.0, *ns*.

Examination of the phenotype:genotype correlation at a marker near each linkage peak illustrates the phenotypic effect and the dominance relationships of the alleles at each QTL (Table 1). Thus, *Ctrq-1* and *Ctrq-3* are, respectively, recessive and dominant susceptibility alleles from the susceptible parent (C3H/HeJ). *Ctrq-2*, meanwhile, is a dominant allele from C3H/HeJ that seems to confer relative resistance to *C. trachomatis*.

To determine the feasibility of fine mapping studies aimed at identifying the relevant polymorphic gene(s) under each linkage peak, we constructed a B6.C3H congenic mouse line that carries a portion of Chromosome 11 from C3H/HeJ between D11Mit20 and D11Mit245 within a C57BL/6J background. This congenic interval contains the entire ±1.5 LOD support interval for *Ctrq-3* (Figure 4). We injected heterozygous

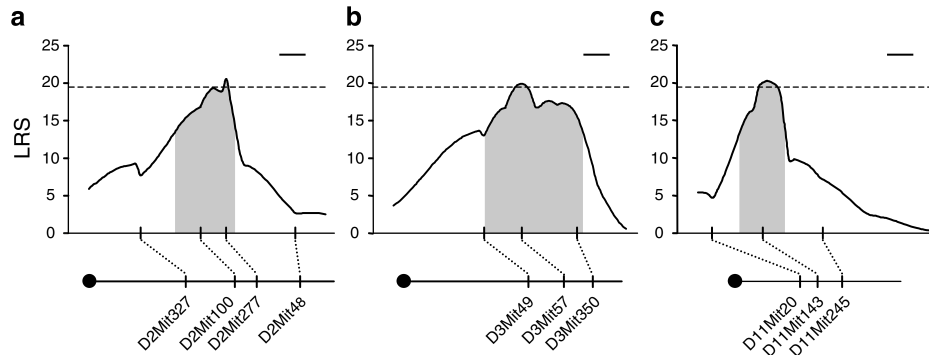

Figure 4 Charts depicting linkage to our *Chlamydia* susceptibility phenotype on Chromosomes 2, 3, and 11 (**a**, **b**, and **c**, respectively). The likelihood ratio statistic (LRS) is calculated every 1 cM along the genetic map. Corresponding physical positions of several genetic markers are shown as tick marks along the representation of each chromosome shown below the figures. Shaded regions correspond to support interval for each peak (1.5 LODs on either side of linkage peak[24]). Values greater than 19.4 are significant at $P<0.01$ (dashed line). Scale bar in the upper right corner of each chart represents 6 cM on the genetic map and 20 megabases on the physical map.

Table 1 Average corrected phenotype by genotype at each linkage peak

QTL	*Marker*	*LRS*	*%Variance*[b]	*Phenotype by Genotype*[a]		
				B/B	*B/C*	*C/C*
Ctrq-1	D2Mit277	20.5	11	−0.64	−1.29	3.02
Ctrq-2	D3Mit57	19.9	11	2.81	−0.63	−1.77
Ctrq-3	D11Mit143	19.5	11	−3.25	0.67	1.30

[a]B/B = homozygous C57BL/6J; C/C = homozygous C3H/HeJ; B/C = heterozygous.
[b]Amount of the total trait variance explained by this QTL.

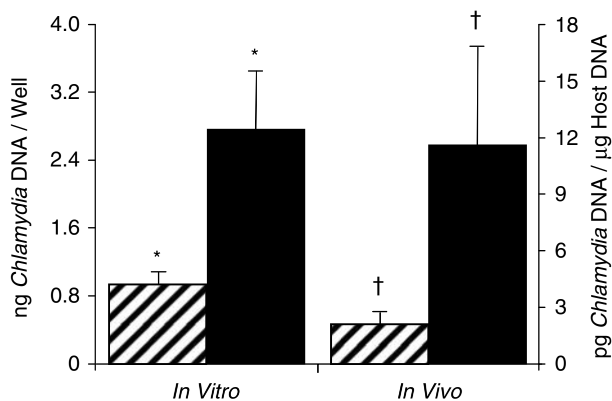

Figure 5 Effect of Chromosome 11 congenic interval on susceptibility to *C. trachomatis*, both *in vitro* (left) and *in vivo* (right). Data for control noncarrier samples are shown as hatched bars, whereas data for congenic samples are shown as solid black bars. *In vitro* data are the amount of *Chlamydia* DNA amplified from embryonic fibroblasts infected for 28 h with *C. trachomatis* L2 after interferon-γ treatment, as described in Materials and methods. *In vivo* data are the amount of *Chlamydia* DNA per unit weight of host DNA amplified from the spleens of infected mice 29 h after intravenous delivery of *C. trachomatis* L2. Each bar is mean of at least three data points; error bars represent one standard deviation. Samples with identical symbol above error bars are significantly different at $P<0.01$.

