Increased Susceptibility to Collagen-Induced Arthritis in Female Mice Carrying Congenic *Cia40/Pregq2* Fragments

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Abstract

Background

Collagen-induced arthritis (CIA) in mice is a commonly used experimental model for rheumatoid arthritis (RA). We have previously identified a significant quantitative trait locus denoted *Cia40* on chromosome 11 that affects CIA in older female mice. This locus co-localizes with another locus, denoted *Pregq2*, known to affect reproductive success. The present study was performed to evaluate the role of the *Cia40* locus in congenic B10.Q mice, and identify possible polymorphic candidate genes, which may also be relevant in the context of RA.

Methods

Congenic B10.Q mice carrying a NFR/N fragment surrounding the Cia40/*Pregq2* loci were created by 10 generations of backcrossing (N10). The congenic mice were investigated in the CIA model, and the incidence and severity of arthritis were recorded as well as the serum levels of anti-collagen II (CII) antibodies.

Results

Significant effects on onset, incidence, severity and anti-CII antibody titres were observed in female mice carrying a heterozygous congenic *Cia40/Pregq2* fragment of NFR/N origin, containing one or more polymorphic genes. Congenic male mice did not show increased incidence of CIA, but males carrying a heterozygous fragment showed a significant increase in severity in comparison to wildtype B10.Q males (littermates).

Conclusions

The *Cia40/Pregq2* locus at chromosome 11 contains one or more polymorphic genes of NFR/N origin that significantly influence both incidence and severity of CIA in heterozygous congenic mice of the B10.Q strain. The major polymorphic candidate genes for the effects on CIA are *Cd79b*, *Abca8a* and *Map2k6*. The congenic fragment also contains polymorphic gene/s that affect reproductive behavior and reproductive success. The *Sox9* gene, known to influence sex reversal, is a candidate gene for the reproductive phenotype.

Introduction

Collagen-induced arthritis (CIA) is a commonly used animal model for rheumatoid arthritis (RA). Although CIA shares several features with RA, there are some obvious differences between the mouse model and the human disease [1-3]. One such dissimilarity is the reversed sex susceptibility. Characteristic for RA is a female predominance [4], while the opposite situation commonly is the case in mice developing CIA. Because of the male predominance of CIA in most strains of mice, including B10.Q, most published CIA experiments have been performed on males.

We have previously performed a genetic linkage analysis on multiparous female mice from an N2 cross between NFR/N and B10.Q, with the aim to find CIA loci that are linked to disease development in females [5]. We identified one novel significant CIA–associated locus on chromosome 11, which is now denoted *Cia40*. No other CIA loci/genes have previously been found in this region, but the central part of chromosome 11 is known to contain a number of inflammation loci, such as *Eae22*, *Eae6b, Eae23* and *Eae7* [6,7,8] However, none of the experimental autoimmune encephalitis (EAE) loci are located close to the *Cia40* linkage peak, indicating that other polymorphic genes might be of importance.

Interestingly, in an additional QTL analysis with females of the same cross (N2 generation of NFR/N and B10.Q), we detected a highly significant QTL close to *Cia40* on chromosome 11 linked to the trait “pregnancy frequency” [9]. This locus is denoted *Pregq2*, and controls the frequency of successful pregnancies following

successful copulation (successful coitus recorded by the detection of the ”vaginal plug”). In the initial QTL analysis heterozygous mice carrying NFR/N genes at the *Pregq2* locus suffered from an increased frequency of pregnancy failures [9]. We hypothesized that the *Cia40/Pregq2* region of chromosome 11 may contain polymorphic genes that influence both CIA incidence and breeding success.

Although our original QTL analysis was performed on (aged) female mice with the hope of finding CIA loci with female predominance there would still be a possibility that the *Cia40* locus is of equal importance in both sexes. In the present paper we present results indicating that *Cia40* congenic females are more affected by CIA than males. We also show that the *Cia40/Pregq2* locus is linked to a disturbed reproductive behaviour and reduced breeding performance in females.

