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***Cdcs1* a major colitis susceptibility locus in mice; subcongenic analysis reveals genetic complexity**

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Abstract

Background—The cytokine-deficiency induced colitis susceptibility (*Cdcs1*) locus is a major modifier of murine IBD and was originally identified in experimental crosses of interleukin-10-deficient (*Il10*^{−/−}) mice. Congenic mice, in which this locus was reciprocally transferred between IBD-susceptible C3H/HeJBir-*Il10*^{−/−} and resistant C57BL/6J-*Il10*^{−/−} mice, revealed that this locus likely acts by inducing innate hypo- and adaptive hyperresponsiveness, associated with impaired NFκB responses of macrophages. The aim of the present study was to dissect the complexity of *Cdcs1* by further development and characterization of reciprocal *Cdcs1* congenic strains and to identify potential candidate genes in the congenic interval.

Methods—In total, 15 reciprocal congenic strains were generated from *Il10*^{−/−} mice of either C3H/HeJBir or C57BL/6J genetic backgrounds by 10 cycles of backcrossing. Colitis activity was monitored by histological grading. Candidate genes were identified by fine mapping of congenic intervals, sequencing, microarray analysis and a high-throughput real-time RT-PCR approach using bone marrow-derived macrophages.

Results—Within the originally identified *Cdcs1*-interval, three independent regions were detected that likely contain susceptibility-determining genetic factors (*Cdcs1.1*, *Cdcs1.2*, and *Cdcs1.3*). Combining results of candidate gene approaches revealed *Fcgr1*, *Cnn3*, *Larp7*, and *Alpk1* as highly attractive candidate genes with polymorphisms in coding or regulatory regions and expression differences between susceptible and resistant mouse strains.

Conclusions—Subcongenic analysis of the major susceptibility locus *Cdcs1* on mouse chromosome 3 revealed a complex genetic structure. Candidate gene approaches revealed attractive genes within the identified regions.

Keywords

Mucosal Immunology; Animal Models of IBD; Adaptive Immune System in IBD; Innate Immune System in IBD; Inflammation in IBD

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INTRODUCTION

In the interleukin-10-deficient (*Il10*^{-/-}) mouse model of Inflammatory Bowel Disease (IBD), 10 quantitative trait loci (QTL) have been shown to be associated with colitis susceptibility by linkage analyses on experimental crosses of highly susceptible C3H/HeJBir (C3Bir)-*Il10*^{-/-} and partially resistant C57BL/6J (B6)-*Il10*^{-/-} mice.^{1,2} The strongest locus (C3Bir-derived cytokine deficiency-induced colitis susceptibility [*Cdcs1*] on chromosome [Chr] 3) controlled multiple colitogenic subphenotypes and contributed the vast majority to the phenotypic variance in cecum and colon. This was demonstrated by interval-specific Chr 3 congenic mice wherein defined regions of *Cdcs1* from C3Bir or B6 were bred into the IL-10-deficient reciprocal background and altered the susceptible or resistant phenotype. While the longest congenic regions reproduced the donor background phenotypes faithfully, reduction in the length of the congenic interval significantly attenuated such phenotypic effects.^{3,4} The finding of weaker effects contributed by multiple subcongenic intervals coupled with the finding of multiple linkage peaks across the *Cdcs1* confidence interval in the original mapping study indicated a very complex genetic structure of the Chr 3 locus. Interestingly, the *Cdcs1*-linkage to colitis was replicated in the *Gnai2* (also known as *Gα_{i2}*)-deficient mouse model of IBD,⁶ underlining the crucial role of this Chr 3 locus in mucosal homeostasis.

In subsequent immunological studies it was shown that the colitogenic Chr 3 haplotype containing the C3Bir *Cdcs1* allele impaired proinflammatory cytokine response of bone marrow-derived macrophages (BMDM) to toll-like receptor (TLR) ligands and in turn skewed the adaptive CD4⁺ T-cell immune response toward compensatory hyperresponsiveness and chronic intestinal inflammation.³ This innate hyporesponsiveness was associated with a constitutively higher expression of NFκB p50, an NFκB-product associated with hyporesponsiveness of monocytic cells to bacterial stimulation,⁷ and with a diminished NFκB-response after TNF-stimulation.³ This finding is in line with recent studies showing that impaired innate immune responses caused by epithelial cells lacking NFκB activation,⁸ or lamina propria dendritic cells with disturbed TNF secretion due to T-bet deficiency,⁹ lead to mucosal barrier defects and subsequent IBD. Importantly, *NFκB1* haplotypes and functional promoter polymorphisms are associated with IBD-phenotypes.^{10,11}

The aim of the present study was to dissect the complexity of *Cdcs1* by further development and characterization of reciprocal subcongenic strains (now backcrossed 10 generations to the IL-10-deficient parental backgrounds). Furthermore, we aimed to refine the list of candidate genes by identifying polymorphic genes in the subcongenic strains and by gene expression analyses of BMDM cultures using microarray and regional specific array technology.

