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Immunity comes first: The effect of parasite genotypes on adaptive immunity and immunization in three-spined sticklebacks

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ABSTRACT

Adaptive immunity in vertebrates can confer increased resistance against invading pathogens upon re-infection. But how specific parasite genotypes affect the temporal transition from innate to adaptive immunity under continual exposure to parasites is poorly understood. Here, we investigated the effects of homologous and heterologous exposures of genetically distinct parasite lineages of the eye fluke *Diplostomum pseudospathaceum* on gene expression patterns of adaptive immunity in sticklebacks (*Gasterosteus aculeatus*). Observable differences in gene expression were largely attributable to final exposures while there was no transcription pattern characteristic for a general response to repeated infections with *D. pseudospathaceum*. None of the final exposure treatments was able to erase the distinct expression patterns resulting from a heterologous pre-exposed fish. Interestingly, heterologous final exposures showed similarities between different treatment groups subjected to homologous pre-exposure. The observed pattern was supported by parasite infection rates and suggests that host immunization was optimized towards an adaptive immune response that favored effectiveness against parasite diversity over specificity.

1. Introduction

The vertebrate immune system is capable of specific responses to parasites. While specificity has traditionally been attributed to adaptive MHC-molecule based immunity, recent years have seen growing evidence of genotype-specific innate immune responses (De Roode and Altizer, 2010; Haase et al., 2014; Lazzaro and Little, 2009; Rauch et al., 2006; Seppälä et al., 2009). It is increasingly recognized that both parts of the immune system are intertwined. While the innate immune response has long been known to activate cell proliferation for an adaptive response (Janeway et al., 2008), adaptive regulation of the innate system is also being increasingly recognized (Shanker, 2010). Studies have shown that there is reciprocal interaction between innate and adaptive immune components (Kurtz et al., 2006; Shanker, 2010; Wegner et al., 2007) and that there are genotype × genotype interactions influencing parasite infection and innate immune responses in fish (Haase et al., 2014; Rauch et al., 2006). Interestingly, there seems to be cross-reactivity advantageous for hosts exposed to a larger variety of parasite genotypes, leading to reduced infection rates regardless of parasite genotype pre-exposure (Rellstab et al., 2013; Scharsack and Kalbe, 2014). It is thus timely to move beyond the artificial dichotomy between the innate and adaptive system but rather obtain an integrative understanding of immunity (Criscitiello and de Figueiredo, 2013; Gardy et al., 2009; Tort et al., 2003). Nevertheless, the extent to which the genetic diversity within single parasite species influences the activity of immune gene expression in the subsequent activation of adaptive and innate immune responses is still poorly understood.

We approached this gap and used distinct parasite lineages to subject hosts to a continuous exposure of identical (homologous) or

Abbreviations: GO, Gene ontology; RNA-seq, RNA sequencing; FDR, false discovery rate.

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alternative (heterologous) parasite genotypes. We measured parasite-genotype specific infection rates and differences in host gene expression by high-throughput sequencing. This allowed us to examine the specificity of host immune gene expression and the transition from innate to adaptive immunity. Specifically, we used an RNA sequencing approach to investigate underlying expression patterns in three-spined sticklebacks, a model fish species with increasing genomic and transcriptomic resources (Gibson, 2005; Haase et al., 2014; Jones et al., 2012; Lenz et al., 2013). The adaptive immune response was triggered by consecutive monoclonal and multiclonal infections with larvae of the eye fluke *Diplostomum pseudospathaceum*. The parasite has a complex life cycle involving freshwater snails and fish as intermediate hosts, before undergoing sexual reproduction in the intestine of piscivorous birds (Chappell et al., 1994). Snail hosts harbor a clonal reproduction stage released as free-swimming cercariae, making trematodes ideal candidates to investigate genotype-specific performance of parasites (Koehler and Poulin, 2012). When successfully infecting a fish host, the cercariae penetrate skin or gills and migrate to the eye lens where it causes cataracts and blindness, thus hampering feeding efficiency and predator avoidance (Crowden and Broom, 1980; Owen et al., 1993). Since the parasite quickly invades the fish's eye lens, where it is protected from the immune system, it is of paramount importance for the host to be able to mount a fast and effective innate immune response.

