Innate immune responses in raccoons after raccoon rabies virus infection

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Zoonotic wildlife diseases pose significant health risks not only to their primary vectors but also to humans and domestic animals. Rabies is a lethal encephalitis caused by rabies virus (RV). This RNA virus can infect a range of terrestrial mammals but each viral variant persists in a particular reservoir host. Active management of these host vectors is needed to minimize the negative impacts of this disease, and an understanding of the immune response to RV infection aids strategies for host vaccination. Current knowledge of immune responses to RV infection comes primarily from rodent models in which an innate immune response triggers activation of several genes and signalling pathways. It is unclear, however, how well rodent models represent the immune response of natural hosts. This study investigates the innate immune response of a primary host, the raccoon, to a peripheral challenge using the raccoon rabies virus (RRV). The extent and temporal course of this response during RRV infection was analysed using genes predicted to be upregulated during infection (IFNs; IFN regulatory factors; IL-6; Toll like receptor-3; TNF receptor). We found that RRV activated components of the innate immune system, with changes in levels of transcripts correlated with presence of viral RNA. Our results suggest that natural reservoirs of rabies may not mimic the immune response triggered in rodent models, highlighting the need for further studies of infection in primary hosts.

INTRODUCTION

Unprecedented and rapid landscape and environmental changes are altering the distributional and dispersal patterns of many wildlife species and their associated pathogens (Harvell et al., 2002; Daszak et al., 2000). Recent examples include the expansion of several infectious diseases such as avian influenza, severe acute respiratory syndrome, West Nile fever/encephalitis, chronic wasting disease, tuberculosis and Lyme disease (Daszak et al., 2000, 2001). These diseases pose a significant health risk not only to their primary host, but also non-target species including humans, companion animals, livestock and species of conservation concern (Daszak et al., 2000). An in-depth understanding of the interaction between pathogen and host can help in the development of tools to mitigate such threats. However, due to the many challenges associated with studying mechanisms of host–pathogen interactions in natural populations, model systems are often employed as surrogates. Although vital information can be gained from surrogate studies, model systems often do not incorporate the effects of co-evolutionary pressures as found in natural hosts and their pathogens, and thus may not provide information indicative of the disease’s true pathology.

Rabies virus is the type species of the genus Lyssavirus, family Rhabdoviridae. Rabies virus (RV) is a highly neurotropic virus that infects mammals and almost invariably causes a fatal encephalopathy (Jackson, 2007). Although significant
advances have been made in rabies prevention and control, it continues to pose significant public health risks. A conservative estimate of 55,000 human deaths are annually attributed to this disease (World Health Organization, 2005), but mortality numbers may be higher than reported because of limited reporting and diagnosis in developing countries (Knobel et al., 2005). Dogs are the most important rabies reservoirs in Asia and Africa, where the majority of human rabies cases occur (Fu, 1997; Knobel et al., 2005). In North America, canine rabies has been controlled through pet vaccination and other control measures but terrestrial reservoirs (raccoons, foxes and skunks) and bats continue to present serious threats given the high densities of these species in urban areas (Totton et al., 2002; Rosatte & Allan, 2009; Rosatte et al., 2010, 2011).

Rabies is typically the result of a bite from an infected animal with the deposition of virus-laden saliva in the wound. The virus infects local sensory and motor neurons and then ascends to the brain by retrograde transport (Tsiang et al., 1986; Shankar et al., 1991; Prosniaik et al., 2001). Uncontrolled replication of virus in the central nervous system (CNS) leads to disease and ultimately death. Host defence against early stages of infection is provided by an innate immune response, which may prevent viral invasion and replication before the adaptive immune response is established (Haller et al., 2006; Johnson et al., 2006; Zhao et al., 2011). RV often evades the host’s immune system, and establishes the disease in the brain, explaining the near 100% mortality rate in humans if timely post-exposure treatment is not administered (Jackson, 2007; Wang et al., 2005). Therefore, it is important to understand the innate immune response following rabies infection; such data not only aids in developing and refining post-exposure treatments, but also effective treatment once clinical disease has established.

