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REVIEW

Toll-like receptors in domestic animals

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Abstract Toll-like receptors are pattern recognition receptors with which hosts recognize pathogen-associated molecular patterns (PAMP). This recognition process is translated rapidly into a meaningful defense reaction. This form of innate host defense is preserved in the animal kingdom: invertebrates heavily depend on it; higher vertebrates also have an adaptive immune system. Both adaptive and innate immune systems are intertwined in that the former also depends on an intact innate recognition and response system. Members of the TLR system cover recognition of parasitic, bacterial or viral germs. Due to the constraints imposed by the necessity to recognize PAMP and to interact with downstream signaling molecules, the TLR system is relatively conserved in evolution. Nevertheless, subtle species differences have been reported for several mammalian TLR members. Examples of this will be given. In all mammalian species investigated, part of the coding sequence is available for the most important TLR members, thus allowing study of expression of these TLR members in various tissues by reverse-transcription polymerase chain reaction in its classical (RT-PCR) and quantitative real time RT-PCR (qRT-PCR) form. In some species, the whole coding sequences of the most important or even all TLR members are known. This allows construction of cDNA and transfection of common host cells, thus permitting functional studies. Extensive investigations were devoted to the study of non-synonymous single nucleotide polymorphisms. In a few cases, expression of a given amino acid in the extracellular (ligand-binding) portion of TLR members could be associated with infectious diseases. This will be discussed below.

Keywords Toll-like receptors · Pattern recognition receptors · PRR · TLR · Pathogen-associated molecular patterns

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Introduction

Toll-like receptors (TLR) are a family of germ-line-encoded receptors of the innate immune system. Collectively, they cover the recognition of a wide variety of pathogens (viruses, bacteria, blood-borne parasites), thereby inducing a fast and appropriate host defense reaction against these. Being conserved in evolution, they represent the prime host defense mechanism of invertebrates and lower vertebrates. In higher vertebrates (from gnathostomes up), they are not only essential for sensing microbes by the innate immune

system but also for inducing a clonally restricted, antigen-specific response in the adaptive immune system, mediated by B and T cells (Medzhitov et al. 1997). This chapter briefly reviews how interest arose in TLR members, then lists some burning questions in different species, summarizes the approaches that have to be taken when studying the TLR system in domestic animals, and finally goes more specifically into what is known in species of domestic animals.

Early investigations in invertebrates

The term "toll-like receptors" suggests that there is also a "toll". Indeed, when a doctoral student in the laboratory of Nuesslein-Volhard found that, in a *Drosophila* mutant in which a gene was lacking, the dorsoventral orientation of larvae was ablated, she expressed, in her German, "toll" (great), and thus a gene important for the larval development of *Drosophila melanogaster* was named (Anderson et al. 1985). The ligand of that receptor was not a pathogen, but a cleaved fly glycoprotein with the name of "spaetzle" (Morisato and Anderson 1994). Later on, it was realized that the cell-membrane-expressed receptor "Toll" also has an important function in adult flies by mediating the recognition of fungi and inducing a meaningful host response against these (Lemaitre et al. 1996). This fundamental observation was the reason for also looking for "Toll" in higher vertebrates (see below).

Early investigations in vertebrates

There are two early traces of linking mammals to TLR: (1) the seminal discovery that TLR4 is the signaling part of the lipopolysaccharide (LPS) receptor (Poltorak et al. 1998) alluded to in more detail by Van der Pool (2010, this issue), and (2) the observation that innate immune recognition by the TLR is essential for the mounting of an adaptive immune response (Medzhitov and Janeway 1997). After these pivotal observations, the area virtually exploded, and rapid progress was made particularly with regard to innate recognition of pathogens and their products. Fascinatingly, Toll homologues or TLR are highly conserved between invertebrates and vertebrates. A great number of different TLR have been identified, and the number of expressed receptors varies between species from 1 in the nematode *Caenorabditis elegans* (Kanzok et al. 2004) to 222 in the sea urchin *Strongylocentrotus purpuratus* (Roach et al. 2005). In higher vertebrate species, 13 TLR members have been identified, subsumed in the following as the TLR system. For 10 of them, natural ligands of pathogens are known. Subsequently, discoveries were made rapidly in mice and humans, particularly with regard to the binding specificity of members of the TLR family, and to the signaling chains involved. To knockout genes involved either in the sensing of pathogens or in signaling and mediating a defense reaction was central. There are a number of excellent reviews of these findings (Akira et al. 2001; Kaisho and Akira 2006; Beutler et al. 2006; Werling and Coffey 2007); one might also study this special issue. However, the expression and function of TLR by domestic animals lagged behind. This area has been comprehensively reviewed by Turin and Riva (2008). While there are a total of 13 TLR members described in mammals, there are 10 genes in most mammals, 10 genes in the chicken, 3 of which are unique to birds (Temperley et al. 2008), and approximately 17 genes in all bony fishes investigated so far (Rebl et al. 2010).

Receptors for pathogen- or microbe-associated molecular patterns?