MATERIAL AND METHODS

Mice and Interval-Specific Congenic Strains

Mice were produced at The Jackson Laboratory (JAX) in a standard conventional specific pathogen free vivarium, free of mouse pathogens except for *Helicobacter hepaticus* and *Klebsiella oxytoca*. Reciprocal congenic strains were generated from *Il10*^{tm1Cgn} (*Il10*^{-/-}) mice of either C3H/HeJBir (C3Bir) or C57BL/6J (B6) backgrounds as described previously,³ and backcrossed for a total of 10 generations (N10) for this study. A summary of the congenic elements that were transferred into the subcongenic B6.C3Bir-*Cdcs1* (BC-R) or C3Bir.B6-*Cdcs1* (CB-R) *Il10*^{-/-} lines and the markers used for genotyping are depicted in Figure 1. For fine mapping, some strains (see below) were transferred into the vivarium of

the Hannover Medical School and maintained in individually ventilated cages as described elsewhere.¹²

Histological scoring and statistical analysis

Colitis activity was monitored by histological grading of intestinal lesions on hematoxylin & eosin stained specimens according to the “JAX-score”.⁵ Briefly, colon and cecum tissues were scored based on lesion severity, ulceration, hyperplasia and percentage of area involved, resulting in a maximum possible score of 12 for the cecum and 36 for the colon. Cecum and colon scores were used to compare disease susceptibility between the *Il10*^{-/-} background and congenic strains. As Bartlett’s test for equal variances revealed dissimilar variances between the groups, the Median test was used for statistical analysis instead of ANOVA or Mann-Whitney-U-tests as recommended by Kasuya,¹³ and exact p-values were calculated as proposed by Mundry & Fisher.¹⁴ Significance levels were set to $\alpha \leq 0.05$ (indicative) and ≤ 0.0064 (Fig. 1A) or ≤ 0.0073 (Fig. 1B) for significance; the latter two values determined by using the Dunn-Šidák correction to correct for multiple testing ($\alpha = 1 - [1 - 0.05]^{1/\gamma}$; γ = number of comparisons). In the case of BC-R7 and CB-R6, variances did not differ after separating for sexes and ANOVA with subsequent Newman-Keuls Multiple Comparison Test were used for analysis with significance levels set to $\alpha \leq 0.05$.

Derivation of BMDM and generation of cDNA for Affymetrix chip analysis

BMDM were obtained from 3 male mice per genotype (IL-10-deficient C3Bir and B6 parental males and the reciprocal CB-R1 and BC-R3 congenics; see Fig. 1). Cells were cultured in polystyrene 6-well culture plates (Corning, Corning, NY) at a density of 3×10^6 /mL in RPMI1640 medium plus 10% fetal calf serum for six days as described previously.³ Culture medium was changed every third day. On the last day of culture, 3 wells per plate were stimulated with CBir1 flagellin (1 μ g/mL culture medium; kindly provided by Dr. C. Elson, University of Alabama, Birmingham).¹⁵ After incubation for additional 4 hours, media were removed and replaced with 2 mL of PBS.

For RNA extraction, cells from each donor in medium only and medium + flagellin (3 wells each) were harvested and pooled in a total of 2 mL Trizol (Invitrogen, Carlsbad, CA.). After passing the lysate through a 20G needle 5–6 times, RNA was extracted following the manufacturer’s protocol. RNA quality and yield was determined using the Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip assay (Agilent Technologies Inc, Palo Alto, CA). Following reverse transcription with an oligo(dT)-T7 primer (Affymetrix, Santa Clara, CA), double-stranded cDNA was synthesized with the Superscript double-stranded cDNA synthesis custom kit (Invitrogen). The cDNA was linearly amplified and labeled with biotinylated nucleotides (Enzo Diagnostics, Farmingdale, NY) in an *in vitro* transcription reaction using T7 RNA polymerase.

Microarray Experiment

Microarray experiments were performed as triplicates. Biotin-labeled and fragmented cRNA (15 μ g) was hybridized onto MOE430v2.0 GeneChipTM arrays (Affymetrix) for 16 hours at 45°C. Post-hybridization staining and washing were performed according to manufacturer’s protocols using the Fluidics Station 450 instrument (Affymetrix). Finally, the arrays were scanned with a GeneChipTM Scanner 3000 laser confocal slide scanner. The images were quantified using GeneChip(TM) Operating Software (GCOS) v1.2 and expression values summarized using the Robust MultiChip Average function,¹⁶ in the R/affy package.¹⁷ An analysis of variance (ANOVA) model was applied and F1, F2, F3 and Fs test statistics were constructed along with their permutation p-values.¹⁸ False discovery rate (FDR),¹⁹ was then assessed using the R/qvalue package to estimate q-values from calculated test statistics.

Cluster analysis was performed using Genesis.20 Functional annotations were assigned using Gene Ontology (<http://www.informatics.jax.org/function.shtml>).

Regional specific quantitative expression profile

RNA was obtained from BMDM from both the B6 and C3Bir wildtype male mice as described above. Expression of 46 genes was analyzed using real-time RT-PCR and the Global Pattern Recognition (GPR) algorithm as described previously.²¹ In summary, synthesis of cDNA from 5–10 μ L of total RNA was carried out using the Retroscript kit (Ambion) following the manufacturer's recommendations. Primer sets (MWG Biotech; sequences are available on request) were designed using Primer Express v1.5 (Applied Biosystems, ABI). Specificity to the desired gene target was ensured by bidirectional sequencing of PCR products and by generating SYBR Green dissociation curves. Quantitative real-time RT-PCR was carried out using a SYBR Green Master Mix (ABI) and data were collected on the ABI Prism 7700 Sequence Detection System v1.7 using the default reaction conditions. The baseline and threshold were set to experimentally determined values and the Experimental Report data (a table of Ct values for each reaction) were exported for GPR analysis that compared the Ct of each candidate gene individually with the Ct of every other gene in the data set that is eligible as a normalizer. For each gene-normalizer combination, the individual Δ Ct values generated for the two mouse strains were compared by a two-tailed heteroscedastic (unpaired) Student's t-test, and a "hit" was recorded if the p-value from the t-test failed below defined p-values (0.05 and 0.01).²¹