Immune pathways and associated genes are well characterized in mice and have been utilized as primary model systems to expand our knowledge of immune responses to rabies infection. Several studies using rodent models have reported upregulation of several cytokines and receptors associated with innate immune responses following infection with fixed laboratory RV strains (Marquette et al., 1996; Prosniaik et al., 2001; Baloul & Lafon, 2003; Saha & Rangarajan, 2003; McKinnie et al., 2005; Wang et al., 2005; Johnson et al., 2006; Mansfield et al., 2008; Zhao et al., 2011). While these studies have yielded a significant understanding of the mechanisms associated with rabies pathogenesis and the host’s response, mice are not reservoirs for rabies, so events observed in this species may not closely resemble the disease under natural conditions. In this study, we focused on the interaction between the raccoon strain of rabies virus (RRV) and its natural reservoir host, the raccoon (Procyon lotor), to gain a better understanding of innate immune responses triggered by virus infection. RRV was first noted in Florida around the 1940s where it remain localized until the late 1970s, when a second outbreak facilitated rapid spread along the eastern seaboard of North America (Biek et al., 2007). High densities of raccoons in urban and suburban areas (Totton et al., 2002) and the concomitant increase in potential human and pet exposures present a serious threat to human health. Contrary to the common belief that exposure to RV is always lethal, empirical studies have suggested that raccoon exposure to RRV does not always result in clinical disease, perhaps due to inter-individual differences in innate immune responses (Carey & Mclean, 1983; Szanto, 2009). For example, 5–40% of raccoons survive RRV challenge (K. Knobles, personal communication; Carey & Mclean, 1983), and the incubation period can vary among raccoons and vaccinated animals that have not demonstrated seroconversion (Rupp, Precht et al., 1988). The culmination of these factors illustrates that there is variability in the immune response of raccoons to RRV exposure that may result in abortive infections.

We investigated the host innate immune response to a peripheral challenge with RRV by determining the extent and temporal course of changes in transcript levels of a limited but crucial set of genes that are known to be generally upregulated during viral infection (Wang et al., 2005; Johnson et al., 2006; Mansfield et al., 2008). These included: (1) two IFNs, IFN-α, which is involved in the production of an antiviral state, and IFN-γ, which is involved in inflammatory responses; (2) IFN regulatory factors (IRF-1 and IRF-7), selected for their important role in the development of antiviral state and inflammation response respectively (Haller et al., 2006); (3) IL-6, as it is known to be involved in inflammatory response to viral infection (Frei et al., 1989); (4) Toll-like receptor 3 (TLR-3), chosen for its role in recognizing dsRNA (an intermediary of viral replication), and activating the production of IFNs (Alexopoulos et al., 2001); and (5) TNF receptor (TNF-R), which binds TNF involved in cytolysis of infected cells and in inducing IFNs in response to viral infections (Camelo et al., 2000; Faber et al., 2005). We examined changes in transcript levels of these components in several different individuals inoculated with the same strain of virus to explore the extent of host response variation. Further, we evaluated cytokine expression levels from a temporal perspective to determine the earliest time point to detect the immune response following peripheral inoculation, which closely mimics the natural route of transmission. From a spatial perspective, we also investigated where, in the route of transmission, the immune response was first detected. In addition, differences between individuals’ genes responsible for the innate immune response were assessed to explore possible effects on disease outcome. Information gained from this study aims to not only provide information on host response to this deadly virus, but also insight into how some animals may be able to resist fatal infection.
RESULTS