Those who coined the expression "pathogen-associated molecular patterns (PAMP)" meant that protection against certain pathogenic microbes is achieved by innate recognition and an appropriate defensive response against these microbes. Put another way, their wording suggests that it is the immune system that discriminates between benign microbes and harmful pathogens compromising the health of the host. That this concept cannot be maintained, in the case of the TLR system, was realized soon thereafter, and the term "microbe-associated molecular pattern (MAMP)" was coined (Mackey and McFall 2006). In the case of a viral infection, one could argue that any intrusion of a virus into host cells in vivo is inappropriate regardless of the pathogenicity of the virus, and therefore any virus has to be regarded as pathogenic. Thus, in the case of a viral infection, the terms PAMP and MAMP have the same meaning. But what discriminates a harmless bacterial intruder from a truly pathogenic bacterium? Both are recognized by the TLR system. That there is a difference between PAMP and MAMP becomes immediately obvious in the case of the gastrointestinal tract harboring myriads of benign bacteria but depending—so it is believed—on the TLR system to cope with a potentially harmful pathogen. Rakoff-Nahoum et al. (2004) even reported that stimulation of the TLR system is absolutely required for a normal development of the gastrointestinal tract. There is no doubt that non-pathogenic bacteria also trigger the TLR system, which consequently fails to discriminate between benign and pathogenic bacteria. What exactly characterizes pathogenic bacteria or blood parasites and distinguishes them from harmless counterparts is unknown. It has been suggested that it is the strategic positioning that allows

discrimination (Gewirtz et al. 2001). Accordingly, benign bacteria are localized only in the colon lumen, where they are disregarded by the TLR system. In contrast, the flagellin of pathogenic Salmonellae gets access to the basolateral side of the epithelium where it is sensed by basolaterally expressed TLR5. (This, however, does not explain why in fishes there is a soluble variant of TLR5, TLR5S; see below.) The finding of Burgener et al. (2008), that dysregulated TLR expression of dogs with inflammatory bowel disease is not normalized after these dogs are treated and get clinically better, is illustrative in this context. It suggests that a healthy, not dysregulated, TLR system in the gastrointestinal tract is finely balanced in order to protect a host from infection on one hand and to avoid inflammatory sequelae triggered by the immune system in its fight against the intruder on the other hand. Be this as it may, the very properties of PAMP sensed by pattern recognition receptors (PRR) are incompatible with a discrimination between benign and pathogenic bacteria. For investigators of the gastrointestinal tract and its immunology, it is advised to give priority to the term MAMP over PAMP.

Binding specificity of mammalian TLR members

It is agreed that three preconditions are required for a PAMP to qualify as such and to be recognized by PRR such as TLR: (1) its broad expression by large groups of potential pathogens; (2) its essentiality for survival and successful propagation of the pathogen in question; and (3) its absence from tissues of the host species. The TLR system of higher vertebrates, which is discussed here, perfectly qualifies as a pattern recognition system that covers sensing of the full spectrum of microbes. (Although more PAMP were reported to be recognized by the TLR system, some of which are probably endogenous, and only the most important ones are summarized here, and a discussion as to whether other potential TLR agonists exist is avoided.) TLR4 is the first identified and probably best-studied TLR. In cooperation with three other proteins, the lipopolysaccharide binding protein (LBP), CD14 and MD-2, it is responsible for the sensing of lipopolysaccharide (LPS), the so-called endotoxin of gram-negative bacteria (Poltorak et al. 1998; Medzhitov et al. 1998; Sauter et al. 2007). The heterodimer formed between TLR2 and TLR6 or TLR1 is able to recognise diacylated or triacylated lipopeptides (LP) found on the surface of all bacteria, respectively (Takeda et al. 2003; Kawai and Akira 2006). TLR5 recognizes flagellin of bacteria (Hayashi et al. 2001). TLR9 binds motifs of CpG DNA, i.e. stretches that are frequently found in microbial DNA whereas they are rare in higher vertebrates, and if expressed, they are methylated and are therefore inaccessible for TLR9 recognition (Hemmi et al. 2000). TLR3 recognizes double-stranded RNA (Alexopoulou et al. 2001). TLR7 and 8 recognize imidazoquinolines used as antiviral compounds (e.g., imiquimod and resiquimod). Later, it was first realized by Heil et al. (2004) that TLR7 and 8 recognize single-stranded RNA, as often occurring in viruses. TLR3, 7, 8 and 9 are expressed in endosomal vesicles (Fig. 1), thereby being separated from host nucleic acids. TLR11, present only in the murine system, senses uropathogenic bacteria, although the exact PAMP, at the molecular level, is unknown. Ligands for TLR10 and members with numbers >11 are not yet known in mammals, but have been identified in chicken and fish, potentially representing the homologues of mammalian TLR.

In the literature, one finds the statement that TLR2 heterodimers recognize lipoteichoic acid (LTA) and peptidoglycan (PG) (Schwandner et al. 1999). The TLR

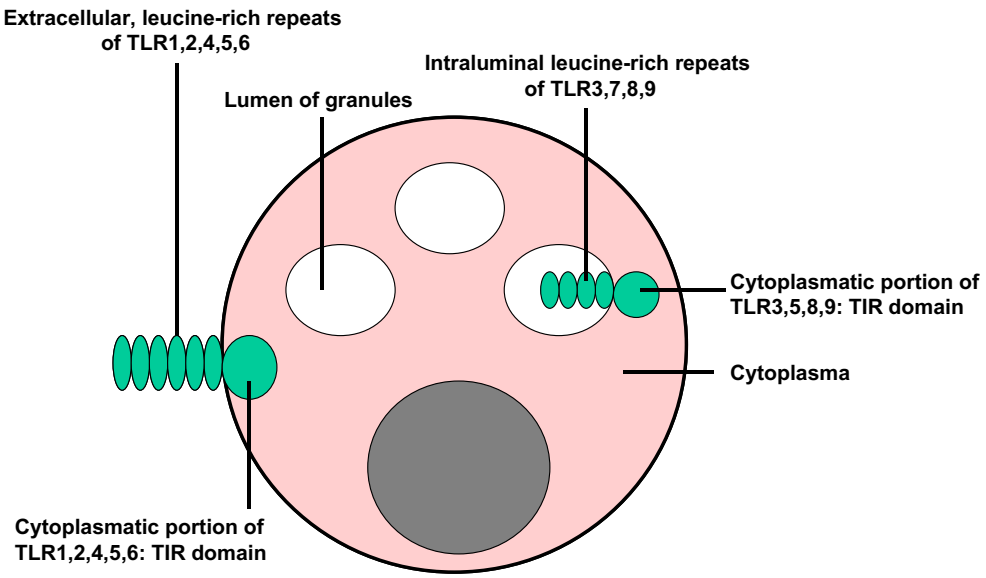

Fig. 1 Illustration of localization of various mammalian TLR in cells. TLR consist either of extracellular or intraluminal LRR recognizing the PAMP (*upright green ellipses*), of a short transmembrane region (not shown) and of cytoplasmatic TIR domains (*green circles*). Endosomal vesicles: *white circles*. Cell nucleus: *dark gray circle*

