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Heligmosomoides polygyrus infection is associated with lower MHC class II gene expression in *Apodemus flavicollis*: Indication for immune suppression?

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ABSTRACT

Due to their key role in recognizing foreign antigens and triggering the subsequent immune response the genes of the major histocompatibility complex (MHC) provide a potential target for parasites to attack in order to evade detection and expulsion from the host. A diminished MHC gene expression results in less activated T cells and might serve as a gateway for pathogens and parasites. Some parasites are suspected to be immune suppressors and promote co-infections of other parasites even in other parts of the body. In our study we found indications that the gut dwelling nematode *Heligmosomoides polygyrus* might exert a systemic immunosuppressive effect in yellow-necked mice (*Apodemus flavicollis*). The amount of hepatic MHC class II *DRB* gene RNA transcripts in infected mice was negatively associated with infection intensity with *H. polygyrus*. The hepatic expression of immunosuppressive cytokines, such as transforming growth factor β and interleukin 10 was not associated with *H. polygyrus* infection. We did not find direct positive associations of *H. polygyrus* with other helminth species. But the prevalence and infection intensity of the nematodes *Syphacia stroma* and *Trichuris muris* were higher in multiple infected individuals. Furthermore, our data indicated antagonistic effects in the helminth community of *A. flavicollis* as cestode infection correlated negatively with *H. polygyrus* and helminth species richness. Our study shows that expression analyses of immune relevant genes can also be performed in wildlife, opening new aspects and possibilities for future ecological and evolutionary research.

1. Introduction

Parasites are omnipresent in wild mammalian populations and often share a long evolutionary history with their particular hosts, on which they can cause strong selection by having impacts upon their fitness and survival (Anderson and May, 1979; May and Anderson, 1979). Our understanding of the mechanisms that are involved in host–parasite coevolutionary processes is primarily based on laboratory rodents that live under non-natural pathogen-free and stress-free conditions (Jackson et al., 2009). But laboratory models are simplified models of reality and might be poor images of the wildlife that faces the constantly changing and challenging conditions of natural environments. Focusing just on laboratory models could mean working at the expense of ecological and evolutionary realism (Feder and Mitchell-Olds, 2003). Furthermore, most interaction studies have focused on single parasite species and their host disregarding interspecific interactions between co-infesting parasites or the cumulative effect of co-infections (Bordes and Morand, 2009a). However, the occurrence of such effects and associations has been demonstrated in model organisms (Graham, 2002, 2008) and wildlife species (Salvador et al., 2011). Multiple simultaneous parasite infections are the norm in nature (Behnke, 2008; Bordes and Morand, 2009b) and might exert a different pressure upon host immunity than single parasite infections (Bordes and Morand, 2009a) as the selection might be bidirectional. In-situ studies on naturally infected wild populations that transfer and validate knowledge gained in the laboratories might give new insights into how the immune system functions in its natural context (Bradley and Jackson, 2008; Jackson et al., 2009).

Helminth parasites comprise the nematodes, trematodes and cestodes. Despite their unrelatedness they provoke a stereotypical modified T helper cell 2 response and can provoke fatal diseases in their hosts (Moreau and Chauvin, 2010). For this reason helminths have often been used as health indicators in ecological and co-evolutionary studies and to explain variability in the pattern of expression of immune relevant genes in the host (e.g. Axtner and Sommer, 2007; Lenz et al., 2009; Schwensow et al., 2011). Genetic variability can be crucial to resistance of populations against parasites and pathogens (Altizer et al., 2003). The most diverse genes in vertebrates are the immune related genes of the major histocompatibility complex (MHC) that act at the interface of infection detection and immune defence (Piertney and Oliver, 2006). The so called 'classical' MHC genes code for membrane glycoproteins that bind to and present foreign antigens to T cells and thereby

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trigger an immune response (Hughes and Yeager, 1998). MHC genes can be divided in two classes, the class I molecules are constitutively expressed in almost every somatic cell, whereas class II glycoproteins are generally restricted to a subset of antigen presenting cells, such as dendritic cells, B cells or macrophages (Piertney and Oliver, 2006).

Some microparasites like *Chlamydia*, *Mycobacterium* spp. or *Toxoplasma* directly influence the expression of MHC class II genes (Wojciechowski et al., 1999; Zhong et al., 1999; Noss et al., 2000; Lüder et al., 2003; Pai et al., 2003) by avoiding immune cell activation and T cell initiation (LeibundGut-Landmann et al., 2004). A reduced MHC expression is advantageous for the parasite as it increases the probability of establishing a constant infection. The probable mechanism used by parasites to lower MHC expression is to interfere with the expression of the class II transactivator CIITA, which controls and orchestrates the interaction of various promoters involved in MHC expression (Muhlethaler-Mottet et al., 1997; Harton and Ting, 2000; Ting and Trowsdale, 2002; LeibundGut-Landmann et al., 2004). Also, some macroparasites like helminths are known to manipulate the mammalian immune defence to evade immune reactions and avoid expulsion from their host (reviewed in Maizels et al., 2004). For example, heligmosomoid nematodes are suspected to act in an immunosuppressive way (Jenkins and Behnke, 1977; Behnke et al., 1978, 1983). The outcome of this is an attenuation of deleterious inflammations (Bazzone et al., 2008) and pronounced patterns of bystander infections in their natural hosts like *Apodemus* spp. (Behnke et al., 2009; Jackson et al., 2009). This immune suppression seems to be systemic rather than limited to the site of infestation as it affects different parts of the host's body (Jenkins and Behnke, 1977; Su et al., 2005; Wilson et al., 2005; Bazzone et al., 2008). Potential agents of heligmosomoids that influence the host's immunity are excretory–secretory antigens that induce regulatory T (T_{reg}) cells via the transforming growth factor β (*Tgfb*) signalling pathway (Grainger et al., 2010). These induced T_{reg} cells provoke elevated levels of the immunosuppressive cytokines *Tgfb* and interleukin 10 (*Il10*) (Metwali et al., 2006; Finney et al., 2007) which in turn reduce MHC class II presentation in antigen presenting cells (Knolle et al., 1998; Romieu-Mourez et al., 2007). Although there is ample evidence of an association between MHC class II alleles and varying levels of susceptibility to helminths (Sommer, 2005; Goüy de Bellocq et al., 2008), only few studies have investigated whether helminths might be able to interfere with MHC class II expression using laboratory models (e.g. Silva et al., 2006; Perrigoue et al., 2009) and are almost missing in wildlife (Schwensow et al., 2011).

