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A rapid and transient innate immune response to avian influenza infection in mallards

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ABSTRACT

The vertebrate innate immune system provides hosts with a rapid, non-specific response to a wide range of invading pathogens. However, the speed and duration of innate responses will be influenced by the co-evolutionary dynamics of specific host-pathogen combinations. Here, we show that low pathogenic avian influenza virus (LPAI) subtype H1N1 elicits a strong but extremely transient innate immune response in its main wildlife reservoir, the mallard (*Anas platyrhynchos*). Using a series of experimental and methodological improvements over previous studies, we followed the expression of retinoic acid inducible gene 1 (*RIG-I*) and myxovirus resistance gene (*Mx*) in mallards semi-naturally infected with low pathogenic H1N1. One day post infection, both *RIG-I* and *Mx* were significantly upregulated in all investigated tissues. By two days post infection, the expression of both genes had generally returned to basal levels, and remained so for the remainder of the experiment. This is despite the fact that birds continued to actively shed viral particles throughout the study period. We additionally show that the spleen plays a particularly active role in the innate immune response to LPAI. Waterfowl and avian influenza viruses have a long co-evolutionary history, suggesting that the mallard innate immune response has evolved to provide a minimum effective response to LPAIs such that the viral infection is brought under control while minimising the damaging effects of a sustained immune response.

1. Introduction

Avian influenza A virus (AIV) is an economically and medically important pathogen, given that it can cause lethal outbreaks of disease in poultry, humans and other species (Burns et al., 2006; McLeod, 2010; McLeod et al., 2011). AIVs have a broad host range, with detections reported in 12 orders and 105 species of birds (Munster et al., 2007; Olsen et al., 2006; Stallknecht et al., 2008), as well as various mammalian species (Reperant et al., 2009). However, only waterfowl (Anseriformes) and shorebirds (Charadriiformes) serve as maintenance hosts for the majority of naturally circulating AIV subtypes and lineages (Clark and Hall, 2006; Stallknecht, 2003a; Webster et al., 1992; Arnal et al., 2015), with mallards (*Anas platyrhynchos*) being identified as the primary reservoir host (Olsen et al., 2006; Runstadler et al., 2013). This has been confirmed both through wild bird surveillance (Munster et al., 2007; Stallknecht, 2003b; Hoye et al., 2010; Cheung et al., 2009; Pannwitz et al., 2009) and studies of viral evolution (Spackman et al., 2005; Wille et al., 2013; Dugan et al., 2008).

AIVs are classified as low pathogenic (LPAI) or highly pathogenic (HPAI), based on their pathogenicity in gallinaceous birds (Alexander, 2000), whereby the majority of mortality and morbidity in poultry is caused by HPAI viruses belonging to the H5, H7 and H9 subtypes (Olsen et al., 2006; Alexander, 2000). In reservoir hosts, the majority of infections are caused by LPAI viruses, but HPAI viruses can occur, either as spill-over from domestic birds, or as sustained epizootics with large geographic spread (Feare, 2010; Verhagen et al., 2015). Intriguingly, HPAI infections in non-reservoir species can lead to dysregulation and uncontrolled production of cytokines (a so-called 'cytokine storm', Tisoncik et al., 2012; Yuen and Wong, 2005) which is a leading cause of AIV-induced mortality in chickens (Vervelde et al., 2013; Ranaware et al., 2016; Karpala et al., 2011a), mice (Bi et al., 2015) and humans (Yuen and Wong, 2005; Cheung et al., 2002; Chen et al., 2013). In contrast, HPAI infections in ducks can result in increased and sustained expression of cytokines but dysregulation and subsequent over-production is not observed (Huang et al., 2012). Thus, one can speculate that reservoir hosts experience lower infection-induced

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immunopathology because they have a longer history of co-evolution with the virus. For example, sustained coexistence may have promoted evolution of optimised immune responses in reservoir hosts such that they are largely asymptomatic carriers of AIV (van Dijk et al., 2015). Given the physiological and immunopathological costs of mounting an immune response (reviewed in Lochmiller and Deerenberg, 2000; Zuk and Stoehr, 2002; Cressler et al., 2015), selection in hosts should favour the evolution of a minimal immune response sufficient to avoid negative fitness costs of infection. Indeed, modelling has shown that when a pathogen is common but has low virulence (defined as the ability to cause disease-induced mortality), low investment in immune response by hosts can be an evolutionarily stable strategy (van Baalen, 1998).

However, while a stable equilibrium between pathogen virulence and host immune response may be reached at the organismal level, evolution at the molecular level can still be an intense driver of co-evolutionary dynamics (Hill and Runstadler, 2016). Antibody-mediated immunity in the host is considered the main driver of AIV antigenic evolution (Hensley et al., 2009; Koel et al., 2013; Wille et al., 2017). In particular, antigenic drift – the accumulation of single nucleotide mutations to the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins – can allow AIVs to escape antibody-mediated neutralisation (Koel et al., 2013). This, in turn, leads to evolution of an array of HA- and NA-specific antibodies in hosts, which function to block AIV infection by preventing attachment to host cell receptors (HA) or viral particle release (NA) (Sylte et al., 2007; Clarke et al., 1985). Mathematical modelling suggests that an intermediate host immune response will promote the highest rate of pathogen adaption (*i.e.* an intense co-evolutionary arms race) and that this is the most parsimonious explanation for the antigenic shifts and rapid turnover of strains observed in AIV dynamics (Grenfell et al., 2004). Unfortunately, empirical tests of these hypotheses are largely lacking, including for the waterfowl-LPAI system.

