

# The role of Intelectin-2 in resistance to *Ascaris suum* lung larval burdens in susceptible and resistant mouse strains

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## SUMMARY

The underlying mechanism of predisposition to *Ascaris* infection is not yet understood but host genetics are thought to play a fundamental role. We investigated the association between the Intelectin-2 gene and resistance in F<sub>2</sub> mice derived from mouse strains known to be susceptible and resistant to infection. *Ascaris* larvae were isolated from murine lungs and the number of copies of the Intelectin-2 gene was determined in F<sub>2</sub> mice. Intelectin-2 gene copy number was not significantly linked to larval burden. In a pilot experiment, the response to infection in parental mice of both sexes was observed in order to address the suitability of female F<sub>2</sub> mice. No overall significant sex effect was detected. However, a divergence in resistance/susceptibility status was observed between male and, female hybrid offspring. The responsiveness to *Ascaris* in mice is likely to be controlled by multiple genes and, despite a unique absence from the susceptible C57BL/6j strain, the Intelectin-2 gene does not play a significant role in resistance. The observed intra-strain variation in larval burden requires further investigation but we hypothesize that it stems from social/dominance hierarchies created by the presence of female mice and possibly subsequent hormonal perturbations that modify the intensity of the immune response.

Key words: *Ascaris suum*, mouse model, C57BL/6j, CBA/Ca, Intelectin-2, resistance.

## INTRODUCTION

*Ascaris lumbricoides* and *Ascaris suum* are widespread parasitic nematodes of humans and pigs respectively (O'Lorcain and Holland, 2000; Crompton, 2001). Predisposition to heavy and light worm burdens has been demonstrated in longitudinal studies for ascariasis in both humans (Elkins *et al.* 1986; Haswell-Elkins *et al.* 1987; Thein-Hlaing *et al.* 1987; Holland *et al.* 1989; Forrester *et al.* 1990; Chan *et al.* 1992; Hall *et al.* 1992) and pigs (Boes *et al.* 1998), and can be detected over multiple rounds of chemotherapy (Holland *et al.* 1989; Chan *et al.* 1992). The bases for heterogeneity of infection or predisposition are not yet fully understood yet there are lines of evidence indicating that the underlying mechanism of resistance/susceptibility to *Ascaris* infection is influenced by host genetics and the host's immune repertoire, which is ultimately under genetic control (Holland *et al.* 1989, 1992; Peng *et al.* 1996; Williams-Blangero *et al.* 1999, 2002, 2008).

Convincing evidence for the genetic control of predisposition in humans requires pedigree studies (Quinnell, 2003), which facilitate the separation of the effects of genetic relatedness and the shared

household environment. Family-based linkage and association studies coupled with genome scanning have been undertaken on a single Jirel pedigree population in the Jiri region of eastern Nepal (Williams-Blangero *et al.* 1999, 2002, 2008). Williams-Blangero and coworkers demonstrated that 30–50% of variation in worm burdens is attributable to genetic factors (Williams-Blangero *et al.* 1999) and later identified 3 significant and 3 suggestive quantitative trait loci (QTL) which influenced infection intensity (Williams-Blangero *et al.* 2002, 2008). Recently, Nejsum and coworkers measured heritability for the intensity of *A. suum* infection in pigs (0.44), and found this to be similar to that reported for *A. lumbricoides* infection in humans (0.3–0.5) (Williams-Blangero *et al.* 1999; Nejsum *et al.* 2009).

Associational studies conducted in the past have demonstrated the HLA 30/31, STAT 6 and  $\beta_2$ -adrenoreceptor genes all play a role in susceptibility to *Ascaris* infection in humans (Holland *et al.* 1992; Ramsay *et al.* 1999; Peisong *et al.* 2004), highlighting the multigenic mechanism of resistance. Furthermore, a recent candidate gene study, undertaken in an *Ascaris*-endemic Columbian population cited 2 genes as being key to resistance to *Ascaris* infection, one of which is located in one of the QTL detected in the Jirel pedigree population (Williams-Blangero *et al.* 2002, 2008; Acevedo *et al.* 2009). However, in none of these studies is it clear whether resistance is directed

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at establishing larvae, tissue-migrating stages or the adult worms.

Alongside these studies in human populations and in pigs, work has been undertaken also in a mouse model, which is particularly useful for dissecting the factors affecting the initial establishment of larvae in the gut and their migration through the liver and lungs. Mouse strains differ markedly in the degree to which they support the migration of larvae to the lung stage of the infection (Lewis *et al.* 2006). It is known that there are strong MHC-dependent antibody responses to *A. suum* which differ between strains, but whether these underpin host-protective immunity is not clear, and indeed doubtful, since the antibody responses in MHC haplotypes were assessed at 14 and 28 days post-infection (p.i.), long after the worms would have disappeared from the lungs and intestines (Kennedy *et al.* 1986). Coupled with this Mitchell *et al.* (1976) did not detect a link between MHC haplotypes and susceptibility in early *A. suum* infection in mice. The resistance of strong responder strains is not dependent on T cells since nude, hypothyroid BALB/c mice fail to develop intense pulmonary worm burdens (Mitchell *et al.* 1976), and immunosuppression of CBA/Ca mice with the anti-inflammatory steroid hydrocortisone failed to enhance worm burdens in CBA/Ca mice (Lewis *et al.* 2007). A particularly marked contrast between strains supporting high and low worm burdens is seen in C57BL/6j and CBA/CA mice, respectively (Lewis *et al.* 2006, 2007; Dold *et al.* 2010). Following Mitchell *et al.* (1976), Lewis *et al.* (2006) confirmed that C57BL/6j mice are unique among strains in their susceptibility to infection and contrast with all other tested inbred strains which develop much lower worm burdens, similar to those of CBA/Ca. In this context, a particularly interesting recent finding was reported by Pemberton *et al.* (2004b) who showed that, in contrast to most other inbred mouse strains, C57BL mice lack the *Intelectin-2* (*Itln-2*) gene.

