

Evidence for genes controlling resistance to *Heligmosomoides bakeri* on mouse chromosome 1

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SUMMARY

Resistance to infections with *Heligmosomoides bakeri* is associated with a significant quantitative trait locus (QTL–*Hbnr1*) on mouse chromosome 1 (MMU1). We exploited recombinant mice, with a segment of MMU1 from susceptible C57Bl/10 mice introgressed onto MMU1 in intermediate responder NOD mice (strains 1094 and 6109). BALB/c (intermediate responder) and C57Bl/6 mice (poor responder) were included as control strains and strain 1098 (B10 alleles on MMU3) as NOD controls. BALB/c mice resisted infection rapidly and C57Bl/6 accumulated heavy worm burdens. Fecal egg counts dropped by weeks 10–11 in strain 1098, but strains 1094 and 6109 continued to produce eggs, harbouring more worms when autopsied (day 77). PubMed search identified 3 genes (*Ctla4*, *Cd28*, *Icos*) as associated with ‘*Heligmosomoides*’ in the B10 insert. Single nucleotide polymorphism (SNP) differences in *Ctla4* could be responsible for regulatory changes in gene function, and a SNP within a splice site in *Cd28* could have an impact on function, but no polymorphisms with predicted effects on function were found in *Icos*. Therefore, one or more genes encoded in the B10 insert into NOD mice contribute to the response phenotype, narrowing down the search for genes underlying the *H. bakeri* resistance QTL, and suggest *Cd28* and *Ctla4* as candidate genes.

Key words: *Heligmosomoides polygyrus bakeri*, NOD mice, *Ctla4*, *Icos*, *Cd28*, C57BL/10, resistance QTL, worm burdens, fecal egg counts.

INTRODUCTION

It is now widely accepted that variation in resistance to infectious diseases between individual hosts is largely, although not exclusively, determined by key genes. However, among parasitic infections there are still few instances where relevant genes have been unequivocally identified, and their alleles defined. In contrast to earlier views that control is based on so called ‘immune response genes’, it is now apparent that in most cases multiple genes are involved (polygenic control), and that their inter-relationships are complex (Abel and Dessein, 1997; Cooke and Hill, 2001; Quinnell, 2003; Kwiatkowski, 2005; Vieira Benavides *et al.* 2014).

Despite enormous efforts to identify genes that control helminth infections in domestic animals, humans and in mouse models, few clear candidate genes have emerged to-date from such studies (Williams-Blangero *et al.* 2002; Keane *et al.* 2008; Levison *et al.* 2013). However this is an important area of research, since developing more resistant breeds of domestic livestock is one of the proposed

solutions for the rapidly spreading resistance to chemotherapy (Kloosterman *et al.* 1992; Bishop and Stear, 2003; Stear *et al.* 2007). Anthelmintic resistance among nematode parasites is now widespread throughout all the major pastoral regions of the world, and there are already many cases of triple resistance on farms, where none of the 3 classes of anthelmintics that dominate the markets, are effective any longer (van Wyk, 1990; Jackson *et al.* 1992; Coles *et al.* 1994; Kaplan, 2004; Gilleard, 2006; Wrigley *et al.* 2006).

In parallel with studies in livestock, laboratory models have been used to search for candidate genes for resistance to infectious diseases, because progress can be much more rapid in these systems (Klementowicz *et al.* 2012; Hurst and Else, 2013; Levison *et al.* 2013). One convenient model is the mouse-*Heligmosomoides bakeri* system, which has been exploited effectively to identify chromosomal regions that harbour genes involved in the control of infections with this parasite (Iraqi *et al.* 2003; Behnke *et al.* 2006a, 2010a; Harris *et al.* 2014). Major quantitative trait loci (QTL) have been described on chromosome 1 (MMU1, *Hbnr1*) and 17 (MMU17, *Hbnr2* and *Hbnr3*) (Behnke *et al.* 2006a). These 2 chromosomal regions undoubtedly harbour the major genes for resistance in mice, and the additive effects are huge. One of the QTL (*Hbnr2*) on

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MMU17 overlies the major histocompatibility complex (MHC) region; a finding that is consistent with much of the earlier work based on syngeneic and congenic mouse strains (Enriquez *et al.* 1988; Behnke and Wahid, 1991). The QTL on MMU1 covers a relatively gene poor region and a recombination cold spot. Thus QTL mapping based on F6/7 generations of strong and poor responder mouse strains, whilst confirming the importance of this QTL, failed to refine the confidence limits relative to the earlier F2 study. Interestingly, a study using a quite different nematode parasite of rodents, *Trichinella spiralis* in rats concluded that there was a significant QTL for resistance on rat chromosome 9 (Suzuki *et al.* 2006), which is orthologous with MMU1.