Materials and Methods

Mice

Inbred NFR/N mice were originally obtained from National Institute of Health (Maryland, USA) and the B10.Q mice were originally from the animal colony of Prof. Jan Klein. (B10.Q × NFR/N) × B10.Q N_{10} mice were bred in the animal house, Dept. of Pathology, Lund University, Sweden. The animals were fed standard rodent chow and water in a photoperiod of L:D 12:12. All mice used in the present study had clean health monitoring protocols according to the Federation of European Laboratory Animal Sciences Association (FELASA) recommendations. Ethical permission: M236-06 (reproduction and arthritis).

The *Cia40* congenic mice and the fragment

To confirm the previously identified linkage on chromosome 11, we backcrossed the NFR/N strain to the (more) CIA resistant strain, B10.Q. Mice heterozygous for the congenic region (a small fragment from the NFR/N strain on B10.Q background) were chosen for additional backcrossing for 10 generations (Figure 1). All mice were derived from the same set of parents. Subsequently, the congenic mice were intercrossed. Mice heterozygous for NFR/N markers between D11Mit70 (93.8 Mb) and D11mit214 (114.8 Mb) were intercrossed two times in order to produce the congenic line *Cia40.* All the mice, homozygote for *Cia40* in the study, have equal

fragment size (Figure 1). However, the heterozygote animals differ slightly in fragment length among the individuals (1-2 Mb).

Genotyping

Genomic DNA was isolated from the tip of the tail according to a previously described protocol [10]. Nine fluorescence-labelled polymorphic micro-satellite markers (INTERACTIVA, Ulm, Germany) were used to cover the heterozygous fragment derived from the NFR/N as previously described [10] (Figure 1). The PCR products were analysed on a MegaBACE™ 1000 (Amersham Pharmacia Biotech) according to the manufacturers protocol. Data were analysed with Genetic Profiler 1.1.

Induction and evaluation of CIA

To induce CIA, 8-12 weeks old mice were immunized subcutaneously at the base of the tail with 100 μg rat collagen type II (CII) emulsified in 0.1 M acetic acid in Complete Freund's Adjuvant (CFA) (Difco Laboratories, Detroit). After 30 days, a booster injection was given containing 50 μg CII emulsified in 0.1 M acetum in Incomplete Freund's Adjuvant (IFA) (Difco Laboratories, Detroit). The clinical scoring of arthritis (was) commenced 25 days after the first immunization. The scoring system is based on the number of inflamed joints, ranging from 1 to 15 for each affected paw. Each affected ankle/wrist was given score 5, and each inflamed knuckle and toe was given 1 point. The scores of the four paws were added, yielding

a maximum total score of 60 points for each mouse. The severity trait is the maximum score observed in each individual female. Mice that did not develop CIA were given a score of 0 for the traits of severity, onset and incidence. The onset is the number of days calculated from the first immunization to the first clinical signs of arthritis excluding unaffected animals.

Enzyme-Linked Immunosorbent Assay

The mice were sacrificed at day 90 and sera were collected. Anti-CII antibody titers in sera were analyzed by a sandwich ELISA technique [11]. In short, immunosorbent plates were coated with collagen type II (10 μg/ml) over night at 4°C. Bovine serum albumin (Sigma) was used for blocking, and thereafter different dilutions of control sera (purified mouse anti-collagen type II antibodies), test sera, positive and negative controls were added. The presence of CII-specific IgG was visualized by peroxidase-conjugated goat anti-mouse IgG.

Statistical analysis

Statistical comparison between the different experimental groups was performed by using the Mann Whitney U test.

Results

Increased Incidence, onset and Severity of CIA in Heterozygous *Cia40* Congenic Female Mice

Heterozygous and homozygous *Cia40* congenic mice and corresponding littermate controls of both sexes were immunized with rat CII and monitored three times a week for 90 days. Serum samples for anti-CII-antibody analyse were collected at the end point of the experiment.

Results presented in Table 1 show that heterozygous *Cia40* congenic mice suffer from an elevated incidence of the disease. This increase in incidence was particularly obvious, and significant, in the group of females ($P<0.05$). Surprisingly, no significant differences in incidence were observed in homozygous *Cia40* congenic females or males in comparison with the corresponding controls.

The onset of the disease was significantly quicker in heterozygous females in comparison to wildtype B10.Q and homozygous congenic littermates. There were no significant differences in onset between the different groups of males.