Fine mapping and sequencing

Congenic elements of selected strains (see Fig. 1) were fine mapped by PCR detection of microsatellite markers and sequencing of single nucleotide polymorphisms (SNPs; primer sequences and conditions used available on request). Microsatellite markers were either chosen from the The Jackson Laboratory's online Mouse Genome Informatics resource (www.informatics.jax.org) with physical map positions confirmed by using the UCSC genome browser (genome.ucsc.edu), or microsatellites were chosen from the UCSC genome browser and primers were designed for amplification using Primer3 (<http://fokker.wi.mit.edu/>).

SNPs between C3H/HeJ and B6 were selected using the The Jackson Laboratory's Mouse Phenome Database (<http://phenome.jax.org/pub/cgi/phenome/mpdcgi?rtn=docs/home>). Detection of these SNPs in our strains was achieved by amplification and sequencing of 200–400 bp products using primers created with Primer3 or Limstall (limstall.niob.knaw.nl). PCR was carried out using using REDExtract-N-AMP-PCR ReadyMix (Sigma-Aldrich, Munich, Germany) and PCR products were gel purified using the Nucleo Spin Extract II Kit (Machery&Nagel, Düren, Germany) and sequenced by Sequence Laboratories Göttingen GmbH (Göttingen, Germany). Sequences were translated (www.expasy.ch/tools/dna.html) and secondary structures predicted (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html).

RESULTS

Characterization of reciprocal congenic strains

In total, 15 *Il10*^{-/-} strains subcongenic for Chr 3 intervals were used for a detailed analysis of alterations in disease susceptibility associated with the transfer of these genetic elements. The most significant phenotypic deviations from the respective parental control backgrounds were seen in subcongenic mice necropsied between 5–9 weeks of age (Fig. 1) although similar differences were also detected in necropsies of older mice (not shown). Figure 1A shows histopathologic scores for cecum and colon for *Il10*^{-/-} subcongenic strains produced

on the nominally resistant B6 background and carrying C3Bir-derived intervals around *Cdcs1* (B6.C3Bir-*Cdcs1* [BC-R] *Il10*^{-/-} lines). As depicted in Figure 1A, only mice harboring C3Bir alleles between 87.1 and 131.1 mega base pairs (Mbp) showed increased colitis susceptibility. Mice of the strains BC-R1 and BC-R3 only differed in the marker allele at 89.1 Mbp but showed similar colitis-susceptible histological scores and were therefore combined for the analysis (denoted as BC-R1/R3). It should be noted that the strain BC-R2 was previously reported to be free of colitis lesions.³ It was subsequently found that the targeted *Il10* allele had been inadvertently lost, an error that has since been corrected by reintroduction of the targeted allele.⁴ Although only a few mice of the strain BC-R2 were available for comparison because of poor fecundity in the Bar Harbor colony, they clearly showed accelerated onset and more severe inflammation than B6 mice (Fig. 1A); this was also seen in BC-R2 mice imported to the Hannover Medical School (not included in the analysis). BC-R7 mice showed a B6-*Il10*^{-/-} like phenotype in the colon; however, mice of this strain, especially male mice, were very susceptible for histological lesions in the cecum and displayed similar or even higher typhlitis scores than C3Bir-*Il10*^{-/-} mice (see below).

The finding that not only strains with elements covering the complete interval from 87.1 to 131.1 Mbp (BC-R1, BC-R2, and BC-R3), but also shorter interval congenics in this region (BC-R7 and BC-R8) became susceptible (at least partially in the case of BC-R7) indicates the presence of multiple contributing loci. In Figure 1A–B, the *Nfkb1* locus is marked because of electrophoretic mobility gel shift assay differences in NFκB in parental strain macrophages reported previously³. However, C3Bir genome spanning the *Nfkb1* structural gene alone was insufficient to elicit colitic lesions, as evidenced by the BC-R4 and BC-R6 strains.

Figure 1B compares phenotypes of mice of the susceptible C3Bir background carrying B6-derived resistance elements on Chr 3 (C3Bir.B6-*Cdcs1* [CB-R] *Il10*^{-/-} lines). The data indicate that an interval between 89.1 and 126.7 Mbp likely confers resistance. Strains CB-R5.1 and CB-R5.2 harbor similar donor intervals with the exception that the B6 *Nfkb1* allele is included in the donor interval for CB-R5.1. The finding of comparably severe histopathology scores in these two strains (combined for analysis) despite different origins of the *Nfkb1* locus, again shows that the *Nfkb1* locus itself is not a primary determinant of phenotype.

After separating for sexes, we observed that male mice of the strain BC-R7 and CB-R6, two strains carrying C3Bir elements in the proximal part of the candidate gene interval and being resistant in the colon, showed susceptibility to disease in the cecum (Fig. 1C–D). Considering that strains on both genetic backgrounds harboring C3Bir alleles throughout this region appear fully susceptible, and also that the strain BC-R8 with C3Bir genotype in the distal part of the candidate gene interval appears fully susceptible, this indicates that modifiers selective for cecal lesions may exist that are located in the proximal part of the candidate gene interval and that are influenced by sex.