carriers of this congenic interval with *C. trachomatis* and found that at 29 h postinfection, there was significantly more *Chlamydia* in the spleens of these mice than in the spleens of noncarrier littermates (Figure 5). Furthermore, MEFs that are heterozygous for this congenic interval support more chlamydial replication in our *in vitro* assay than do noncarrier controls (Figure 5).

Discussion

C. trachomatis is a significant cause of disease in a variety of human populations. In an effort to identify novel host genes involved in resistance to acute *C. trachomatis* infection, we have adapted a systemic model of *C. trachomatis* infection in mouse for use in genetic analysis. In this study, we demonstrated that although *C. trachomatis* L2 colonizes the spleens of C57BL/6J and C3H/HeJ mice to a similar extent, more *C. trachomatis* is present in the spleens of C3H/HeJ mice relative to C57BL/6J mice starting at 14 h postinfection and persisting beyond 17 days postinfection (Figures 1 and 2). This difference correlates with an *in vitro*, interferon-γ-dependent difference in susceptibility between fibroblasts isolated from the two strains (Figure 3). We applied standard genetic mapping to the *in vivo* strain difference, and identified three significant QTL that segregate with the bacterial load in the spleens of F2 mice at 29 h postinfection (Figure 4). These QTL represent the first host loci involved in the *in vivo* response to *C. trachomatis* to be identified using an unbiased genome-wide approach.

As in other genetic screens, the QTL that emerged from our screen are almost certainly a function of the

particular features of the infection model that we employed. Thus, a defining characteristic of our mapping project is that we chose to focus on early events in the infection, thereby offering unique insight into factors that regulate initial and presumably critical steps in the infection process. These steps might include colonization of *C. trachomatis* in the spleen, attachment and entry into the relevant cell type(s), proper intracellular targeting, and various other intracellular events that lead to productive chlamydial replication. The nature of our screen did not allow the identification of genes that are specifically involved in acquired immunity against *Chlamydia* infection. However, genes that act early may nonetheless shape or participate directly in later stages of the immune response; thus, despite our focus on early events in the infection, we may have in fact identified loci that function in more chronic infections as well. Indeed, we observed a strain difference in a chronic model of infection, a difference that may be related to the acute differences that we mapped. Eventual identification and analysis of our QTL will be critical in determining their relevance in chronic *C. trachomatis* infection.

Infections caused by *C. trachomatis* are diverse and cannot be encapsulated in a single model system. Still, the model used in our genetic analysis captures elements of the lymphatic colonization and replication that typifies infection with a lymphogranuloma venereum biovar of *C. trachomatis*. Furthermore, it is worth emphasizing that the effects we mapped may generalize to other types of infections, such as those of the ocular and genital tract epithelia by the trachoma biovars of *C. trachomatis*. For example, the particular inbred mouse strains that we analyzed have similar documented differences in susceptibility to other *Chlamydia* infections, including respiratory tract infection with *C. trachomatis* MoPn and genital tract infection with *C. trachomatis* MoPn and serovar E.[11,15,16] These findings suggest that the mechanisms regulating the difference in susceptibility between C57BL/6J and C3H/HeJ may be relatively general and that the effects of the loci we identified will not be specific to splenic infection with *C. trachomatis* L2.

One reason why C57BL/6J and C3H/HeJ exhibit differences in susceptibility across a range of infection models may be related to the susceptibility difference that we observed between fibroblasts prepared from these two strains (Figure 3). Indeed, given that the *Chlamydia* developmental cycle is remarkably well-conserved across different *Chlamydia* serovars and species,[2] it seems likely that the host–pathogen interactions that occur in fibroblasts infected with *C. trachomatis* L2 would be preserved across a variety of cell types infected with different serovars of *C. trachomatis*. This allows for the possibility that polymorphisms in host genes that interact with the chlamydial developmental cycle might positively or negatively affect growth and replication across a variety of different cell types.