The severity of the disease was elevated in heterozygous *Cia40* congenic mice of both sexes, as shown in Figure 2 a-b. Homozygous mice showed a minor increase in severity in comparison to wildtype B10.Q littermates, but this difference was not

significant. The heterozygous congenic males showed a higher severity in the beginning of the disease, while heterozygous females showed higher severity in the latter part of the disease. The heterozygous congenic females developed a more severe arthritis than the heterozygous congenic male mice. The heterozygous congenic females also showed a significantly shorter onset ($p<0.05$) of CIA than corresponding controls, and all other groups (Table 2).

Heterozygous *Cia40/Pregq2* congenic mice show increased anti-CII antibody levels

Anti-CII antibody titers in serum were analysed at the end of the experiment (Table 3). The results showed that heterozygous *Cia40* congenic females develop significantly higher anti CII antibody titres than wild type and homozygous congenic mice ($P<0.05$) of the same sex. No significant differences in anti-CII titres were observed between the different groups of males. This shows that the antibody titres follow the disease phenotype in the congenic mice.

Reduced Breeding Performance and Disturbed Breeding Behaviour in *Cia40/Pregq2* Congenic Mice

The *Cia40/Pregq2* congenic mice were difficult to breed and congenic mice of both sexes showed disturbed breeding behaviour. Congenic females showed a reduced

frequency of successful pregnancies, and pups were frequently killed and eaten shortly after delivery.

Figure 3A, shows that the mean litter size (surviving pups) of *Cia40* congenic females crossed with B10.Q males is significantly reduced (P=0.041) compared to the litter size of wild type littermate females crossed with B10.Q males. Figure 3B shows the frequency of litters containing dead pups (the exact numbers were normally not possible to count) in breeding cages containing *Cia40/Pregq2* congenic female mice and breeding cages exclusively containing wild type littermate females. The frequency of litters containing dead pups was dramatically higher in breeding cages containing *Cia40/Pregq2* congenic females compared to those containing wild type females (P=0.0069).

These data show that the majority of the litters that were born by the congenic females contained non-surviving pups. The high neonatal mortality among the pups from the congenic females appeared to be due to behavioural disturbance characterized by maternal ignorance and a tendency towards attacking and eating their own pups.

Discussion

The results of the present study indicate that one or more polymorphic genes in the congenic *Cia40/Pregq2* fragment affect severity, onset and incidence of CIA, as well as the reproductive performance of B10.Q mice. Interestingly, the increased incidence and severity are pronounced traits in heterozygous mice only, and the influence of the congenic fragment is particularly obvious in the heterozygous females, which actually show a much higher incidence than the males. This is striking, since females of the strain B10.Q normally show a very low incidence of arthritis (around 15%). The female predominance in incidence of CIA makes polymorphic genes in the congenic fragment particularly interesting, since female predominance is characteristic for RA in humans.

None of the genes close to the calculated position of the *Cia40/Preqq2* locus are known to be involved in the regulation of inflammation (Table 1). For this reason, we believe that polymorphic or mutated regulatory genes, that in turn affect the activity of several enzymes, could be particularly interesting candidate genes. One such candidate gene is mitogen activated protein kinase, *Map2k6*, which has been reported to affect the function of the immune system. For instance, Ehlting and co-workers recently reported that the regulation of a suppressor of cytokine signalling 3´ (SOCS3) mRNA stability by TNF-alpha, involves the activation of the Map kinase cascade [12]. Table 4 shows possible gene candidates, based on SNP polymorphic data in this particular fragment on chromosome 11 in between inbreed strains of NMRI and C57BL/10 mice from Wellcome Trust database (gscan) [13].

We have previously speculated that the same gene(s) might affect both arthritis incidence and pregnancy failure [5]. This assumption is supported by the fact that the incidence of autoimmune CIA is elevated in females but not males, and that the elevated severity is particularly obvious in females. A modified gene that increases the risk of developing autoimmune inflammation in females can also be expected to interfere negatively with pregnancy success. Some types of early pregnancy failures could actually be caused by increased autoimmune reactivity. Again, it is possible that the map kinase is involved in the success of implantation. This assumption is strengthened by a recent observation that map kinase cascade indeed affects pre-implanted embryos [14].

Still, it might be more likely that different mechanisms and genes are involved in the regulation of arthritic inflammation and the regulation of pregnancy success. If true, this would make it possible to separate *Cia40* gene/s from the breeding-suppressing *Pregq2* gene/s, which would be of great advantage for the future characterization of arthritis controlling *Cia40* gene/s.

