Published in final edited form as:

Vet Res Commun. 2014 June ; 38(2): 165–170. doi:10.1007/s11259-014-9592-3.

Characterization of toll-like receptors 1-10 in spotted hyenas

Andrew S. Flies^{1,2,3,*}, Matthew Maksimoski¹, Linda S. Mansfield⁴, Mary L. Weldele⁵, and Kay E. Holekamp^{1,2}

¹Department of Zoology, Michigan State University, East Lansing, MI 48824-1317, U.S.A

²Interdisciplinary program in Ecology, Evolutionary Biology and Behavior, Michigan State University, East Lansing, MI 48824, USA

³School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA, 5000, Australia

⁴Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA

⁵Department of Psychology, University of California, Berkeley, CA 94720, USA

Abstract

Previous research has shown that spotted hyenas (*Crocuta crocuta*) regularly survive exposure to deadly pathogens such as rabies, canine distemper virus, and anthrax, suggesting that they have robust immune defenses. Toll-like receptors (TLRs) recognize conserved molecular patterns and initiate a wide range of innate and adaptive immune responses. TLR genes are evolutionarily conserved, and assessing TLR expression in various tissues can provide insight into overall immunological organization and function. Studies of the hyena immune system have been minimal thus far due to the logistical and ethical challenges of sampling and preserving the immunological tissues of this and other long-lived, wild species. Tissue samples were opportunistically collected from captive hyenas humanely euthanized for a separate study. We developed primers to amplify partial sequences for TLRs 1-10, sequenced the amplicons, compared sequence identity to those in other mammals, and quantified TLR expression in lymph nodes, spleens, lungs, and pancreases. Results show that hyena TLR DNA and protein sequences are similar to TLRs in other mammals, and that TLRs 1-10 were expressed in all tissues tested. This information will be useful in the development of new assays to understand the interactions among the hyena immune system, pathogens, and the microbial communities that inhabit hyenas.

Keywords

hyena; disease; toll-like receptor; TLR

*Corresponding author: Tel.: +61 08 8222 3271, Andrew.Flies@unisa.edu.au.

Introduction

Pathogens are ubiquitous in most environments, and organisms at all trophic levels have evolved to defend themselves against microbial exploitation. Identification and eradication of pathogens is necessary to maintain host health, and individuals who cannot quickly identify and eliminate pathogens will succumb to infectious disease. However, not all microorganisms are pathogenic, and the ability of an organism to distinguish pathogens from commensal and mutualistic microorganisms is critical for survival and reproduction. Excessive or unnecessary responses against non-pathogenic microorganisms can waste critical host resources, inflict collateral damage on the host itself, and kill potentially helpful microorganisms (Medzhitov et al., 2012). Further complicating the issue, some microorganisms are beneficial when they are sequestered in one organ system, such as digestion-aiding bacteria in the gut or odor-producing bacteria in scent glands, whereas the same microorganism can cause life-threatening damage in other organ systems, such as the nervous system.

Toll-like receptor (TLR) genes are highly conserved across vertebrate genomes and the proteins that they encode function as sentinels of the immune system. Recognition of conserved pathogen-associated molecular patterns (PAMPs) by TLRs is often the first step in initiating both innate and adaptive immune responses (Barton and Kagan, 2009). Due to the variable immune challenges confronted by different tissue types, TLR expression is also variable among host tissues (Siegemund and Sauer, 2012). Furthermore, TLR expression is modulated in response to exposure levels. For example, TLR4, the receptor for lipopolysaccharides (LPS) derived from gram-negative bacteria, is expressed at low levels in the gut, where the immune system is tolerant to the abundance of gram-negative bacteria (Abreu et al., 2001), but at higher levels in other types of tissues. This trade-off of resistance and tolerance extends more broadly to immune function as a whole. Resistance, the limiting of pathogen burden in a host, and tolerance, the limiting of health impacts of pathogens, are two common immune defense strategies (Medzhitov et al., 2012; Schneider and Ayres, 2008).