To address this gap, we chose wild-caught yellow necked mouse (*Apodemus flavicollis*) as our non-classical model organism. The MHC class II *DRB* gene of *A. flavicollis* have been characterised (Musolf et al., 2004) and its alleles show manifold associations to nematode susceptibility (Meyer-Lucht and Sommer, 2005, 2009); and *Heligmosomoides polygyrus* is a ubiquitous helminth in this host species (Ferrari et al., 2004, 2009; Klimpel et al., 2007). We employed quantitative real-time PCR (qPCR) to measure messenger RNA levels of MHC class II *DRB* gene, *Tgfb* and *Il10* in liver samples of wild *A. flavicollis*. Due to its immunological function of mediating tolerance to antigens but also immune responses to antigens entering the body from the gastrointestinal tract (Selmi et al., 2007; Tiegs and Lohse, 2009), the liver comprises a large reservoir of antigen presenting cells (Racanelli and Rehermann, 2006; Nemeth et al., 2009) making it an ideal target to study systemic effects of *H. polygyrus* infection on MHC class II expression. Furthermore, liver resident T_{reg} cells have been demonstrated to mediate a systemic antigen specific immune tolerance by a hepatic pathway in transgenic mice (Lüth et al., 2008). Our aim was to investigate whether heligmosomoid infections are associated with immune gene expression under natural conditions and if a *H. polygyrus* infection influence co-infection patterns. This study contributes to close the gap between our understanding of immune regulation in laboratory models and its application to ecological studies in free-ranging mammal populations naturally confronted by multiple parasite species.

2. Methods

2.1. Sample collection

We trapped yellow-necked mice (*Apodemus flavicollis*) of a single population using Sherman live traps (7.7 cm × 7.7 cm × 30.5 cm) in a deciduous forest approximately 35 km North–East of the city of Hamburg, Germany. Animals were anesthetized at the trapping site using isoflurane and then killed immediately by cervical dislocation. Only adult animals with a body weight above 16 g (Jüdes, 1979) that were neither pregnant nor lactating were sampled. Liver samples were stored in RNA-Later (Sigma) and kept at 4 °C for 24 h and were subsequently frozen at −20 °C until RNA extraction. The intestinal tract was removed and stored in 75% ethanol until parasitological screening. The stomachs and intestines of all animals were screened for helminth infestations under a microscope. Nematodes were assigned to the genus level at least, whereas all cestodes were pooled as they could not be further distinguished. Infection intensity was assessed as number of adult worms per individual. All research reported in this manuscript was carried out in accordance with the legal requirements of Germany and complied with the protocols approved by the state office for Agriculture, Environment and Rural Areas of Schleswig–Holstein (Reference No.: LANU 315/5327.74.1.6).

2.2. RNA extraction and cDNA synthesis

The whole liver was homogenised (2 × 10 s at 5000 rpm, Precellys, Bertin Technologies) in QIAzol lyses reagent (Qiagen). From each homogenate we placed 0.5 ml in two 1.5 ml tubes and treated each of these aliquots separately as independent replicates of a sample (A and B) for the subsequent procedures. Total RNA was extracted following the QIAzol lyses reagent protocol and dissolved in 87.5 μl of water. Genomic DNA was digested with DNase I (RNase-free DNase Kit, Qiagen) followed by a clean-up using RNeasy spin columns (Qiagen) according to the manufacturer's protocol. Extracted total RNA was finally eluted in 100 μl of water; concentration and purity were assessed three times by a Nanodrop 1000 (Thermo Scientific) and averaged. RNA quality was checked on agarose gels by electrophoresis. Two micrograms of total RNA were reverse transcribed with Oligo-dT_{18} primers (5 μM). Reverse transcription was run in a 20 μl reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. Complementary DNA (cDNA) was diluted 1:3 prior to quantitative real-time RT PCR (qPCR) with water.

2.3. Quantitative real-time RT PCR

Quantitative PCR was performed for the three target genes (MHC-*DRB* and the regulatory cytokines *Il10* and *Tgfb*, Supplement 1) and for the four reference genes (ribosomal protein S18, calnexin, cytoplasmic actin gamma 1, phosphoglycerate kinase 1). Details on reference gene validation and reference gene primers are given elsewhere (Axtner and Sommer, 2009). We used intron-spanning primers to avoid the amplification of genomic DNA contaminants.

We used the SensiMix SYBR No-ROX Kit (Quantace) in a 25 μl volume on a Rotor Gene 3000 (Corbett Research). To avoid inter-run variation and to control RT differences we analysed the A and B replicates of a sample in the same qPCR run. All reactions were run in triplicates (in total six reactions per individual) with