That the *in vitro* difference in susceptibility to *C. trachomatis* is interferon-γ-dependent suggests a strain difference in interferon responsiveness between fibroblasts isolated from C57BL/6J and C3H/HeJ. Interferon-γ is a major inflammatory cytokine involved in the host response to *Chlamydia* infection and regulates aspects of both innate and acquired immunity against *Chlamydia*.[18] Marked interstrain differences in interferon responsiveness under similar conditions have been described once previously, when Qiu *et al*.[11] found that growth of *C. trachomatis* MoPn was less inhibited by interferon-γ in peritoneal macrophages isolated from C3H than in macrophages isolated from C57BL/6. This difference was associated with greater nitric oxide production in C57BL/6 macrophages relative to C3H macrophages, though nitric oxide production was not sufficient to fully explain the susceptibility difference between the two strains. Nelson *et al*.[26] recently showed that the small interferon-γ-inducible GTPase Iigp1 was a critical component of the inhibitory effect of interferon-γ on growth of *C. trachomatis* L2 in primary mouse epithelial cells. The role of Iigp1 in the susceptibility difference we observed between C57BL/6J and C3H/HeJ MEFs remains unclear; however, none of our QTL map in the vicinity of *Iigp1*, suggesting that we have identified novel host loci involved in cellular resistance to *C. trachomatis* replication (see below).

Identification of the causative polymorphism(s) underlying our three QTL will require construction of congenic mice that harbor an interval of donor DNA within the genomic background of a recipient strain. After confirming a phenotypic effect of this larger interval, recombination analysis can then be carried out to identify the smallest interval still capable of conferring the donor phenotype. This method has been used in the mouse to localize and identify QTL involved in type 1 diabetes, tumor susceptibility, and a variety of other multigenic or continuously varying traits (reviewed in Flint *et al*.[27]). To commence a similar analysis in this study, and to validate our mapping results, we introgressed more than 30 megabases of C3H/HeJ DNA around the *Ctrq-3* locus into a C57BL/6J background and found that this interval confers increased susceptibility to *C. trachomatis*, both *in vivo* and *in vitro* (Figure 5). This result reinforces our genetic mapping data and affirms our ability to perform fine mapping studies to identify the causative polymorphisms that affect susceptibility *C. trachomatis* in our model.

It is also significant that the *Ctrq-3* congenic interval confers susceptibility both in mice infected systemically and in MEFs infected in culture. This implies that the resistance mechanism governed by *Ctrq-3* is cell autonomous in nature and can function in intracellular pathways affecting resistance to *C. trachomatis*. Additionally, this finding validates our model system as a useful representation of *Chlamydia* infection as it shows that our *in vivo* genetic screen was successful in identifying at least one QTL that seems important for intracellular replication of *C. trachomatis*. The fact that this locus can function *in vitro* should also accelerate identification and characterization of this QTL.

Finally, it is important to note that despite our identification of three significant QTL, the size of our cross precluded the identification of polymorphic genes that might have more subtle effects on susceptibility to *C. trachomatis*. Whereas an approximation of the experimental noise in our cross is complicated by an apparent effect of genotype on trait variance, we estimate that our three QTL explain about 50% of the total phenotypic variation that is attributable to genetic differences among our F2 progeny (see Materials and methods). This suggests the existence of other polymorphic genes of

small effect that went undetected in our cross. It may be possible to utilize mice congenic for the *Ctrq* loci in additional screens for genes that may have been masked by these QTL of larger effect.

In sum, we utilized an unbiased genetic approach to identify three loci that influence susceptibility to *C. trachomatis* in mouse during the acute phase of infection. At least one of these loci seems to also influence susceptibility of embryonic fibroblasts to *C. trachomatis*, as mice and MEFs that are congenic for the susceptible allele of one of the QTL are rendered more susceptible to *C. trachomatis*. We anticipate that the identification of these loci will provide novel insight into the host–pathogen interactions that occur during the acute phase of *C. trachomatis* infection in mouse. A thorough understanding of these processes will be valuable in understanding the immunological and pathological mechanisms of chlamydial infection and disease in humans.