Two congenic strains followed only partly the pattern described above, illustrating the complex genetics of the model. CB-R2 mice showed an intermediate phenotype in the cecum despite presence of B6 alleles throughout the candidate gene interval on Chr 3 (Fig. 1B). This finding remained stable even in older mice. Less than 10 week-old mice of the strain BC-R5 (that carried C3Bir alleles from the *Nfkb1* locus to *D3Mit17*, data not shown) were susceptible for lesions in the cecum but showed resistance in the colon. In contrast, BC-R5 mice older than 11 weeks showed resistance also in the cecum, indicating remission of inflammation.

Fine mapping and positional candidate genes identified by sequencing

For further dissection of *Cdcs1*, fine mapping of selected congenic regions (those of BC-R2, BC-R4, BC-R8, and BC-R7 as well as CB-R6 and CB-R8) was performed using microsatellite markers and sequencing of SNPs. Three independent regions were detected that likely contain susceptibility-determining genetic factors. The first interval (*Cdcs1.1*; 88.1 to 108.8 Mbp, Fig. 2) spans the congenic element of the susceptible BC-R7 strain and overlaps the congenic element of the highly susceptible BC-R2 strain. This region contains 46 genes with coding non-synonymous SNPs (Supplementary Table 1). Among these genes some stand out as candidates because of their function: *Muc1*, *Fcgr1*, *Cd2* and *Cd160*.

The second region (*Cdcs1.2*; 120.0 to 123.0 Mbp, Fig. 3) resulted from comparing B6 and C3Bir alleles of CB-R6 and CB-R8 mice and contains a total of 27 genes; none of which harbor coding non-synonymous polymorphisms according to the Mouse Phenome Database. We verified six SNPs within putative regulatory regions of four candidate genes, *Tmem56*, *Alg14*, *Cnn3* and *Slc44a3* (Table 1). As expression differences were detected for *Cnn3* by microarray analyses (see below), polymorphisms detected in the promoter region might indeed modify expression of this gene.

Phenotypes of BC-R2 and BC-R8 mice indicate a third interval (*Cdcs1.3*; 125.6 to 128.0 Mbp, Fig. 4) that contains a total of 13 genes; five of which were verified by sequencing to contain coding non-synonymous SNPs (*Ank2*, *LOC100043364*, *Larp7*, *4930422G04Rik*, and *Alpk1*, Table 2). *In silico* analyses revealed that all SNPs (one in *Ank2* and *Larp7*, two in *Alpk1* and *4930422G04Rik*, and five in *Loc100043364*) change the secondary structure of the respective proteins except one SNP in the *4930422G04Rik* gene. Both SNPs in *Alpk1* are located in a region which is conserved between mice, humans, and rats, and one of them is located in a functional region coding for alpha kinase. The SNPs in the *4930422G04Rik* gene are also located in a conserved area, and one changes the secondary structure of the DNA/RNA helicase of this gene.

A complete list of sequencing results is shown in Supplementary Table 2. A list showing the names of all genes mentioned is provided in Supplementary Table 3.

Candidate genes identified by microarray analysis

As strain specific, *Cdcs1*-inherited differences in innate immune response were observed after stimulation of BMDM with CBir1 flagellin,³ we used the same approach to detect global gene expression differences. Therefore, BMDM of C3Bir-*Il10*^{-/-} and B6-*Il10*^{-/-} as well as the *Il10*^{-/-} reciprocal congenic strains CB-R1 and BC-R3 (harboring the long congenic elements covering the whole *Cdcs1*-region) were cultured and stimulated with CBir1 flagellin or left unstimulated.

A total of 3939 probesets were found to be differentially expressed (defined as at least ± 1.5 fold expression differences) between C3Bir-*Il10*^{-/-} and B6-*Il10*^{-/-} BMDM after comparing expression values in either unstimulated or stimulated cells of both strains (2284 probesets in unstimulated, 560 in stimulated, and 1095 in both stimulated and unstimulated cells). These probesets were selected for cluster analysis, and genes of certain clusters were subsequently annotated using Gene Ontology (GO; results are shown in Supplementary Fig. 1). In summary, it became obvious that the genetic background explained most of the differential gene expression observed. However, a large number of the genes whose expression was influenced by the congenic interval likely share common processes such as DNA-metabolism, cell adhesion, cell organization and biogenesis, and development (including development of hematopoietic and lymphoid organs) like the candidate genes *Fcgr1* and *Cnn3*.

We subsequently selected genes that were both affected in their expression by the congenic interval and located in the *Cdcs1* region (indicating cis-regulation) to identify further candidate genes. These genes had to show at least 1.5 fold expression difference not only between the background strains, but also between congenic strains, demonstrating the impact of *Cdcs1* on their regulation. These genes are listed in Table 3. By taking the candidate gene intervals identified by subcongenic analyses into account, the list of candidates includes besides the already mentioned *Fcgr1* and *Cnn3* also *Olfml3*, *Chi3l3/Chi3l4*, *9030201C23Rik*, and *Ccdc109b*.

Concerning global gene expression, we observed that the majority of genes were found to be down regulated in C3Bir BMDM, including a variety of genes involved in immune processes. This also applied to a cluster of genes that showed a similar but much more pronounced response to stimulation in B6 compared to C3Bir BMDM. This is in line with the previous observation of an innate hyporesponsiveness in C3Bir. As expected, genes with known allelic differences in B6 and C3Bir were detected in the respective clusters, like *Gbp1* (located in the congenic interval and down-regulated in B6) or *H2-Ea* (which contains a mutation in B6 and is down-regulated in this strain).