Other QTL have been reported on chromosomes 5 (MMU5), 8 (MMU8) and 11 (MMU11) and these probably play a lesser, but nevertheless, important role in resistance (Behnke *et al.* 2010a). A further range of QTL have been described for the accompanying immune responses (Menge *et al.* 2003), but which genes underlie these QTL, their hierarchical relationships with one another and how their constituent alleles differ in orchestrating host-protective immunity is still far from clear. An approach to identifying the genes that contribute to each QTL is to exploit recombinant mouse strains which have been bred in a way that allows entire QTL to be switched between responder and non-responder strains or alternatively sub-sections of each QTL. The former approach has been extremely useful in elucidating the hierarchical relationships between the 3 candidate QTL in trypanosomiasis (Kemp *et al.* 1997; Iraqi *et al.* 2000) and the latter has been used to dissect the QTL regions in non-obese-diabetes (NOD) mice, seeking the underlying genes responsible for this condition (Yamanouchi *et al.* 2007). The NOD series of recombinant mouse strains is now extensive and fortuitously some of these are based on introgression of alleles from poor responder donors onto MMU1.

In contrast with studies on helminth resistance there has been significant progress in mapping genes for susceptibility to autoimmune diseases such as diabetes using the NOD mouse model (Makino *et al.* 1980). By introgression of fairly short sections of the chromosomes from C57BL/10 (B10) mice (a strain that is not prone to diabetes) onto the NOD background and assessing the extent to which these alleviate, or delay the onset of diabetes, NOD researchers have been able to refine their understanding of the genes responsible for insulin-dependent diabetes mellitus (IDDM) in NOD mice. A number of genes or regions responsible for susceptibility to type 1 diabetes have now been identified including the MHC on MMU17, and a region on MMU1, *Idd5.1*, which overlaps with *Hbnr1* (Maier and Wicker, 2005; Hunter *et al.* 2007; Yamanouchi *et al.* 2007). Of the genes contained in *Idd5.1* on MMU1, those encoding the co-stimulatory molecules CTLA4 and ICOS are

likely candidates for involvement in both resistance to helminth infection and susceptibility to diabetes (Redpath *et al.* 2013). Indeed the molecular basis of the *Idd5.1* region was confirmed to be due to a single nucleotide polymorphism (SNP) in *Ctla4* which affects the levels of expression of a ligand-independent isoform of this molecule (Araki *et al.* 2009; Stumpf *et al.* 2013).

Little is known about the response of the NOD parental mouse strain to *H. bakeri*, but they have been found to harbour infections for longer than BALB/c mice, although the duration of infections to complete clearance was not reported (Saunders *et al.* 2007). Thus on the basis of this single report, and judging by the worm burdens in weeks 22–23, they can be considered tentatively to be intermediate-slow rather than rapid responders. It is also pertinent that this strain is not entirely inbred and that some variation in responses can be expected. In contrast, the inbred C57Bl/10 mouse is an extremely poor responder to *H. bakeri* (Behnke *et al.* 2003), so introgression of C57Bl/10 alleles onto a NOD background would be expected to slow down further, the already slow response to *H. bakeri* in congenic strains, relative to pure NODs.

Here we report an experiment in which 2 NOD congenics with introgressed B10 alleles in a region of MMU1 that overlaps with the major QTL for resistance to *H. bakeri* were assessed for resistance to *H. bakeri* and compared against a strain that had B10 inserts on MMU 3, where no *H. bakeri* resistance genes are known to lie, and against other mouse strains known to be extremely susceptible and resistant to infection. In an effort to further refine the identity of the candidate genes underling the QTL on MMU1, we also report the outcome of an analysis of SNPs, between the original 2 strains (CBA and SWR) that were used to identify the QTL for resistance to *H. bakeri* and between NOD and C57BL/10 for the congenic region, using novel recently developed software.

MATERIALS AND METHODS

Parasites

Mice were infected with the trichostrongyloid intestinal nematode *H. bakeri*. Until recently, this species was known as *Heligmosomoides polygyrus* and *H. polygyrus bakeri* (Cable *et al.* 2006; Behnke *et al.* 2009; Behnke and Harris, 2010). In older literature this parasite has also been referred to as *Nematospiroides dubius* (Behnke *et al.* 1991). We used a repeated infection protocol described by Iraqi *et al.* (2003) and characterized immunologically by Behnke *et al.* (2003), based on the administration of 125 infective larvae (L3) once weekly for 7 weeks. In fact in this case the protocol was extended to week 11 for reasons explained below in the 'Results' section.