The observation that the heterozygous *Cia40* congenic mice show a quicker onset, and in the case of males, also develop a more severe disease, raises questions about the molecular mechanisms controlling arthritis. A polymorphism, leading to an amino acid substitution, in one allele could have strong effects on the function of a di- or multimeric protein and polymorphisms in non-coding regulatory regions could result in skewed transcription and altered protein levels. The observed phenotypic effects due to heterozygous alleles might be helpful in the identification of candidate

genes. The heterozygous effect has previously been reported in a study of CIA development, where mice with heterozygous alleles in a congenic fragment on mouse chromosome 15 were much more affected by the disease than homozygous littermates [15].

We have only found a limited number of genes in the vicinity of the *Cia40* and *Pregq2* peaks, which show polymorphism between B10 and NMRI. In addition to *Mapk6*, we have also focused some attention on *Abca8a* gene and *CD79b* gene. The role of the *Abca8a* gene in the context of reproduction and immunity is largely unknown, while the *CD79b* gene is of importance primarily in the context of B cell development [16]. At present it is not possible to speculate about the possible influence of these two genes for the phenotypes observed, but the function of these genes does not make them being our main candidate genes.

The interesting reversal of sex susceptibility to arthritis, and the observation that congenic males show impaired development of genital organs, and females are more aggressive and less caring mothers, has made us paying attention to the *Sox9* gene. The *Sox9* gene has been reported to cause sex reversal [17], which is a highly relevant phenotype in the context of the *Cia40/Pregq2* congenic mice. The possible presence of a *Sox9* polymorphism/mutation on chromosome 11 in our congenic mice is under investigation.

Conclusions

The present results show that the *Cia40* locus on chromosome 11 contains one or more polymorphic genes that particularly influence incidence and severity of CIA in female mice. These effects are significant in congenic B10.Q female mice carrying heterozygous *Cia40* fragments of NFR/N origin. Congenic mice carrying heterozygous fragments also show quicker onset of the disease. The major polymorphic candidate genes in the congenic fragment are *Cd79b*, *Abca8a* and *Map2k6*. The NFR/N fragment present in the congenic mice also contains a locus denoted *Pregq2*, which causes a change in reproductive behaviour and reduces pregnancy success. This effect is significant in congenic B10.Q females carrying a homozygous NFR/N fragment. The *Sox9* gene, known to influence sex reversal, is a candidate gene for the reproductive phenotype.

List of abbreviations

CIA = collagen-induced arthritis, RA = Rheumatoid arthritis, QTL = quantitative trait locus, CFA = complete Freund's adjuvant, IFA = incomplete Freund's adjuvant, CII = collagen type II, EAE = Experimental autoimmune encephalitis.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

M Liljander is responsible for genotyping, phenotyping, analyses and together with R Mattson, Å Andersson and R Holmdahl interpretation and writing the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1.

The dark area indicates the genetic region from NFR/N, in the congenic strain *Cia40*.

The markers are placed according to Mouse Ensemble built 36 [17].

