Published in final edited form as:

Mamm Genome. 2011 October ; 22(0): 544–555. doi:10.1007/s00335-011-9343-5.

Quantitative trait loci in a bacterially induced model of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBDs) are complex disorders caused by a combination of environmental, microbial, and genetic factors. Genome-wide association studies in humans have successfully identified multiple genes and loci associated with disease susceptibility, but the mechanisms by which these loci interact with each other and/or with environmental factors (i.e., intestinal microbiota) to cause disease are poorly understood. *Helicobacter hepaticus*-induced intestinal inflammation in mice is an ideal model system for elucidating the genetic basis of IBD susceptibility in a bacterially induced system, as there are significant differences in *H. hepaticus*-induced disease susceptibility among inbred mouse strains. Infected A/J mice develop acute overexpression of proinflammatory cytokines followed 2–3 months later by chronic cecal inflammation, whereas infected C57BL/6 mice fail to develop cecal inflammation or increased cytokine expression. The goal of this project was to use quantitative trait locus (QTL) mapping to evaluate genetic factors that contribute to the differential disease susceptibility between these two mouse strains. Using acute cecal IL-12/23p40 expression as a biomarker for disease susceptibility, QTL analysis of *H. hepaticus*-infected F₂ mice revealed involvement of multiple loci. The loci with the strongest association were located on Chromosome 3 and Chromosome 17, with logarithm of odds (LOD) scores of 6.89 and 3.09, respectively. Cecal expression of IL-12/23p40 in *H. hepaticus*-infected C57BL/6J-Chr3^{A/J}/NaJ chromosome substitution mice had an intermediate phenotype, significantly higher than in resistant C57BL/6 but lower than in susceptible A/J mice, confirming the importance of this locus to the immune response to *H. hepaticus* infection.

Introduction

The inflammatory bowel diseases (IBDs) are complex disorders with multiple factors contributing to disease pathogenesis. It is widely accepted that IBD results from a dysregulated intestinal mucosal immune response in genetically susceptible individuals and that inflammation is induced or exacerbated by various environmental factors, including, but not limited to, smoking status, diet, and intestinal microbiota (Podolsky 2002). In recent years, much attention has been devoted to understanding the roles that various genes play in the pathogenesis of the two most common forms of IBD: Crohn's disease and ulcerative colitis. Through multiple genome-wide association studies in humans, at least 70 loci have been associated with susceptibility to Crohn's disease and 30 loci have been associated with ulcerative colitis (Barrett et al. 2008; Franke et al. 2010; McGovern et al. 2010). Roughly half of the loci associated with ulcerative colitis are also associated with susceptibility to Crohn's disease, suggesting that some genes play a general role in intestinal inflammation, while others are more specific to the pathogenesis of either Crohn's disease or ulcerative colitis (McGovern et al. 2010). The identification of so many putative IBD genes with relatively small effects suggests that interactions between multiple genes may be of critical importance to IBD pathogenesis (Franke et al. 2010). Investigations using mouse models of IBD have also helped identify loci and candidate genes associated with intestinal inflammation (de Buhr et al. 2006, 2009). Importantly, because mice of varying genetic architectures can be rapidly produced, these models are amenable to studies of complex interactions not possible with human populations.

The composition of the intestinal microbiota is another key factor involved in the development of IBDs. Multiple bacterial species have been implicated in disease pathogenesis but no single pathogenic agent has been consistently identified. Several mouse models have shown a requirement for the intestinal microbiota in the development of disease. To this end, when mice are rendered germ-free, these models do not develop intestinal inflammation, but when they are subsequently colonized under "specific-pathogen-free" conditions, or with specific bacterial species such as *Helicobacter hepaticus*, they develop inflammation (Nell et al. 2010; Sellon et al. 1998). Given the likely complex interaction between genetic susceptibility and intestinal bacteria, we sought to identify genes involved in susceptibility to bacterially induced inflammation in an established mouse model of inflammatory bowel disease.

We and others have observed a distinct difference between the response of A/J and C57BL/6 mouse strains to infection with the bacterium *H. hepaticus* (Fox et al. 1996; Ihrig et al. 1999; Myles et al. 2007; Whary et al. 1998). A/J mice develop chronic cecal inflammation and increased cecal mucosal expression of Th1-type inflammatory cytokines and chemokines, whereas C57BL/6 mice do not develop inflammation or upregulate cytokine and chemokine expression (Myles et al. 2007). Like human IBD, cytokine genes dysregulated in this model include IFN γ , TNF α , and IL-12/23p40, which is shared by IL-12 and IL-23. Importantly, blockage of IFN γ or IL-12/23p40 abrogates disease indicating a definitive role for these genes in disease pathogenesis (Kullberg et al. 2001; Myles et al. 2007; Neurath et al. 1995; Peyrin-Biroulet et al. 2008). Moreover, IL-12/23p40 expression is elevated in susceptible strains as early as 4 days post-inoculation, making it an ideal biomarker for disease susceptibility.

We sought to use quantitative trait locus (QTL) analysis to identify genes that contribute to differential disease susceptibility between the A/J and C57BL/6 mouse strains. This analysis identified two loci on Chromosomes 3 and 17 that are associated with strain differences in expression of IL-12/23p40. We further confirmed, through the use of the C57BL/6-Chr3^{A/J} chromosome substitution mouse strain, that the presence of a Chromosome 3 from the

susceptible A/J mouse on an otherwise resistant C57BL/6 mouse strain resulted in increased IL-12/23p40 expression upon inoculation with *H. hepaticus*, but the presence of this chromosome alone was insufficient to result in intestinal inflammation. These findings suggest that other loci are also necessary to reach an underlying susceptibility threshold for inflammation. Thus, this model will be ideal for future studies investigating the genetic interactions necessary for disease susceptibility.