Chronic exposure to pathogens may lead to the induction of immune tolerance in order to avoid excessive inflammation. The behavioral ecology of spotted hyenas (*Crocuta crocuta*) suggests that exposure to pathogens from scavenging on putrid carcasses and suffering wounds inflicted by conspecifics, competitors, and prey animals should be commonplace, and so the development of immune tolerance may be adaptive in this species. By contrast, the human immune system is extremely sensitive to microbial antigens, and autoimmune and other immunopathological disorders are commonplace in humans (Opal and Huber, 2002). It has been well-documented that spotted hyenas are capable of surviving exposure to pathogens that are lethal to sympatric species (East et al., 2001; Harrison et al., 2004; Lembo et al., 2011). Additionally, it has recently been demonstrated that symbiotic bacteria in hyenas are associated with sex and reproductive state-specific odors (Theis et al., 2013), but little is known about the underlying mechanisms that regulate microbial communities and immune function in spotted hyenas (Flies et al., 2012).

TLRs have been well-characterized in humans and traditional laboratory animals such as mice, but much less is known about the role of TLRs in wildlife (Raja et al., 2011). Our objective was to characterize the sequences and expression of TLR genes in spotted hyenas in order to begin elucidating the mechanisms of disease resistance and tolerance observed in the wild. Here we report partial sequences for hyena TLRs 1-10, and assess homology between TLRs in hyenas and other mammals. We also quantify relative expression of these TLRs in four hyena tissue types as a first step in mapping function of TLRs in this species.

2. Materials and Methods

2.1. Tissue collection

The captive spotted hyenas used in this study were born and housed at the Field Station for Behavioral Research (FSBR) of the University of California, Berkeley (UCB) (Animal Use Protocol # R091-0609R). Tissue samples were collected from two purpose-bred hyenas that were euthanized as part of a different study at UCB. Hyena 1 was 18 years old and hyena 2 was 13 years old, and both hyenas were in overall good health. Prior to euthanasia, the animals were immobilized via intramuscular injections of ketamine (4–6 mg/kg) and xylazine (1 mg/kg) delivered by blow dart. Following immobilization, hyenas were heavily sedated with diazepam, and euthanized with 1 mL / 4.5 kgs of Euthasol (Virbac AH, Inc, Ft. Worth, Texas). Lung, lymph node, pancreas, and spleen tissues were excised by a clinical veterinarian from the Office of Laboratory Animal Care at the UCB, then rinsed with phosphate-buffered saline to remove red blood cells, and immediately immersed into RNAlater solution. Tissue samples were stored overnight at ambient temperature, then cooled to 4 °C, and frozen at -80°C until further use.

Genomic DNA (gDNA) from wild spotted hyenas that had been collected as part of a long-term study in the Maasai Mara National Reserve, Kenya was used for primer testing and as a control for cDNA testing (Animal Use Protocol # 07/08-099-00). Immobilization, blood collection, and gDNA extraction were completed as previously described (Engh et al., 2002; Van Horn et al., 2004). Bobcat (*Lynx rufus*) gDNA was provided by Dr. Kim Scribner (Michigan State University) and used as a positive control during initial primer testing.

2.2. RNA isolation

RNAlater-preserved samples were cut into small pieces (< 0.5 cm) to preserve excess tissue and improve processing efficiency. RNA was extracted following the manufacturer's instructions for the PrepEase RNA Spin Kit (USB, #78767) except for the cell lysis procedure. For cell lysis, tissues were homogenized using 0.3 g of homogenizing beads (MO BIO Laboratories, #13113-50) and a bead beater homogenizer (Biospec Products, #693) for 45 seconds, then centrifuged at $10,000 \times g$ for 30 seconds, and the supernatant collected. Turbo DNase (Life Technologies # AM2238) was used in addition to the DNase step provided in the PrepEase protocol to ensure complete digestion of residual gDNA.

Extracted RNA concentrations were determined using a Nanodrop spectrophotometer, and only samples with 260/280 ratios greater than 1.8 were included in the study. RNA solutions were then diluted to 1 ng/μL using RNase free water and frozen at -80 °C until further use. RNA integrity was assessed using an Agilent 2100 Bioanalyzer. Only samples with RNA

integrity numbers (RINs) over 7.5 were used for cDNA creation and quantification of gene expression.