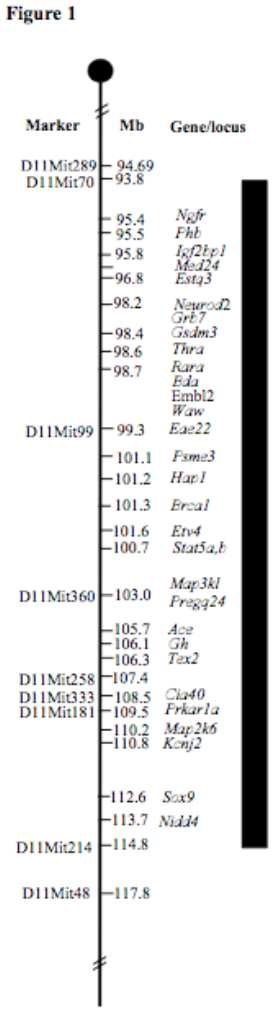
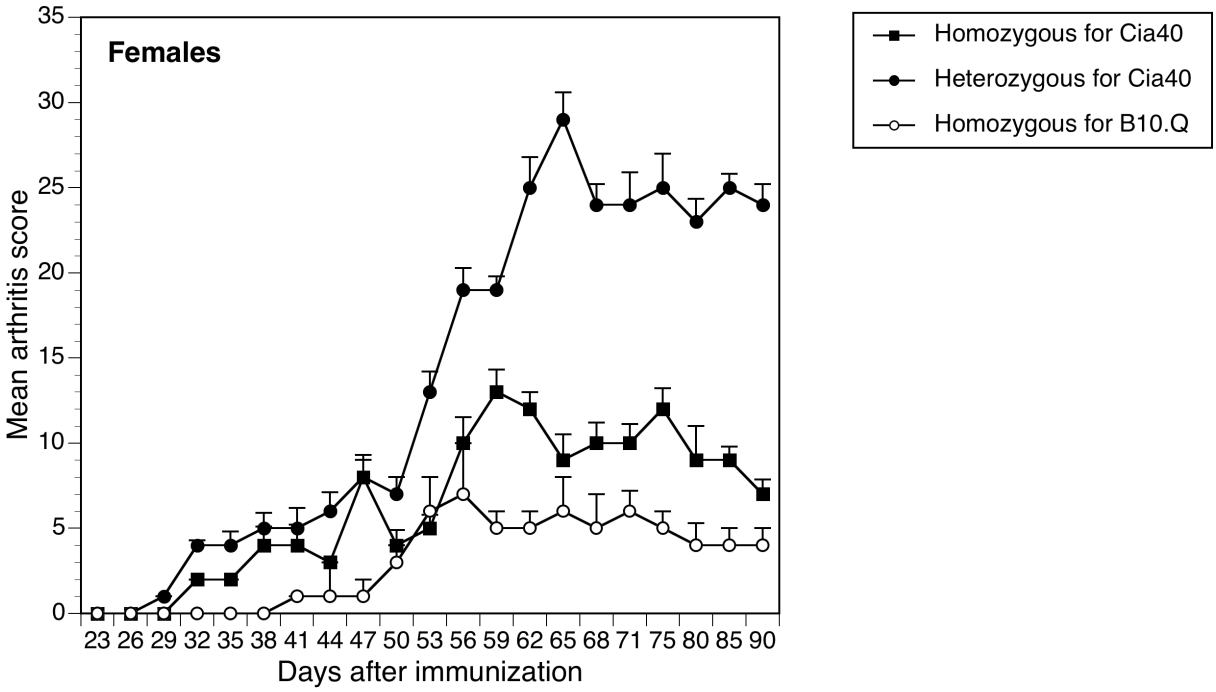


Figure 2.

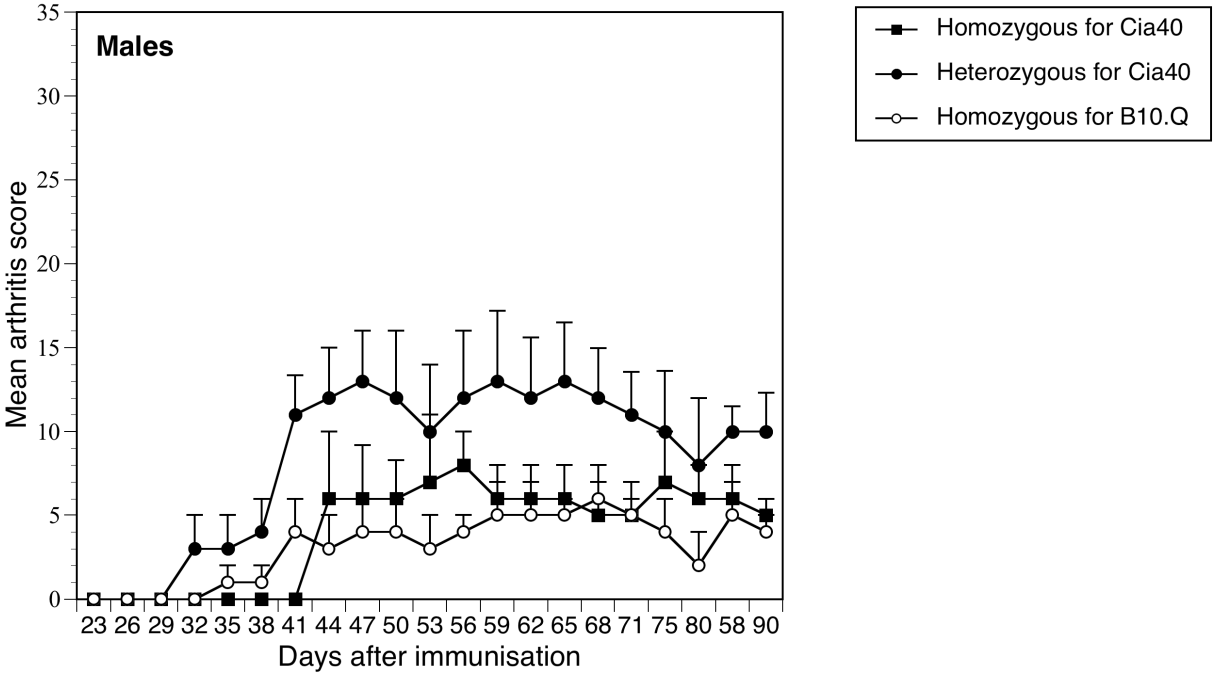
Severity of CIA in *Cia 40* congenic male and female mice.

