Polymorphisms at the innate immune receptor TLR2 are associated with Borrelia infection in a wild rodent population

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The discovery of the key role of Toll-like receptors (TLRs) in initiating innate immune responses and modulating adaptive immunity has revolutionized our understanding of vertebrate defence against pathogens. Yet, despite their central role in pathogen recognition and defence initiation, there is little information on how variation in TLRs influences disease susceptibility in natural populations. Here, we assessed the extent of naturally occurring polymorphisms at TLR2 in wild bank voles (Myodes glareolus) and tested for associations between TLR2 variants and infection with Borrelia afzelii, a common tick-transmitted pathogen in rodents and one of the causative agents of human Lyme disease. Bank voles in our population had 15 different TLR2 haplotypes (10 different haplotypes at the amino acid level), which grouped in three well-separated clusters. In a large-scale capture–mark–recapture study, we show that voles carrying TLR2 haplotypes of one particular cluster (TLR2c2) were almost three times less likely to be Borrelia infected than animals carrying other haplotypes. Moreover, neutrality tests suggested that TLR2 has been under positive selection. This is, to our knowledge, the first demonstration of an association between TLR polymorphism and parasitism in wildlife, and a striking example that genetic variation at innate immune receptors can have a large impact on host resistance.

1. Introduction

Parasites, by definition, are harmful to their hosts and should therefore impose selection for enhanced resistance. Despite this, there is typically significant genetic variation for resistance to parasites and pathogens in natural populations [1]. To elucidate the evolutionary causes and consequences of such variation, a better understanding of which genes actually contribute to variation in resistance is desirable [2–4]. However, whereas there is a considerable body of literature on the genetic basis of resistance in humans [5], knowledge from other animals, and in particular from natural vertebrate populations, is as yet very limited (but see [6]).

The principal weapon that hosts of pathogens have evolved to fight off pathogens is the immune system, which in vertebrates consists of two main parts, innate and acquired immunity [7]. Invading infectious agents are initially recognized by the innate branch of the immune system through pattern-recognition receptors (PRRs). PRRs recognize structures that are specific to microbes (microbe- or pathogen-associated molecular patterns). After stimulation by the ligand, PRRs activate an intracellular signalling cascade, which initiates innate and acquired immune responses [8–10]. An important class of PRRs are Toll-like receptors (TLRs), which were discovered in vertebrates as late as 1997 [11]. Most mammals have 10–13 different TLRs, each recognizing different ligands [12]. TLRs have been found to play a key role in pathogen recognition and initiation of immune responses in humans and laboratory animals [13,14], and there is an increasing number of studies in humans showing associations between TLR polymorphisms and disease. The discovery of the key role of Toll-like receptors (TLRs) in initiating innate immune responses and modulating adaptive immunity has revolutionized our understanding of vertebrate defence against pathogens. Yet, despite their central role in pathogen recognition and defence initiation, there is little information on how variation in TLRs influences disease susceptibility in natural populations. Here, we assessed the extent of naturally occurring polymorphisms at TLR2 in wild bank voles (Myodes glareolus) and tested for associations between TLR2 variants and infection with Borrelia afzelii, a common tick-transmitted pathogen in rodents and one of the causative agents of human Lyme disease. Bank voles in our population had 15 different TLR2 haplotypes (10 different haplotypes at the amino acid level), which grouped in three well-separated clusters. In a large-scale capture–mark–recapture study, we show that voles carrying TLR2 haplotypes of one particular cluster (TLR2c2) were almost three times less likely to be Borrelia infected than animals carrying other haplotypes. Moreover, neutrality tests suggested that TLR2 has been under positive selection. This is, to our knowledge, the first demonstration of an association between TLR polymorphism and parasitism in wildlife, and a striking example that genetic variation at innate immune receptors can have a large impact on host resistance.

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and infectious diseases [14–16]. Yet, TLR polymorphisms have, unlike polymorphisms at the major histocompatibility complex [17], thus far received little attention from ecologists investigating host–parasite interactions and wildlife disease (but see, e.g. [6,18,19]).