Materials and methods

Mice

This study was conducted in accordance with guidelines set forth by the Guide for the Care and Use of Laboratory Animals and approved by the University of Missouri-Columbia Animal Care and Use Committee. C57BL/6Cr and A/JCr mice were obtained from Frederick Cancer Research and Development Center, Frederick, MD. Reciprocal F₁ crosses were generated by mating female A/JCr mice to male C57BL/6 mice (AB6 F₁) and C57BL/6 females to A/J males (B6A F₁). F₂ mice were generated by brother × sister matings of all combinations of F₁ animals (AB6 × AB6, B6A × B6A, AB6 × B6A, and B6A × AB6). Breeding trios of C57BL/6J-Chr3^{A/J}/NaJ and C57BL/6J-Chr17^{A/J}/NaJ (JAX stock Nos. 004381 and 004395, respectively) were obtained from the Jackson Laboratory (Bar Harbor, ME) to establish breeding colonies.

Bacterial culture and mouse inoculation

Isolation and growth of *H. hepaticus* strain MU-94 has previously been described (Livingston et al. 2004). Briefly, 1.5 ml of a stock solution containing approximately 5 × 10⁸ bacteria/ml of *H. hepaticus* was diluted in 15 ml of brucella broth (Becton Dickinson, Franklin Lakes, NJ), divided equally between three sheep blood agar plates, and incubated for 24 h at 37°C in a microaerobic environment with 90% N₂/5% H₂/5% CO₂. After 24 h, the broth containing bacteria was transferred to a 250-ml Erlenmeyer flask along with 35 ml of fresh brucella broth supplemented with 10% of fetal bovine serum (Sigma-Aldrich Co., St. Louis, MO). The culture was incubated for another 24 h at 37°C under the same microaerobic conditions with constant stirring. Three- to four-week-old mice of both sexes were individually inoculated via gavage with 0.5 ml of culture containing 5 × 10⁸ bacteria/ml. Fecal colonization was confirmed in all mice after inoculation and at necropsy by *H. hepaticus*-specific PCR (Riley et al. 1996).

Necropsy and sample collection

Depending on the study, mice were euthanized for sample collection at either 4 days or 90 days post-inoculation. At the time of sacrifice, ceca were removed, laid on note cards, and opened longitudinally along the mesenteric border. The cecal contents and feces were removed and used to confirm *H. hepaticus* colonization by PCR. The cleaned cecum was then split longitudinally. One half was rinsed with sterile PBS then flash frozen in liquid nitrogen for use in cytokine gene expression analysis. The other half was fixed in zinc fixative and embedded in paraffin. Five-micron sections were prepared and stained with hematoxylin and eosin for histologic examination and lesion scoring.

IL-12/23p40 cecal expression

Frozen ceca from all mice were thawed in a solution of phenol and guanidine isothiocyanate (TRIzol reagent, Invitrogen, Carlsbad, CA). Cecal sections were homogenized using a Tissuelyser (Qiagen, Valencia, CA) for 2 min at 30 Hz. Total RNA was isolated as per the manufacturer's instructions (Invitrogen). The extracted RNA was then dissolved in 75 µl of DEPC-treated water (Sigma-Aldrich, St. Louis, MO). Quantity and quality of RNA were

assessed by measuring the absorbance at 260 and 280 nm (NanoDrop-1000 spectrophotometer, NanoDrop Technologies, Wilmington, DE).

Five micrograms of total RNA was reverse-transcribed using the Super Script III kit (Invitrogen) by following the oligo(dT) primer protocol. The resultant cDNA was diluted with DEPC-treated water to a final expected concentration of 20 ng/μl.

Real-time PCR was performed on the Roche Light Cycler 2.0 using Qiagen Quantitect SYBR® Green. All expression was normalized to cecal levels of *HPRT* expression. Run conditions for real-time PCR have been previously described and primers used included (1) *HPRT* forward, 5'-GTAATGATCAGTCAACGGGGGAC-3'; (2) *HPRT* reverse, 5'-CCAGCAAGCTTGCAACCTTAACCA-3'; (3) *IL-12/23p40* forward, 5'-ACTCACATCTGCTGCTCCAC-3'; (4) *IL-12/23p40* reverse, 5'-GGGAAGTCTACTGCTCTTGA-3' (Myles et al. 2007).

Genotyping

Tail biopsies, collected at necropsy, were digested in Proteinase K, and DNA was isolated with the Qiagen DNeasy spin columns following the manufacturer's protocol. A total of 86 fluorescently labeled microsatellite primers, with an average spacing of 16 cM along each chromosome, were obtained from Applied Biosystems (ABI, Carlsbad, CA). Microsatellites were amplified via PCR following the manufacturer's protocol and analyzed using an ABI 3100 Genetic Analyzer and Gene Mapper software version 4.0. Additional SNP genotyping was performed using the Illumina LD Linkage Panel consisting of 377 SNP markers (Illumina, San Diego, CA) following the manufacturer's protocol. Samples were genotyped on the Illumina Bead-Array Reader. Analyses were carried out using the Illumina BeadStudio software and included control samples from A/J, C57BL/6, and F₁ mice. A total of 244 SNPs informative between A/J and C57BL/6 were used for subsequent analysis.