requirement for LTA recognition is controversial (Schwandner et al. 1999; Takeuchi et al. 1999). LTA preparations are notoriously contaminated by lipopeptides (LP); and there is evidence that the active components of LTA are contaminating LP (Hashimoto et al. 2006). Likewise, there is published evidence that PG are recognized by NOD1/NOD2, but not by TLR2. Instead, an LP contamination of PG and present in most PG preparations appears to be recognised by TLR2 (Travassos et al. 2004). Thus, a contamination by LP is underestimated by most studies. Farhat et al. showed that commercial LPS preparations but not ultra-pure LPS preparations from *E. coli* are recognised by boTLR2- and boTLR1-transfected cells (in preparation; Fig. 2), although all LPS preparations tested, including the ultra-pure LPS, proved to be agonists of TLR4. These authors concluded that LP contaminations are widespread and may confuse interpretation of the results. It has been suggested that TLR2 is not a promiscuous receptor for gram-positive bacteria and many blood-borne parasites; instead, its heterodimers are receptors for the recognition of di- or triacylated LP and LP-contaminated preparations (Zähringer et al. 2008). TLR10, which is expressed in humans, is closely related to TLR-1 and 6, and appears to also recognize triacylated LP, but the signaling mechanisms are apparently different for the TLR1/2 and TLR-2/10 heterocomplexes (Guan et al. 2010).

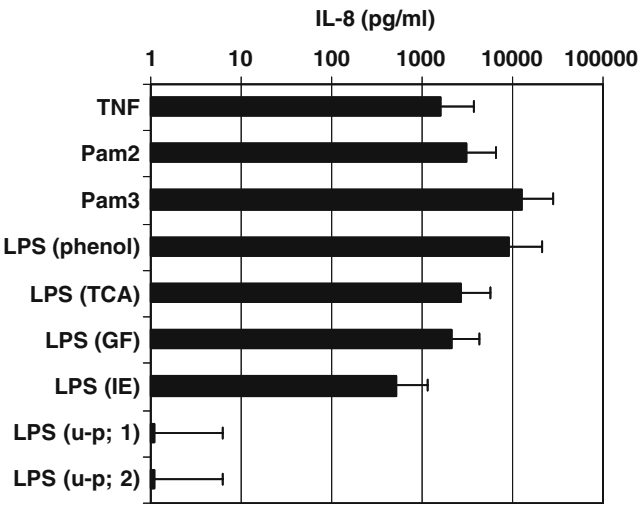

Fig. 2 The production of IL-8 by HEK293 cells stably transfected with both boTLR1 and boTLR2 and stimulated with 100 nM of either commercial or ultra-pure (*u-p*) *LPS* from *E. coli*. The numbers *1* and *2* refer to two lots of LPS kindly provided by Dr. H. Brade, Borstel Research Center. Commercial LPS include LPS from *E. coli* and purified by phenol extraction (*phenol*), precipitated by trichloroacetic acid (*TCA*), purified by gel filtration (*GF*) or by isoelectric focussing (*IE*). Controls were transfected cells stimulated by 10 mM TNF, 100 mM dipalmitoylated (*Pam2*) or 100 mM tripalmitoylated (*Pam3*) cysteyl-seryl-lysyl-lysyl-lysyl-lysine (CSK_4). IL-8 production was measured 24 h after stimulation, and means ± 2SD were plotted. The graph shows a representative experiment

Signaling through TLR (see Pierce (2010) in this special issue for details)

After discovery of the TLR system, a lot of effort went into characterizing its signaling pathways (Kaisho and Akira 2006). It is amazing how conserved these are, as the one for Toll in *Drosophila* and the most widespread signaling pathway in mammals are homologous to each other. Whereas TLR2 forms heterodimers with TLR1 or TLR6, and TLR4 probably forms multimeric complexes, upon engagement, it is not clear whether TLR3, TLR5, and TLR 7–9 also require multimerization for activating a signal transduction chain, At least in the case of TLR9, evidence for an allosteric effect has been provided (Latz et al. 2007). What is, however, clear is that all TLR contain a Toll-interleukin-1-receptor (TIR) domain that binds another intracellular adapter protein, called MyD88, with the exception of TLR3. In the case of all known TLR members except TLR3, the engagement/activation of MyD88 results in activation of the kinase IRAK-4 (interleukin-1-receptor associated kinase number 4) which phosphorylates/activates IRAK-1, leading to Traf6 (TNF receptor-associated factor number 6) activation. This ultimately results in the cleaving of the NF-κB-IκB complex. Whereas IκB is phosphorylated and degraded, the liberated NF-κB has access to the nucleus where it acts as a transcription factor, resulting in the up-regulation and/or induction of many host defense-related genes. TLR2 and TLR4 engagement preferentially use a TIR adapter molecule other than MyD88, namely Mal, in order to activate NFκB. In the case of TLR3, the activated TIR domain interacts with still another intracellular adapter molecule called TRIF, resulting in the induction of type-1 interferon (IFN). In the case of TLR4, one of five TIR adaptors (MyD88, Mal, TRIF, TRAM, SARM; see review of Kenny and O'Neill 2008) can be activated which culminates in both nuclear translocation of NF-κb and induction of IFN. IFN is exported and binds to its receptor (IFNAR) of producing and/or neighboring cells, which induces the production of more type-1 IFN. Thus, both signaling cascades lead either to activation of NFκB (all mammalian TLR members except TLR3) or to type-I-IFN induction (mammalian TLR3 and 4). NF-κB is associated with the induction of many genes having a role in immunity, and in the activation of prominent cytoplasmatic enzymes (Remer et al. 2003), up-regulation of surface-expressed molecules and induction of dendritic cell maturation. The latter protects neighboring target cells from viral infection. Type-1 IFN is antiviral, but whether it also has a protective effect in bacterial infections is controversial. The older literature also ascribes to type 1 IFN a protective role in bacterial (e.g., listerial) infections. There are three more recent papers suggesting that type-1 IFN has a harmful effect in listerial infection (Auerbuch et al. 2004; Carrero et al. 2004;

O'Connell et al. 2004). Whether this can be generalized to any bacterial infection is an open question.