Variable response in raccoons following RRV challenge

The course of infection in raccoons was variable and peripheral inoculation did not always result in disease or CNS invasion, as determined by the detection of viral RNA in the brain (Table 1). Viral RNA was detected in the brain and salivary glands using real-time PCR, with RRV-specific primers and probes (Szanto, 2009). The two raccoons that were euthanized 4 days prior to the experiment had no detectable virus in any of the tissues. In the test group, where two raccoons were euthanized at 2, 4, 8, 15 and 32 days post-inoculation (p.i.), viral RNA was only detected in the brain of the sample that was euthanized at 8 days p.i. (Szanto, 2009). Virus was not detected in any of the other samples from this group in the tissues tested, nor did they exhibit any clinical symptoms (Szanto, 2009). The remaining 10 animals (challenge group) were kept under observation until they exhibited clinical symptoms at which point they were euthanized. Six of the 10 animals showed clinical symptoms at 20, 21 and 23 days p.i. Viral RNA was detected in the brains of all these samples, and in the salivary glands of two of these samples (21+1 and 22+1; Szanto, 2009). Four of the 10 raccoons showed no clinical symptoms for the duration of the challenge study and were euthanized at 533 days p.i. Although these samples did not exhibit clinical symptoms of RRV, low levels of the virus was detected in the brains of two raccoons (533+2 and 533+3; Szanto, 2009).

Temporal expression of immune genes during RRV challenge

Following peripheral infection with RRV, a number of transcriptional changes were observed in the brain. The earliest point that we were able to detect an indication of an innate immune response was at 8 days p.i. (Fig. 1), however, it was not statistically significant (P>0.05). Once the clinical signs of rabies were apparent (20–24 days), expression of all genes under investigation increased significantly (Fig. 1). IFN-α transcripts increased fivefold in expression (P=0.002), and this was much lower compared with the other IFN genes that were examined. The expression of IFN-α was lower than other genes, at earlier time points and different tissues that were examined in this study. IFN-γ demonstrated a 130-fold increase in the brain once clinical signs were apparent (P=0.002), and this increase was much higher than that noted for other genes. Transcripts for IFN regulatory factors (IRF-1 and IRF-7) increased by 50- and 120-fold, respectively (P<0.001). Viral RNA was

Table 1. Time course of RRV infection in raccoons

Viral detection data was obtained from the doctoral thesis of Szanto (2009).

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<th>Sample*</th>
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*Sample shows days p.i. and the sample numbers that were euthanized that day.
†Viral RNA detected by quantitative PCR <45 Ct.
changes in the challenge study animals. Innate immune gene transcripts were not detected at sites of inoculation in any of the samples (Fig. 2). However, samples from individuals that displayed clinical symptoms of RRV showed a significant increase in the expression of the genes investigated in the spinal cord (Fig. 2). IFN-α demonstrated a seven-, five- and twofold increase in the spinal cord, brain and salivary glands, respectively. Transcripts of IFN-γ increased by 116-fold, 130-fold and eightfold in the spinal cord, brain and salivary glands, respectively (Fig. 2). The expression of IRF-1 increased by 70-fold, 53-fold and twofold and IRF-7 increased by 160-fold, 120-fold and twofold in the spinal cord, brain and salivary glands, respectively (Fig. 2). Transcripts of IL-6 showed a 16-fold increase in the spinal cord and 20-fold increase in the brain, and a twofold increase in the salivary glands of the samples that showed clinical symptoms (Fig. 2). The expression of TLR-3 increased by 20-fold, 16-fold and 2.5-fold, in spinal cord, brain and salivary glands, respectively (Fig. 2). TNF-R showed a 16-fold increase in the brain followed by 14-fold increase in the brain and 1.5-fold increase in the salivary glands (Fig. 2). The increases noted in spinal cord and brains of the samples that showed clinical symptoms were statistically significant (P<0.05) (Fig. 2). Virus was also detected in all the samples and tissues that showed a significant increase in the immune gene transcripts.

**Genetic variation**

We examined variation in the coding regions of the gene amplicons selected for expression analysis to see if any underlying genetic variation influenced expression of these genes. We found no variation within the IFNs (IFN-α, IFN-γ, IRF-1 and IRF-7), IL-6 or TNF-R. The TLR-3 fragment had one nucleotide difference (C→T) in four samples; however, it did not result in an amino acid change (data not shown).