Lesion scoring

Cecal lesions in mice sacrificed at 90 days post-inoculation were evaluated using a previously described protocol (Myles et al. 2003). Briefly, lesions were scored blindly for intensity (0 = none, 1 = mild, 2 = moderate, and 3 = severe), longitudinal extent (1 = one or two small foci, 2 = patchy, and 3 = diffuse), and vertical extent of inflammation (1 = basal mucosal, 2 = full-thickness mucosal, and 3 = transmural inflammation). In addition, lesions were scored for hyperplasia by using the following criteria: the presence of basophilic staining "crypt" epithelial cells in at least the lower two thirds of the gland or at least doubling of the height of the mucosal epithelium. Focal hyperplasia was given a score of 1 and diffuse hyperplasia was given a score of 2. The scores for intensity of inflammation, longitudinal and vertical extents of inflammation, and hyperplasia were added to give a total score for each animal. Because the minimum inflammation score using this system is 3 (mild, focal, and basal), inflammation and hyperplasia were not on comparable scales. To rectify this bias, lesion scores of >2 were adjusted by subtracting 2 from the total score to obtain a total adjusted score.

Data Analysis

IL-12/23p40 expression and cecal lesion scores were compared using one-way ANOVA with Student Newman-Keuls *post-hoc* analysis. Differences among groups were considered significant if $p < 0.05$ (SigmaPlot ver. 11, San Jose, CA). QTL analysis of log₂ transformed *IL-12/23p40* levels from F₂ mice was performed utilizing R ver. 2.9, R/QTL, and J/QTL software package ver. 1.3.0 (The Jackson Laboratory, Bar Harbor, ME) (Broman et al. 2003; Smith et al. 2009). In J/QTL, a parametric one QTL scan (Multiple Imputation model) was used to calculate the logarithm of odds (LOD) score, and 10,000 phenotype permutations

were run to empirically determine the genome-wide significance threshold. LOD scores were considered significant if $p < .05$ genome-wide.

Results

Response to *Helicobacter hepaticus* in F₁ mice

Our lab and others have previously reported a distinct, strain-dependent immune response to *H. hepaticus* (Fox et al. 1996; Ihrig et al. 1999; Myles et al. 2007; Whary et al. 1998). *H. hepaticus*-infected A/J mice develop increased inflammatory cytokine expression (IL-12/23p40 and IFN γ) within 4 days of inoculation and histologic signs of disease accompanied by increased inflammatory cytokine expression are seen by 90 days post-inoculation. C57BL/6 mice are resistant to *H. hepaticus* and do not develop inflammation or increased cytokine expression despite colonization levels equivalent to A/J mice (Myles et al. 2007). To determine if susceptibility or resistance to *H. hepaticus*-induced inflammation was inherited additively, reciprocal male and female F₁ mice (AB6 and B6A F₁) were inoculated with *H. hepaticus*. Cecal expression of IL-12/23p40 mRNA was measured in animals at 4 days and 90 days post-inoculation. Cecal inflammation was also assessed by histology in F₁ mice 90 days post-inoculation.

At 4 days post-inoculation (Fig. 1a), A/J mice had significantly higher cecal expression of IL-12/23p40 than the infected C57BL/6 mice. IL-12/23p40 expression levels in B6A F₁ mice were significantly lower than in the A/J controls, but significantly higher than in the C57BL/6 controls. IL-12/23p40 expression levels in AB6 F₁ mice were significantly lower than in the A/J controls, but not significantly different than levels in C57BL/6 mice. These differences in expression are not a result of differing basal expression as there was not a significant difference in IL-12/23p40 levels in uninoculated A/J, C57BL/6, AB6 F₁, or B6A F₁ mice (data not shown).

By 90 days post-inoculation, A/J mice had significantly elevated IL-12/23p40 expression and lesion scores when compared to C57BL/6 mice, and, as previously reported (Livingston et al. 2004; Myles et al. 2007), female A/J mice had higher expression levels and lesion scores when compared to males. Lesion scores and cytokine expression levels of infected F₁ mice of all crosses were similar to those of the C57BL/6 parental strain (Fig. 1b, c). Collectively, these results show that the response to *H. hepaticus* is complex and nonadditive and suggests that maternal factors also affect the disease response.

QTL analysis reveals two loci associated with increased IL-12/23p40 expression

To map the loci responsible for susceptibility to *H. hepaticus*-induced inflammation, we examined the expression of IL-12/23p40 in F₂ mice at 4 days post-inoculation. Since there was a difference in the early expression of IL-12/23p40 between the AB6 and the B6A F₁ mice, we generated F₂ animals from all possible parental strain combinations. Ninety-one AB6 $\times$ AB6, 95 B6A $\times$ B6A, 78 AB6 $\times$ B6A, and 50 B6A $\times$ AB6 mice were inoculated with *H. hepaticus* and assessed for cecal IL-12/23p40 expression (Fig. 2a). Expression of p40 within the F₂ population varied widely from high (A/J-like) to low (C57BL/6-like).