Regional specific quantitative expression profile

A high-throughput real-time RT-PCR strategy and the GPR algorithm,²¹ were used to analyze quantitative expression of a set of genes specific for the *Cdcs1* interval in BMDM. B6 and C3Bir wildtype mice were used for isolating BMDM for this approach, because differences in *Cdcs1* expression at the macrophage level should not require IL-10-deficiency. Significantly differentially expressed genes from the GPR output are shown in Table 4. Of these genes, *Larp7* and *Alpk1* were noted previously because of coding non-synonymous SNPs (Table 2), and *Ccdc109b* because of expression differences detected by microarray analysis (Table 3). The most striking expression difference detected by this analysis was observed for *Ugt8a*. This gene carries a coding non-synonymous SNP (GLU→LYS) in exon 5 that was confirmed by sequencing. Interestingly, all three probes used to detect *Nfkb1* expression were differentially expressed at a significance level of $p < 0.05$.

DISCUSSION

The *Cdcs1* locus is a major modifier of murine IBD and was originally identified in experimental crossings of *Il10*^{-/-} mice. The C3Bir allele of *Cdcs1* (that confers colitis susceptibility) codes genetic factors that impair innate immune responses of BMDM to TLR-ligands, but enhance adaptive CD4⁺ T-cell responses. *Cdcs1* was originally positioned within a minimum 7 Mbp interval containing the NFκB p50 coding gene (*Nfkb1*).³ As C3Bir BMDM showed constitutively higher expression of this transcription factor and a diminished NFκB-response after TNF-stimulation, *Nfkb1* appeared as an attractive candidate.³ However, further subcongenic analysis revealed that the effective congenic region was considerably larger,⁴ expanding the list of potential candidates markedly.

Breeding of additional congenic IL-10-deficient strains and backcrossing of all strains to N10 enabled us to refine the *Cdcs1* interval in this study. By histological scoring of intestinal lesions, we observed the most informative phenotypes in mice less than 10 weeks old. In general, the C3Bir congenic interval mediated a more distinct colitogenic effect in mice of the B6 background compared to the protective effect of B6 alleles within the C3Bir background. This explains more concise phenotypes in BC-R than in CB-R congenic mice, likely due to additional modifiers in the remaining background, particularly the C3Bir inherited QTLs *Cdcs2*, 3, 7, 9, and 10.

Our analysis revealed that a congenic interval between 87.1 and 131.1 Mbp on Chr 3 modified IBD susceptibility and excluded *Nfkb1* as major primary factor. Interestingly, also strains that were partially congenic in these intervals changed their susceptibility according to the inherited congenic alleles, indicating the presence of multiple contributing loci. The finding of a major QTL breaking up into several smaller loci has been shown in other experimental models.²² Fine mapping of selected congenic intervals allowed a further dissection of *Cdcs1* and revealed three regions that likely contain genetic modifiers: *Cdcs1.1* from 88.1 to 108.8 Mbp; *Cdcs1.2* from 120.0 to 123.0 Mbp; and *Cdcs1.3* from 125.6 to 128.0 Mbp. Positional candidate genes were identified in these regions by sequencing and included *Fcgr1*, *Cnn3*, *Larp7*, and *Alpk1*.

In microarray experiments on C3Bir flagellin stimulated or unstimulated BMDM of parental and congenic *Il10*^{-/-} strains, we have observed that the genetic background explained most of the differential gene expression. Furthermore, the majority of genes were found to be down regulated in C3Bir BMDM, including a variety of genes involved in immune processes. This corroborates the concept of innate hyporesponsiveness in C3Bir leading to disturbed intestinal homeostasis. To identify potential genes underlying *Cdcs1* linkage, we selected genes that were both affected in their expression by the congenic interval and located in the *Cdcs1* region (indicating cis-regulation). Genes located within the defined candidate gene intervals included above mentioned *Fcgr1* and *Cnn3*, as well as *Ccdc109b*. It has to be stated that although previous studies showed strain specific differences in innate immune response of macrophages especially after flagellin stimulation, other cell types and mechanisms likely play also a role in *Cdcs1*-mediated colitis susceptibility.

Further candidate gene analysis was performed using a high-throughput real-time RT-PCR strategy for genes within the *Cdcs1* interval. For this analysis, BMDM of B6 and C3Bir wildtype mice were used instead of the *Il10*^{-/-} strains as we expected to detect background effects on gene expression in cells without IL-10 mutation. Interestingly, *Nfkb1* showed higher expression levels in C3Bir macrophages on the mRNA level, which corresponds to the mobility shift assays performed previously. Using a long congenic strain, it was also demonstrated that the strain specific *Nfkb1* expression was determined by the haplotype of the transferred congenic element.³ It remains to be determined whether strain specific *Nfkb1* expression is caused by cis-regulation or by a genetic/epigenetic factor located within the defined *Cdcs1* interval, e.g. by a detailed analysis of *Nfkb1* expression in long and short congenic strains. Of the remaining 17 genes that showed statistically significant expression differences in this GPR analysis, *Ugt8a* stood out with a 38.78 fold higher expression in B6 BMDM. This gene is located at most 0.5 Mbp proximal to *Cdcs1.3*, carries a coding non-synonymous SNP, and encodes a key enzyme for synthesis of glycosphingolipides that, besides being an integral part of myelin sheaths,²³ are essential for formation of lymphoid-specific stromal niches in the bone marrow and therefore for lymphopoiesis.²⁴ The real-time RT-PCR approach confirmed differential expression of *Ccdc109b* detected by microarray analysis. This gene is located just distal to *Cdcs1.3*. Expression analyses revealed high expression on the mRNA level in cells of the macrophage lineage (<http://biogps.gnf.org>);²⁵ however, the function of the gene product is unknown.