**DISCUSSION**

Living organisms are continuously exposed to a stream of pathogens that have the potential to disrupt biological processes. Humans and other vertebrates rely on an array of defensive measures produced by the immune system for constant and continued protection. The clinical outcome of viral infections depends on the balance between viral replication and host response. Innate immune responses are the host’s first line of defence against infections. During pathogen–host co-evolution, many viruses have developed strategies to successfully evade the host’s innate immune system (Samuel, 2001). The majority of previous studies investigating immune response to RV infection, which have focused on murine models and laboratory strains of rabies, have shown that the expression of several genes involved in the innate immune response increased (Wang et al., 2005; Johnson et al., 2006; Mansfield et al., 2008).
Fig. 2. Host transcriptional response to infection with RRV in different tissues of raccoons. Each group contained two raccoons, except for the 20–24 days group, which had six, and the 533 days group, which had four. Results are normalized against β-actin and are presented as fold change relative to the control. Each bar shows the mean ± SEM of the group; significantly different values (unpaired t-test, \(P<0.05\)) were found only in samples obtained at 20–24 days p.i.
While these studies are valuable, providing insight into mechanisms triggered by RV, it is important to note that the response elicited in a natural reservoir of the virus may be different than in surrogate model systems. Accordingly, we examined host response triggered by RRV in its primary vector, the raccoon. Our findings indicate that the timing of the immune response plays a crucial role in the pathogenesis of RRV, highlighting the need for further studies in primary hosts of rabies.

This study has shown that raccoons infected with RRV and exhibiting clinical signs displayed statistically significant upregulation of transcripts of several genes associated with the innate immune response in the brain. Samples from exposed animals not exhibiting clinical symptoms of viral infection did not show such a response. In the brain, the earliest time point at which activation of innate immune response was detected was 8 days p.i., however the increase noted was not statistically significant. Previous studies using mouse models have detected the immune response in the brain as early as 4 days after peripheral inoculation (Johnson et al., 2006; Mansfield et al., 2008; Zhao et al., 2011). The distance from the site of inoculation to the brain is greater in raccoons than mice, which may explain the delay noted in raccoons, as there is a time-dependent movement of virus along the peripheral nerves from the site of inoculation to the CNS (Kelly & Strick, 2000). There may also be variation in the length of time the virus remains localized at or near the site of inoculation before entry into the CNS (Charlton et al., 1997; Shankar et al., 1991).

Components critical to innate immunity include the IFNs, which induce chemo-attractive and inflammatory responses in the infected cells and play a crucial role in setting up an antiviral environment (Wang et al., 2005; Chelbi-Alix et al., 2006; Faul et al., 2010; Rieder et al., 2011). Similar to previous studies (Wang et al., 2005; Johnson et al., 2006; Mansfield et al., 2008; Zhao et al., 2011), this study found type I and II IFNs (IFN-α and IFN-γ) as well as IFN regulatory factors (IRF-1 and IRF-7) to be upregulated in RV-infected tissues. Compared with the expression of other genes involved in the IFN pathway, the expression of IFN-α was weak, however, similar results were found in other studies examining IFN-α in response to rabies infections (Wang et al., 2005; Johnson et al., 2006). Similar results with weaker and delayed IFN-α expression compared with other IFN genes were found in other viruses including influenza A virus and Sendai virus (Marie et al., 1998; Osterlund et al., 2005). The importance of IFNs in response to rabies is illustrated by previous studies that found that the administration of IFN-inducing poly(I-C) resulted in various degrees of protection against RV in mice, hamsters, rabbits and monkeys (Harmon et al., 1974; Hilfenaus et al., 1975). Similarly, it has been noted that IFN-α/β receptor knockout mice had a higher virus titre than immunologically intact mice after infection with RV (Hooper et al., 1998). These data suggest that the IFN pathway plays a crucial role in RV resistance through innate immune response.