Recently, it was found that certain factors limit a TLR2- or TLR4-mediated NF-κB activation. The first molecule identified in such an inhibitory loop was Tollip (Burns et al. 2000). The Tollip molecule phosphorylated by IRAK1 ubiquitinylates IRAK1, thereby marking it for degradation by the proteosomal degradation pathway and producing less free NF-κB.

Approaches to be taken to study the TLR system in domestic animals

In the following, it will be summarized which methods are generally used, and are most successful when studying TLR members of domestic animals. These include reverse transcription-polymerase-chain-reaction (RT-PCR) both in its classical form and the more quantitative real time-RT-PCR (qRT-PCR) form, transfection, flow cytometry, immunohistochemistry (IHC), electron-microscopic immunocytochemistry (EMICC), and stimulation in vitro by putative TLR agonists. The sequencing, which of course is very important, is not especially mentioned.

RT-PCR and qRT-PCR

If part of the nucleotide sequence of the TLR member concerned is available, one can use either RT-PCR or qRT-PCR to look for expression of the TLR member concerned at the mRNA level. This method is meaningful, particularly for purified cells in solution (e.g., blood cells), or for tissues available in highly purified form. It does not consider the heterogeneity with regard to tissue and cell content of organs. The expression of a given TLR member by heterogenous tissue requires IHC or EM-ICC in order to identify the tissue concerned, which means that antibodies must be available (see below), or in situ hybridization, which is not sensitive enough. The study of the expression of the TLR system in various tissues is therefore in its infancy.

Transfection

If the whole coding sequence of a given TLR member is available, one can clone the TLR in question and make DNA constructs to be used for transfection. Usually, in cells not expressing a certain trait but acquiring it when the cDNA is transfected, it can be measured whether TLR-mediated activation of cells is co-transfected. Care has to be taken in interpreting the data since the recipient cell may express endogenous TLR members heterodimerizing with the transfected TLR member, thereby activating the cell line in the presence of an appropriate MAMP. For example, bovine TLR2 transfected alone was active upon stimulation of the cells with diacylated lipopeptides, but it was suspected that transfected bovine TLR2 heterodimerized with endogenous (human) TLR6 in the HEK293 cells studied (Farhat et al. 2008, 2010). This promising approach has been taken up by several groups (e.g., Schwarz et al. 2007; Yang et al. 2008; Farhat et al. 2008).

Anti-TLR antibodies

If TLR-specific antibodies are available, one can localize expression of the TLR member concerned, for example using flow cytometry, IHC or EMICC. Various TLR-specific antibodies, including monoclonals, are sold commercially, and they recognize specifically TLR members. Sadly enough, not many of these are useful for studying the TLR system of domestic animals. This could have two reasons: (1) due to the large degree of conservation, not enough antigenic differences between the species of antibody induction and the antigen chosen is observed; (2), and more likely, due to the focusing on the study of the murine and the human TLR system, domestic animal species have the role of a Cinderella and are insufficiently investigated. A study of Burgener and Jungi (2008) provided evidence that commercial antibodies recognizing, with high affinity, human TLR and useful in flow cytometry, also cross-react with canine TLR. By immunizing rabbits with peptides derived from canine TLR members, a specific staining of canine mesenterial lymph node was obtained, and it is suggested that these represent the canine variant of TLR members (Fuog et al., submitted). Thus, polyclonal antibodies against TLR members of domestic animals can be made. The laboratory of Dominguez was successful in generating monoclonals specific for porcine TLR2 (Alvarez et al. 2008). Unfortunately, this monoclonal failed to recognize bovine cells (Jungi and Dominguez, unpublished). Others were successful in generating polyclonal pig TLR2-specific (Tohno et al. 2005a) and pig TLR9-specific antibodies (Shimosato et al. 2005). Due to a lack of antibodies specific for TLR members of domestic animals, one often resorts to quantitative real-time RT-PCR to study the expression of TLR members (see below). As outlined below, for most species, complete or partial coding sequences of the most important TLR members are known.

Cultivation of cells in the presence of TLR agonists

Cells expressing TLR (either autochthonous or transfected) are stimulated with highly purified agonists of TLR [LPS, synthetic diacylated or triacylated LP, poly(I:C), single

stranded or double-stranded RNA, CpG DNA] or growth-arrested whole gram-positive or gram-negative bacteria (Farhat et al. 2008), and induction of an appropriate effector function is measured, depending on the cells stimulated. This can range from measurement of a cytokine by ELISA, up-regulation of transfected surface molecules such as ELAM-1 by flow cytometry (Schwandner et al. 1999), to any other function whose expression in the cells studied is TLR-dependent.

Analysis of TLR in domestic animals

Being a conserved system, it is clear that one cannot expect dramatic inter-species changes for the TLR system, as most domestic animal species are mammals. However, in evolutionary terms, interesting observations were made in birds, in teleostean fishes and in lamprey. The review of Turin and Riva (2008) also alluded to some of these species. To allow an estimation on evolutionary dynamics, homologies of DNA of genes are indicated.