*Intelectins* (*Itlns*) are a family of galactose-binding lectins with homologues documented in sea squirts, fish, frogs and mammals (Chang *et al.* 2004; Chang and Nie, 2007). Differential expression of *Itln-2* has been documented in mouse models of *Trichinella spiralis* and *Trichuris muris* coupled with heightened expression levels coinciding with expulsion (Pemberton *et al.* 2004a; Datta *et al.* 2005; Artis, 2006). The dominant expression of *Itlns* following *T. spiralis* and *T. muris* infections in mouse strains resistant to infection led to the suggestion that *Itlns* may play an anti-nematode role (Pemberton *et al.* 2004a, b; Artis, 2006). Therefore, given the knowledge on differential expression of *Itln-2* in susceptible and resistant mice in other mouse models of helminth infection and the natural deletion of the *Itln-2* gene in the susceptible C57BL/6j strain (Pemberton *et al.* 2004a), it was logical to hypothesize that the *Itln-2*

gene is important in regulating susceptibility and resistance to *A. suum* infection in the mouse model, and that its lack endowed C57BL/6j mice with their uniquely high levels of susceptibility to the helminth.

This hypothesis was tested in the experiments described in this paper. We predicted that the more copies of the *Itln-2* gene a mouse strain possesses the stronger should be its resistance to infection with *A. suum* and we designed backcross experiments of the progeny of C57BL/6j and CBA/Ca mice to test this hypothesis. Equally, if this single gene makes the dominant contribution to response phenotype we would expect the response phenotypes of the backcrosses to follow simple Mendelian genetics with clear segregation consistent with a single gene hypothesis.

#### MATERIALS AND METHODS

In order to maximize the efficiency of the breeding colony, both male and female F<sub>2</sub> progeny were included in the key experiment reported in this paper. In earlier published work on the *A. suum* mouse model (Lewis *et al.* 2006, 2007; Dold *et al.* 2010), only male mice had been used and therefore it was necessary first to investigate whether there is a difference in the phenotypic response to infection between male and female mice of both parental strains. For this reason, a pilot experiment was undertaken in which the effect of sex on infection intensity in both strains was investigated. The protocol detailed for both experiments was approved by the Bioresources ethics committee, Trinity College Dublin and the Department of Health and Children, Ireland.

#### *Pilot sex experiment*

*Experimental animals.* A total of 60 (30 C57BL/6j and 30 CBA/Ca, 1:1 sex ratio) inbred mice was purchased from Harlan UK Ltd at 7 weeks of age. They were allowed to acclimatize to animal house conditions prior to infection at 8 weeks of age. Mice were housed in an animal maintenance room in the Bioresources Unit, Trinity College Dublin for the duration of the experimental procedure. The room was maintained at approximately 22 °C with a daily 12 h light/dark photoperiodicity. Water and commercial pelleted food were supplied *ad libitum*, and cages were cleaned on a regular basis. The mice were individually weighed on arrival, and randomly assigned, within each strain, to groups of 4 per cage.

*Parasite and infection of mice.* Approximately 5 000 000 embryonated *Ascaris suum* ova (batch no. AP 04.04) were supplied by the Danish Centre for Experimental Parasitology (CEP), Copenhagen. Individual doses were adjusted in order to contain 1000 fully embryonated ova in 1 ml of 0.1 M H<sub>2</sub>SO<sub>4</sub> as

described previously (Lewis *et al.* 2006). At 8 weeks of age, mice were administered an individual dose of 1000 *A. suum* ova.

*Post-mortems, collection of tissues and larval counts.* On days 6, 7 and 8 p.i., five female and five male mice of each strain were sacrificed and the lungs were removed. Living larvae were recovered from the lungs by means of the Modified Baermann technique, which has been described previously (Lewis *et al.* 2006). A pellet of the isolated viable larvae was suspended in 5 ml of a solution of 0.9% saline and 6% formalin. The solutions were agitated and 2 ml were pipetted into the chamber of a nematode-counting slide (Chalex Corporation). The number of larvae present in the grid area, which represents 1 ml, was counted under  $\times 40$  magnification. The number of larvae in a 1 ml solution was multiplied by the total volume in order to estimate the number of larvae in the tissue sample.

#### Backcross breeding experiment

*Mouse strains and breeding.* The C57BL/6j (50 female and 15 male) and CBA/Ca inbred strains were used to produce, first F<sub>1</sub> and then F<sub>2</sub> offspring. Parental strains were purchased from Harlan UK Ltd at 7 weeks of age and were allowed to acclimatize for 1 week prior to breeding. Parental reference strains were bred simultaneously to the F<sub>2</sub> progeny. All progeny were weaned at approximately 21 days old and all breeding was undertaken in the Bioresources Unit, Trinity College Dublin.