Apart from the IFN signalling pathway, increased expression of other genes encoding inflammatory responses, as well as viral recognition receptors, was observed. Inflammatory cytokine IL-6 and TNF-R, which is involved in recruiting lymphocytes to the infection, were both upregulated in the samples from animals with clinical symptoms of RRV. Similar results have been found in other studies, which suggest inflammatory response and infiltration of lymphocytes play a major role in response against RV (Wang et al., 2005; Johnson et al., 2006; Mansfield et al., 2008; Solanki et al., 2009). RRV also induced the expression of TLR-3 in the CNS of the raccoons, which was correlated with the detection of the viral RNA. In mouse models, TLR-3 was found to be expressed earlier than other genes, suggesting that activation of this receptor is an early marker of the innate immune response to RV (McKimmie et al., 2005; Mansfield et al., 2008). We did not detect expression of TLR-3 at earlier time points suggesting that RRV may evade early detection by this receptor.

Analysis of samples taken in close proximity to the site of inoculation did not reveal upregulation of any of these gene transcripts. Under natural conditions animals are known to experience long and variable incubation periods (Baer & Cleary, 1972). There is uncertainty about the events that occur during the incubation period (Jackson, 2007), but based on an experimental study using skunks, the virus was found to remain localized at or near the site of inoculation (Charlton et al., 1997). Charlton et al. (1997) did not detect an inflammatory response at the site of inoculation, which suggests that an immune response was not triggered by the low dosage of virus used in that study. In this study, no innate immune response was detected, either because this response was highly transitory and localized or non-existent. In the present study, an increase in the expression of the suite of genes at the spinal cord in individuals that showed clinical symptoms was detected. It was interesting to note that in some of these animals, the level of expression in the spinal cord was higher than in the brain, whereas for the remainder, the opposite was true. This suggests that immune response triggered once the virus reaches the CNS may not be enough to control the disease. There was a slight increase in the transcripts in the salivary glands of two of the animals that showed clinical symptoms. The decrease noted in the expression of the transcripts with the progression of the virus suggests failure of the immune system to control the infection. Similar results were found in a previous study, where slight increase of gene expression is indicative of innate immune response was noted in the salivary glands, with a decrease after the onset of clinical symptoms (Mansfield et al., 2008).

Although chemo-attractive and inflammatory responses are often present in the RV-infected brain (Wang et al., 2005; Chelbi-Alix et al., 2006; Johnson et al., 2006; Mansfield et al., 2008; Lafon et al., 2008; Zhao et al., 2011), like most viruses, RV has developed strategies to evade the immune system. Specifically, the RV phosphoprotein (P) can impair the IFN...
signalling pathway by both interfering with transcriptional activation of IFN and by disrupting the function of STAT-1, involved in transcriptional activation of many secondary products that contribute to an antiviral state (Conzelmann, 2005; Brzózka et al., 2006; Chelbi-Alix et al., 2006; Vidy et al., 2007). The findings in this study and others show that transcripts from a range of genes involved in the IFN signalling pathway increase upon RV infection, which suggests that this inhibition by RV P is not absolute, but it may contribute to a reduced antiviral response over time. Future proteomics studies to examine regulation of IFN-related genes in RV-infected tissues may provide insight into the kinetics of this process and the role of RV P in impairing the signalling cascade. Such studies can also provide insight into the kinetics of this process and the role of RV P in impairing the signalling cascade.

While we found evidence of induction of the innate immune response following RRV infection in raccoons, there was variability in response between different individuals. The genes examined in this study were upregulated in the brains of all individuals that showed clinical symptoms; however, the expression of these genes in the spinal cord and salivary glands differed. Similarly, in previous studies using murine models, variation was noted in immune responses and histopathological changes caused by the virus (Jackson & Reimer, 1989; Johnson et al., 2006; Mansfield et al., 2008; Zhao et al., 2011). Although several factors, such as the strain of RV and differences in the route of infection used by the virus may contribute to these differences, host genetics may also play a crucial role in disease outcome. We found no variation within the coding regions of the genes examined; however, more comprehensive analysis of the non-coding regions that affect the translation and transcription of these genes may provide insight into variations that influence disease outcome. Several studies examining promoter regions, introns and transcription binding sites of innate immune genes have found variation that is linked to various disease outcomes as a result of infection with smallpox virus and hepatitis C virus (Ovsyannikova et al., 2012; Cussigh et al., 2011).