Guinea pig

In the guinea pig, TLR2, 3, 4, 7 and 8 have been worked on (Kuno et al. 2009; Astakhova et al. 2009; Kawahara et al. 2001). This is a species of lesser importance in present-day immunology. Nevertheless, one of the first studies using a pharmacological TLR4 antagonist has been reported in this species (Kuno et al. 2009).

Rabbit

Only TLR3, 4, and 7 have been addressed by researchers of rabbits (Astakhova et al. 2009; Zhou et al. 2007), as this is also a species of lesser interest in present-day immunology.

Cattle

This is one of the well-investigated species of domestic animals. Thus, the complete coding sequence of TLR1–10 is known (Menzies and Ingham 2006; Seabury et al. 2007, 2010; Turin and Riva 2008), with genes mapped to chromosomes (McGuire et al. 2006). Research has addressed additional questions such as the degree of polymorphism (see below). The nucleotide homology to human TLR genes is, on average, 65–77%, and 98% to the closely related nilgai or water buffalo genes in the same subfamily (amino acid similarity is 97%; Turin and Riva 2008). This enabled Vaharan et al. (2008) to establish primers for assessing nilgai TLR mRNA and to study expression by RT-PCR of TLR genes in various nilgai organs.

Small ruminants

The sheep genome was found to contain, as is typical for mammals, TLR1–10 and partial coding sequences are available for sheep (Menzies and Ingham 2006; Bhide et al. 2009; Chang et al. 2009; Turin and Riva 2008). It was reported to express the "human" rather than the "murine" type of TLR members (Nalubamba et al. 2007). Primers of ovine TLR to be used in qRT-PCR were published, based on partial sequences (Menzies and Ingham 2006), and expression in various tissues has been reported (Table 1). For both ovine TLR7 and 8, two variant genotypes are available. For all TLR genes in the ovine genome, the homology to bovine orthologues is >95%.

Based on the high degree of relatedness between sheep and goat, Tirumurugaan et al. (2010) succeeded in defining primers available for RT-PCR of goat TLR1-10 and in measuring the level of expression in various tissues (Table 1). Sequencing the amplified products revealed, as was expected, that there is a close match between ovine and caprine TLR (Tirumurugaan et al. 2010).

Pig

Genes expressing all TLR members known in humans and porcine MyD88 have been cloned (Bergman et al. 2010; Astakhova et al. 2009; Shinkai et al. 2006a; Bailey 2009; Tohno et al. 2007; Turin and Riva 2008) showing a nucleotide homology of DNA of 65–77% with human TLR orthologues. The gene coding for porcine MyD88 is 87% homologous to the human MyD88 gene.

Horse

Equine TLR2, 3, 4, 5, 7 and 8 have been fully sequenced (Turin and Riva 2008; Astakhova et al. 2009). For TLR9, part of the sequence is known, allowing qRT-PCR to be performed (Schneberger et al. 2009; Sharma and Maheshwari 2009). Compared with human TLR genes, the equine counterparts have a nucleotide homology of 65–77%.

Cat

In the cat, the first 9 TLR members have been partially cloned and sequenced, and the genes have been analyzed (Ignacio et al. 2005; Turin and Riva 2008). Again, compared with human TLR genes, homology is somewhere between 65 and 77% (TLR4) and 90% (TLR9).

Dog

Complete cDNA sequences are available only for TLR2, 4, and 9 (Asahina et al. 2003; Hashimoto et al. 2005; Ishii

Table 1 Expression of TLR members by various organs from mammalian domestic animal species

Species	Organ	Finding	Method	Reference
Cattle (Ruminantia)	Skin	TLR7,2>3,5,8>1>6,9,10; 4 low, 6 lacking	qRT-PCR	Menzies and Ingham (2006)
	Lung	TLR4 expressed; PIM have high LE	IHC, EMICC	Wassef et al. (2004)
	Small intestine	TLR4 expressed	IHC, EMICC	
	Skeletal muscle	Negative for TLR4	IHC, EMICC	
	Liver	TLR4 expressed	IHC, EMICC	
	Spleen	TLR4 expressed	IHC, EMICC	
Nilgai (Ruminantia)	Mononuclear cells	TLR2-10 expressed	RT – PCR	Vaharan et al. (2008)
	Neutrophils	TLR1-10 expressed except TLR3	RT – PCR	
	Spleen	TLR1-10 expressed	RT – PCR	
	Kidney	TLR2,5,7,9 expressed	RT – PCR	
	Lung	TLR1-10 expressed	RT – PCR	
	Liver	TLR1-10 expressed	RT – PCR	
	Heart	All TLR expressed except TLR10	RT – PCR	
	Uterus	TLR2,5,7-10 expressed	RT – PCR	
	Ovary	TLR2-10 expressed	RT – PCR	
Sheep (Ruminantia)	Jejunum	TLR6>7,10>TLR2>TLR3-5,8,9>TLR1	qRT – PCR	Menzies and Ingham (2006)
	Peyers patches	Same as above, but weaker	qRT – PCR	
	Mesent. lymph node	Same as above, but weaker, still	qRT – PCR	
Goat (Ruminantia)	Blood	High LE of TLR1-9; TLR10 lacking	RT – PCR	Tirumurugaan et al. (2010)
	Lung	High LE of TLR1-9, TLR10 lacking RT-PCR	RT – PCR	
	Lymph node	High expression of TLR1-10	RT – PCR	
	Jejunum	High expression of TLR1,5,7-9>TLR6,10	RT – PCR	
	Skin	Low expression of TLR2,3,4,8,9,10	RT – PCR	
Pig (Perisso-dactyla)	Intestinal M cells	TLR2 LE high>TLR1,3-10	qRT-PCR, IHC	(Tohno et al. 2005b)
	Peyers patches	TLR2 and TLR9 LE high	qRT-PCR, IHC	(Tohno et al. 2006)
	Lung	TLR4 expressed; PIM have high LE of TLR4	ICH, EMICC	Wassef et al. (2004)
	Small intestine	TLR4 expressed	IHC, EMICC	
	Skeletal muscle	Negative for TLR4	IHC, EMICC	
	Liver	TLR4 expressed	IHC, EMICC	
	Spleen	TLR4 expressed	IHC, EMICC	
	Mesent. lymph node	TLR2 LE high		
Horse (Perisso-dactyla)	Lung	TLR 9 expressed; LE high in PIM, subject to modulation	qRT-PCR	Schneberger et al. (2009)
	Lung	TLR4 in lungs; TLR2 only in PIM, subject to modululation	qRT-PCR, IHC, EMICC	Singh Suri et al. (2006)
Dog (Carnivora)	Lung	TLR4 expressed; PIM LE high	IHC, EMICC	Wassef et al. (2004)
	Small intestine	TLR4 expressed	IHC, EMICC	
	Skeletal muscle	Negative for TLR4	IHC, EMICC	
	Liver	TLR4 expressed	IHC, EMICC	
	Spleen	TLR4 expressed	IHC, EMICC	
	Synovial joint	TLR2 and 4 expressed	qRT-PCR; IHC (TLR4)	Kuroki et al. (2010)
		Only TLR4 subject to modulation by disease		
	Intestine	TLR2,4,9 expressed, subject to modulation	qRT-PCR	Burgener et al. (2008)
	1°CEC	TLR2 and 4 expressed; subject to modulation	RT-PCR	Swerdlow et al. (2006)
Cat (Carnivora)	Lymphocyte subsets	TLR1-9 expressed, subsets variable	qRT – PCR	Ignacio et al. (2005b)
	Mesent. lymph node	TLR1-9 HE, except TLR1,4,6	qRT – PCR	