Here, we investigated the role of naturally occurring TLR2 polymorphisms in mediating parasite resistance (here defined as the ability to prevent and/or clear infection, and measured as the presence/absence of infection) in a population of wild-living bank voles (Myodes glareolus) by testing for associations between TLR2 genotype and Borrelia afzelii infection status. Borrelia afzelii is a common tick-transmitted pathogen in rodents [20], and one of the causative agents of human Lyme borreliosis in Europe [21,22]. Lipopeptides, which are central components of the cell walls of Borrelia, are ligands for TLR2, and knock-out studies with laboratory mice have shown that TLR2 plays an important role in the recognition and the initiation of immune responses against Borrelia [23–27]. Moreover, there is evidence that a common single nucleotide polymorphism (SNP) in the human TLR2 affects susceptibility to Lyme disease [28]. By transferring this immunological background knowledge into an ecological context, we here show that polymorphisms at TLR2 are associated with B. afzelii infection in a natural rodent population, highlighting the important role of TLRs in mediating disease susceptibility in wildlife.

2. Material and methods

(a) Study species

The bank vole (M. glareolus, Rodentia) is one of the main hosts of B. afzelii in Europe. Borrelia afzelii is transmitted by the sheep tick (Ixodes ricinus) between hosts [29]. We captured bank voles in 2008 in Kalvs Mosse (N 55° 42.470', E 13° 29.216'), a homogeneous, deciduous woodland of about 0.25 km² southeast of Revingeby, Skåne, Southern Sweden using live-traps (Ugglan Special No1, Grahnab, Gnosjö, Sweden). Animals were caught during trapping sessions in May (n = 31; 100% adults), June (n = 171; 45% adults), August (n = 252; 43% adults), September (n = 320; 29% adults) and October (n = 350; 29% adults). They were weighed (+0.1 g), and the number of tick larvae on the ears was counted as a proxy for infestation with nymphs, the main infective stage [30]. Molecular sexing was performed by amplifying a fragment of the male-specific sex-determining region Y as described in Wandeler [31]. At first capture, animals were individually marked with subcutaneous transponder tags (Trovan ID-100B, AEG ID, Ulm, Germany) to allow for the identification of individuals upon recapture. We obtained ear biopsies from 726 individuals during the trapping sessions. Samples were stored in 70 per cent ethanol for later DNA extraction (as described in [19,32]), TLR2 genotyping and determination of Borrelia infection status. All animal procedures were performed under licences M101-06 and M141-10 issued by the Malmö/Lund, Sweden ethical board for animal experiments.

(b) Toll-like receptor 2 (TLR2) genotyping

Mammals have a single functional copy of TLR2 [12]. There is a TLR2 pseudogene in humans and dogs, but not in mice [12]. In bank voles, the entire TLR2 coding region is 2352 bp long [19]. For this study, we sequenced a 1173 bp long fragment of TLR2 from bp 691 to 1863 as described in Tschirren et al. [33]. There was no indication that we amplified more than one locus (at most two peaks per site in the chromatograms), and we found no sign of pseudogenes (no stop codons or frame shift mutations). The sequenced part of TLR2 contains most of the functionally relevant sites involved in pathogen–recognition and TLR2–TLR1 heterodimerization [34,35], and we previously demonstrated molecular signatures of positive selection during the evolutionary history of rodents [19], as well as strong population differentiation and isolation by distance across bank vole populations within this gene region [33]. The amplicon consisted of coding sequence only. Sequences were processed, assembled and aligned using Geneious v. 5.0.4 [36], and all polymorphisms were examined by eye. TLR2 haplotypes were reconstructed with PHASE v. 2.1 [37,38] using the default settings of a thinning interval of one, 100 burn-in iterations and 100 main iterations. Haplotypes were submitted to NCBI GenBank (see table 1 for accession nos).

To test whether patterns of haplotype frequencies and tree topology are consistent with neutral expectations, we performed two neutrality tests: Fay & Wu’s H [39] and Li’s maximum frequency of derived mutations (MFDM) [40]. For the former, the empirical distribution of the test statistics was generated using neutral coalescent simulations in DNaSP [41], based on the observed number of segregating sites, 20,000 replicates and the assumption of no recombination (no recombination was detected with the MFDM test). The results of this analysis did not change qualitatively when allowing for moderate levels of recombination (data not shown). For both tests, we used the Mus musculus (NM_011900-3), Apodemus flavicollis (JN674549.1) and Myodes rufocanus (HM215593.1) TLR2 sequences as outgroups. Deviation from neutrality detected by Fay & Wu’s H can be caused by selection and/or demographic processes such as population expansion or bottlenecks [40,42]. Li’s MFDM is robust against population size changes, but can be sensitive to admixture events [40].