Materials and methods

Animals

Mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained under specific-pathogen-free conditions until infection. For some experiments, mice were housed and infected in a facility designed for holding pathogen-infected animals. Congenic mice described herein were commissioned from The Jackson Laboratory and were created through use of marker-assisted elimination of donor DNA; mice were phenotyped at generation N6.

Chlamydia trachomatis

C. trachomatis serovar L2 434/Bu was propagated in the McCoy murine fibroblast cell line. McCoy cells were grown in Eagle's MEM supplemented with 10% fetal calf serum (FCS), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 1.5 g/l sodium bicarbonate. *C. trachomatis* elementary bodies were isolated from infected McCoy cells by density gradient centrifugation as previously described.[28]

Infection

Mice, 6- to 8-week-old, were injected into the tail vein with *C. trachomatis* L2 in 200 μl of sucrose-phosphate-glutamic acid buffer (SPG, 220 mM sucrose, 12.5 mM phosphate, 4 mM l-glutamic acid, pH 7.5). Owing to technical difficulties associated with the calculation of titer values, the number of inclusion-forming units (IFUs) injected was variable though known: 10^7 IFUs were used in the time course experiments and for the congenics; 10^6 IFUs were used in the F2 cross. At various times postinfection, the mice were killed by CO_2 euthanasia, and their spleens were removed. For titering experiments, the spleens were promptly pressed through a mesh screen into sucrose-phosphate buffer (200 mM sucrose, 20.6 mM phosphate, pH 7.5) supplemented with 10% FCS, gentamicin, vancomycin, nystatin, and amphotericin B, and then flash-frozen. For quantitative PCR assessment of chlamydial load, intact spleens were flash-frozen. All samples were stored at -80°C until processing.

Titering

The number of infectious *C. trachomatis* organisms present in the spleens of infected mice was determined as previously described.[29] Briefly, spleen homogenates were thawed, sonicated, and overlaid onto McCoy cell monolayers. Approximately 30 h later, the monolayers were fixed and stained with the Pathfinder *Chlamydia* Culture Confirmation System (Bio-Rad, Hercules, CA, USA) to visualize developing inclusions. The number of inclusions present on the monolayer was then used to calculate the number of IFUs per spleen.

Mouse embryonic fibroblasts

Embryonic day 12.5–14.5 pups were removed from pregnant females that had been killed by CO_2 euthanasia. After removal of the head and fetal liver tissue, the pups were individually minced, trypsinized, and plated in DMEM supplemented with 10% FCS, 0.1 mm nonessential amino acids, and 0.1% β-mercaptoethanol. Cells were expanded and passaged in the same media in a humidified environment maintained at 37°C, 5% CO_2/95% air. After two or more passages, cells were plated for infection; for some experiments, the media was supplemented with 10 U/ml of recombinant mouse interferon-γ (Invitrogen, Frederick, MD, USA) for 15 h before infection. Cells were infected with *C. trachomatis* L2 at a multiplicity of infection of 2:1 in SPG by centrifugation at 1928 g for 1 h at 37°C, and then returned to standard media. For analysis of *Chlamydia* growth, the monolayers were trypsinized, pelleted, and prepared for real-time PCR as described below. For immunofluorescent visualization of chlamydial inclusions, cells were plated and infected as above on coverglass, fixed with paraformaldehyde, permeabilized with Triton X-100, and stained using a mouse monoclonal antibody against *C. trachomatis* major outer membrane protein (Accurate Chemical & Scientific Corp., Westbury, NY, USA) and a rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were viewed at 500 $\times$ magnification using a Zeiss Axioskop 2 microscope.

Quantitative PCR

Total nucleic acid was prepared from homogenized spleens or trypsinized cell pellets using the QIAamp DNA Mini Kit from Qiagen (Valencia, CA USA). Samples were then subjected, in duplicate, to singleplex (cells) or multiplex (spleen) quantitative, real-time PCR on an ABI 7000 Sequence Detection System to assess the amount of *Chlamydia* and host DNA in the sample. *Chlamydia* 16 s DNA was detected through use of the following primer sequences: forward primer 5′-GGA GGC TGC AGT CGA GAA TCT-3′, reverse primer 5′-TTA CAA CCC TAG AGC CTT CAT CAC A-3′, and dual-labeled probe 5′-[6-FAM]-TCG TCA GAC TTC CGT CCA TTG CGA-[TAMRA]-3′. Mouse GAPDH DNA was detected using the Rodent GAPDH Control Reagent Kit from Applied Biosystems (Foster City, CA, USA). Standard curves were generated in parallel from known amounts of *C. trachomatis* and murine DNA, and these curves were used to calculate the amount (pg) of *Chlamydia* DNA per unit weight (μg) of mouse DNA in the samples.