Table 1. Strains of mice used in the current work and their characteristics

Strain reference number	NOD locus replaced by B6 alleles	Insert on chromosome	Flanking markers of insert ^a		Position of insert		
					cM	Bases $\times 10^6$	
1094	Idd5	1	D1mit	478	NOD	26.58	51.981
				124	B10	28.83	57.746
				132	B10	39.51	77.143
				134	NOD	41.24	80.264
1098	Idd3	3	D3Nds	36		17.67	36.464
				76		18.44	37.272
6109 ^b	Idd3 + Idd5	1 and 3	–			–	–
C57Bl/6-	–	–	–			–	–
BALB/c-	–	–	–			–	–

^a The flanking markers differ between the 2 strains of mice, B6 (C57BL/10) and NOD.

^b Position of insert on chromosome 1 is exactly the same as that for strain 1094 and that for chromosome 3 as in strain 1098.

The methods used for infection, and the accompanying changes in worm burdens and fecal egg counts (FEC) have all been thoroughly documented in earlier publications (Behnke *et al.* 2003, 2006b).

Mice

NOD congenics were provided by Linda Wicker (University of Cambridge) and were imported from the Taconic Farms in the USA to the ILRI by air. We used 3 strains, as shown in Table 1 (Further details of strains can also be found in Hunter *et al.* 2007 and Yamanouchi *et al.* 2007). The position of the B10 inserts was obtained from <http://www.t1dbase.org/page/DrawStrains> and details of the markers (genetic distances in cM and physical position on the chromosomes in millions of base-pairs) from the Mouse Genetics Database (<http://www.informatics.jax.org/>). Strain 1098 (Idd3 mice) which has B10 alleles on MMU3 (insert length is 0.808 Mb; no response genes were identified on MMU3 in earlier work) acted as our NOD control strain, and we predicted that infections in this strain should be rejected more quickly than in the other 2 strains. Strain 1094 (Idd5 mice) has a B10 insert that is between 19.4 and 28.3 Mb in size bounded by markers with NOD alleles D1Mit478 (Chr1:51 981 710) and D1mit134 (Chr1:80 264 451). If the genes in the B10 interval are involved in resistance to *H. bakeri* we would expect infections in this strain to be longer lasting. Figure 1 shows the exact position of the B10 insert superimposed on *Hbnr1* the QTL for resistance to *H. bakeri*, taken from Behnke *et al.* (2006a). Strain 6109 (which has both Idd3 and Idd5 inserts) was expected to behave much like strain 1094 retaining worms longer than strain 1098. Our experiment was further controlled by the inclusion of C57Bl/6 (B6) mice, which we expected to accumulate heavy worm burdens under the repeated infection protocol and by BALB/c mice which are intermediate responders, and would be expected to reject worms more rapidly than NOD mice (Saunders *et al.* 2007). We began

the experiment with 15 individuals of each strain other than B6 ($n = 16$), and lost two B6s and one 6109 mouse before necropsy.

PubMed search for candidate genes in the B10 insert region

The database PubMed was searched to obtain the number of titles or abstracts that contained the Ensembl 'External ID' and any of the words 'Helminth', '*Heligmosomoides*', 'Diabetes' or 'Inflammation' (a list of the genes is included in IDD5 Supplementary data.xls). It is possible that any polymorphism that affects gene function will affect the development of both diabetes and helminth infection, since both are in some degree inflammatory conditions.

Annotated gene list

An annotated list of all SNP between C57BL/6 and NOD/LtJ in the insert region in Ensembl v67 (NCBI37) was obtained from Biomart. Gene descriptions and external IDs were added with a local Perl script. Counts of hits for gene names and *Heligmosomoides* were also obtained with a different local Perl script that used the National Center for Biotechnology Information, Bethesda, USA (NCBI) E-utilities API to access PubMed (Both local Perl scripts are available from the authors). Polyphen annotation for non-synonymous SNP was obtained using a local Perl script to submit jobs to the Polyphen website (Ramensky *et al.* 2002). This analysis was also extended to compare CBA mice with SWR in the entire QTL region originally described for F2 and F6/7 crosses of these 2 strains (Iraqi *et al.* 2003; Behnke *et al.* 2006a).