Combining the results of all four candidate gene approaches reveals four attractive candidate genes: *Fcgr1* (*Cdcs1.1*), *Cnn3* (*Cdcs1.2*), *Larp7* and *Alpk1* (*Cdcs1.3*). The *Fcgr1* gene encodes a high-affinity Fc- γ receptor (also known as CD64) that is constitutively expressed on monocytes and macrophages. This receptor plays a pivotal role in immune responses, including immune-complex mediated phagocytosis and antigen presentation to T cells. Interestingly, absence of functional Fc- γ receptor 1 leads to elevated antibody responses.²⁶ Furthermore, signalling via this receptor was essential for flagellin induced Th1 IBD response in mice.²⁷ The h3-isoform of calponin that is encoded by the *Cnn3* gene is

abundantly expressed in non-smooth muscle tissue.²⁸ While the role of calponins in smooth muscle contractility is well known, its function in non-muscle cells is less well understood. Interestingly, the h2-isoform has recently been shown to modify the number of peripheral neutrophils and monocytes, the rate of proliferation and migration of macrophages as well as their phagocytotic activity.²⁹ **Larp7** encodes a negative regulator of RNA-polymerase II (Pol II) that catalyze transcription of DNA to synthesize precursors of mRNA, most snRNA and microRNA. Larp7 inhibits via stabilization of the 7SK ribonucleoprotein the positive transcription elongation factor (P-TEF) β that is recruited by NF κ B and regulates a subset of NF κ B dependent genes, those that are characterized by TNF-induced Pol II recruitment.³⁰ Accordingly, reduction of LARP7 by RNA interference enhanced transcription from cellular Pol II promoters, including the acute phase protein HSP70.³¹ Therefore, *Larp7* represents an attractive candidate that might play a role in innate hyporesponsiveness and *Nfkb1* dysregulation in C3Bir mice. **ALPK1** is essential for the transport of lipid rafts carrying vesicles from the Golgi-apparatus to the plasma membrane probably by phosphorylation of myosin Ia, a function that has been identified using polarized epithelial cell lines.³² However, taking into account that *Alpk1* mRNA is ubiquitously expressed including in macrophages and other immune cells (<http://biogps.gnf.org>),²⁵ and that lipid rafts play an important role in immune cells including TLR-signalling, TNF-release, antigen presenting, and phagocytosis in macrophages as well as B- and T-cell receptor signalling in lymphocytes, this gene might be of great importance for responses to bacterial stimuli and mucosal homeostasis.^{33,34} Homologs of *Larp7* and *Alpk1* are located within a human susceptibility region for IBD on Chr 4,³⁵ that, however, has not been confirmed by current genome wide association studies.³⁶

By analyzing 15 reciprocal congenic strains that were generated from *Il10*^{-/-} mice of either C3Bir or B6 genetic backgrounds by 10 cycles of backcrossing, we have observed that a major colitis susceptibility locus in mice split up in at least three independent regions, demonstrating the complex structure of this QTL. Subcongenic analyses enabled us to define candidate gene intervals within the *Cdcs1* region. Besides fine mapping of the defined congenic intervals, identification of candidate genes was achieved by sequencing, microarray analysis, and high-throughput real-time RT-PCR. Combining results of these analyses, we identified four attractive candidate genes that fit well into the concept that impaired mucosal barrier functions, processing of bacteria, and/or immunoregulation plays an important role in the etiology of IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Genotype and phenotype of *Il10*^{-/-} Chr 3 interval-specific congenic mouse strains. Different subcongenic **B6.C3Bir-*Cdcs1* (BC; 1A & 1C)** or **C3Bir.B6-*Cdcs1* (CB; 1B & 1D)** lines (denoted as R1-R8), the markers used for genotyping with positions in mega base pairs (Mbp), cecum/colon total scores, numbers (N) of mice used for analysis, and detailed p-values (Fig. 1A–B) are shown. The candidate gene intervals determined by the respective congenic set of mice are highlighted in Fig 1A and 1B. Resistant (R), susceptible (S), intermediate (I) was defined based on results of the Median test, determining whether a given congenic strain differed from either the B6-*Il10*^{-/-} or the C3Bir-*Il10*^{-/-} parental strain or both. Figures 1C and 1D show typhlitis scores (single values and medians) of male or female B6-*Il10*^{-/-}, C3Bir-*Il10*^{-/-}, and BC-R7 (Fig. 1C) or CB-R6 (Fig. 1D) mice and p-values determined by the Newman-Keuls Multiple Comparison Test; ns = not significant; na = not applicable.