To identify clusters of host haplotypes we constructed a TLR2 haplotype network in TCS 1.21 [43]. Homology models for the most common haplotype of each cluster (haplotype 1 and 6, see §3) were generated based on the human TLR1–TLR2 lipopetide crystal structure [35]. Alignments were generated with ClustalW [44], manually curated where necessary, and served as the input for program MODELER 9 v. 4 [45]. Figures were prepared using PyMol (http://www.pymol.org/).

(c) Borrelia infection status

To determine whether voles were infected with B. afzelii, we performed TaRE real-time PCR assays as described in Råberg [46] (n = 1124 samples). Samples with a melting temperature
between 78.15°C and 78.75°C and a Ct value corresponding to greater than or equal to 1 B. afzelii spirochaete were considered positive. The PCR assay is specific for B. afzelii [46].

Borrelia afzelii is the only Lyme borreliosis-causing Borrelia species observed in our bank vole population. The relapsing fever-causing Borrelia species, B. miyamotoi is also found, although at very low prevalence [46].

Whereas B. afzelii infection status is highly repeatable (once infected, an individual stays infected for life [47]), we found that an individual’s infection intensity (number of B. afzelii spirochaetes per unit host tissue) varied considerably over time. Therefore, we focused on infection status only.

(d) Statistical analyses
The reconstructed TLR2 haplotype network revealed that TLR2 haplotypes grouped in three clearly separated clusters, one of which was very rare (see §3). To increase the statistical power to detect differences in B. afzelii infection among individuals with functionally different TLR2, we focused on the two common clusters and determined for each individual bank vole if it carried two TLR2 haplotypes belonging to the first cluster (TLR2c1; green), haplotypes 6, 7, 8, 9 grouped into a second major haplotype cluster (TLR2c2; red). Haplotype 10 formed a third group.

**Figure 1. TLR2 haplotype network.** Reconstructed bank vole TLR2 haplotype network based on 726 individuals. Circle sizes reflect the number of haplotype copies found in the population. The circle size for 50, 150 and 350 copies is given as a reference. The numbers along the connecting lines indicate the positions of the (synonymous and non-synonymous) substitutions that separate different haplotypes. Different colours (and numbers) indicate that haplotypes differ at the amino acid level. Haplotypes 1, 2, 3, 4, 14 grouped into one major haplotype cluster (TLR2c1; green), haplotypes 6, 7, 8, 9 grouped into a second major haplotype cluster (TLR2c2; red). Haplotype 10 formed a third group.
Analyses were run in R v. 2.14.1 [49] using the glmer function, part of the lme4 package [50]. For all analyses, the significance of the fixed effects was determined by comparing two nested models, with and without the factor of interest, using likelihood-ratio tests.

In addition, we also used a model selection procedure using Akaike information criterion with a correction for finite sample sizes (AICc) to determine which model best explains variation in *Borrelia* infection status. Candidate models contained combinations of TLR2 genotype, age class and sex. All candidate models contained individual ID and trapping session as random effects. Model selection was performed using the MuMIn package in R v. 2.14.1 [49].

3. Results

(a) TLR2 diversity in wild-living bank voles

TLR2 diversity was high in the surveyed bank vole population with 15 unique DNA haplotypes, of which 10 differed at the amino acid sequence. The most common amino acid haplotype (haplotype 1) occurred in six variants (1a–f), which differed at the nucleotide, but not the amino acid level (i.e. only synonymous substitutions). No synonymous variants were observed in the other nine haplotypes. The frequencies of the different TLR2 haplotypes are shown in table 1 (n = 726 individuals).

Both neutrality tests indicated that positive selection has shaped TLR2. Fay & Wu’s test detected an excess of high-frequency derived haplotypes in the population (*Mus musculus* as outgroup: \( H = -14.95, p < 0.001; Apodemus flavicollis* as outgroup: \( H = -15.21, p = 0.012; Mysodes rufocanus* as outgroup: \( H = -13.27, p = 0.023)\). Similarly, Li’s MFD test, which uses tree topology to infer selection, was significant \( (p < 0.009)\).