2.3. Primers and sequences

Felids are the closest phylogenetic relatives to hyenas for which TLRs have previously been sequenced, so we first attempted to use feline TLR primers (Ignacio et al., 2005) with hyena gDNA. Bobcat gDNA was used as a positive control during our initial primer testing. New primers were designed as needed using the Integrated DNA Technologies' primer design tools based on the genome of the domestic cat (*Felis catus*). TLR10 primer sequences were obtained from Mercier et al. (2012) and feline primer sequences for the reference gene HPRT were obtained from Penning et al. (2007). PCR products for each gene were purified using a PCR clean-up system (Promega, #A9281) and sequenced at the MSU Research Technology Support Facility. Consensus sequences were created using CLC Sequence Viewer version 6.7.1 (Knudsen et al., 2012). The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) was used to calculate percent identity between hyena sequences and sequences from domestic cats, domestic dogs (*Canis lupus familiaris*), humans (*Homo sapiens*), and house mice (*Mus musculus*).

2.4. Real-time quantitative reverse transcription PCR (qRT-PCR)

RNA (0.5 µg) was reverse transcribed to cDNA using random and oligo(dT) primers following the manufacturer's instructions for the qScript cDNA synthesis kit (VWR, # 101414-098) to a final volume of 20 µL. cDNA was then stored at 80°C until use. All qPCR reactions were carried out using a Bio-Rad iQ5 iCycler and iQ5 software. PCR-grade water, PerfeCTa SYBR Green Fastmix (VWR, # 101414-262), primers, and cDNA template were aliquoted into 96-well plates and sealed. Each combination of tissue sample and target gene was assayed in triplicate. Primers were used at 200 nM with 2 µL of cDNA product per 25 µL reaction. We used a no template control and a no reverse transcriptase RNA control for each tissue sample tested. All qRT-PCR reactions used the following conditions: 2 minutes at 95°C; followed by 45 cycles of 15 seconds at 95°C, 30 seconds at T_a , and 60 seconds at 72°C. Following the final extension of 120 seconds at 72°C, a stepwise melt curve from 45°C to 95°C was performed. HPRT was used as a reference gene to normalize expression of target genes using the C_t method (Livak and Schmittgen, 2001). Amplification efficiency was assessed using 10,000-fold serial dilutions of purified PCR products, and efficiency was calculated using Bio-Rad iQ5 software.

3. Results and Discussion

We amplified and sequenced cDNA for TLRs 1-10 and the reference gene HPRT in spotted hyenas. See Table 1 for primer sequences, amplicon base pair lengths, and annealing temperatures. As expected, domestic cats and hyenas, which both belong to the Feliformia suborder of the Carnivora order, exhibited a high degree of DNA sequence identity; TLR sequence identity between domestic cats and spotted hyenas ranged from 92-98% (Table 2). Nucleotide sequence identity between hyenas and other mammals in order of highest to lowest sequence similarity was: domestic cats, domestic dogs, humans, and mice. The only

exception to this order was that hyena TLR5 was more similar to the human TLR5 than it was to the domestic dog TLR5.

Hyenas also generally showed highest AA sequence similarity to domestic cats (Table 2). However, the AA sequence of hyena TLR3 was more similar to domestic dogs than it was to that of domestic cats. Hyena TLR5 and TLR6 AA sequences were more similar to human sequences than to dog sequences. Interestingly, domestic cat TLR5 was also more similar to human TLR5 than it was to domestic dog TLR5, suggesting that TLR5 function may be altered in dogs (Mercier et al., 2012) as compared to other carnivores. Mouse AA similarity with hyenas was the lowest in all cases. The HPRT reference gene AA sequence identity was greater than 95% for all species tested in this study.

Our data show that hyena TLRs are highly conserved relative to those of other mammals. Roach et al. (2005) suggested that TLRs are highly conserved across taxa because their PAMP ligands, such as flagellin, cannot be easily mutated without reducing the microorganism's fitness. The TLR1 family, which includes TLRs 1, 2, 6, and 10, form extracellular heterodimers that recognize lipopeptides (Roach et al., 2005). An analysis of vertebrate TLRs suggested that TLR2 in many species may be under strong stabilizing selection, whereas TLR2 heterodimer mates, such as TLR6 may be under fewer evolutionary constraints than TLR2 itself (Roach et al., 2005). Our data support this hypothesis, as hyena and cat TLR6 and TLR10 had the lowest AA sequence identities of the ten TLRs we assessed, 84% and 88% respectively, whereas TLR2 AA sequence identity between hyenas and cats (98%) was the highest, suggesting selection favoring conservation of TLR2.