Statistical analysis

Where appropriate, data are presented as mean \pm standard error of the mean (S.E.M.). For worm counts

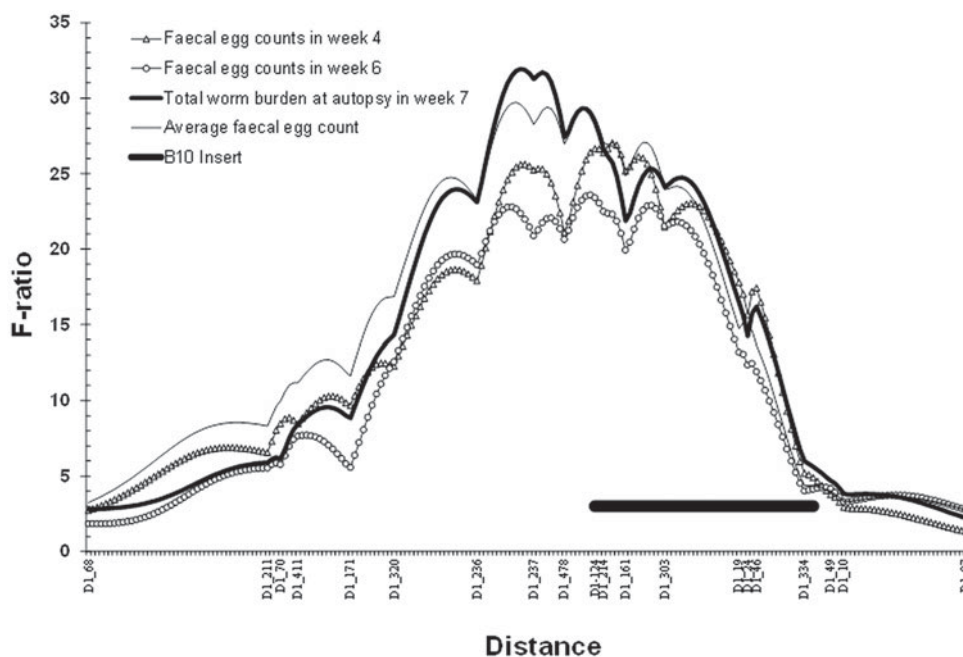


Fig. 1. The QTL for total worm counts and FEC in weeks 4 and 6 and averaged, on chromosome 1, identified in Behnke *et al.* (2006a), and showing the location and length of the inserts from B10 mice onto the NOD background. Three of the markers (D1mit 124, 132 and 134) were not used in the original QTL study but together with D1mit 478 are the flanking markers for the B10 insert. D1mit 132 and 134 are not illustrated on the figure to avoid overlaying markers on the x-axis, but they are located just distal to D1mit 334 and proximal to D1mit 49.

and FEC raw data were transformed ($\log_{10}(x+10)$ and $\log_{10}(x+25)$, respectively) to normalize the distribution. Tests of significance were mostly on transformed values, but in the text we refer to geometric means (GM, calculated by back transformation and adjustment by subtraction of 10 or 25, respectively). The GMs for FEC are in units of eggs per gram and for worm burdens as worm counts.

FEC were analysed by repeated measures analysis of variance (rmGLM in SPSS for Windows, release 16.0.0) with time as the within subject factor and strain as the between subject factor. When the data did not meet the requirement of sphericity (Mauchly's test of sphericity) we employed the Huynh-Feldt adjustment in degrees of freedom to calculate probability levels, erring on the side of caution. Worm burden data were analysed by 1-way GLM in SPSS, with worm burden (transformed by $\log_{10}(\text{number of worms} + 10)$) as the dependent variable and strain as the explanatory factor. In one case we used the non-parametric Mann-Whitney *U* test in SPSS because the data did not conform to the requirements of normality and could not be suitably transformed. $P < 0.05$ was taken as the cut-off for significance.

RESULTS

Faecal egg counts

Figure 2A shows the mean FEC recorded week by week for each group of mice. B6 mice showed consistently increasing FEC, with EPG (eggs per

gram of feces) values rising steadily from week 2 onwards. In contrast in BALB/c mice FEC declined consistently from week 2, both much as expected from previous work (Behnke *et al.* 2006b). All 3 recombinant groups showed intermediate FEC at this time, in agreement with Saunders *et al.* (2007), but by day 42 no clear separation between these strains was evident. Therefore, the experiment was continued for longer, beyond day 43, when normally we would have ended such experiments. However, because the FEC were rising continuously in B6 mice, and threatening their survival, for humane reasons we necropsied both control groups on days 51–53. No statistical analysis is required to support the divergent FEC with time in the 2 control groups representing slow/poor responders (B6) and intermediate/fast responders (BALB/c), which by week 7 differed by more than 2 orders of magnitude.