A reconstructed haplotype network revealed two major TLR2 clusters (TLR2a and TLR2c; figure 1). The main difference between the two clusters was six linked, non-synonymous SNPs that separated the two major haplotype clusters (figure 1). The six-site amino acid combination ‘276Ala/417Gly/453Thr/484Leu/536Ile/565Asp’ occurred in six variants (1a–f), which differed at the nucleotide, but not the amino acid level (i.e. only synonymous substitutions). No synonymous variants were observed in the other nine haplotypes. The frequencies of the different TLR2 haplotypes are shown in table 1 (n = 726 individuals).

(b) Structural differences between TLR2 haplotypes

To investigate whether any of the six linked SNPs that separated the two major haplotype clusters could potentially affect ligand binding, we modelled the structure of the bank vole TLR2. Based on the human TLR2–TLR1 lipopeptide crystal structure [35], we generated homology models for the most common TLR2 haplotype of each cluster, haplotype 1 and 6. These models confirmed a high degree of structural conservation between the human and bank vole TLR2–TLR1 complex (55% sequence identity for TLR2 residues 206–549). Furthermore, all residues that are involved in the TLR1–TLR2 interface are conserved between humans and bank voles, suggesting that human and bank vole TLR2 possess similar modes of action. None of the polymorphic sites are located directly in the TLR1–TLR2 interface, but the polymorphic site at amino acid position 276 in LRR 10 (see the electronic supplementary material, S1) is likely to have a significant impact on ligand binding. The side chain of the amino acid at position 276 is pointing into the hydrophobic core of the TLR2–TLR1 complex and is located in close proximity (10.3 Å) to the putative binding site for the *Borrelia* lipopeptide (figure 2). The amino acids found at this position (276Ile in humans) in the two haplotype clusters (276Thr versus 276Ala) differ markedly in size and polarity, which, given their location, is likely to affect the size of the hydrophobic pocket, and thereby ligand binding.

The polymorphic sites 417, 453 and 484 in the TLR2–TLR1 complex are located more than 18 Å away from the ligand-binding site, and the side chains of residues at positions 417 and 453 are pointing towards the solvent, which makes it unlikely that they influence ligand-binding directly. However, they could still affect the thermodynamic stability of TLR2. Polymorphic sites 536 and 565 were outside the template structure (human TLR2–TLR1 complex; [35]) and could not be modelled. However, because of their location in LRR 20 and the C-terminal domain, we would not expect a prominent effect of these amino acid mutations on ligand affinity [35].

(c) TLR2 polymorphisms are associated with *Borrelia* infection status

Overall prevalence of *B. afzelii* infection reached 34.1 per cent in adult bank voles (more than 20 g), but was markedly lower in subadults (15–20 g; 10% infected) and juveniles (less than 15 g; 5.6% infected; \( \chi^2 = 99.38, p < 0.001 \)). Similar results were obtained when analysing differences in *Borrelia* prevalence across age classes for each trapping session separately (see the electronic supplementary material, S2). The low *Borrelia* prevalence in juveniles and subadults is probably owing to limited *Borrelia* exposure rather than higher resistance. Indeed, subadults and juveniles were infested with more than four-times fewer ticks than adult voles (\( \chi^2 = 92.67, p < 0.001 \)). Again, similar results were obtained when analysing differences in tick load across age classes for each trapping session separately (see the electronic supplementary material, S3). Because of differential *Borrelia* exposure, associations between genetic determinants of resistance and *Borrelia* prevalence are predicted to be pronounced in adults, but much weaker, or absent, in juveniles and subadults. In line with this prediction, there was a significant interaction effect between age class and TLR2 genotype on *Borrelia* infection status (\( \chi^2 = 10.26, p = 0.036 \)). No significant association between TLR2 clusters and *Borrelia* infection was observed in juveniles (\( \chi^2 = 2.55, p = 0.279 \)) or subadults (\( \chi^2 = 0.24, p = 0.886 \)). Adult bank voles had a significantly higher prevalence of *Borrelia* infection than subadults and juveniles (\( \chi^2 = 9.48, p = 0.002 \)) and adults were more likely to be infected with *Borrelia* than subadults (\( \chi^2 = 4.65, p = 0.031 \)).
voles, however, differed significantly in *Borrelia* infection status depending on their TLR2 genotype ($\chi^2 = 12.27, p = 0.002$; figure 3). *Borrelia* prevalence was lowest in adult voles carrying two haplotypes belonging to cluster TLR2c1, and highest in individuals carrying two haplotypes belonging to cluster TLR2c2. Voles with one haplotype of each cluster had intermediate infection prevalence (figure 3).