We next assessed TLR expression in lung, pancreas, and spleen tissues from two hyenas, and sternal and inguinal lymph nodes from one hyena. Transcripts for TLRs 1-10 and HPRT were detected in all tissues, although some occurred at relatively low levels. No amplification of non-reverse-transcribed RNA was observed, indicating there was no gDNA contamination. Additionally, the HPRT primer set spanned an intron and only band sizes that corresponded to the mRNA transcript were observed.

Robust statistical analysis was not possible due to the small sample size ($n = 2$); the results from the tissue-specific mRNA expression analysis are presented graphically (Fig. 1). Despite members of the TLR1 family (TLR1, TLR2, TLR6, TLR10) often forming homodimers or heterodimers with TLR2, our results do not appear to show a relationship among the TLR1 family members. Expression of TLR1 and TLR10 was in highest in the lymph nodes, and lowest in the lungs. TLR2 expression was also relatively high in the lymph nodes, but was also highly expressed in the lungs and minimally expressed in the spleen. TLR6 was moderately expressed in all tissues.

Of particular interest is how spotted hyenas survive regular exposure to rabies virus (East et al., 2001), whereas survival after rabies virus infection is extremely rare in most other mammals, with the notable exception of bats (Cowled et al., 2011). Rabies virus is a negative-sense single-stranded RNA virus that replicates primarily in neurons, eventually making its way to the salivary glands, where it can be secreted with saliva and transmitted to

new hosts. TLR3 is an intracellular transmembrane protein that recognizes double-stranded RNA (dsRNA) and host mRNA, and is a negative regulator of axonal growth (Cameron et al., 2007). TLR3^{-/-} mice are more resistant to rabies virus than mice with functional TLR3 (Ménager et al., 2009).

TLR3 was expressed at moderate levels relative to other TLRs in all hyena tissues tested in this study. In black flying foxes (*Pteropus alecto*), TLR3 is highly expressed in the liver, moderately expressed in salivary glands, and minimally expressed in the brain (Cowled et al., 2011). It would be interesting to assess TLR3 expression in hyena neurons and salivary glands, as these are the tissues in which the rabies virus is most likely to be detected. Inoculating long-lived animals with virulent pathogens such as rabies virus is not feasible, but comparing expression dynamics in response to different types of rabies and non-rabies vaccines might shed light on how spotted hyenas are able to survive rabies exposure to the rabies virus. A vaccine consisting of a killed Pasteur strain rabies virus (i.e. IMRAB), which is commonly used for vaccinating domestic animals, should more closely mimic a natural rabies infection than a Vaccinia-Rabies Glycoprotein (V-RG) recombinant oral rabies virus vaccine, which has been successfully used on large scales for vaccinating wildlife. Comparison of TLR3 dynamics in response to these two rabies vaccines and a non-rabies vaccine should provide insight into the relative importance of TLR3 for resistance to rabies.

TLR4, the primary receptor for lipopolysaccharide from gram-negative bacteria, was expressed at low-to-moderate levels in most tissues tested. TLR4 down-regulation is commonly observed in response to repeated exposure, and is often associated with endotoxin tolerance (Nomura et al., 2000). Spotted hyenas regularly feed on both fresh and decaying carcasses, increasing the chance of exposure to additional commensal and pathogenic bacteria found in their food. Additionally, spotted hyenas regularly engage in aggressive encounters with conspecifics and sympatric species such as lions (*Panthera leo*), which often results in open wounds by which bacteria can enter a host. It would be interesting to compare TLR4 dynamics in response to LPS challenge in captive hyenas inhabiting a relatively clean environment with the TLR4 dynamics of wild hyenas. Additionally, TLR4 and TLR2 signaling may play a role in mediating the bacterial community-associated odors observed in wild hyenas.