The figure shows that in weeks 10 and 11, FEC began to drop sharply in strain 1098, in which the B10 insertion was on MMU3, a chromosome that has not been identified as bearing genes involved in genetic resistance to *H. bakeri*. However, strains 6190 and 1094, both of which had B10 inserts in the QTL region on MMU1, continued to produce high levels of parasite eggs, suggesting that protective immunity in these strains was slower to develop. Statistical analysis by rmGLM, incorporating all the days on which FEC had been conducted, indicated that FEC among these 3 groups differed significantly ($F_{2,39} = 4.2$, $P = 0.022$) and *post hoc* analysis indicated that strain 1098 differed significantly from strains

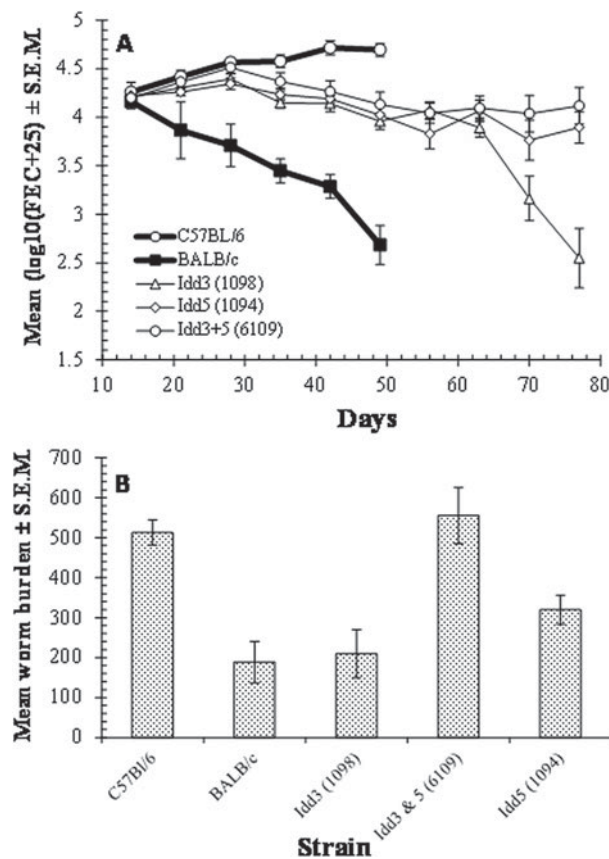


Fig. 2. (A) FEC during the course of the experiment by experimental group, and (B) worm burden at necropsy. For BALB/c and C57Bl/6 mice this was day 51–53, and for the congenic strains it was day 77.

6109 but that strain 1098 could not be separated clearly from strain 1094.

However, the data were heavily biased by the first 7 weeks when the strains had overlapping and similar mean FEC. Confining the analysis to the last 4 weeks (days 56, 63, 70 and 77) when strains began to diverge, again gave a highly significant main effect of strain ($F_{2,39} = 5.0$, $P = 0.012$) and indicated that the strains diverged significantly (2-way interaction time * strain, $F_{4,2,82,1} = 10.6$, $P < 0.001$). *Post hoc* tests (LSD) indicated that FEC in strain 1098 were significantly different from those of strain 1094 ($P = 0.021$) and of strain 6109 ($P = 0.005$).

Worm burdens

BALB/c and the C57Bl/6 mice, which acted as responder and non-responder controls, respectively, were culled in week 8 on days 51–53 because some of the C57Bl/6s were beginning to lose condition through having developed very high worm burdens at this time (Fig. 2B and Table 2), ranging from 278 to 682 worms. BALB/c were also culled at this time to enable comparison, their FEC on average being extremely low, but as it turned out whilst, most had rejected their worms (10 had worm burdens of fewer than 50 worms), there were some with surprisingly

high worm burdens, including 3 worm burdens of 450, 549 and 603). However, FEC even in these animals were low (600, 3600 and 1800, respectively) indicating that their worm burdens were mostly non- or low-fecund, and likely to be rejected within a few more days, had the experiment been continued for longer. This variation in BALB/c mice is consistent with earlier work where they have been found to rank as intermediate responders (Behnke *et al.* 2006b). There was a highly significant difference in worm burdens between these 2 strains at necropsy (Mann–Whitney *U* test, 1-tailed predicting higher worm burdens in C57Bl/6 mice, $z = 3.45$, $P < 0.001$).

Figure 2B and Table 2 show also that among the NOD congenics, strain 1098 had lower worm burdens than the other strains. Confining analysis to the 3 congenic groups gave a highly significant effect of strain (1-way GLM with strain on log₁₀(worm burden + 10), $F_{2,41} = 11.074$, $P < 0.001$) and *post hoc* tests (LSD) indicated that strain 1098 differed significantly from strain 1094 ($P = 0.002$) and from strain 6109 ($P < 0.001$), but there was no difference between strains 1094 and 6109.

PubMed search for candidate genes in the B10 insert region

There were 9 genes associated with ‘Helminth’, of which 3 were also associated with ‘*Heligmosomoides*’ (Table 3) and 42 were associated with ‘Diabetes’ (‘IDD5 Supplementary data.xls’). *CD28* (61Mb) had the highest number of PubMed hits for ‘Helminth’ and ‘*Heligmosomoides*’.