The 93 F₂ mice with the highest and the 93 mice with the lowest IL-12/23p40 expression were genotyped using a combination of 86 microsatellite and 244 SNP markers. Linkage analysis of all F₂ animals identified two QTLs with significant LOD scores (LOD > 2.9) associated with IL-12/23p40 expression levels (Fig. 2b). The QTL with the largest LOD score of 6.89 was on Chromosome 3. A second QTL with a LOD score of 3.09 was located on the proximal end of Chromosome 17. The QTL on Chromosome 3 explained 10% of the total variance of IL-12/23p40 expression in the F₂ population. The peak signal for this QTL was at SNP *mCV23483645* located at 123 Mbp (NCBI build 37). The flanking markers

defined a 20-Mbp region from 116.7 to 136 Mbp. The QTL on Chromosome 17 explained 4% of the variance in IL-12/23p40 expression in the F₂ population. The peak signal for this QTL was at SNP *rs6239530* at 3.8 Mbp and the flanking region consisted of a 12.7-Mbp region on the proximal end of Chromosome 17 (results summarized in Table 1).

Sex influences IL-12/23p40 expression-associated QTL

We have previously observed a sex influence in the extent of intestinal inflammation of *H. hepaticus*-infected A/J mice. Infected female mice consistently develop more severe intestinal inflammation and increased expression of inflammatory cytokines in the cecum (Livingston et al. 2004). To test if sex influenced IL-12/23p40-associated QTLs in the F₂ population, the data were partitioned based on sex and analyzed separately. Genotypes for 97 female mice were included in this analysis, which revealed a strong QTL on Chromosome 3 with a significant LOD score of 4.23 (Fig. 3a). This locus was in the same position (i.e., SNP *mCV23483645*) identified as when males and females were jointly analyzed (Fig. 2b). Another, previously unidentified QTL at approximately 58 Mbp on Chromosome 11 was also detected in the female population. This QTL was at SNP *rs13481045* and had a LOD score of 3.16.

Analysis of the 88 genotyped male F₂ mice revealed two QTL peaks on Chromosome 3 (Fig. 3b). The QTL with the highest LOD score of 3.13 was at marker *D3Mit98* (86 Mbp). Another QTL at marker *D3Mit57* (116 Mbp) also reached significance with a LOD score of 3.09. The second locus was within 8 Mbp of *mCV23483645*, the SNP identified in the joint analysis of all F₂ animals. Given its proximity to SNP *mCV23483645*, it is likely that the locus associated with *D3Mit57* is the same locus detected in the original analysis of the entire F₂ population. It is also possible that the QTL at marker *D3Mit98* may be a false positive due to a reduced sample size from the original analysis. Analysis of the male samples also identified a significant QTL on Chromosome 17 at SNP *rs6239530* (3.8 Mbp), with a LOD score of 3.47. This QTL was previously identified in the analysis of all F₂ animals. Comparison of the male and female analyses showed that the QTL on Chromosome 3 is conserved and not influenced by the sex of the mice. The previously identified QTL on Chromosome 17 appears to influence the inflammatory response only in males, while a previously unidentified QTL on Chromosome 11 appears to influence only the female response.

Parent of origin influences IL-12/23p40 expression-associated QTL

Because the IL-12/23p40 expression in the reciprocal F₁ crosses differed between B6A and AB6 F₁ mice, the influence of the direction of the cross on susceptibility to *H. hepaticus*-induced gene expression was further assessed. F₂ mice with a B6A mother (B6A × B6A and B6A × AB6 F₂ mice) had significantly higher IL-12/23p40 expression than mice with an AB6 mother (AB6 × AB6 and AB6 × B6A F₂ mice) (Fig. 4a). To assess the presence of genetic loci with parent-of-origin effects, AB6 × AB6 and the B6A × B6A F₂ mice were analyzed individually (Fig. 4b, c). Analysis of 78 AB6 × AB6 mice identified the QTL previously seen in the global analysis (Chromosome 3 at SNP *mCV23483645*) with a LOD score of 5.76 (Fig. 4b). Analysis of the reciprocal B6A × B6A F₂ population (*n* = 80) also identified a significant QTL on Chromosome 3 at marker *D3Mit320* (LOD 3.56), 20 Mbp downstream of the locus identified in the original scan (Fig. 4c). In the B6A × B6A cross, a previously unidentified QTL was observed on Chromosome 7 at SNP *mCV24269234*, with a LOD score of 2.89. This QTL may contribute to the differences in IL-12/23p40 expression observed in the F₁ and F₂ populations of mice as this QTL was identified only in the B6A maternally derived population.

Presence of A/J alleles on Chromosome 3 increases cecal IL-12/23p40 expression

Because the QTL on Chromosome 3 was consistently detected regardless of the cross used to produce the F₂, we sought to test its affect on susceptibility to *H. hepaticus* utilizing the C57BL/6 chromosome substitution mice with an A/J Chromosome 3 (C57BL/6J-Chr3^{A/J}/NaJ) developed in the laboratory of Dr. Joe Nadeau (Singer et al. 2004). These mice were infected with *H. hepaticus* and assessed for cecal IL-12/23p40 expression and/or inflammation at 4 and 90 days post-inoculation. At 4 days post-inoculation IL-12/23p40 expression in infected B6.A-Chr3 mice was significantly higher compared to expression in C57BL/6 mice and significantly lower than expression in the A/J mice (Fig. 5a). At 90 days post-inoculation, the IL-12/23p40 expression in B6.A-Chr3 mice was again intermediate to the two parental strains (Fig. 5b). However, even with the moderate increase in IL-12/23p40 expression, B6.A-Chr3 mice failed to develop cecal inflammation, and lesion scores in these mice were not significantly different from those in C57BL/6 controls (Fig. 5c). Taken together, these data show that the presence of A/J alleles at this susceptibility locus on Chromosome 3 on an otherwise resistant background moderately increases IL-12/23p40 expression, but these alleles are not sufficient to render mice susceptible to disease. This suggests that disease susceptibility is polygenic.