qRT-PCR Quantitative real-time reverse transcription-polymerase chain reaction, *PIM* pulmonary intravascular macrophages, *LE* level of expression, *IHC* immunohistochemistry (includes detection by fluorescence), *EMICC* electron microscope immunocytochemistry, *RT-PCR* reverse transcription-polymerase chain reaction, *mesent*. mesenteric, *CEC* colonic epithelial cells.

et al. 2006). Compared with human TLR genes, the nucleotide homology is between 65 and 77% (TLR4) or 88% (TLR2, 9).

Birds

We are now coming to non-mammalian species. Due to the larger distance to any given mammal, the phylogenetic aspect of a system putatively being under evolutionary pressure becomes more obvious (Temperley et al. 2008). The new names, as suggested by Temperley et al. (2008), are used. The chicken, i.e. the bird studied best, is used as representative. Some of the genes have orthologues in mammals (TLR3, 4, 5, 7), while TLR8 and TLR9 are lacking, and the mammalian TLR1, 6 and 10 being in mammals on the same chromosome are replaced by

TLR1LA and LB, and so is TLR2, replaced by TLR2A and TLR2B as a result of gene duplication. There is also TLR15 (unique to birds) and TLR21 (shared with fishes). Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9 (Brownlie et al. 2009; Keestra et al. 2010). In the zebra finch, an additional gene duplication occurred in TLR7 (Brownlie et al. 2009). Thus, the recognition spectrum of PAMP.is almost identical in birds and mammals.

As far as downstream signaling is concerned, the MyD88-mediated pathway appears to be expressed, but the TRIF-TRAM-mediated pathway may be missing (Keestra and van Putten 2008). This has been related to markedly lower sensitivity to LPS of birds compared to mammals.

Interestingly, linkage analyses suggested that certain forms of chicken TLR4 are associated with resistance/ suseptibility to systemic infection of young chickens with *Salmonella enterica* serovar *Typhimurium* (Leveque et al. 2003).

Teleostean fishes

Despite their high degree of variation, teleostean fishes all have a similar TLR system and share functional properties with those of mammals in principle, but some distinct features are noted (Rebl et al. 2010). Bony fishes tend to have more TLR members than mammals. In approximately a dozen teleostean species, 17 TLR members were found (TLR1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23) (Rebl et al. 2010). They may be grouped in 6 major families (TLR1/2, 3, 4, 5, 7, 11) (Roach et al. 2005). There is also a soluble TLR5 variant (designated TLR5S). The TLR7 family comprises TLR9 that has specificity for CpG DNA rather than single-strand RNA, as have other representatives of this group. In some fish species, there is extensive gene duplication (e.g., TLR8.1 and TLR8.2), and use of different splice variants (e.g., TLR9.1 and TLR9.2). The two-digit TLR members are within the TLR11 family except TLR-14 (TLR-1/2 family; Roach et al. 2005), and they are referred to as "fish TLR", as this order experienced extensive gene duplication in some teleost species, whereas in the mouse, it is represented by murine TLR11, 12 and 13 only, and in humans, there is only a pseudogene of TLR22. TLR22 has been shown in the fugu (*Takifugu rupripes*) to induce type-1-IFN, thereby being a functional analog of TLR3. As stated above, a PAMP at the molecular level is not known for this family in mammals. Quite clearly, there are more gene duplications in teleosts than in mammals, and the redundancy appears to be higher.

It has been pointed out by Rebl et al. (2010) that these TLR, in order to function like mammalian TLR, have to interact with down-stream signal transduction molecules. There is functional evidence that piscine MyD88 is interacting with TIR domains (van der Sar et al. 2006), but the piscine IFN-activating pathway differs from the mammalian one. The Tollip molecule has also been found in teleosts either in one or several different variants (Rebl et al. 2008)

Lamprey

The Japanese lamprey genome contains 16 TLR genes, some of which are M-type ("mammalian-type"), and F-type ("fish-type"), respectively. Remarkably, neither IRF3 nor IRF7 was expressed, two essential signaling molecules in the mouse, leading to type-I-IFN production, although the upstream molecules of the signaling chain were highly conserved between lamprey and mammals (Kasmatsu et al. 2010).