Despite the vast number of microorganisms inhabiting the gut, skin, and mucous membranes of most mammals, most sites within the body are free of microorganisms in healthy individuals. Systems with no direct exposure to the host's external environment, such as the pancreas, should remain free of microorganisms. TLR5, which recognizes the bacterial protein flagellin, expression in the pancreas was the highest in any of the tissues we tested. TLR5 is also expressed at high levels in the feline pancreas (Franchini et al., 2010), suggesting that this critical tissue may be highly sensitive to flagellated bacteria.

TLR7 and TLR8 both detect single stranded RNA and are believed to be the result of a gene duplication event. Our results show that in three of the four tissues tested, TLR7 expression is low when TLR8 is high, or the reverse that TLR7 expression is high when TLR8 is low. A similar pattern of TLR7 and TLR8 expression was observed in domestic cats (Ignacio et al., 2005), supporting the hypothesis that given the shared evolutionary history and similar

binding patterns of these two receptors, some of the functions of these receptors may be redundant (Croizat and Beutler, 2004). TLR9, which recognizes unmethylated CpG motifs, is another endosomal receptor that forms an evolutionary cluster with TLR7 and TLR8 (Croizat and Beutler, 2004). Similar to TLR7, expression of TLR9 also appeared to be inversely related to TLR8 expression.

Prior to this study, it was unknown which, if any, TLRs were expressed in hyena tissues. Here we have characterized partial sequences of spotted hyena TLR1-10 genes, examined hyena TLR homology to other mammals, and documented the expression patterns in tissues from two hyenas; characterization of the TLR genes in this long-lived species provides a critical starting point for elucidating the mechanisms of the remarkable disease resistance observed in hyenas. Future studies may be able to build on this work by comparing the TLR expression patterns between captive and wild hyenas, collecting peripheral blood samples for *in vitro* studies, and taking advantage of the myriad natural infections suffered by wild hyenas.

Acknowledgments

We would like to thank the Dr. Stephen E. Glickman and the staff of the Field Station for Behavioral Research at UC Berkeley for their assistance with tissue collection from the captive hyenas, Dr. Julia Bell for her advice on laboratory procedures, and Dr. Barry Williams for his comments on the research. This work was supported by Award No. W911NF-08-1-0310 from the Army Research Office and NSF Grant IOS0819437 to KEH, NSF Grant IOS0809914 to SEG, NIH grant K26RR023080 to LSM, grants-in-aid-of research from Sigma Xi and the American Society of Mammalogists to ASF, veterinary student scholars program from the Morris Animal Foundation, and a NSF Graduate Research Fellowship to ASF.

References

- Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *The Journal of Immunology*. 2001; 167:1609–1616. [PubMed: 11466383]
- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. 1997; 25:3389–3402. [PubMed: 9254694]
- Barton GM, Kagan JC. A cell biological view of toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol*. 2009; 9:535–542. [PubMed: 19556980]
- Cameron JS, Alexopoulou L, Sloane JA, DiBernardo AB, Ma Y, Kosaras B, Flavell R, Strittmatter SM, Volpe J, Sidman R, Vartanian T. Toll-like receptor 3 is a potent negative regulator of axonal growth in mammals. *The Journal of Neuroscience*. 2007; 27:13033–13041. [PubMed: 18032677]
- Cowled C, Baker M, Tachedjian M, Zhou P, Bulach D, Wang LF. Molecular characterisation of Toll-like receptors in the black flying fox *Pteropus alecto*. *Dev Comp Immunol*. 2011; 35:7–18. [PubMed: 20692287]
- Croizat K, Beutler B. TLR7: A new sensor of viral infection. *Proc Natl Acad Sci U S A*. 2004; 101:6835–6836. [PubMed: 15123819]
- East ML, Hofer H, Cox JH, Wulle U, Wiik H, Pitra C. Regular exposure to rabies virus and lack of symptomatic disease in Serengeti spotted hyenas. *Proc Natl Acad Sci U S A*. 2001; 98:15026–15031. [PubMed: 11742089]
- Engh AL, Funk SM, Horn RCV, Scribner KT, Bruford MW, Libants S, Szykman M, Smale L, Holekamp KE. Reproductive skew among males in a female-dominated mammalian society. *Behavioral Ecology*. 2002; 13:193–200.