Although the expression levels of innate immune genes in raccoons were similar to previous published studies using mouse models, some differences were noted in both timing and level of expression, highlighting the need to replicate these studies in natural reservoirs of rabies. While this study attempted to examine host response to rabies infection in natural reservoirs, it is important to highlight that the virus was propagated in murine cells, and the effect that this has on the infection is not known. Future studies using virus propagated in a cell line of the host will provide a more comprehensive understanding of host–pathogen interaction. While evidence of the initiation of the immune response was noted in raccoons, it was variable among individuals suggesting that host genetic factors may have a crucial role in the pathogenesis of RV. The genes upregulated in this study may have a critical role in response against RV, however, the timing of the upregulation of these genes and nature of these genes (inflammatory response) suggests that they may also contribute to the pathogenesis, resulting in encephalopathy. In all the animals that succumbed to RRV in the challenge study, genes related to innate immune response were initially upregulated, followed by a decrease in expression levels with viral propagation and disease progression. This suggests that immune response needs to be initiated before entry of the virus into the brain in order for the host to successfully clear it.

Our findings enhance the understanding of the pathways triggered by RRV in their primary vector, raccoons. We found several genes indicative of the innate immune response to be upregulated in the brains of raccoons following RV infection. However, further knowledge of the factors contributing to the pathogenesis is needed to gain insight into which host factors may provide immunity to this virus. These results provide a basic framework for further studies investigating genes associated with both the innate and the adaptive immune response. In the samples from animals that showed clinical symptoms of RRV, we found the presence of RRV to be strongly correlated with the innate immune response; which suggests that the immune response in the host may be more effective if the pathogen is detected early in the infection. Therefore in-depth analysis of the roles of receptors responsible for detecting viruses and mechanisms that RRV employs to bypass these receptors will also prove valuable. Greater understanding of the interaction between a host’s immune response and the rabies virus variant associated with it may aid in the development of antiviral therapies to augment innate immune system function and prevent fatal disease.

**METHODS**

The initial premise of the challenge experiment was to examine viral progression in raccoons after inoculation with RRV (Szano, 2009); therefore, the challenge study, experimental design and virus preparation were performed by Canadian Food Inspection Agency (CFIA) in conjunction with Ontario Ministry of Natural Resources (OMNR). This study started with RNA extraction from previously collected tissues.

**Challenge study.** Twenty-two raccoons were captured in southern Ontario from regions that had not been exposed to RRV, by staff of the OMNR (lack of prior exposure to rabies was confirmed through serology tests). These animals were transported to a biocontainment level 3 animal care facility at the CFIA, in Nepean Ontario where the challenge study was conducted. All animal experimentation was performed according to the guidelines of the Canadian Council on Animal Care with the approval of the institute’s animal care committee. Additional details of the challenge study can be found in Szanto (2009).

**Virus preparation and experimental design.** An RRV stock was propagated in murine neuroblastoma cells as described previously (Szano, 2009). Two raccoons were euthanized 4 days prior to the start of the experiment to serve as negative controls. Twenty raccoons were inoculated into the right hind leg muscle with 0.5 ml virus stock (106.7 TCID50). Raccoons were housed individually and monitored daily for clinical signs of rabies. Two raccoons were chosen at random
on days 2, 4, 8, 12 and 32 p.i. and euthanized (test group of 10 animals). The remaining 10 animals were kept under observation until they exhibited clinical signs of rabies (challenge group), at which point they were euthanized. Animals that did not show clinical symptoms during the duration of the challenge study were euthanized at 533 days p.i. Tissues collected from all animals included: muscle tissue from the innolated right leg muscle, a section of lumbar spinal cord, cerebral cortex and salivary gland. Samples were immediately frozen and stored at −80 °C until use.