In a previous experiment we treated naïve fish with a single exposure to different genotypes of *D. pseudospathaceum* to examine the specificity of innate immunity. That study identified general patterns of gene expression as well as specific responses to individual parasite clones (Haase et al., 2014). Here, we complement our findings on genotype specific immune responses and investigated transcriptomic patterns of continually exposed sticklebacks to mimic natural conditions in the field. To this end, we repeatedly subjected sticklebacks to monoclonal or multiclonal mixes of *D. pseudospathaceum* cercariae. We pre-exposed sticklebacks over several weeks to a certain parasite infection, and terminated the experiment using a final exposure with either the same or a different genotype treatment. We investigated parasite infection rates and expression patterns (RNAseq) induced by repeated infection with distinct parasite lineages. Switched final infections allowed us to investigate whether the genetic identity of the parasite affects the outcome of the final infection in terms of parasite load and gene expression. By switching the final infection treatment to a different clonal lineage, we were able to compare transcription patterns induced by homologous and heterologous infections in order to address any specific priming of immunity by the innate components. In accordance with our previous results, we expected to find genes shared between all consecutive treatments (i.e. homologous and heterologous exposures), as well as specific responses to different *D. pseudospathaceum* genotypes. In addition, we expected to identify genes differentially expressed as a result of distinct pre-exposures, as well as genes being affected by final treatments. By examination of differences and similarities of gene expression patterns induced by consecutive infection treatments and those in single infections, we additionally aimed for the transition from the initial innate immune response to the development of adaptive immunity. Overall, this study revealed how specific different parasite genotypes influenced immune memory in fish and how this is reflected in the interaction of innate and adaptive immune responses.

2. Material & methods

2.1. Infection experiment

For experimental infection, clonal parasite lineages of *D. pseudospathaceum*, and four lab-bred fish families of three-spined sticklebacks (*Gasterosteus aculeatus*) were used as described earlier (Haase et al., 2014; Rieger et al., 2013). Genotypes of cercariae emerging from snails were determined via polymorphisms of microsatellite loci (Diplo08, Diplo09, Diplo23 and Diplo29; (Reusch et al., 2004)). For DNA extraction and PCR conditions see Haase et al., 2014 (Haase et al., 2014). Fish were selected randomly for infection trials 6 months after hatching and 36 fish per sibship were placed in three 16 L aquaria in groups of 12 fish each. Over the course of 5 weeks fish were exposed weekly to either clone I, clone XII or a mix of clonal lineages, containing inbred and outcrossed parasite genotypes (Rieger et al., 2013). For this purpose, cercariae of snails infected with the same parasite clone were pooled and parasite density per ml was estimated. During weekly exposure a cercariae suspension consisting of 240 parasites (20 per fish) was added to each tank. The procedure will be termed “pre-exposure” throughout this study. Two weeks after the fifth pre-exposure, 6 fish per family pre-exposed to the clone mix and 12 fish per family pre-exposed to single clones ($6 \times 4 + 12 \times 4 \times 2 = 120$) were placed into 1 L aquaria. Three fish per sibship per pre-exposure were either exposed to controlled doses of 100 cercariae of clone I, clone XII, clone mix or not infected for use as control (Fig. 1); this procedure will be referred to as “final exposure”. The clone mix was produced by mixing cercaria of different *D. pseudospathaceum* clones as described in Rieger et al. (2013). In this manuscript, we will refer to the effect of different clone combinations in the gene expression analysis by naming the groups “pre-exposure − final exposure”. This results in three control groups, I-C, XII-C and M-C, and five treatment groups. Three of those were exposed homologous, I-I, XII-XII and M-M. Two were exposed heterologous, I-M and XII-M. The use of clone I, clone XII and clone mix will refer to a general effect of a parasite treatment.

Penetrating *D. pseudospathaceum* larvae migrate to the fish‘s eye lens within 24 h. This part of the stickleback shows no activity of the immune system, thus protecting the parasite (Chappell et al., 1994). Fish used for RNA extraction were sacrificed 4 h after the final treatment by immediate decapitation and directly stored in RNA later (Qiagen). To reliably assess infection rates, a subset of fish was sacrificed 2 days after final exposure. The fish heads were kept in 0.64% physiological NaCl solution for later assessment of infection rates. We decided to examine gene expression patterns in head kidneys, one of the major immune organs in fishes (Press and Evensen, 1999; Rauta et al., 2012) and gills, one of the preferred spots for cercarial penetration (Whyte et al., 1990). RNA extraction on gill and head kidney tissue was performed using the Macherey-Nagel NucleoSpin 96 RNA kit following the standard protocol. Quantity and quality measurements of RNA as well as library preparation and final sequencing were conducted as described in Haase et al., 2014 together with the samples analyzed in that study (Haase et al., 2014). We sequenced a 96 sample setup on one flow cell (Illumina HiScanSQ) with 12 samples per lane distributed over 8 lanes respectively for 2×101 cycles (paired-end). Sequence data has been submitted to the NCBI short read archive (SRA) BioProject ID PRJNA276419, infection data and supporting information are available at http://doi.pangaea.de/10.1594/PANGAEA.843814.

2.2. Data analysis

All statistical analyses of parasite infection data were carried out using R, version 2.14.1 (R Development Core Team, 2015). A full negative binomial generalized linear model was fitted to the infection rate data, including pre-exposure, treatment, fish sibship and interactions thereof as explanatory variables (Venables et al., 2002). Assumptions for generalized linear models were met. We