Discussion

Inflammatory bowel diseases result from a dysregulated intestinal mucosal immune response in genetically susceptible individuals that is induced or exacerbated by various environmental factors. Recent genome-wide association studies have implicated over 70 genes in the pathogenesis of Crohn's disease and 30 genes in the pathogenesis of ulcerative colitis, the two most prevalent forms of IBD (Franke et al. 2010). Infection of mice with *H. hepaticus* provides an ideal animal model for the study of inflammatory bowel diseases. In particular, this model is very amenable to the study of genetic factors of disease because different strains of mice exhibit differing susceptibilities that occur without genetic manipulation affecting immunomodulation as with other models (e.g., *IL-10* knockouts). Specifically, infected A/J mice develop a chronic inflammatory disease of the cecum while similarly infected C57BL/6 mice do not. Intestinal inflammation is characterized by a dysregulated Th1-driven immune response, which manifests as segmental inflammation in the intestinal tract. Unlike many models of IBD which are acute, disease in this model is chronic, with lesion development occurring at approximately 90 days post-inoculation. However, changes in cecal inflammatory gene expression can be seen as early as 4 days post-inoculation (Myles et al. 2007). Thus, this model is ideal for the identification of genes that may be involved at preinflammatory stages of disease. Using this model and expression of IL-12/23p40 as a biomarker of disease, the studies performed herein identified a major QTL associated with susceptibility to bacterially induced intestinal inflammation and two additional QTLs that were associated with inflammation in subsets of the examined animals.

We first determined the mode of inheritance of disease susceptibility by examining the immune response to *H. hepaticus* infection in F₁ mice. Like their C57BL/6 parents, infected F₁ mice did not develop intestinal inflammation or dysregulated gene expression 90 days post-inoculation. Analysis of the early IL-12/23p40 expression in response to infection in F₁ mice again demonstrated a response similar to that of C57BL/6 mice; however, significant differences were seen between B6A and AB6 F₁ mice, suggesting that the parental pairing and/or maternal environment influenced this response.

Utilizing reciprocal F₂ populations of mice from crosses between A/J and C57BL/6 mice, two QTLs associated with increased early expression of IL-12/23p40 in response to *H. hepaticus* infection were identified. The first locus was located on the distal end of Chromosome 3 in a region approximately 20 Mbp in size from 116.7 to 136 Mbp. The

second was located within a 12.7-Mbp region on the proximal end of Chromosome 17. The QTL on Chromosome 3 was detected in all F₂ subpopulations regardless of sex or direction of cross. Subsequent testing of *H. hepaticus*-infected C57BL/6J-Chr3^{A/J}/NaJ chromosome substitution mice showed that the presence of the susceptible A/J Chromosome 3 in an otherwise resistant C57BL/6 mouse leads to increased IL-12/23p40 expression after inoculation.

Several studies in other mouse models have also identified loci on Chromosome 3 associated with intestinal inflammation, strengthening the evidence that this locus plays a critical role in aberrant intestinal inflammation (Table 2). For example, C3H/HeN mice deficient for *Gnai2*^{-/-} develop severe colitis while C57BL/6 mice with this deficiency do not. Analysis of an F₂ cross between *Gnai2*^{-/-} C3H/HeN and *Gnai2*^{-/-} C57BL/6 mice revealed multiple QTLs responsible for the development of colitis. Included was a major QTL on Chromosome 3 (*Gpdc1*) between markers *D3Mit316* and *D3Mit348* from 120 to 127 Mbp, similar to the location of the Chromosome 3 QTL identified in the present study (Borm et al. 2005). In the IL-10-deficient mouse model of colitis, QTL analysis revealed at least ten QTLs (Bleich et al. 2010). A large-effect QTL on Chromosome 3 (*Cdcs1*) was detected in F₂ mice derived from C3H/HeJBir and C57BL/6 IL-10-deficient mice, which differ in susceptibility to spontaneous intestinal inflammation. This QTL was subsequently confirmed in an N₂ population of the same IL-10-deficient mice (Farmer et al. 2001). This locus was associated with cecal and colonic inflammation and overlaps the location of the QTL identified in the *Gnai2*^{-/-} mice and the studies described herein.

The 20-Mbp QTL region on Chromosome 3 identified in the present study contains 200 SNPs polymorphic between A/J and C57BL/6 mice, located in the coding regions of nearly 40 genes (<http://phenome.jax.org/SNP/>). Many of these genes are excellent candidates that have previously been associated with inflammatory responses. For example, *Nfkb1*, a gene located at 135 Mbp, encodes NF- κ B, a transcription factor responsible for controlling various immune responses such as the response to pathogenic organisms. Perturbations in NF- κ B have also been associated with inflammatory bowel diseases in both mice and humans, and mice deficient in NF- κ B are highly susceptible to *Helicobacter*-induced intestinal inflammation (Erdman et al. 2001). Moreover, studies of the human syntenic region harboring *Cdcs1* have revealed certain human *Nfkb1* haplotypes enriched in Crohn's disease patients. Patients with these haplotypes have increased circulating anti-flagellin antibodies and decreased NF- κ B expression and function, resulting in an inability to appropriately respond to pathogenic organisms in the gut (Takedatsu et al. 2009).