*Phenotyping of mice.* At 8 weeks of age, 120 F<sub>2</sub> and 60 parental strain mice were infected with 1000 *A. suum* ova (Table 1). All mice were euthanized on day 7 p.i. The entire lungs from each individual mouse were removed and larvae were isolated using the modified Baermann technique (Lewis *et al.* 2006) and enumerated as described above.

*Intelectin-2 gene screening.* At post-mortem, a tail snip was removed from each individual mouse and placed in 1.5 ml of 20% dimethyl sulfoxide (DMSO) and stored at  $-80^{\circ}\text{C}$ . DNA was extracted from tail snips of all F<sub>2</sub> mice and 4 reference parental mice (2 C57BL/6j and 2 CBA/Ca mice) using the QIAamp Tissue Kit (Qiagen). Samples were stored at  $-20^{\circ}\text{C}$ .

Since the *Itln-2* gene is absent in C57BL/6j mice, an *Itln-2* specific marker was not used to establish the *Itln* locus genotype in each F<sub>2</sub> mouse. A differential marker and subsequent primers for the *Itln* locus were previously designed by means of comparing the locus of C57BL/6j, sequenced by Pemberton *et al.* (2004a) and 129S7 [GenBank: HM370554] mice.

A unique region of the neighbouring gene, SLAMF7 differs in length in C57BL/6j and 129S7

Table 1. Number of F<sub>2</sub> and parental strain progeny infected with 1000 *Ascaris suum* ova

Female parental strain	Male parental strain	Number of progeny	
		Male	Female
C57BL/6j	C57BL/6j	15	15
CBA/Ca	CBA/Ca	15	15
C57BL/6j	F <sub>1</sub>	15	15
CBA/Ca	F <sub>1</sub>	15	15
F <sub>1</sub>	C57BL/6j	15	15
F <sub>1</sub>	CBA/Ca	15	15

mice. Furthermore, this region was found to be of a similar length in CBA/Ca mice as in 129S7 mice, when SLAMF7 primers were tested on CBA/Ca genomic DNA (Steven Wright, unpublished data) (Fig. 1). Therefore the SLAMF7 gene was considered a suitable marker for the *Itln* locus in the two mouse strains in the present study and primers were designed for this region.

The sequence of the SLAMF7 primers used were as follows: Forward primer: TG TAGAGGTGGA-TGGTGCTG; Reverse primer: TGGGGTTCCA-TTCTGAGTTT. Using SLAMF7 marker primers, C57BL/6j mouse DNA gives a 200 bp product, whereas 129S7 and CBA/Ca mouse DNA gives a 218 bp product. In F<sub>2</sub> mice these primers yielded 200 bp and/or 218 bp PCR fragments, depending on the number of copies of the *Itln-2* gene in the individual mouse analysed. Since the *Itln* genes and SLAMF7 are closely linked, the presence of *Itln* genes from either parental strain can be inferred from the marker sizes following PCR of F<sub>2</sub> mouse genomic DNA. F<sub>2</sub> mice with a C57BL/6j parent were determined to be homozygous and so have zero copies of the *Itln-2* gene, if there is no evidence of the 218 bp SLAMF7 product, or heterozygous, and so have 1 copy of the derived *Itln-2* gene, if both 200 bp and 218 bp products are detected. F<sub>2</sub> mice with a CBA/Ca parent were determined to be homozygous, so have 2 copies of the *Itln-2* gene, if only the higher 218 bp product can be seen, or heterozygous, so to have 1 copy of the *Itln-2* gene, if both 200 bp and 218 bp products are detected.

The common forward primer for *Itln-1* and *Itln-2* gene, MITLNComf1 (5'-GGTTCCTGCCATTA-CTCAGC-3'), and the *Itln-2* specific reverse primer, MITLN2r1-r (5'-TTTATCATGATTGCCACG-AGAGT-3'), were used to confirm the results of the SLAMF7 marker analysis (data not shown). These two primers span intron 1, giving a 350 bp product for *Itln-2* whereas the paralogous sequence for *Itln-1*, gives no product.

To confirm the presence or absence of the *Itln-2* gene in the genome of the F<sub>2</sub> mice, PCR was undertaken on all extracted DNA samples. Samples which

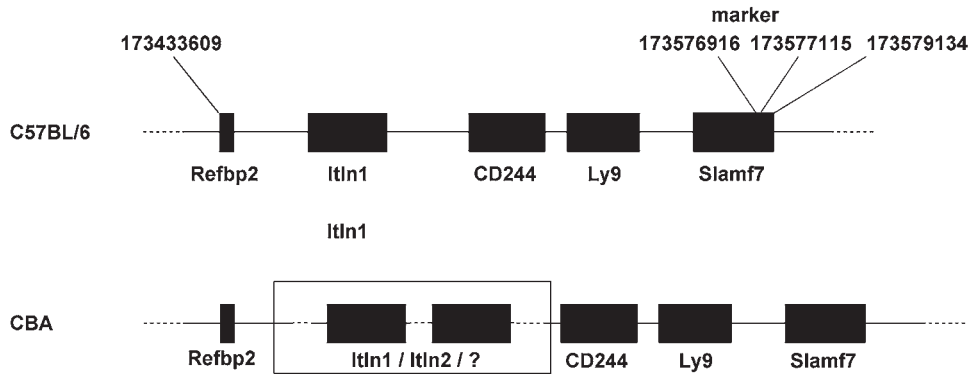


Fig. 1. Schematic diagram of *Itln* locus and neighbouring genes (*Refbp2*, *CD244*, *Ly9*, *Slamf7*) on chromosome 1 of C57BL/6j and CBA/Ca mice.

were tested using *Itln* specific primers were loaded on agarose gels, while those tested using SLAMF7 primers were loaded on polyacrylamide gels. Resultant PCR products were imaged using Quantity One<sup>®</sup> software.