Functional variation of TLR in mammals

Being a well-conserved system, one may not expect great differences in TLR functions since most domestic animals are mammals and thus are relatively close to each other in phylogeny. Nevertheless, there are some species-specific differences in ligand recognition (Werling et al. 2009; Fitzgerald et al. 2004). The lipid A analog C406 acts as an agonist for murine but as an antagonist for human TLR4 (Tamai et al. 2003). LPS from *Rhodobacter sphaeroides* was shown to activate horse and hamster TLR4 but not human and murine TLR4 (Lohmann et al. 2007). TLR2/6 and TLR2/1 heterodimers recognize diacylated and triacylated LP, respectively. Depending on the species, fatty acids of a maximal length of 6, 12 or l4 C atoms are recognized by the mouse, cattle or human, respectively (Farhat et al. 2010). The requirements for optimal recognition of the murine, the bovine or the human TLR9 differs between species (Zhang et al. 2001). One of the receptors for the recognition of *Salmonella enterica* serovar *Typhimurium* is TLR5 as it recognizes bacterium-derived flagellin. Chicken and mouse TLR5 were consistently more sensitive to flagellin from this organism than human TLR5, a species-restricted particularity controlled by one single amino acid of this type of flagellin (Keestra et al. 2008). Thus, although the TLR system is conserved in principle in mammals, some subtle species-specific variations are observed.

TLR expression by various tissues

The interface between a potential host and a microbe coming from the surroundings are either immune cells or epithelia. So, expression of TLR members must be highest

and most varied in mononuclear phagocytes, neutrophils/heterophils, dendritic cells and epithelial cells such as enterocytes. However, dendritic cells are not uniform, and expression levels of TLR are quite different between types of dendritic cells (Werling et al. 2006). The same applies to mononuclear cells. Several studies refer to expression of TLR members by ocular tissue (Pearlman et al. 2010; Redfern and McDermott 2010). Although it is generally assumed that a high level of expression of a given TLR member indicates an efficient response to a PAMP, the study of expression is complicated by several factors: (1) it is not always clear whether nonexisting reports on certain TLR member expression means the virtual absence of a given TLR member from this tissue or organ, or a lack of relevant tools for analysis, or simply lacking information; (2) it is difficult to delineate experimentally a threshold between scarce and lacking expression, and using RT-PCR or qRT-PCR it is difficult to estimate the level of expression at the protein level; (3) the expression levels can be modulated by various factors, e.g., cytokines or PAMP themselves (see below); and (4) in whole organs with many different tissues, it is not clear whether this means a given TLR member is highly expressed in this organ at large, or whether a high-expressing tissue (e.g., blood cells) causes this result. Nevertheless, for quick information, an attempt to summarize what is known on TLR expression levels in domestic animals is presented in Table 1, which may be consulted for getting an impression of the most important organs. Most revealing are such data if antibodies are available, allowing the study of protein expression by individual cells rather than mRNA expression of the organ at large. Therefore, the method by which a result was obtained is also given in Table 1. Most work has been done on TLR2 and TLR4, as these are regarded as major PRR for gram-positive and -negative bacteria, respectively. As the TLR system is conserved, in mammals, one may, as a first estimate, extrapolate from one species to another. Looking at Table 1, it is surprising that both partners of heterodimers are not always coexpressed, i.e. that TLR2 may be expressed, but neither TLR1 nor TLR6.

TLR4, the best-studied TLR member, appears to be expressed in various species (pig, dog, cattle) by macrophages and epithelial cells of the normal lung, small intestine, liver, spleen, kidney, cornea, but not in the normal skin and in skeletal muscles (Wassef et al. 2004; Tirumurugaan et al. 2010). For details, the reader is referred to Turin and Riva (2008).

The laboratory of Baljit Singh succeeded in assessing expression of TLR2, 4 and 9 in the unstimulated horse lung (Singh Suri et al. 2006; Schneberger et al. 2009). Moreover, they had antibodies crossreacting with equine TLR4. That TLR2 is expressed in pulmonary vascular macrophages exclusively is based on circumstantial evidence. Also, for TLR4. the level of expression was higher in PIM than in other lung cells.

Quite recently, evidence for expression of TLR2, 4 and 9 was reported for a porcine cell line (IPEC-J2) (Burkey et al. 2009). Another study showed that porcine TLR2 is expressed by intestinal M cells (Tohno et al. 2005b), and that TLR9 is also expressed in swine Peyer's patches (Tohno et al. 2006). These authors had access to TLR2-specific and TLR9-specific polyclonal antibodies (Tohno et al. 2005b; Shimosato et al. 2005).

Modulation of TLR expression and activity

Under modulation, we understand up- or down-regulation of TLR expression and function. There are several ways how to modulate TLR expression: (1) specific modulation by TLR agonists; (2) specific modulation of other immune-related parameters as a result of TLR triggering; (3) non-specific modulation of TLR expression by any compound, or by the underlying disease; and (4) induction of regulatory T cells (T_{reg}) (Nyirendra et al. 2009). A word of warning is also appropriate here. It may be that the level of expression and the function are dissociated. Thus, we observed in bovine mononuclear cells expression of both TLR2 and TLR4, but the levels of expression were uninfluenced by IFN-γ, quite in contrast to their response to LPS (Jungi and Sauter, unpublished).