Mbp	Marker	BC-R2	BC-R7
87.1	<i>D3Mit40</i>	B6	B6
88.1	<i>D3Mit175</i>	B6	B6
89.1	<i>D3Mit49</i>	C3Bir	C3Bir
93.0	<i>D3Mit141</i>	C3Bir	C3Bir
96.4	<i>D3Mit101</i>	C3Bir	C3Bir
100.4	<i>D3Mit11</i>	C3Bir	C3Bir
101.2	<i>D3Mit312</i>	C3Bir	C3Bir
103.7	<i>D3Mit266</i>	C3Bir	C3Bir
108.8	<i>D3Mit286</i>	C3Bir	B6
111.2	<i>D3Mit78</i>	C3Bir	B6
Phenotype	Cecum	S	S

FIGURE 2.

Subcongenic strain pair illustrating the *Cdcs1.1* interval. Comparison of the highly susceptible BC-R2 and partially susceptible BC-R7 mice, the latter showing susceptibility in the cecum (males), indicates genetic modifiers in a region from 88.1 to 108.8 Mbp on Chr 3. Mbp = mega base pairs; S = susceptible; R = resistant; ex. = exon.

Mbp	Marker	CB-R6	CB-R8
120.0	<i>D3Mia120.0</i>	C3Bir	C3Bir
120.9	<i>Tmem56</i>	B6	C3Bir
121.0	<i>Alg14</i>	B6	C3Bir
121.2	<i>Cnn3/Slc44a3</i> (4 SNPs)	B6	C3Bir
121.2	<i>D3Mit43</i>	B6	C3Bir
122.3	<i>D3Mia122.3</i>	B6	C3Bir
123.0	<i>Sec24d</i> (ex. 5 & 11)	B6	B6
123.2/123.4	<i>Ndst3</i> (ex. 1 & 11)	B6	B6
123.7	<i>D3Mit199</i>	B6	B6
Phenotype	Colon	R	S

FIGURE 3.

Subcongenic strain pair illustrating the *Cdcs1.2* interval. Colon phenotypes of CB-R6 and CB-R8 mice suggest alleles that contribute to disease susceptibility between 120.0 and 123.0 Mbp on Chr 3. Mbp = mega base pairs; S = susceptible; R = resistant; ex. = exon

Mbp	Marker	BC-R4	BC-R8	BC-R2
123.7	<i>D3Mit 199</i>	B6	B6	C3Bir
124.1	<i>1700006A11Rik</i>	B6	B6	
125.6	<i>Ugt8a</i>	B6	B6	
126.1	<i>D3Mia126.1</i>	B6	C3Bir	C3Bir
126.6	<i>Ank2</i>	B6	C3Bir	C3Bir
126.7	<i>D3Mit348</i>	B6	C3Bir	C3Bir
127.2	<i>LOC100043364</i>		C3Bir	C3Bir
127.2	<i>Larp7</i>	B6	C3Bir	C3Bir
127.3	<i>4930422G04Rik</i> (ex. 23 & 24)	B6	C3Bir	C3Bir
127.4	<i>Alpk1</i> (ex. 2 & 5)	B6	C3Bir	C3Bir
128.0	<i>EG668828</i>	B6	C3Bir	B6
128.0	<i>D3Mit319</i>	B6	C3Bir	B6
129.3	<i>D3Mia129.3</i>	C3Bir	C3Bir	B6
Phenotype	Cecum Colon	R R	S S	S S

FIGURE 4.

Subcongenic strain pair illustrating the *Cdcs1.3* interval. Phenotypes of BC-R2 and BC-R8 mice indicate genetic factors that contribute to disease susceptibility between 125.6 and 128.0 Mbp on Chr 3. This candidate gene interval results from the presumption that BC-R8 and BC-R2 share common modifier; however, if BC-R8 mice carry independent modifier genes, a maximum of 1.3 Mbp (including the gene *EG668828*) have to be added to the candidate gene interval, as BC-R4 mice are resistant and carry C3Bir alleles at 129.3 Mbp. Mbp = mega base pairs; S = susceptible; R = resistant; ex. = exon

TABLE 1

Polymorphisms detected in putative regulatory regions of candidate genes

Mbp	Gene	RefSNP ID	Down-/upstream*	Nucleotide
				B6 C3Bir
120.9	<i>Tmem56</i>	rs30109541	Down	G A
121.0	<i>Alg14</i>	rs47441003	Up	A T
121.2	<i>Cnn3/Slc44a3</i>	rs13464154**	Down	G A
121.2	<i>Cnn3/Slc44a3</i>	rs6212614**	Down	T C
121.2	<i>Cnn3/Slc44a3</i>	rs49731762**	Down	C Del.
121.2	<i>Cnn3/Slc44a3</i>	rs30203293**	Down	G A

* Within 2000 bp down- or upstream of the start or stop codon, respectively.

** These SNPs are located between the genes *Cnn3* and *Slc44a3*.

The positions of the genes on chromosome 3 are given in mega base pairs (Mbp). Del = deletion

TABLE 2

Polymorphisms detected in the coding region of candidate genes

Mbp	Gene	RefSNP ID	Exon	Nucleotide		Amino acid	
				B6	C3Bir	Pos.	B6 → C3Bir
126.9	<i>Ank2</i>	rs30540733	1	C	T	213	ASN→ASP
127.2	<i>Loc100043364</i>	rs31724259	1	A	C	7	LEU→ARG
127.2	<i>Loc100043364</i>	ENSMUS SNP4125122	1	T	C	27	ARG→GLY
127.2	<i>Loc100043364</i>	rs30699516	1	C	T	31	ALA→THR
127.2	<i>Loc100043364</i>	rs31195770	1	G	A	56	PRO→LEU
127.2	<i>Loc100043364</i>	rs31640604	1	G	A	106	PRO→SER
127.3	<i>Larp7</i>	rs13462046	7	C	G	242	VAL→LEU
127.3	<i>4930422G04Rik</i>	rs31431528	23	A	G	801	SER→GLY
127.3	<i>4930422G04Rik</i>	rs31344677	24	G	A	848	VAL→ILE
127.4	<i>Alpk1</i>	rs45651673	12	T	C	1097	ILE→VAL
127.4	<i>Alpk1</i>	rs31148189	2	G	C	16	GLN→GLU

The positions of the genes on chromosome 3 are given in mega base pairs (Mbp).