*A posteriori* tests revealed that individuals carrying one haplotype of each cluster were significantly less likely to be *Borrelia* infected than individuals with two haplotypes of cluster TLR2c1 ($\chi^2 = 5.99, p = 0.014$). However, they were not significantly different from individuals with two haplotypes of cluster TLR2c2 ($\chi^2 = 2.39, p = 0.122$), probably owing to a large confidence interval (CI) in this latter group (figure 3).

Males were more likely to be *Borrelia* infected than females ($\chi^2 = 4.51, p = 0.034$), but the association between TLR2 clusters and *Borrelia* infection did not differ significantly between the sexes ($\chi^2 = 3.17, p = 0.155$). There was no significant difference in tick load between TLR2 clusters ($\chi^2 = 2.43, p = 0.296$). Including tick load as a covariate in the model of *Borrelia* prevalence (see above) did not change the significant association between TLR2 clusters and *Borrelia* infection ($\chi^2 = 7.44, p = 0.024$).

A model selection procedure based on AICc values largely confirmed these results (except for the sex differences in infection) and revealed that a model containing TLR2 genotype, age class and the interaction between TLR2 genotype and age class best explained variation in *Borrelia* infection status of voles. All other candidate models had a $\Delta$AICc $\geq 2$. Model averaging revealed a significant effect of TLR2 genotype, with individuals with a TLR2c1/TLR2c2 (95% CI: $-3.57$ to $-0.33$) and a TLR2c2/TLR2c2 (95% CI: $-6.46$ to $-0.29$) genotype being significantly less likely to be *Borrelia* infected. Furthermore, juveniles (95% CI: $-6.67$ to $-1.22$) and subadults (95% CI: $-4.44$ to $-1.82$) were significantly less likely to be *Borrelia* infected.
4. Discussion

Using a candidate gene approach, we have shown that polymorphisms at the innate immune receptor TLR2 are associated with *B. afzelii* infection status in a natural rodent population. Genetic diversity at TLR2 was high in the studied bank voles, and a reconstructed haplotype network revealed that TLR2 variants grouped in well-defined clusters. The same clusters were also found in other bank vole populations in southern Sweden (0.3–342 km apart) [33], indicating that this unusual haplotype network is not specific to our study population.

Homology modelling based on the human TLR2–TLR1 complex indicated that the polymorphism at position 276 (Thr276Ala), which was one of the six linked non-synonymous mutations that defined the two major haplotype clusters, may have pronounced functional consequences for ligand binding of the TLR2–TLR1 complex [35]. Consistent with the hypothesis that Thr276Ala, or linked amino acid mutations, affect the function of the TLR2–TLR1 complex, we observed marked differences in *Borrelia* prevalence in adult voles with different TLR2 genotypes. Animals with two haplotypes belonging to cluster TLR2c2 were almost three times less likely to be *Borrelia* infected compared with animals with two haplotypes belonging to cluster TLR2c1. Whereas there were clear differences in *Borrelia* infection status, we found no difference in tick load between TLR2 haplotype clusters, suggesting that TLR2 genotype influences the hosts’ resistance to *B. afzelii* rather than their rate of exposure (e.g. through indirect effects of TLR2 genotype on host behaviour). Adult males were more heavily infected with *B. afzelii* than adult females. This might be owing to a higher moving activity of males, which influences *Borrelia* encounter [51], and/or higher testosterone levels, which negatively influences parasite control [52].