While *Nfkb1* is an attractive candidate gene, other genes may be more important in other subsets of inflammatory bowel disease (and their respective animal models). For example, microarray analysis of mouse strains that are susceptible (C3H/HeJBir) and resistant (C57BL/6) to intestinal inflammation in the IL-10-deficient model revealed no differences in *Nfkb1* expression. Rather, differences in the expression of the Chromosome 3 gene *Gbp1* were seen (de Buhr et al. 2006). Interestingly, microarray analysis of *H. hepaticus*-infected parental strains used in the present study (A/J and C57BL/6) also revealed dys-regulation of the *Gbp1* and *Gbp2* genes (Myles et al. 2007). How dysregulation of *Gbp* genes might result in inflammatory bowel disease is unknown, but at least two possibilities exist. Genes in the guanylate binding protein family (GBP) are induced by INF γ signaling and mediate the effects of proinflammatory cytokines (Guenzi et al. 2001). Thus, dysregulation could result in an uncontrolled proinflammatory immune response. Knockdown of *Gbp1* with siRNA also increases permeability of the intestinal epithelium and epithelial apoptosis (Schnoor et al. 2009). Because defects in intestinal permeability have been demonstrated in humans with IBD, this may represent an additional mechanism by which dysregulation of this gene increases susceptibility to intestinal inflammation (Salim and Söderholm 2011).

The role of *Cdcs1* in the pathogenesis of intestinal inflammation in IL-10-deficient mice was further assessed in reciprocal congenic mice containing the *Cdcs1* locus. These studies showed that the presence of the C3H/HeJBir susceptibility locus in resistant C57BL/6 mice conferred susceptibility to disease and altered the response of in vitro stimulated immune cells (Beckwith et al. 2005). Similarly in our studies, *H. hepaticus*-infected C57BL/6J-Chr3^{A/J}/NaJ chromosome substitution mice developed moderately increased IL-12/23p40 expression. However, the presence of this susceptibility chromosome did not result in the development of histologic evidence of inflammation in infected mice. This discrepancy between our findings which used IL-10-competent mice and those seen in IL-10-knockout mice highlight the complexity of inflammatory bowel disease pathogenesis and further suggests that multiple genes likely interact to render mice and people fully susceptible to disease.

Further analysis of congenic mouse strains generated from the IL-10-deficient C3H/HeJBir and C57BL/6 mice suggests that *Cdcs1* may actually consist of at least three distinct regions involved in disease susceptibility (*Cdcs 1.1*, *Cdcs 1.2*, and *Cdcs 1.3*). Through the microarray analysis of susceptible congenic mice, additional candidate genes in this region were identified, including *Fcgr1*, *Cnn3*, *Larp7*, and *Alpk1* (Bleich et al. 2010). Future analysis of the expression and function of these genes in the *H. hepaticus*-induced model of intestinal inflammation will elucidate if any of these genes contribute to disease susceptibility. Collectively, the identification of overlapping QTLs on Chromosome 3 among multiple models of intestinal inflammation (IL-10 deficiency, *Gnai2* deficiency, and *H. hepaticus*-induced intestinal inflammation) indicates the importance of this region in controlling intestinal inflammation.

A QTL on Chromosome 17 was also identified in the primary scan of all F₂ mice and in the male subpopulation. This is a novel QTL that has not been identified in any previous mapping studies in mouse models of inflammatory bowel disease. The most attractive candidate gene in this region is the CC chemokine receptor 6 (*Ccr6*). *Ccr6* encodes a chemokine receptor that is upregulated on dendritic cells and memory T cells during inflammatory responses and allows these cells to home in on sites of inflammation. Polymorphisms in this gene have been associated with Crohn's disease, adding weight to the prospect that *Ccr6* is a key candidate for this QTL (Barrett et al. 2008). Additionally, in the female F₂ samples, a QTL on Chromosome 11 was identified. This QTL is in a location similar to that identified in dextran sulfate sodium-induced colitis and is very close to two attractive gene candidates that encode the cytokines interleukin 5 and interleukin 3 (Mähler et al. 1999). Both of these cytokines have been shown to be important in eosinophil survival and have been implicated in ulcerative colitis pathogenesis (Lampinen et al. 2004).

An unexpected but interesting finding of this study was that reciprocal F₁ and F₂ populations responded differently to infection with *H. hepaticus*. B6A F₁ mice expressed IL-12/23p40 intermediate to the controls, significantly higher than the resistant C57BL/6 mice but significantly lower than A/J mice. In contrast, the reciprocal AB6 F₁ mice had the same expression levels as C57BL/6 controls. F₂ mice derived from B6A mothers had higher mean expression of IL-12/23p40 than F₂ mice derived from AB6 mothers. We cannot attribute these changes in expression to a sex-linked locus as there were no differences in expression detected between males and females within strains in either generation. We speculate that either paternally imprinted genes or the maternal environment are responsible for this phenomenon. Unfortunately, with the F₂ strategy used, we were unable to identify the parent-of-origin alleles and thus could not test for the presence of imprinted loci. Future studies utilizing backcross (N₂) populations will allow for the distinction of the parental origin of alleles and will aid in understanding this phenomenon.