Genotyping and mapping

SSLP markers for mapping were selected from the Broad Institute (http://www.broad.mit.edu) and Mouse Genome Informatics (MGI, http://www.informatics.jax.org). Genetic positions were obtained from the composite genetic map at MGI, and physical positions from the genomic assembly at The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Many of the markers with polymorphism sizes$\geqslant$6 basepairs were amplified by standard PCR methods, and electrophoresed on 4% agarose gels; remaining markers were analyzed by fluorescent PCR on an ABI 3700 DNA Analyzer according to protocols furnished by the manufacturer. For genetic analysis, the genotype and corrected phenotype data (see Results) were entered into MapManager QTX.[23]

Statistics and analysis

Statistical regression was initially used to identify markers with linkage to the trait; where significant linkage was found, interval mapping was used to generate linkage data for regions between markers. The level of significance was based on a distribution of the linkage statistic generated from 10^5 random permutations of the data. Estimates of nongenetic variance (σ^2_{noise}) were used to infer the amount of the total variance attributable to genetic differences among our F2 animals ($\sigma^2_{\text{genetic}}$), and were based on 15 C57BL/6J and C3H/HeJ control animals injected in parallel with the F2 animals. Where

$$\sigma^2_{\text{total}} = \sigma^2_{\text{noise}} + \sigma^2_{\text{genetic}},$$

the percent genetic contribution to the total variance is given by

$$(\sigma^2_{\text{total}} - \sigma^2_{\text{noise}}/\sigma^2_{\text{total}}).$$

σ^2_{noise} is the variance among the control animals and had values of 3.21×10^{-6} for the C57BL/6J animals and 1.48×10^{-5} for the C3H/HeJ animals. Thus, on average, 68% of the variance among our F2 animals could be attributed to genetic differences. Each of our QTL explained roughly 11% of the variance among our F2 animals; therefore, half of the genetic variance was accounted for by the three loci that we detected in our cross.

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References

1 Schachter J. Infection and disease epidemiology. In: Stephens RS (ed). *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*. ASM Press: Washington DC, 1999, pp 139–169.

2 Hackstadt T. Cell biology. In: Stephens RS (ed). *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*. ASM Press: Washington DC, 1999, pp 101–138.

3 Brunham RC. Human immunity to chlamydiae. In: Stephens RS (ed) *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*. ASM Press: Washington DC, 1999, pp 211–238.

4 Witkin SS, Linhares I, Giraldo P, Jeremias J, Ledger WJ. Individual immunity and susceptibility to female genital tract infection. *Am J Obstet Gynecol* 2000; **183**: 252–256.

5 Kimani J, Maclean IW, Bwayo JJ, MacDonald K, Oyugi J, Maitha GM *et al*. Risk factors for *Chlamydia trachomatis* pelvic inflammatory disease among sex workers in Nairobi, Kenya. *J Infect Dis* 1996; **173**: 1437–1444.

6 Cohen CR, Gichui J, Rukaria R, Sinei SS, Gaur LK, Brunham RC. Immunogenetic correlates for *Chlamydia trachomatis*-associated tubal infertility. *Obstet Gynecol* 2003; **101**: 438–444.

7 Geisler WM, Tang J, Wang C, Wilson CM, Kaslow RA. Epidemiological and genetic correlates of incident *Chlamydia trachomatis* infection in North American adolescents. *J Infect Dis* 2004; **190**: 1723–1729.

8 Wang C, Tang J, Geisler WM, Crowley-Nowick PA, Wilson CM, Kaslow RA. Human leukocyte antigen and cytokine gene variants as predictors of recurrent *Chlamydia trachomatis* infection in high-risk adolescents. *J Infect Dis* 2005; **191**: 1084–1092.