TABLE 3

Likely Cis-regulated genes located in the transferred congenic intervals

Gene	Probe Set ID	Fold change				Position (Mbp)
		Background strains (C3Bir- vs B6-III0 ^{-/-})		Congenic Strains (CB-R1 vs BC-R3)		
		Unstim.	Stim.	Unstim.	Stim.	
<i>Fcgr1</i>	1417876_at	-1.7	-1.1	1.3	1.7	96.1
<i>Olfml3</i>	1448475_at	-5.1	-7.5	5.6	5.2	103.5
<i>Chi3l3/Chi3l4</i>	1425451_s_at	-1.3	-1.5	6.6	5.3	106.0
<i>9030201/C23Rik</i>	1454503_at	-1.4	-1.7	1.6	1.5	116.6
<i>Cmn3</i> *	1426724_at	-2.2	-1.2	1.6	1.2	121.1
	1456380_x_at	-2.4	-1.1	1.5	1.2	
	1455570_x_at	-3.0	-1.3	2.0	1.5	
	1436836_x_at	-3.3	-1.3	2.2	1.4	
	1436759_x_at	-3.4	-1.3	2.3	1.5	
<i>Ccdc109b</i>	1418778_at	-2.8	-2.8	1.6	1.6	130.0
<i>Gbp1</i>	1420549_at	34.6	16.8	-9.8	-16.0	142.2
<i>Cyr61</i> *	1438133_a_at	-2.1	-1.3	2.4	1.4	145.3
	1416039_x_at	-2.4	-1.4	2.1	1.3	
<i>Mcoln3</i> *	1450148_at	-1.3	-1.5	1.9	2.4	145.8
	1437540_at	-1.9	-1.8	3.2	2.5	
<i>Ifi44</i>	1423555_a_at	3.6	15.4	-3.7	-2.7	151.4
<i>H28</i> *	1421596_s_at	29.0	30.1	-26.4	-26.8	151.4
	1425917_at	16.8	16.4	-19.4	-18.0	
	1450301_at	1.7	1.6	-1.8	-1.5	

* Represented by more than one probe set.

Only probe sets showing ≥ 1.5 fold expression difference in at least one background strain comparison (stimulated or unstimulated) **and** one congenic strain comparison (stimulated or unstimulated) are listed in this table. The positions of the genes on chromosome 3 are given in mega base pairs (Mbp).

TABLE 4

Cdcs1 specific expression profile determined by real-time RT-PCR and GPR analysis with scores of differentially expressed genes at significance levels $p < 0.05$ and $p < 0.01$

Gene	Score ($p < 0.01$) [*]	Score ($p < 0.05$) [*]	Fold Change ^{**}	Chr. 3 Mbp
<i>Ugt8a</i>	0.913	0.978	38.78	125.86
<i>Larp7</i>	0.848	0.957	-5.02	127.53
<i>Dkk2</i>	0.826	0.913	6.30	132.02
<i>Alpk1</i>	0.804	0.870	2.88	127.66
<i>Ccdc109b</i>	0.717	0.870	-3.24	129.91
<i>Ccdc109b</i>	0.717	0.826	-2.43	129.91
<i>Arsj</i>	0.478	0.826	4.01	126.36
<i>Camk2d</i>	0.413	0.587	-1.31	126.59
<i>Nfkb1</i>	ns	0.739	-2.18	135.52
<i>Rap1gds1</i>	ns	0.565	-1.40	138.86
<i>Papss1</i>	ns	0.565	1.33	131.5
<i>Papss1</i>	ns	0.543	1.41	131.5
<i>Slc39a8</i>	ns	0.522	1.30	135.76
<i>Nfkb1</i>	ns	0.500	-1.17	135.52
<i>Tspan5</i>	ns	0.500	1.34	138.68
<i>Nfkb1</i>	ns	0.500	-1.22	135.52
<i>Pla2g12a</i>	ns	0.478	1.41	129.87
<i>Ube2d3</i>	ns	0.478	-1.19	135.38
<i>Casp6</i>	ns	0.457	1.24	129.89
<i>Papss1</i>	ns	0.435	1.21	131.5
<i>Mttp</i>	ns	0.435	2.34	138.02
<i>H2afz</i>	ns	0.413	-1.08	137.8
<i>EG668831</i>	ns	0.413	-1.34	128.95

^{*} The GPR score indicates the fraction of normalizer genes against which a given gene was found to be significantly different; a threshold of at least 40% (score 0.4) was set to identify genes with significant change.21 ns = not significant

^{**} Fold change with respect to 10 best normalizers. Negative values indicate C3Bir upregulation, positive values B6 upregulation.

Genes were represented by up to three primer-sets for each gene. The positions of the genes on chromosome 3 are given in mega base pairs (Mbp).