#### Statistical analysis

**Pilot experiment comparing sexes.** Larval burdens in the pilot experiment designed to assess the effect of host sex were more variable than had been expected and even after transformations did not conform to the assumptions of parametric statistics. The experiment included 3 main factors (sex, strain and day after infection) but since no three-way non-parametric ANOVA (np ANOVA) is available, we analysed the data in stages by two-way np ANOVA, using bespoke software based on Meddis (1984) and applied as described by Barnard *et al.* (2007). Where possible we tested *a priori* predictions based on earlier published observations (e.g. that larval burdens should be higher in C57BL/6j mice, compared with CBA/Ca and that male mice should harbour more larvae than females) and in these we give the value of  $z$  and the corresponding  $P$ . Where specific predictions were not possible we applied a general test and give the value of  $H$  and its corresponding  $P$ . For all tests we considered a  $P$  value of 0.05 and lower, as indicating significant difference.

**Backcross breeding experiment.** Data recorded for the  $F_2$  mice and C57BL/6j and CBA/Ca parental strains were analysed at 2 levels. First, we considered any mouse that had more than 45 larvae as susceptible, using the highest burden recorded in the resistant CBA/Ca mice. This allowed  $F_2$  hybrid mice to be classified as either resistant or susceptible to *A. suum*. The percentages of resistant mice in relevant subsets are shown with 95% confidence limits, calculated as described by Rohlf and Sokal (1995) employing bespoke software. The factors affecting the percentage of resistant mice were analysed by

maximum likelihood techniques based on log linear analysis of contingency tables using the software package SPSS (Version 16.0.1.).

An initial full factorial model incorporated the following factors: parental strain with which the  $F_1$  were crossed (2 levels, CBA/Ca or C57BL/6j), sex of the  $F_2$  hybrid (2 levels, male or female), and number of copies of the *Itln-2* gene (3 levels, zero, 1 or 2 copies). Then, beginning with the most complex model involving all possible interactions, those combinations that did not contribute significantly to explaining variation in the data were eliminated in a stepwise fashion beginning with the highest-level interaction (backward selection procedure). A minimum sufficient model was then obtained, for which the likelihood ratio of  $\chi^2$  was not significant, indicating that the model was sufficient in explaining the data (these values are given in the legends to the figures as relevant). The importance of each term (i.e. interactions involving resistant/susceptible status and other relevant terms) in the final model was assessed by the probability that its exclusion would alter the model significantly and these values are given in the text. The remaining terms in the final model are summarized for completion, in the legends to appropriate figures, together with the likelihood ratio for the final model.

The larval burden data were highly overdispersed, and therefore non-parametric procedures were employed throughout. Non-parametric statistics cannot deal with 3 explanatory factors but a two-way non-parametric ANOVA based on Meddis (1984), has been described by Barnard *et al.* (2007) and was employed using bespoke software. We analysed the  $F_2$  hybrids separately in 2 steps, the first being the  $F_1$  to C57BL/6j hybrid offspring, and the second the  $F_1$  to CBA/Ca hybrid offspring. This approach is justified because the former hybrids can only have zero or 1 copy of the gene, and the latter can only have 1 or 2 copies, and unless a model can be fitted that controls for the parental strain (C57BL/6j or CBA/Ca), the outcome is likely to be heavily influenced by copy number in the parents. In each case we tested 2

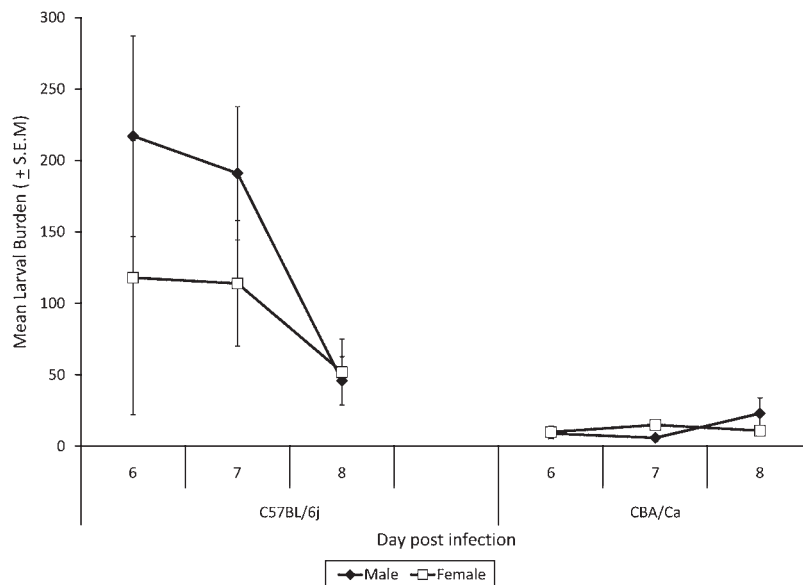


Fig. 2. Changes in mean larval burden ( $\pm$  S.E.M.) in the lungs of male and female C57BL/6j and CBA/Ca mice, following inoculation with 1000 *Ascaris suum* ova.