Environmental factors have also been demonstrated to impact parental effects and could conceivably modulate a difference in susceptibility among offspring of different parents. For example, studies of autoimmune disease models have shown that the postnatal maternal environment can affect disease phenotype. In mouse models of autoimmune ovarian disease and multiple sclerosis, cross-fostering of A/J pups to C57BL/6 mothers resulted in increased disease severity in both models. The authors speculated that these findings reflected differences in components of maternal milk (Case et al. 2010). In a mouse model of colitis, T-bet and Rag2 double knockout mice develop spontaneous colitis that requires the presence of intestinal microbiota. Cross-fostering of wild-type mice to double-knockout mothers resulted in the development of colitis, and the authors suggested that this was the result of transfer of maternal intestinal microbiota (Garrett et al. 2007). Moreover, commensal microbiota has been shown to be a critical factor in the pathogenesis of *Helicobacter*-triggered murine models of inflammatory bowel disease (Jergens et al. 2007). Similarly, pilot studies in our laboratory have shown that susceptibility to *H. hepaticus*-induced IL-12/23p40 expression increased in A/J pups cross-fostered to C57BL/6 mothers (data not shown), and that the latter had a higher incidence of colonization by bacteria of the family *Enterobacteriaceae*. It is conceivable that C57BL/6 mice harbor a more proinflammatory microbial population that when transferred to A/J mice renders them more susceptible to intestinal inflammation. Similarly, the increased expression of IL-12/23p40 in pups originating from C57BL/6 mothers or grandmothers may also be due to environmental factors such as differences in intestinal microbiota. Regardless, these findings have revealed potential new targets for investigation of inflammatory bowel pathogenesis.

Highlighting the complex nature of this disease, this study identified a major QTL on Chromosome 3 contributing 10% of the phenotypic variance in IL-12/23p40 expression in the analyzed F₂ mice. Moreover, two additional sex-specific QTLs were identified in subpopulations of the F₂ mice. Importantly, use of the *Helicobacter*-induced model will allow for the future investigation of complex gene interactions that contribute to susceptibility to intestinal inflammation. To this end, our future studies will first focus on identifying the gene or genes on Chromosome 3 that underlie the detected QTL. These studies will yield congenic mice that can be used to identify additional interacting loci that further enhance susceptibility to disease.

Acknowledgments

We thank Dr. Stephanie McKay of the Division of Animal Sciences, University of Missouri, for her assistance with the Illumina genotyping platform, and members of the Research Animal Diagnostic Laboratory for their assistance with this project. We also thank Dr. Aaron Ericsson of the Research Animal Diagnostic Laboratory for his assistance in preparing the manuscript. This work was supported by NIH grant K26 RR018811, a grant from the University of Missouri, Department of Veterinary Pathobiology and Research Animal Diagnostic Laboratory research funds.

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Fig. 1.

Susceptibility to *Helicobacter hepaticus* in F₁ crosses at 4 and 90 days post-inoculation. **a** Consistent with previous studies, A/J mice expressed significantly higher mean levels of IL-12/23p40 normalized to HPRT ($p < 0.05$) than did C57BL/6 mice at 4 days post-inoculation with *Helicobacter hepaticus*. Mean expression levels of IL-12/23p40 in AB6 F₁ mice were similar to those seen in C57BL/6 mice. In contrast, B6A F₁ mice had a slight but significant ($p < 0.05$) increase in p40 expression when compared to C57BL/6 controls. At 90 days post-inoculation, A/J mice had significantly higher lesion scores (**b**) and IL-12/23p40 expression (**c**) when compared to all other strains. Female A/J mice had higher lesion scores and cytokine expression than males. *F* female, *M* male

Fig. 2. Early expression of IL-12/23p40 in F₂ mice and QTL analysis. Expression of IL-12/23p40 at 4 days post-inoculation was used as a biomarker for disease susceptibility in 314 *Helicobacter hepaticus*-infected F₂ mice. **a** Distribution of phenotypes in the F₂ samples ranged from high (A/J-like) to low IL-12/23p40 expression (C57BL/6-like). **b** QTL analysis of 314 F₂ mice revealed significant QTLs on Chromosome 3 at *mCV23483645* and Chromosome 17 at *rs6239530*, with LOD scores of 6.89 and 3.09, respectively, associated with increased expression of IL-12/23p40 upon exposure to *H. hepaticus*. Hatch marks along the X axis indicate the position of individual markers and vertical dotted lines indicate chromosomal boundaries. Loci were considered significant if the LOD score reached an threshold >0.05

Fig. 3.

Influence of sex on IL-12/23p40 expression-associated QTL. **a** Linkage analysis of the 97 F₂ female mice revealed two QTLs with significant LOD scores: a QTL on Chromosome 3 at *mCV23483645* with a LOD score of 4.23 and a QTL on Chromosome 11 at *rs13481045* with a LOD score of 3.16. **b** Analysis of 88 F₂ male mice revealed two QTLs on Chromosome 3 at *D3Mit98* and *D3Mit57* with LOD scores of 3.13 and 3.09, respectively. In addition, a QTL was identified on Chromosome 17 at marker *rs6239530* with a LOD score of 3.47.

Fig. 4.