SNP analysis

Ensembl variation 67 contains 17834 SNP within 284 genes in the region between D1Mit478 (Chr1:51 981 710) and D1mit134 (Chr1:80 264 451) when comparing mouse strain C57BL/6 with NOD/LtJ (‘IDD5 Supplementary data.xls’). There were only 2 positions in these genes where C57BL/6J differ from C57BL/10 so C57BL/6 should be a good proxy for C57BL/10; however, sequence coverage for C57BL/10 may be limited. The only obvious loss of function polymorphism amongst the SNP was a premature stop codon in *Gm5528* at 72 Mb, but this gene is annotated in Ensembl as a processed pseudogene so the premature stop codon is unlikely to have any functional consequences. There were 115 non-synonymous SNP within 34 genes so these SNP alone are not very useful for prioritizing genes and therefore we combined the SNP data with the lists of genes with known associations with helminths.

The genes with literature associations with ‘Helminths’ and ‘*Heligmosomoides*’ were scanned for ‘interesting’ SNP (Functional SNP in Table 3). These were classified as the SNP that were most likely to have functional consequence (See Table 3)

Table 2. Arithmetic, log and geometric means of worm burdens at necropsy of the 5 strains of mice utilized in the experiment

Strain	No. of mice	Arithmetic	Log	Geometric	
		Mean \pm S.E.M.	Mean \pm S.E.M.	Mean	Range
1094	15	319.6 \pm 36.7	2.46 \pm 0.071	275.1	56–467
6109	14	555.3 \pm 69.7	2.70 \pm 0.060	494.7	145–1061
1098	15	209.4 \pm 60.1	1.86 \pm 0.202	61.8	0–594
C57Bl/6	14	512.6 \pm 31.4	2.71 \pm 0.030	498.2	278–682
BALB/c	15	188.6 \pm 51.6	2.07 \pm 0.123	108.6	4–603

For statistical analysis see text.

Table 3. Counts of hits in PubMed for genes in the region associated with the 4 search terms and numbers of SNP associated with each gene

Gene	Helminth	<i>Heligmosomoides</i>	Diabetes	Inflammation	SNP count	Functional SNP
<i>Cd28</i>	14	8	131	436	118	1
<i>Icos</i>	6	1	35	77	33	1
<i>Des</i>	2	0	402	275	55	1
<i>CtLa4</i>	1	1	153	79	33	0
<i>Fev</i>	1	0	109	720	12	0
<i>Stat1</i>	1	0	47	335	3	0
<i>Stat4</i>	1	0	23	78	3	0
<i>Gls</i>	1	0	9	3	7	0
<i>Cxcr2</i>	1	0	6	293	19	0

SNP that have different annotations on different transcripts will have been counted more than once.

Functional SNP had one of the following annotations: INTRONIC&SPLICE_SITE, NON_SYNONYMOUS_CODING, SYNONYMOUS_CODING&SPLICE_SITE, NMD_TRANSCRIPT&INTRONIC&SPLICE_SITE, STOP_GAINED, SYNONYMOUS_CODING&NMD_TRANSCRIPT.

although almost any SNP could have important consequences for gene expression. *Cd28* contained 63 unique SNP (the 118 SNP in Table 3 includes the SNP in multiple transcripts with different annotations) although few of these had support from multiple sources. One SNP was within a splice site, which could have a significant impact on function. Others were in regions that could affect expression or splicing but their potential effects on function are difficult to predict and no studies of the effect of these SNP have been published. *Icos* (*Cd278*) had 31 SNP of which 1 was non-synonymous although this was predicted to be benign by Polyphen (Ramensky *et al.* 2002; Adzhubei *et al.* 2010). In the case of *Ctla4* (*Cd152*) none of the 26 SNP could cause a structural difference in the gene product but all could potentially cause differences in expression. There was a single SNP in an intronic splice site in *Des*, the gene for desmin which is a subunit of the intermediate filaments in the sarcomeres of muscle tissue.

Genes in the QTL region for which no publications were found were also reviewed. The SNP with the greatest potential structural impact are those that cause nonsense mediated decay (NMD). There were 300 SNP that could cause NMD in 15 different genes; the biotype of all these genes was 'nonsense

mediated decay'. For all of these genes the NMD attribute was associated with just one of several transcripts.