RNA extraction and reverse transcription. Total RNA was extracted from all samples using TRIzol (Invitrogen) following the manufacturer’s standard protocol. Precipitated RNA was dissolved in 50 μl DEPC-treated water and quantified using a NanoDrop 8000 spectrophotometer (Thermo Scientific). DNA was removed by treatment with Turbo DNase enzyme (Applied Biosystems) following the manufacturer’s protocol. RNA quality and quantity was assessed again using gel electrophoresis and the NanoDrop 8000 spectrophotometer. DNA contamination was assessed through a mitochondrial DNA cytochrome b gene fragment amplification (primer sequence). PCR was prepared with the components in the following concentrations: 1× PCR buffer (Invitrogen), 0.2 mM each dNTP, 1.5 mM MgCl2, 0.2 μM forward primer, 0.2 μM reverse primer, 0.05 U Taq DNA polymerase (Invitrogen) μl⁻¹, 10 ng RNA and ddH2O as needed for a total volume of 12 μl. Amplification conditions were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; final extension at 60 °C for 45 min; and 4 °C hold. Two microliters of the reaction was subjected to agarose gel electrophoresis to assess contamination. Without prior cDNA generation using a Thermoscript reverse transcription system the amplicons were not detected in the samples, thereby establishing the identity of the obtained sequences.

Gene assay design. Since raccoon genomic information is limited, primer pairs for the initial amplification of the raccoon genes of interest (IFN-α, IFN-γ, IL-6, TLR-3, IFR-1 and -7, and TNF-R) were designed based on the sequences of conserved regions in phylogenetically related carnivores available from GenBank (Table S1 available in JGV Online). DNA extracted from a raccoon from Ontario using a Qiagen kit was used as template. Amplicons were generated using each primer pair as follows: PCR components were in the following concentrations: 1.5× PCR buffer (Invitrogen), 0.2 mM each dNTP, 1.5 mM MgCl2, 0.6 μM forward primer, 0.6 μM reverse primer, 0.05 U Taq DNA polymerase μl⁻¹, 10 ng DNA and ddH2O for a total volume of 12 μl. Two microlitres of the amplified product was subjected to agarose gel electrophoresis to confirm gene amplification. PCR products were purified using ExoSAP (New England BioLabs) following the manufacturer’s instructions. BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the forward primer were used to sequence the fragments. When comparing the gene sequences obtained from raccoons using the Basic Local Alignment Search Tool (BLAST), with respective genes in phylogenetically related carnivores, the high degree of similarity (>84% at the nucleotide level; >89% at the amino acid level; data not shown) strongly implied the identity of the obtained sequences.

Once sequences of these raccoon-specific amplicons were determined, primers and Taqman probes to target them were designed using Custom TaqMan Expression Assays (Applied Biosystems; Table S2).

Gene expression. To determine the expression levels of the selected genes, quantitative reverse transcriptase PCR (RT-qPCR) was performed on RNA extracts. One microlitre of purified RNA was used in a 20 μl reverse transcription reaction using High Capacity Reverse Transcriptase enzyme (Applied Biosystems) following the manufacturer’s protocol; the gene-specific primers listed in Table S2 were used to prime cDNA synthesis. TaqMan PCR was performed on a StepOnePlus instrument, using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s conditions. All experimental samples contained at least three technical replicates with no template controls. All transcripts were normalized against β-actin and expression levels were calculated using the 2−ΔΔCt method (Schmittgen & Livak, 2008). Statistical significance was determined by Student’s t-test, with P values of <0.05 considered statistically significant. Results are presented as mean ± SEM.

Genetic variation. Fragments of the genes selected were amplified in all the experimental samples using primers listed in Table S1 following the PCR conditions described above with cDNA as the template. The amplicons were sequenced from both directions and PCR products were ethanol precipitated, then electrophoresed and visualized on an ABI 3730 DNA Analyzer. Sequences were edited and aligned to the similar gene of other species using MEGA4.1 (Tamura et al., 2007).

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