*a priori* predictions. The first was that worm burdens should be higher in male mice and, crucially, the second that more copies of the *Itln-2* gene should be associated with lower worm burdens.

## RESULTS

### Pilot sex experiment

The changes in lung worm burdens in both sexes of both strains are shown in Fig. 2. Overall mean worm burdens were higher in C57BL/6j mice compared with CBA/Ca mice and this difference between strains was significant on day 6 and day 7, and showed borderline significance on day 8 (2-way np ANOVA, for the specific prediction C57BL/6j > CBA,  $\approx 2.23$  [ $P=0.0129$ ],  $3.41$  [ $P=0.0003$ ] and  $1.63$  [ $0.0511$ ], respectively) at which time larval numbers had declined in both male and female C57BL/6j mice and were only marginally higher than those in CBA/Ca mice.

Larval burdens recorded at individual time-points were highly variable in C57BL/6j mice (on day 6 in males the range was 5–420, and in females 0–500). On day 7 p.i. slightly lower larval numbers were recorded in male and female C57BL/6j mice but by day 8 p.i., worm burdens in both strains had dropped considerably, as expected from earlier studies (Lewis *et al.* 2006, 2007; Dold *et al.* 2010).

In the CBA/Ca mouse strain larval burdens were similar in male and female individuals, throughout the experiment; whereas in C57BL/6j those in male mice appeared to be higher especially on days 6 and 7 p.i. (Fig. 2). However, no significant sex-effect was detected in the two-way np ANOVA (with sex and strain as main effects) at any of the post-mortem time-points. A further test, confined to C57BL/6j mice,

but incorporating all 3 post-mortem time-points gave borderline significance for the comparison between the sexes (2-way np ANOVA, days post-infection and sex of mice as main effects, for the specific prediction that males > females  $\approx 1.56$ ,  $P=0.06$ ), and despite the drop in the mean larval recovery between days 7 and 8 p.i., a general test of the effect of time on larval burdens was not significant ( $H_2=4.8$ ,  $P=0.09$ ).

*Backcross breeding experiment.* Pulmonary larval burdens were recorded on day 7 p.i. in male and female parental reference strains, C57BL/6j and CBA/Ca, and F<sub>2</sub> mice. Since CBA/Ca mice have 2 copies of the *Itln* locus and C57BL/6j mice none, it was expected that F<sub>2</sub> mice generated from crossing F<sub>1</sub> to C57BL/6j mice would have either 1 copy or none, and F<sub>2</sub> from the cross of F<sub>1</sub> to CBA/Ca mice would have either 1 or 2 copies. As shown in Table 2, this expectation was proved correct.

*Analysis based on percentage of responders in each F<sub>2</sub> subset.* As in earlier sections of this paper, responder mice were considered to be those animals that carried 45 or fewer pulmonary larvae at autopsy. Log linear analysis revealed, as expected, that the number of copies of the *Itln-2* gene carried by F<sub>2</sub> hybrids was dependent on the strain to which the F<sub>1</sub> were backcrossed (so either C57BL/6j or CBA/Ca; Table 2;  $\chi^2_2=91.7$ ,  $P<0.001$ ).

Resistant/susceptible status was dependent on the strain to which the F<sub>1</sub> were backcrossed ( $\chi^2_1=14.7$ ,  $P<0.001$ ). Thus comparing the data in Fig. 3 (A) with Fig. 3 (B), the percentage of resistant mice among the F<sub>1</sub> backcross to C57BL/6j mice (Fig. 3A) was 46.7% (range: 36.29 to 57.50), and among F<sub>2</sub> backcrossed to CBA/Ca (Fig. 3B) was 80.0% (70.09 to 87.57).

Table 2. The number of male and female F<sub>2</sub> hybrid offspring with zero, 1 and 2 copies of the *Itln-2* gene

F <sub>1</sub> generation mice	No. of copies of <i>Itln-2</i> gene	Sex	No. of mice
F <sub>1</sub> × C57BL/6j	0	male	15
F <sub>1</sub> × C57BL/6j	0	female	20
F <sub>1</sub> × C57BL/6j	1	male	15
F <sub>1</sub> × C57BL/6j	1	female	10
F <sub>1</sub> × CBA/Ca	1	male	19
F <sub>1</sub> × CBA/Ca	1	female	11
F <sub>1</sub> × CBA/Ca	2	male	10
F <sub>1</sub> × CBA/Ca	2	female	20

Resistant/susceptible status was also independently affected by the sex of the hybrid offspring ( $\chi^2_1 = 5.2$ ,  $P = 0.023$ ). Among male F<sub>2</sub> the percentage of responders was 53.3% (42.50 to 63.71) whereas among females it was 73.3% (62.99 to 81.92). This is best seen among the offspring of F<sub>1</sub> to C57BL/6j crosses (Fig. 3A) where the discrepancy between the sexes was relatively large, but there is also a trend in favour of females among the F<sub>1</sub> to CBA/Ca crosses carrying 1 copy of the *Itln-2* gene although not among those with 2 copies.

In addition to the 3 terms given in the text, there was significant interaction between number of copies of the *Itln-2* gene and host sex reflecting simply the differences in numbers of mice in the subsets given in Table 2. The goodness-of-fit of the overall model was given by the likelihood ratio  $\chi^2_{12} = 6.3$  ( $P = 0.9$ ).