2A.



2A. Mean (S.E) arthritic scores in homozygous *Cia40* congenic females, heterozygous *Cia40* congenic females and wild type littermate females. Exclusively mice that developed arthritis have been included. Heterozygous congenic females show higher severity than wild type B10.Q and congenic homozygous females ($p < 0.05$).

2B.

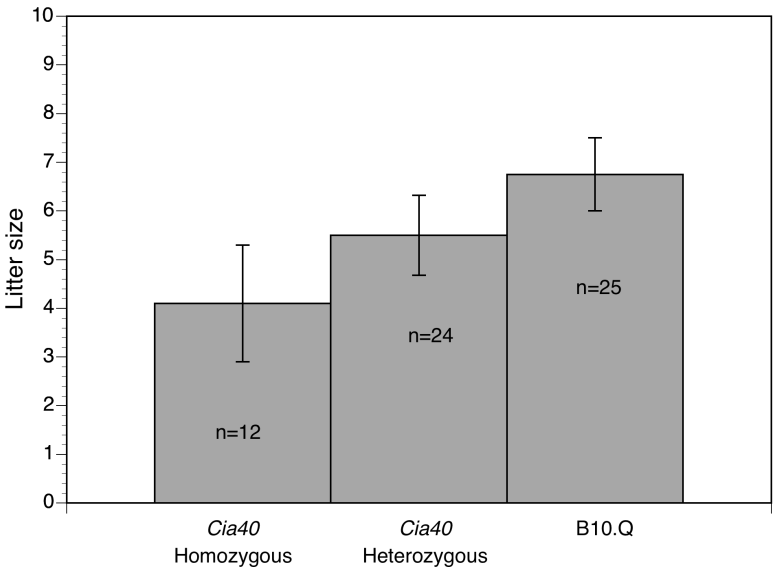


2B. Mean (S.E) arthritic scores in homozygous *Cia40* congenic males, heterozygous *Cia40* congenic males and wild type littermate males. Exclusively mice that developed arthritis have been included. Heterozygous congenic males show significantly higher severity than wildtype B10.Q littermates ($p < 0.05$).

Figure 3.

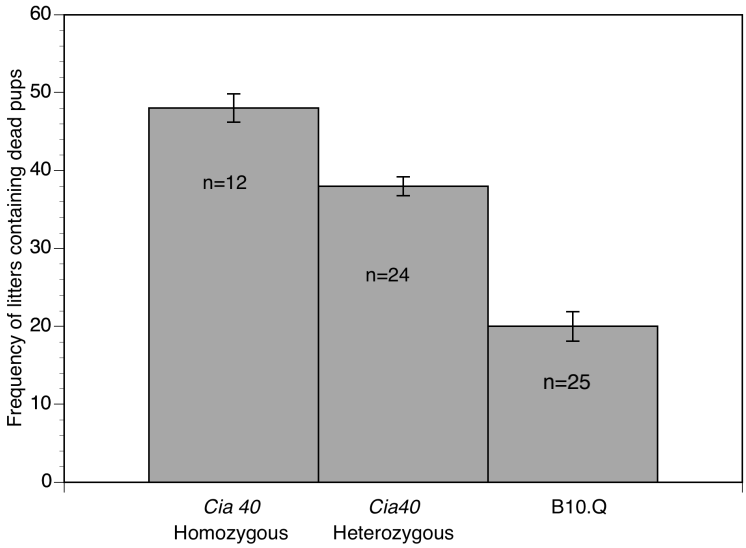
Figure 3 shows the mean litter size and the frequency of litters containing dead pups in Cia40 congenic females and B10.Q wild type littermates.