Haplotype structure of chromosome 1 for CBA and SWR

The QTL on MMU1 in the F6 hybrids lies between D1Mit171 (36 800 667) and D1Mit46 (75 569 122) (Behnke *et al.* 2006a). Therefore, it is possible that the QTL gene/s is outside the NOD congenic region (Table 1). We have previously used haplotypes from the Perlegen mouse SNP to identify candidate genes within QTL regions (Behnke *et al.* 2010; Goodhead *et al.* 2010) and here we used the same approach to identify genes that were on different haplotypes between CBA and SWR mice within the MMU1 QTL. A list of haplotypes where CBA and SWR mice have different alleles and genes within, proximal or distal to those haplotypes is shown in 'IDD5 Supplementary data.xls'. The object of this analysis was to eliminate genes where both strains of interest have the same haplotype and this strategy can dramatically reduce gene lists (Behnke, *et al.* 2010a; Goodhead, *et al.* 2010). On this occasion the number of candidate genes in the QTL region was reduced

from 453 to 326 by this approach. A search of PubMed Titles and Abstracts with these 326 gene names and the same keywords as described above did not identify any genes with published associations with helminths or *Heligmosomoides* beyond those already described within the NOD congenic region. *Cd28*, *Icos* and *Ctla4* (see above) had different haplotypes between CBA and SWR mice; however, both strains had different haplotypes from NOD/LtJ.

DISCUSSION

Despite the relatively slow response of NOD mice to primary infection with *H. bakeri* (Saunders *et al.* 2007) and to repeated infections, this experiment has clearly shown that when the time frame under the latter protocol was extended beyond that normally used under repeated infection regimens (6 weeks), rejection of worms did occur. However, it was further slowed when NOD alleles of genes mapping in the region between D1Mit478 (Chr1:51 981 710) and D1mit134 (Chr1:80 264 451) on MMU1 were replaced by those from the very poor responder strain C57Bl/10. In this experiment the slower response was evident in 2 congenic strains and was supported both by their FEC and by worm burdens at necropsy. This is the first experimental study to show unequivocally that the expulsion of *H. bakeri* worms from mice can be delayed by alleles of genes mapping in this region of mouse chromosome 1. Moreover, it is highly pertinent that this region maps well within the confidence intervals of the *H. bakeri* resistance QTL (*Hbnr1*) described in Behnke *et al.* (2006a). Since the B10 insert is relatively short, it now allows our search for resistance genes to be focused on a few candidates within this region.

Our PubMed search identified *Cd28*, *Icos* (*Cd278*) and *Ctla4* (*Cd152*) as being the genes most linked in the literature with helminth infections. The gene products of *Icos* and *Ctla4* are both members of the CD28 superfamily of costimulatory surface receptor molecules found on T cells which participate in Toll pathways and are important in expansion of Foxp3+ T reg cells (CD278 in mucosal tissues), driving Th2 responses (CD152 in lymphoid tissues) and in downregulating (CD152) the signal arising from the MHC and T-cell receptor interaction (Redpath *et al.* 2013). CD152, like CD28, interacts with B7 (CD80 and CD86) and has far greater affinity for the CD80/86 ligands, whilst CD278 interacts with the ICOS ligand, CD275. Functional CD80/86 and CD152 are particularly important in driving Th2, IL4-dependent antibody responses (Lu *et al.* 1994; Greenwald *et al.* 1997), although CD28 is not, since in CD28 knockout mice the IgG1 and IgE responses to *H. bakeri* are not inhibited (Gause *et al.* 1997). Blockade of the B7 (CD80/86)/CTLA4 (CD152) interaction did not impair the expulsion of *Nippostrongylus brasiliensis* (Harris *et al.* 1999)

nor was acquired protective immunity to *H. bakeri* weakened in mice with antibody blocked CD28 and CTLA4 (CD152)-B7 (CD80/86) interactions (Gause *et al.* 1996). CD28 knockout mice resist challenge effectively, as do also CD80/86 knockouts (Ekkens *et al.* 2002) and their IL-4 secretion by CD4+ T cells, IgG1 and IgE responses to infection are unaffected (Gause *et al.* 1997). Nevertheless, acquired protective immunity to *H. bakeri* is known to be highly dependent on IgG1 antibodies (Pritchard *et al.* 1983; Harris *et al.* 2006, 2014; McCoy *et al.* 2008; Liu, *et al.* 2010), so polymorphisms in these genes with resultant differences in gene products affecting their roles in the immune response, may well explain some of the genetic differences between strong and weak responder mouse strains associated with the QTL on MMU1 and the results of the current experiment. It may be relevant that most of the experimental work with knockouts and antibody blocked responses has been carried out in immunized animals very soon after challenge (usually about day 10), when despite the absence of fecund adults, many worms are known to be still alive but arrested in the mucosal tissues (Behnke and Parish, 1979) and in mouse strains that rank as either poor or intermediate responders (Behnke *et al.* 2006b). It is therefore conceivable that SNP in this family of genes still play some decisive roles in longer term, repeated infection experiments in which differences in immune responsiveness become apparent only after several months of repeated challenge.