Crucially, there was no significant link between number of copies of the *Itln-2* gene carried and resistant/susceptible status (Fig. 3).

*Analysis based on pulmonary larval burdens in each subset.* Among F<sub>1</sub> to C57BL/6j hybrids, male mice carried significantly higher larval burdens than females ( $z = 2.87$ ,  $P = 0.002$ ), as can be seen in Fig. 4 (A). However, there was no significant effect of sex for the F<sub>1</sub> to CBA/Ca hybrids ( $z = 0.7$ ,  $P = 0.24$ , Fig. 4B). In neither case did copy number of the *Itln-2* gene affect the larval burden (for the F<sub>1</sub> to C57BL/6j hybrids  $z = 1.16$ ,  $P = 0.12$ ; for the F<sub>1</sub> to CBA/Ca hybrids  $z = 0.63$ ,  $P = 0.26$ , Fig. 4).

A high degree of variability in the larval burdens was recorded in individual mice, which was particularly evident in male C57BL/6j mice (variance-to-mean ratio: 325.38) and male F<sub>2</sub> mice with male C57BL/6j parental strains (variance-to-mean ratio: 496.17) (Table 3). The dispersion of larval burdens in female mice of these two strains was comparatively uniform (variance-to-mean-ratio in C57BL/6j: 14.99, F<sub>1</sub> × C57BL/6j: 110.19). Individual larval burdens were most consistent in CBA/Ca parental reference strains (variance-to-mean ratio in males: 10.19, females: 7.42) and female F<sub>2</sub> mice with

C57BL/6j mothers (variance-to-mean ratio: 2.34) (Table 3).

## DISCUSSION

The unique susceptibility of the C57BL/6j mouse to developing intense lung burdens with the larvae of *A. suum*, and the observation that this strain in particular has a deletion in the *Itln-2* gene cassette on chromosome 1, suggested to us that these may be causally linked. Equally if just a single gene is responsible we would expect backcross progeny to segregate in their response phenotypes along Mendelian predictions, and in this case in line with resistance being dominant. The results reported in this paper are clearly not consistent with either prediction. Our data show convincingly that the *Itln-2* gene does not underlie the differing response phenotypes of C57BL/6j mice and that susceptibility in this system is controlled by more than a single gene. Unexpectedly our results also emphasize the confounding effect of host sex, particularly when both sexes are housed in separate cages but in close proximity.

In the present study we assessed the contribution of a candidate gene, *Itln-2*, to host variation in the *A. suum* mouse model. Our genomic PCR data are consistent with the presence of *Itln-2* in the *Itln* locus on chromosome 1 of the CBA/Ca genome, but since we did not have the resources to fully characterize the *Itln* locus in the CBA/Ca mouse, which may indeed contain multiple *Itln* genes as compared to a single *Itln* gene (*Itln-1*) in C57BL/6j mice, the results of the *Itln* screening were effectively the number of copies of the CBA *Itln* locus, rather than the *Itln-2* gene itself.

In the backcross experiment undertaken, F<sub>2</sub> mice with varying numbers of copies of the *Itln-2* gene were derived from the parental strains C57BL/6j and CBA/Ca. The single gene hypothesis, based on resistance being dominant, predicts that the outcome of F<sub>2</sub> breeding experiments resulting from 2 parental reference mouse strains, which represent the extremes of resistance and susceptibility, should yield all resistant mice in the backcross to the resistant parent and 50% resistant offspring when backcrossed to the susceptible parent. When mice were separated into subsets based on the cut-off pulmonary burden of 45 larvae, a 50–50 split in responders and non-responders was not evident among F<sub>1</sub> to C57BL/6j hybrid offspring, and some of the F<sub>1</sub> mice backcrossed to CBA/Ca were susceptible. On this basis, it is likely that more than one gene is involved in resistance to *A. suum* infection in the mouse, and the quantitative analysis based on worm burdens concluded that there was no association between the *Itln-2* gene and resistance to *A. suum* infection.

The lack of an association between the candidate gene of interest and resistance to *A. suum* infection