- Flies AS, Grant CK, Mansfield LS, Smith EJ, Weldele ML, Holekamp KE. Development of a hyena immunology toolbox. *Vet Immunol Immunopathol.* 2012; 145:110–119. [PubMed: 22173276]
- Franchini M, Zini E, Osto M, Jablonski K, Kaufmann K, Lutz TA, Reusch CE, Ackermann M. Feline pancreatic islet-like clusters and insulin producing cells express functional Toll-like receptors (TLRs). *Vet Immunol Immunopathol.* 2010; 138:70–78. [PubMed: 20674989]
- Harrison TM, Mazet JK, Holekamp KE, Dubovi E, Engh AL, Nelson K, Van Horn RC, Munson L. Antibodies to canine and feline viruses in spotted hyenas (*Crocuta crocuta*) in the Masai, Mara National Reserve. *Journal of Wildlife Diseases.* 2004; 40:1–10. [PubMed: 15137483]
- Ignacio G, Nordone S, Howard KE, Dean GA. Toll-like receptor expression in feline lymphoid tissues. *Vet Immunol Immunopathol.* 2005; 106:229–237. [PubMed: 15963821]
- Knudsen, B.; Knudsen, T.; Flensburg, M.; Sandmann, H.; Heltzen, M.; Andersen, A.; Dickenson, M.; Bardram, J.; Steffensen, P.J.; Mønsted, S.; Lauritzen, T.; Forsberg, R.; Thanbichler, A.; Bendtsen, J.D.; Görlitz, L.; Rasmussen, J.; Tordrup, D.; Værum, M.; Ravn, M.N.; Hachenberg, C.; Fisker, E.; Dekker, P.; de Meza, J.; Hein, A.M.K.; Sinding, J.B.; Quorning, J.; Hvam, K.; Mikkelsen, S.; Liboriussen, P.; Grydholt, J.; Handberg, H.; Bundgaard, M.; Joecker, A.; Simonsen, M.; Nielsen, P.R.L.; Joecker, A.; Fleischer, P.; Jakobsen, J.; Juul, S.; Appelt, U.; Fejes, A.; Christensen, A.S. CLC Sequence Viewer, 6.7.1. CLC bio; 2012.
- Lembo T, Hampson K, Auty H, Beesley CA, Bessell P, Packer C, Halliday J, Fyumagwa R, Hoare R, Ernest E, Mentzel C, Mlengeya T, Stamey K, Wilkins PP, Cleaveland S. Serologic surveillance of anthrax in the Serengeti ecosystem, Tanzania, 1996-2009. *Emerg Infect Dis.* 2011; 17:387–394. [PubMed: 21392428]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ Ct method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Medzhitov R, Schneider DS, Soares MP. Disease Tolerance as a Defense Strategy. *Science.* 2012; 335:936–941. [PubMed: 22363001]
- Ménager P, Roux P, Mégret F, Bourgeois JP, Le Sourd AM, Danckaert A, Lafage M, Préhaud C, Lafon M. Toll-like receptor 3 (TLR3) plays a major role in the formation of rabies virus negri bodies. *PLoS Pathog.* 2009; 5:e1000315. [PubMed: 19247444]
- Mercier E, Peters IR, Day MJ, Clercx C, Peeters D. Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. *Vet Immunol Immunopathol.* 2012; 145:618–624. [PubMed: 22321737]
- Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S. Cutting Edge: Endotoxin Tolerance in Mouse Peritoneal Macrophages Correlates with Down-Regulation of Surface Toll-Like Receptor 4 Expression. *The Journal of Immunology.* 2000; 164:3476–3479. [PubMed: 10725699]
- Opal S, Huber C. Bench-to-bedside review: Toll-like receptors and their role in septic shock. *Critical Care.* 2002; 6:125–136. [PubMed: 11983038]
- Penning LC, Vrieling HE, Brinkhof B, Riemers FM, Rothuizen J, Rutteman GR, Hazewinkel HAW. A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues. *Vet Immunol Immunopathol.* 2007; 120:212–222. [PubMed: 17904230]
- Raja A, Vignesh AR, Mary BA, Tirumurugan KG, Raj GD, Kataria R, Mishra BP, Kumanan K. Sequence analysis of Toll-like receptor genes 1-10 of goat (*Capra hircus*). *Vet Immunol Immunopathol.* 2011; 140:252–258. [PubMed: 21288575]
- Roach J, Glusman G, Rowen L, Kaur A, Purcell M, Smith K, Hood L, Aderem A. The evolution of vertebrate Toll-like receptors. *Proc Natl Acad Sci USA.* 2005; 102:9577–9582. [PubMed: 15976025]
- Schneider DS, Ayres JS. Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nat Rev Immunol.* 2008; 8:889–895. [PubMed: 18927577]
- Siegemund S, Sauer K. Balancing pro- and anti-inflammatory TLR4 signaling. *Nat Immunol.* 2012; 13:1031–1033. [PubMed: 23080196]
- Theis KR, Venkataraman A, Dycus JA, Koonter KD, Schmitt-Matzen EN, Wagner AP, Holekamp KE, Schmidt TM. Symbiotic bacteria appear to mediate hyena social odors. *Proceedings of the National Academy of Sciences.* 2013; 110:19832–19837.