Overall, ducks mount a muted antibody-mediated immune response to AIV when compared to chickens (Magor, 2011). Instead, tightly regulated expression of innate immune genes in the early stages of infection appear to allow ducks to control infection, mitigating the need for sustained antibody production. For example, *RIG-I* and *Mx* have been shown to be strongly upregulated in response to AIV (Barber et al., 2010; Adams et al., 2013). Retinoic acid inducible gene 1 (*RIG-I*), is a cytoplasmic DEx(D/H) box helicase pattern recognition receptor (PRR) that is present in a variety of organisms such as birds, fish and mammals (Shao et al., 2015). It acts as an RNA sensor, recognising pathogen-associated molecular patterns (PAMPs) associated with intracellular viral RNA (Kolakofsky et al., 2012; Sutejo et al., 2012) and is thus active in the early stages after infection. *RIG-I* signalling is triggered during infection by a wide variety of RNA viruses, as well as by the presence of synthetic RNA transcribed *in vitro* (Kolakofsky et al., 2012; Loo et al., 2008; Yoneyama et al., 2004; Pichlmair et al., 2006). The presence of *RIG-I* in ducks but not chickens has been postulated as a partial explanation for why chickens are more susceptible to the lethal effects of HPAI infection than ducks, whereby a more efficient and rapid stimulation of interferons, ultimately leading to viral clearance, is achieved via the *RIG-I* pathway in ducks (Barber et al., 2010; Barber et al., 2013; Vanderven et al., 2012) than the MDA-5 pathway in chickens (Karpala et al., 2011b; Cornelissen et al., 2012; Liniger et al., 2012). The myxovirus resistance gene (*Mx*) is a large GTPase belonging to the family of interferon (IFN) stimulated genes, and is stimulated by type I (α/β) and type III (γ) IFNs (Haller et al., 2007). It is present in most vertebrates, as well as yeast (Ko et al., 2002) and occasionally in invertebrates (De Zoysa et al., 2007). The antiviral activity of *Mx* is mediated via inhibition of transcription and/or replication in various viral species (Ko et al., 2002; Haller et al., 2009).

While both *Mx* and *RIG-I* have been implicated in the mallard immune response to AIV infections (see Table 1 in Helin et al., 2018), a number of methodological issues may hinder the generality and/or interpretation of results. First, with one exception, all previous studies have been conducted on Pekin ducks (*Anas platyrhynchos domesticus*), which may provide a poor proxy for infection dynamics in the wild reservoir host, because a long history of artificial selection in domestic species may alter natural allele frequencies of immunologically important genes and hence alter the host's capacity to respond to infection. Indeed, the *Mx* gene offers one such example, whereby a SNP associated with antiviral activity in chickens was found to be in Hardy-Weinberg (H-W) equilibrium in natural populations with the protective allele occurring at high frequency (Li et al., 2006). In contrast, this SNP was rare in domestic populations, with allele frequencies deviating significantly from H-W expectations (Li et al., 2006). Second, previous studies of the duck immune response to AIV have used artificial infection techniques whereby large doses of viral particles are flooded into the nostrils and throats of experimental birds. The dosage used tends to be chosen with little regard for the dose required to achieve infection and far exceeds that faced by birds in the wild (Aldous et al., 2010). This may cause unnatural gene expression patterns, especially in the early stages of infection. For example, it is possible that a minimum viral threshold needs to be crossed in order to activate immune defences. If so, previous studies may have underestimated the time taken for natural hosts to respond to infection. Third, in many previous studies the AIV subtype used was chosen without reference to its frequency in the wild. Studies have largely focussed on HPAI, or low pathogenic H5 strains, none of which are common in the reservoir host (Latorre-Margalef et al., 2014; Olson et al., 2014). Fourth, all previous studies of the duck immune response to AIV have used a single, non-validated reference gene (RG) (Table 1 in Helin et al., 2018), which is inappropriate for the normalisation of qPCR results (reviewed in Chapman and Waldenström, 2015). Here, we used a controlled semi-natural infection technique to transmit a LPAI H1N1 virus, which circulates at high frequency in waterfowl in Northern Europe (Latorre-Margalef et al., 2014; Olson et al., 2014), to mallards. This allowed us to assess individual responses to a commonly circulating virus, using natural transmission patterns, in the reservoir host. qPCR results were normalised with multiple RGs previously proven stable for the experimental treatment under consideration (Chapman et al., 2016). Using this solid methodological framework, we analysed patterns of gene expression for the innate immune genes *RIG-I* and *Mx*, which occur at the top of the innate immune cascade, in mallards semi-naturally infected with H1N1. We hypothesised that both genes would show an increase in expression after LPAI infection, but that this immune response would be slower and more muted than has been found in previous studies of HPAI infection. Instead, we found that *RIG-I* and *Mx* expression in our study was rapid and transient, being of a similar magnitude to previous studies.

2. Materials and methods

2.1. Animal experiments

Male mallards were acquired from a commercial breeding facility when they were one day old, and subsequently raised indoors at a biosecurity level two animal facility at the Swedish National Veterinary Institute (SVA). All animal rooms contained a pool for swimming, food and water (*ad libitum*), and were subject to a 12-h day-night cycle. Daily monitoring of ducks confirmed that there were no signs of disease or stress due to captivity or AIV infection, and all individuals remained alive and overtly healthy until their pre-determined endpoint. Cloacal swabs and blood samples (brachial vein) were taken from all individuals prior to the start of the experiment to confirm they were AIV negative via qPCR and AIV antibody negative via ELISA (see Chapman et al., 2016 for experimental details and results of AIV screening). Animal experiments were approved by the Ethical Committee on Animal Experiments in Uppsala (permit number C63/13) and were conducted in accordance with regulations provided by the Swedish Board of Agriculture.