Furthermore, the association between TLR2 clusters and *B. afzelii* infection was pronounced in adults after dispersal, but absent in juveniles before dispersal [53], indicating that the observed pattern is unlikely to be owing to non-genetic factors shared by family members (i.e. *B. afzelii* abundance in a territory). The conclusion that there is a causal relationship between TLR2 polymorphisms and the voles’ resistance to *B. afzelii* is in line with the results of knock-out studies in laboratory mice, which have identified TLR2 as a candidate gene for *Borrelia* resistance [23–27]. Nevertheless, given the correlative nature of our study, we cannot exclude the possibility that a linked locus, rather than TLR2 itself, is driving the observed relationship.

What immunological mechanisms could mediate an association between TLR2 polymorphisms and *B. afzelii* infection status? In principle, improved resistance could be a result of enhanced innate or acquired immune responses. Infections with *B. afzelii* and other Lyme borreliosis spirochaetes typically result in chronic infections in their natural hosts, with low rates of clearance once the infection has established and disseminated [47]. This suggests that the improved resistance conferred by variants of cluster TLR2c2 acts via mechanisms expressed early during infection, that is, effectors belonging to the innate immune system. Recent studies have shown that TLRs can activate the complement system [54], a component of innate immunity known to be important for resistance against *Borrelia* [55]. Thus, one possibility is that a higher affinity of cluster TLR2c2 haplotypes to *B. afzelii* ligands results in a stronger complement response.

Haplotype 6 was the most common TLR2 haplotype in the host population, but unlike the common haplotype of cluster TLR2c1 (haplotype 1), which occurred in six variants, this haplotype has not yet accumulated any synonymous nucleotide substitutions. Synonymous nucleotide substitutions are considered to be selectively neutral (but see [56,57]), and to accumulate over time at a gene specific point-mutation rate [58]. The complete lack of synonymous nucleotide substitutions in haplotype 6, despite its high frequency, is consistent with recent positive selection that has favoured this haplotype. This is also reflected by significant Fay & Wu’s H and Li’s MFDM tests, which both indicate that positive selection has acted on TLR2 [40,59].

Although the latter test is comparably robust against demographic processes [40], it is important to acknowledge that it is difficult to fully disentangle selection and demography with the currently available data. Nevertheless, the structure of the haplotype network and the results of the neutrality tests in combination with the finding that animals carrying haplotype 6 had lower *B. afzelii* prevalence suggests that this TLR2 variant may have increased in frequency as a result of parasite-mediated selection (by *Borrelia* or other pathogens).

While our results are in line with the hypothesis that parasite-mediated selection has shaped TLR2 evolution in bank voles, it is as yet difficult to assess the role of *B. afzelii* as a selective agent because very little is known about the fitness consequences of *Borrelia* infection in natural populations. In white-footed mice (*Peromyscus leucopus*), experimental infection with *B. burgdorferi* sensu stricto in the laboratory led to carditis and multifocal arthritis [60], which probably affects host survival and/or reproduction in the wild. However, the two (correlative) studies performed in wild-living hosts to date, one in white-footed mice [61] and one in black-legged kitiwakes (*Rissa tridactyla*) [62], did not find indication for survival costs of *Borrelia* infection. Yet, the strength of selection required to drive a selective sweep is relatively low. For example, Obbard et al. [63] estimated that the selective advantage driving the evolution of Ago2, one of the fastest evolving immune genes in *Drosophila*, was a mere 0.5–1%. Clearly, it would be very difficult to detect such low levels of selection in a field study.

In conclusion, our study shows that polymorphism at TLR2 has shaped TLR2 evolution in bank voles, it is as yet difficult to assess the role of *B. afzelii* as a selective agent because very little is known about the fitness consequences of *Borrelia* infection in natural populations. In white-footed mice (*Peromyscus leucopus*), experimental infection with *B. burgdorferi* sensu stricto in the laboratory led to carditis and multifocal arthritis [60], which probably affects host survival and/or reproduction in the wild. However, the two (correlative) studies performed in wild-living hosts to date, one in white-footed mice [61] and one in black-legged kitiwakes (*Rissa tridactyla*) [62], did not find indication for survival costs of *Borrelia* infection. Yet, the strength of selection required to drive a selective sweep is relatively low. For example, Obbard et al. [63] estimated that the selective advantage driving the evolution of Ago2, one of the fastest evolving immune genes in *Drosophila*, was a mere 0.5–1%. Clearly, it would be very difficult to detect such low levels of selection in a field study.

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