Van Horn RC, Engh AL, Scribner KT, Funk SM, Holekamp KE. Behavioural structuring of relatedness in the spotted hyena (*Crocuta crocuta*) suggests direct fitness benefits of clan-level cooperation. *Mol Ecol*. 2004; 13:449–458. [PubMed: 14717899]

Fig. 1. Normalized expression of TLRs 1-10. Each bar represents the mean expression ($\pm$ S.E.M.) from tissue samples from two hyenas, except for the lymph nodes; both lymph nodes are from a single hyena (inguinal and sternal).

Table 1

Details of primers, amplicons, and reaction conditions.

Gene	Primer sequence 5' - 3' Forward / Reverse	Amplicon length (bp)	Ta (°C)
TLR1	AGTCAGCACAGCAGTAAACCTGGA GCTTGTATGCCAAACCAACTGGATG	190	54
TLR2	AGACTCTACCAGATGCCTCCTTCT GCGTGAAAGACAGGAATTCACAGG	168	56
TLR3	GACCTGTCAAGCCATTACCTCTGT CAAACCTGCTCTGGCTGTCTGTCTA	255	55
TLR4	GCTGGCAATTCTTTCCAGGACAAC TCTGGAGGGAGTGAAGAGGTTTCAT	208	57
TLR5	TTCCTTCCGCCAGGAGTATTTAGC GGAGTTCGCACTCACAGATGAACT	217	55
TLR6	CTCTCAAACATGTGGAAACAACCTCGG GCTTGATGTCTGAGGACAAAAGCAT	296	56
TLR7	TGGTGGGTAAACCATAACAGAGGTG GAGAAAGAGCCACCGATACGGAAA	172	57
TLR8	GGACCGCTACCAACCTAACCATTT ACGATGCTCTTCCCTCTTTGATCC	169	55
TLR9	CTGGAGGAGCTGAACCTGAG GCGGGCAGGGGTTCCTATAG	100	52
TLR10	TGCCAACAACACATCCTTG GCAAGCACCTGAAAACAGAA	143	52
HPRT	ACTGTAATGACCACTCAACAGGGG TGTATCCAACACTTCGAGGAGTCC	209	51

Table 2
DNA and amino acid (AA) sequence identity among *C. crocuta* and other mammals

Gene	<i>F. catus</i>		<i>C. lupus</i>		<i>H. sapiens</i>		<i>M. musculus</i>	
	DNA	AA	DNA	AA	DNA	AA	DNA	AA
TLR1	97	92	89	79	85	70	74	63
TLR2	98	98	85	89	85	82	76	65
TLR3	95	89	92	92	89	82	81	78
TLR4	94	91	80	74	77	68	72	60
TLR5	93	93	76	70	77	77	70	67
TLR6	93	84	85	66	80	69	70	57
TLR7	98	98	91	83	89	85	83	80
TLR8	96	98	88	93	82	80	77	74
TLR9	92	91	91	88	89	88	80	78
TLR10	95	88	94	83	82	67	*	*
HPRT	96	97	95	95	93	97	90	95

* *M. musculus* do not possess a functional TLR10 gene