Consistent with the above, the SNP analysis did not reveal any obvious SNP that might be unequivocally linked to resistance to *H. bakeri*. In *Cd28* there was only one SNP that might have affected function, but *Cd28* had the strongest association with '*Heligmosomoides*' in the literature. *Icos* only had one non-synonymous SNP that was probably benign. Although ICOS blockade has not been tested in *H. bakeri*, blockade in mice infected with *T. spiralis* does not delay worm expulsion (Scales *et al.* 2004). In the case of *Ctla4*, no structural changes were predicted but many possible regulatory consequences are possible. However, there is a known splice site SNP 77 in *Ctla4* exon 2 that is not present in the BioMart data set (<http://www.biomart.org/martservice.html>). This SNP has been shown to be the QTL SNP for the IDD5.1 diabetes QTL (Araki *et al.* 2009), the A allele of this SNP derived from C57BL/10 mice being associated with higher expression of the ligand-independent isoform of CTLA4 (liCTLA4). Ectopic expression of liCTLA4 in T cells limits T-cell activation, cytokine production and proximal TCR signalling and these effects appear to be protective against type 1 diabetes. Given the demonstrated effect of this SNP on immune function it is perhaps the best candidate SNP. The *Cd28*, *Icos*, *Ctla4* cluster is clearly subject to a publication bias in favour of known immune response genes;

however, in support of a role for these genes in protective immunity to helminth infections, a hypothesis free genome wide survey for SNP associated with helminth infections in humans identified *Cd28* and *Ctla4* as being independently subject to selection for resistance to helminths (Fumagalli *et al.* 2010). The haplotype structure of CBA and SWR genotypes was also markedly different in this region of MMU1, although distinct from that of the NOD mice, and it is possible that one or more combinations of SNP in this region of MMU1, perhaps in the regulating regions rather than structural, play a decisive role in determining the responder status of mice subjected to repeated infection protocols.

An increase in desmin expression has been linked to fibrosis associated with *Trichinella* spp. and *Fasciola hepatica* infections and there is also a SNP in an intronic splice site in this gene (Wu *et al.* 2008; Golbar *et al.* 2013). However, at this stage it is still not clear whether increased fibrosis is linked to either of the alleles expressing the different SNP, or how they may be involved in worm expulsion from the intestine. However, fibrosis is involved in the trapping of larvae during their development in the outer mucosa, under the serosa (Liu, 1965; Jones and Rubin, 1974; Behnke Parish, 1979), and hence variation in the fibrotic response is likely to contribute to response phenotype. Indeed, the granulomatous response (which involves fibrosis) to repeated *H. bakeri* infections, as quantified by visual assessment using a score ranging from 0 to 4, was linked to QTL on chromosomes 4, 8, 11, 13 and 17, but not on chromosome 1 (Menge *et al.* 2003; Behnke *et al.* 2010). Moreover, at 75.356×10^6 bp *Des* is located at the extreme distal end of the QTL region, and is therefore unlikely to be the key gene in the QTL region responsible for response phenotype to *H. bakeri* infection.

It is also possible that the QTL may be attributable to one of the genes in the region for which there are no publications, and therefore genes with structural polymorphism were reviewed. However, among the 300 SNP that were identified, the NMD attribute was associated with just one of several transcripts and therefore could simply be an indicator of an erroneous gene model or even if that transcript was affected it is hard to predict what if any effect NMD would have on phenotype. Nothing more is known about the role of these genes, or the consequences of NMD to their structures, for resistance to *H. bakeri*.

Similarly the biotype for *Gm5528* is 'processed pseudogene' and although BioGPS (<http://biogps.org/#goto=welcome>) shows that it is most highly expressed in dendritic plasmacytoid B220 cells and haemopoietic stem cells and almost nowhere else, it seems unlikely to have a functional role (Su *et al.* 2004). Other genes which are known to play important roles in immune responses such as *Stat1* and *Stat4* were within the region and had literature

associations with helminths but not *Heligmosomoides*. However, these genes only had potentially regulatory SNP rather than structural SNP so the effect of these SNP on gene function is harder to predict.

Finally, based on the available evidence from the present study *Cd28* and *Ctla4* are perhaps the best candidate QTL genes within the region and could control the difference between both NOD and C57BL/6 and between SWR and CBA mice. In addition to the evidence presented above they are also close to the peak of the QTL for FEC, adult worm specific IgG1 antibody (IgG-Ad) and packed cell volume in week 6 (PCV6), which is around 52 Mb (Behnke *et al.* 2006a). However, it should be emphasized that the available evidence is not comprehensive at this stage and other genes could be responsible for or contribute to the observed differences in response. Furthermore, the plots of F ratios across the QTL region have multiple peaks for some phenotypes and different peaks for different phenotypes so there may well be more than one QTL gene within the region (Behnke *et al.* 2006a).

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0031182014001644>.

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