3A.



3A. The mean litter size (S.E) in homozygous *Cia40* congenic females, heterozygous *Cia40* congenic females and wild type B10.Q littermates (n = number of pregnancies). Significant difference between homozygous *Cia40* congenic mice and wild type B10.Q littermates ($P<0.05$).

3B.



3B. The mean (S.E) frequency (%) of litters containing dead pups in homozygous *Cia40* congenic mice, heterozygous *Cia40* congenic mice and wild type B10.Q littermates (n = number of pregnancies). Significant difference between *Cia40* homozygous congenics and B10.Q wild type littermates ($P<0.007$).

Tables

Table 1. Incidence of CIA in *Cia40* congenic male and female mice.

	n	Incidence		
		Wild type B10.Q	Heterozygous *Cia40*	Homozygous *Cia40*
Total	116	12/48 (25 %)	24/47 (51%)	12/36 (33 %)
Females	54	4/24 (17%)	12/15 (80%)[1]	5/15 (33 %)
Males	62	8/24 (33 %)	7/17 (41%)	7/21 (33 %)

[1] Significantly higher incidence in heterozygous congenic females compared with wild type littermates ($p<0.05$).

Table 2. Onset of arthritis in *Cia40* congenic male and female mice.

	n	Onset[1] (range)		
		Wild type B10.Q	Heterozygous Cia40	Homozygous Cia40
Total	116	55 (32, 82)	50 (29, 78)	45 (38, 84)
Females	54	53 (32, 70)	38 (29, 59)[2]	42 (38, 72)
Males	62	59 (35, 82)	56 (32, 78)	52 (47, 84)

[1] Day of onset. Median values for onset calculated on all arthritic mice in the group on day 90. Figures within brackets = maximum and minimum values for onset.
[2] Significantly shorter onset in heterozygous congenic females compared with wild type littermates ($p<0.05$).

Table 3. Anti CII titers in *Cia40* congenic male and female mice.

	n	Anti-CII titers S.E (mg/ml) day 90		
		Wild type B10.Q	Heterozygous Cia40	Homozygous Cia40
Total	161	0.68 ± 0.24	1.29 ± 0.31	0.86 ± 0.25
Females	54	0.71 ± 0.32	1.57 ± 0.34[1]	0.96 ± 0.24
Males	62	0.67 ± 0.38	0.75 ± 0.21	0.70 ± 0.17

[1] Significantly higher antibody titre in heterozygous congenic females compared with wild type littermates ($p<0.05$).

Table 4. Summary of possible candidate genes on chromosome 11 for *Cia40/Pregq2*.

Gene	Position (mb)	Description	Reproductive or inflammatory phenotypes of mutation
Ngfr	95.4	Nerve growth factor receptor	Perinatal lethality.
Phb	95.5	Prohibitin	Lethality before weaning.
Igf2bp1	95.8	Insulin-like growth factor 2	Foetal growth.
Med24	96.5	Mediator complex subunit 24	Pups die prior to birth.
Gsdm3	98.5	Gasdermin	Abnormal skin and hair loss.
Etv4	101.6	Ets variant gene 4 (E1A enhancer binding protein, E1AF).	Mammary gland abnormality, male infertility.
*Cd79b**	106.1	CD79B antigen.	Hematopoietic, immune
Prlar1a	109.5	Protein kinase, cAMP dependent regulatory, type I, alpha.	Embryonic lethality.
*Abca8a**	109.8	ATP-binding cassette, sub-family A (ABC1), member 8a.	Not known
*Map2k6**	110.2	Mitogen activated protein kinase	Abnormal immune system
Sox9	112.6	SRY-box containing gene 9	Perinatal lethality, cartilage formation, sex reversal.

*Polymorfism between inbreed strains of NMRI and C57BL/10 according to gscan, Wellcome Trust Centre for Human Genetics [18]