9 Conway DJ, Holland MJ, Bailey RL, Campbell AE, Mahdi OS, Jennings R *et al*. Scarring trachoma is associated with polymorphism in the tumor necrosis factor alpha (TNF-alpha) gene promoter and with elevated TNF-alpha levels in tear fluid. *Infect Immun* 1997; **65**: 1003–1006.

10 Rupp J, Goepel W, Kramme E, Jahn J, Solbach W, Maass M. CD14 promoter polymorphism -159C>T is associated with susceptibility to chronic *Chlamydia pneumoniae* infection in peripheral blood monocytes. *Genes Immun* 2004; **5**: 435–438.

11 Qiu H, Yang J, Bai H, Fan Y, Wang S, Han X *et al*. Less inhibition of interferon-gamma to organism growth in host cells may contribute to the high susceptibility of C3H mice to *Chlamydia trachomatis* lung infection. *Immunol* 2004; **111**: 453–461.

12 Tuffrey M, Falder P, Gale J, Quinn R, Taylor-Robinson D. Infertility in mice infected genitally with a human strain of *Chlamydia trachomatis*. *J Reprod Fert* 1986; **78**: 251–260.

13 Tuffrey M, Alexander F, Woods C, Taylor-Robinson D. Genetic susceptibility to chlamydial salpingitis and subsequent infertility in mice. *J Reprod Fert* 1992; **95**: 31–38.

14 de la Maza LM, Pal S, Khamesipour A, Peterson EM. Intravaginal inoculation of mice with the *Chlamydia trachomatis* mouse pneumonitis biovar results in infertility. *Infect Immun* 1994; **62**: 4675–4681.

15 Darville T, Andrews Jr CW, Laffoon KK, Shymasani W, Kishen LR, Rank RG. Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response. *Infect Immun* 1997; **65**: 3065–3073.

16 Darville T, Andrews Jr CW, Sikes JD, Fraley PL, Rank RG. Early local cytokine profiles in strains of mice with different outcomes from chlamydial genital tract infection. *Infect Immun* 2001; **69**: 3556–3561.

17 Darville T, Andrews Jr CW, Sikes JD, Fraley PL, Braswell L, Rank RG. Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. *Infect Immun* 2001; **69**: 7419–7424.

18 Rottenberg ME, Gigliotti-Rothfuchs A, Wigzell H. The role of IFN-gamma in the outcome of chlamydial infection. *Curr Opin Immunol* 2002; **14**: 444–451.

19 Buzoni-Gatel D, Bernard F, Pla M, Rodolakis A, Lantier F. Role of H-2 and non-H-2-related genes in mouse susceptibility to *Chlamydia psittaci*. *Microb Pathog* 1994; **16**: 229–233.

20 Dietrich WF. Using mouse genetics to understand infectious disease pathogenesis. *Genome Res* 2001; **11**: 325–331.

21 Buer J, Balling R. Mice, microbes and models of infection. *Nat Rev Genet* 2003; **4**: 195–205.
22 Brunham RC, Kuo C, Chen WJ. Systemic *Chlamydia trachomatis* infection in mice: a comparison of lymphogranuloma venereum and trachoma biovars. *Infect Immun* 1985; **48**: 78–82.
23 Manly KF, Cudmore Jr RH, Meer JM. Map Manager QTX, cross-platform software for genetic mapping. *Mamm Genome* 2001; **12**: 930–932.
24 Ott J. *Analysis of Human Genetic Linkage*. The Johns Hopkins University Press: Baltimore & London, 1991, pp 66–67.
25 Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 1995; **11**: 241–247.
26 Nelson DE, Virok DP, Wood H, Roshick C, Johnson RM, Whitmire WM *et al*. Chlamydial IFN-gamma immune evasion is linked to host infection tropism. *Proc Natl Acad Sci USA* 2005; **102**: 10658–10663.
27 Flint J, Valdar W, Mott R. Strategies for mapping and cloning quantitative trait genes in rodents. *Nat Rev Genet* 2005; **6**: 271–286.
28 Howard L, Orenstein NS, King NW. Purification on renografin density gradients of *Chlamydia trachomatis* grown in the yolk sac of eggs. *Appl Microbiol* 1974; **27**: 102–106.
29 Steele LN, Balsara ZR, Starnbach MN. Hematopoietic cells are required to initiate a *Chlamydia trachomatis*-specific CD8+ T cell response. *J Immunol* 2004; **173**: 6327–6337.