Early expression of IL-12/23p40 in subgroups of F₂ mice and QTL analysis. QTL analysis was performed on F₂ subsamples partitioned according to the maternal cross from which they were derived. **a** The mean IL-12/23p40 expression in F₂ mice derived from a B6A F₁ mother was significantly higher than the mean expression in F₂ mice derived from an AB6 F₁ mother. Analysis of individual F₂ crosses of **b** AB6 × AB6 F₂ mice and **c** B6A × B6A F₂ mice which consistently identified the locus on Chromosome 3 but not the Chromosome 17 QTL. Analysis of the B6A × B6A F₂ cross identified a QTL on Chromosome 7 that was not identified in the original scan

Fig. 5. Response of C57BL/6-Chr3^{A/J} mice to *Helicobacter hepaticus* at 4 days and 90 days post-inoculation. To test the effect of the QTL identified on Chromosome 3, C57BL/6J-Chr3^{A/J}/NaJ chromosome substitution mice (B6.A-Chr3) were inoculated with *Helicobacter hepaticus* and assessed for IL-12/23p40 expression at 4 and 90 days post-inoculation and cecal inflammation 90 days post-inoculation. **a** At 4 days post-inoculation, cecal IL-12/23p40 expression was significantly increased in B6.A-Chr3 mice compared to the resistant C57BL/6 controls. Expression of IL-12/23p40 was also significantly lower in the B6.A-Chr3 mice than in the susceptible A/J controls. **b** Expression of IL-12/23p40 90 days post-inoculation was increased in B6.A-Chr3 mice relative to C57BL/6 controls, but was not

increased to the levels of the susceptible A/J controls. **c** While IL-12/23p40 expression was greater in B6.A-Chr3 mice than in C57BL/6 mice, the increase was not sufficient to increase the overall inflammation in the ceca of infected animals. Inflammation scores in the B6.A-Chr3 mice were not significantly different than in the resistant C57BL/6 controls

Table 1Summary of *Helicobacter hepaticus* susceptibility loci

Cross-analyzed	Chr	Peak marker	Position (Mbp)	LOD score	Contributing allele	Mode of inheritance
F ₂ (all mice)	3	mCV23483645	123	6.89	A/J	Recessive
Female F ₂	17	rs6239530	3.8	3.09	A/J	Additive
	3	mCV23483645	123	4.23	A/J	Recessive
	11	rs13481045	58	3.16	A/J	Recessive
Male F ₂	3	D3Mit98	86	3.13	A/J	Additive
	3	D3Mit57	116	3.09	A/J	Additive
	17	rs6239530	3.8	3.47	A/J	Additive
AB6 × AB6 F ₂	3	mCV23483645	123	5.76	A/J	Recessive
B6A × B6A F ₂	3	D3Mit320	143	3.56	A/J	Recessive
	7	mCV24269234	127	2.89	C57BL/6	Dominant

Table 2

Colitis models and previously identified quantitative trait loci associated with intestinal inflammation

Colitis model	Strains used	Loci identified (chromosome)	QTL location (cM)	Candidate gene(s)	Citation
IL-10 ^{-/-}	C57BL/6 × C3H/HeJBir (F ₂ and N ₂)	<i>Cdcs 1</i> (Chr 3)	61.8	<i>Nrkb1, Egfr, Gbp1</i>	Beckwith et al. (2005), de Buhr et al. (2006, 2009), Farmer et al. (2001), Mähler et al. (2002)
		<i>Cdcs 2</i> (Chr 1)	32.8	<i>Casp8, Cd28, Ctla4</i>	
		<i>Cdcs 3</i> (Chr 2)	65	<i>Il1, Pcnal</i>	
		<i>Cdcs 4</i> (Chr 8)	21	<i>Defb, Sec8</i>	
		<i>Cdcs 5</i> (Chr 17)	19.06	<i>H2-Ea, Tnf, Hspl1a, Hspl1b, Lys66c</i>	
		<i>Cdcs 6</i> (Chr 18)	32	<i>Dec, Apc, Mad2, CD14</i>	
		<i>Cdcs 7</i> (Chr 12)			
		<i>Cdcs 8</i> (Chr 12)			
		<i>Cdcs 9</i> (Chr 4)		<i>Pla2g2a, Gnb1</i>	
		<i>Cdcs 10</i> (Chr 5)		<i>Areg</i>	
<i>Gnat2</i> ^{-/-}	C57BL/6 × C3H/HeN (F ₂)	<i>Gpdc1</i> (Chr 3)	62	<i>Nrkb1, Egfr, Gbp1</i>	Borm et al. (2005)
		<i>Gpdc2</i> (Chr 1)	47		
		<i>Gpdc3</i> (Chr 9)	42		
Dextran sulfate sodium (DSS)	C57BL/6 × C3H/HeJ (F ₂ and N ₂)	<i>Dssc1</i> (Chr 5)	72	<i>Selp1, Pdgrfa</i>	Mähler et al. (1999)
		<i>Dssc2</i> (Chr 2)	47	<i>Iiga4, Il1a, Il1b</i>	
Spontaneous	C57BL/6 × SAMP1/YitFc (F ₂)	<i>Ibdq1</i> (Chr 9)	34	<i>Il18, Il10ra</i>	Kozaiwa et al. (2003)
Trinitrobenzene sulfonic acid (TNBS)	C57BL/6 × SJL/J (F ₂)	<i>Tnbs1</i> (Chr 9)	48		Bouma et al. (2002)
		<i>Tnbs2</i> (Chr 11)	17	<i>Il12b</i>	