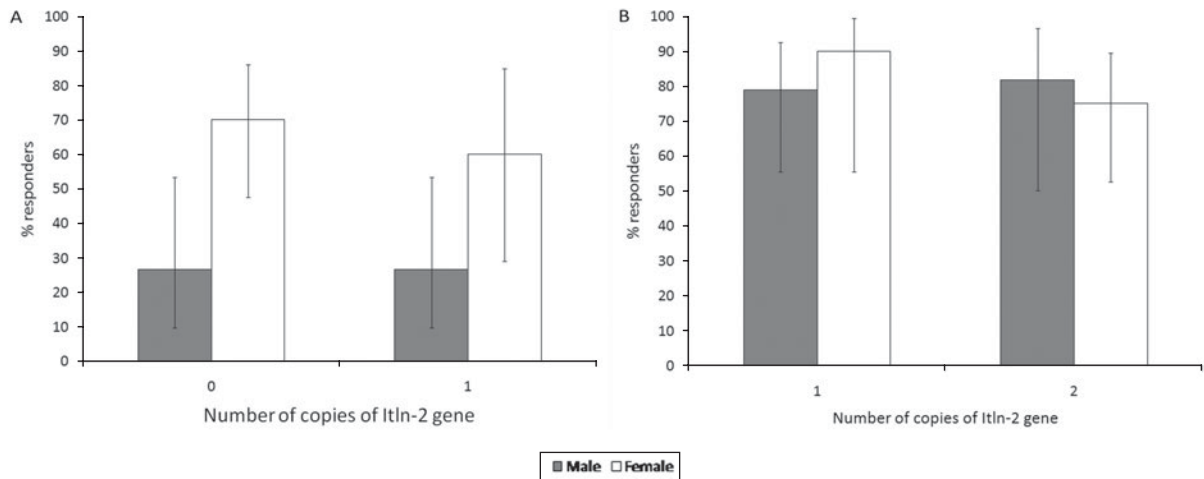


Fig. 3. Percentage of responder mice among F<sub>2</sub> backcross hybrids, by number of copies of the *Itln-2* gene and by sex. (A) F<sub>1</sub> backcrosses to C57BL/6j mice (B) F<sub>1</sub> backcrosses to CBA/Ca mice.

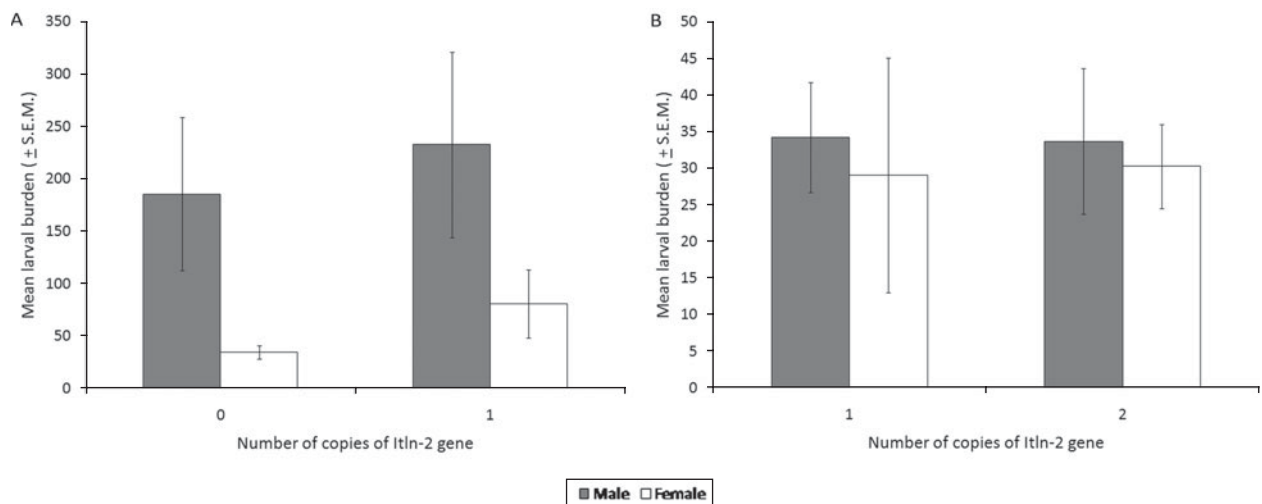


Fig. 4. Mean pulmonary larval burdens among F<sub>2</sub> backcross hybrids, by number of copies of the *Itln-2* gene and by sex. (A) F<sub>1</sub> backcrosses to C57BL/6j mice (B) F<sub>1</sub> backcrosses to CBA/Ca mice.

contrasts with studies in which expulsion of *T. spiralis* and *T. muris* coincides with upregulation of *Itln-2* expression (Pemberton *et al.* 2004a, b; Datta *et al.* 2005; Artis, 2006). Immunolocalization of *Itln-2* protein in Paneth and goblet cells (Pemberton *et al.* 2004b; Artis, 2006) may explain the common role of *Itln-2* in resistance against *T. spiralis* and *T. muris* as both parasites inhabit the intestinal environment at the time of the host immune response, which results in expulsion. However, even though expression of *Itln-2* and another *Itln* gene, *Itln-1* in the small intestine was evident during *Nippostrongylus brasiliensis* infection (Voehringer *et al.* 2007), *Itlns* cannot be the sole mechanism of worm expulsion because C57BL/6j mice expel the parasite with the same kinetics as BALB/c mice (Ishiwata *et al.* 2002). Similarly, *T. muris* parasites are expelled from C57BL/6j mice before adult worms develop (Faulkner *et al.* 1998) despite lacking the *Itln-2* gene (Pemberton *et al.* 2004a). Furthermore, Voehringer *et al.* (2007)

demonstrated that high *Itln* mRNA transcript levels in the lung of transgenic mice did not enhance parasite clearance. It is not yet known whether expulsion of *T. spiralis* and *T. muris* is influenced by *Itln* locus genotype, but we have shown clearly here that resistance to *A. suum* is not. The mechanism of resistance to *A. suum* infection appears to manifest itself in the liver of CBA/Ca mice (Dold *et al.* 2010) and *Itln-2* has not yet been documented to contribute to an immune response against a murine liver infection.

Analysis of the larval burdens observed in the pilot experiment indicated that mouse sex did not significantly affect worm burdens, although there was a clear tendency towards male mice carrying heavier larval worm burdens (Dold *et al.* 2010). In addition, the heightened larval burdens in the susceptible strain were not considered to be a concern because they merely enhanced the divergence between the pulmonary burdens of the two mouse strains of the

Table 3. The distribution of *Ascaris suum* larvae in F<sub>2</sub> and parental reference strain mice

Mother	Father	Sex	Larval burden range	Mean larval burden $\pm$ S.E.M.	Variance	Variance-to-mean ratio	K
C57BL/6j	F1	M	10–155	87 $\pm$ 12.18	2724.52	31.32	49.74
CBA/Ca	F1	M	0–115	35 $\pm$ 9.36	1314.29	37.55	2.91
F1	C57BL/6j	M	0–980	330.67 $\pm$ 104.58	164067.40	496.17	2.64
F1	CBA/Ca	M	5–95	33 $\pm$ 7.51	845.71	25.63	2.22
C57BL/6j	F1	F	5–110	31.67 $\pm$ 6.98	73.95	2.34	5.02
CBA/Ca	F1	F	0–90	20 $\pm$ 6.71	675.00	33.75	1.24
F1	C57BL/6j	F	5–350	67.67 $\pm$ 22.3	7456.67	110.19	5.04
F1	CBA/Ca	F	5–170	39.67 $\pm$ 10.52	1658.81	41.82	5.71
C57BL/6j	C57BL/6j	M	10–990	232 $\pm$ 7.94	75488.57	325.38	9.83
C57BL/6j	C57BL/6j	F	5–80	36.33 $\pm$ 6.03	544.52	14.99	5.11
CBA/Ca	CBA/Ca	M	5–45	19 $\pm$ 3.59	193.57	10.19	4.00
CBA/Ca	CBA/Ca	F	0–35	14.67 $\pm$ 2.69	108.81	7.42	6.91

model in the direction that we expected (i.e. higher worm burdens in C57BL/6j mice). Coupled with this, it was more practical to work with both male and female progeny in the much larger and logistically more complicated backcross experiment, not the least for ethical reasons.

The variability in larval burdens among susceptible individuals was magnified in the larger F<sub>2</sub> study and among the parental reference strain mice. Despite this, divergence of the susceptible C57BL/6j and resistant CBA/Ca mice strains was still clearly evident. The mechanism behind the larval burden intra-strain variability, particularly of the susceptible male individuals, is not fully understood and requires further investigation, but we hypothesize that an important factor may be that, in contrast to earlier work based on only one sex, here mice of both sexes were present in close proximity in the animal house, albeit in separate cages.

Sex differences in immune function are mediated, in part, by steroid hormones (Grossman, 1985; Klein, 2000, 2004). The androgen testosterone is known to have suppressive effects on the immune system (Ahmed *et al.* 1985; Alexander and Stimson, 1988; Hillgarth and Wingfield, 1997). Host gender is an important factor in determining immunity to *Trichuris muris* (Hayes *et al.* 2007; Hepworth and Grecis, 2009) and a recent study conducted by Hepworth *et al.* (2010) demonstrated that endogenous sex steroid hormones have a direct effect on the generation of a Th2 response to the intestinal roundworm. Increased host testosterone levels have led to higher levels of *Strongyloides ratti* (Kiyota *et al.* 1984), *N. brasiliensis* (Solomon, 1966; Bone and Bottjer, 1986), *Nippostrongylus muris* (Haley, 1958) and *T. spiralis* (Charniga *et al.* 1981) and *Heterakis spumosa* (Harder *et al.* 1992), demonstrating that immunosuppression by testosterone is responsible for increased susceptibility to infection. Moreover, as discussed by Bohus and Koolhaas (1991), exposure to environmental and social stressors can lead to immunosuppression and therefore increased susceptibility to infection. In captivity, aggression in male

rodents can be triggered by female scent alone (Barnard *et al.* 1997). Therefore, it is possible that even though the male mice in this study were not housed in cages with females, the scent of the females in close proximity may have increased aggression and investment in courtship behaviours among the male mice. Hence, the stressors associated with the presence of female mice may have also led to increases in gonadal steroids in males. The observed intra-strain variation in larval burdens may be a result of differing responses to an environmental challenge, influenced by social organization (Laudenslager and Kennedy, 2008). Infection in dominance hierarchies has been documented previously in response to the protozoan *Babesia microti* (Barnard *et al.* 1994), but data on dominance were not collected in the current work. Genetic variation and differences in expression of sex steroid-related immunosuppression may account for the varied and increased susceptibility in C57BL/6j but not CBA/Ca individuals. Mice with different MHC haplotypes have significantly different responses to *Heterakis spumosa* infection, which only become apparent after treatment with testosterone (Harder *et al.* 1994).

The dramatically increased larval burdens in a number of male C57BL/6j mice and male F<sub>2</sub> offspring bred from male C57BL/6j parents is of particular interest as previous attempts at immunosuppression, using the steroid hydrocortisone, did not enhance *A. suum* larval burdens in the susceptible mouse strain (Lewis *et al.* 2007). Therefore, it is possible that the stressor resulting from the presence of females in close proximity induced heightened testosterone levels in male individuals, which lead to some form of potent immunosuppression. The contrasting results between work conducted by Lewis *et al.* (2007) and the current study may indicate that a single pulse administration of hydrocortisone does not have as great an influence on the mechanism of resistance as a continuous stressor. The target of this effect, however, remains intriguing since resistance to *A. suum* in the mouse is known to be independent of T-cells (Mitchell *et al.* 1976).



While larval burdens were confounded by an unexpected effect of co-habitation of male and female mice, this study found no evidence that *Itln-2* plays a significant role in preventing the accumulation of *A. suum* larvae in the lungs of resistant strains of mice.

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