In the study reported on dogs with chronic enteropathies (Burgener et al. 2008), there is a general upregulation of TLR2 and TLR4. McMahon et al. (2010) also showed that, in inflammatory bowel disease of the dog, there is an up-regulation of TLR2 in the duodenum, and regarding up-regulation of TLR4, there was a definitive trend but levels of significance were not reached (McMahon et al. 2010). In the latter study, more mildly ill dogs were enrolled than in the former. In dogs with oligoarthritis and degenerative cranial cruciate ligament rupture, the expression of several genes involved in immune responses, among these TLR2 and 9, were found to be elevated on synovial cells (Muir et al. 2007), although there was wide variation. What is surprising in this study is that a comparison with healthy dogs was made, which have a very small volume of synovial fluid and a low number of synovial cells.

In the dog, both TLR2 and 4 appear to be expressed weakly by normal colonic epithelial cells, and they are dramatically up-regulated by prior treatment of the cells with the respective MAMP (Swerdlow et al. 2006). In cattle and horses, TLR2 and TLR4 were up-regulated after exposure to LPS or TLR2 ligands (Ibeagha-Awemu et al. 2008; Singh Suri et al. 2006). A study suggested that activation of TLR3, but not TLR4, leads to increased resistance to infection with porcine reproductive and

respiratory syndrome virus (PRRSV) (Akashi et al. 2000). In some cases, specific stimulation by a TLR agonist has a tolerizing effect. For example, stimulation of TLR5 by *Salnonella* flagellin results in tolerization of these and in blockade of NF-κB-mediated gene induction (Sun et al. 2007). Prolonged or repeated stimulation of TLR5 also leads to their refractoriness. Similarly, prior exposure of human epithelial cells to flagellin of *Pseudomonas aeruginosa* tolerized these in that a subsequent TLR5 stimulation by *P. aeruginosa* no longer had a deleterious effect, and the innate and potentially harmful response was weakened (Kumar et al. 2007). That TLR engagement may be counterproductive has been shown by Lang et al. (2005). It has been found that expression in the pancreas of an antigen to which all transgenic cytotoxic T cells are specific is not attacked (Ohashi et al. 1993), but when infected with the virus (Ohashi et al. 1993), when applying TNF (Ohashi et al. 1993) or when stimulating the TLR system (Lang et al. 2005), tolerance is broken, and autoimmune disease—in this case diabetes—results. Using porcine reproductive and respiratory syndrome virus (PRRSV), it was shown that infection with this virus led to a reduction of LTA-induced IL-1 production and LTA- or LPS-induced IL-6 production by macrophages (Chaung et al. 2008; Liu et al. 2009). Furthermore, infection of bovine alveolar macrophages with *M. bovis* may silence other TLR-related signaling pathways, and may induce some sort of cross-tolerance induction to other PAMP, similar to that described for other species (Piercy et al. 2007).

The expression of 9 TLR members was studied in various feline tissues with a view to investigating modulation by infection with feline immunodeficiency virus (FIV) (Ignacio et al. 2005). A complex pattern of up- or down-regulation induced by FIV for all members of the TLR family was obtained.

Considering that there are roughly 10 domestic animal species, about 10 TLR members, 20 or so tissues to be analyzed, and many potentially modulatory treatments, one grasps immediately that the information available on modulation is sketchy, at best. But diseases are species-specific. It makes sense that one has studied preferentially modulation of TLR expression by disease and their therapy.

TLR and diseases

When the Bern laboratory started working on TLR of domestic animals, there was the theoretical concept of TLR as PRR (Werling and Jungi 2003). But there was no recognized disease associated with a dysregulation of TLR members or TLR signaling. This has dramatically changed. We now know that modulating either TLR expression or TLR-mediated function may alter both innate and adaptive immune reaction. This knowledge can be utilized not only in vaccination protocols to optimize immunostimulatory effects of antigens but also in other situation, e.g., in the control of allergies.

Allergic diseases are governed by a Th2 response, and triggering of TLR favors a Th1 response, thereby inhibiting allergic reactions and other Th2-mediated responses (see review of Gangloff and Guenounou 2003). Although it may not be possible to identify a defunct TLR member or signalling compound, a tolerization of the TLR system occurs, e.g., due to previous stimulation of the TLR system. Alternatively, TLR stimulation may lead to the induction of T_{reg} and dampens an adaptive immune response. Examples of this kind are numerous and are summarized by Nyirendra et al. (2009).

TLR polymorphisms

Here, domestic animals might provide some interesting clues as pathogens cause, in most cases, a species-specific health problem, and it is the coevolution of host and pathogen that shaped TLR sequences, particularly the extracellular, leucine-rich repeats (LRR). The cases reported are not as clearcut as in the case of the C3H/HeJ mouse which contains a missense mutation, rendering the response to LPS an all-or-none question (Poltorak et al. 1998). Nevertheless, cases of an inheritable trait favoring certain diseases have been reported, and we can still learn from non-synonymous single nucleotide polymorphism (NSSNP), although the pathogen(s) shaping TLR LRR is/are most often unknown. The proportion of NSSNP gives us some information as to whether a sequence is genetically stable, or whether evolution drives the amino acid sequence away from the original one. Most often, NSSNP have been listed without association of a given disease.

Ruminants

In genes of many ruminants, including TLR members and TLR-adapter proteins, NSSNP have been identified. At least 54 NSSNP in 11 bovine innate immune genes [TLR1–10, peptidoglycan recognition receptor number 1 (PGLYRP1)] have been identified for 37 cattle breeds, with an average polymorphic density of one alteration per 219 base pairs (Seabury et al. 2010). Interestingly, however, *Bos taurus taurus* and *Bos taurus indicus* breeds appear to have haplotype sharing at every locus. However, the actual number of NSSNP might be considerably higher (Jann et al. 2009), and may also indicate selective pressure in ruminant TLR compared to other mammalian TLR in the region necessary for ligand binding/heterodimerization (Werling et al. 2009). Whereas a direct link of such TLR-

NSSNP in ruminants to a specific disease might be hard to obtain, a recent comarative genomic approach identified eight genes as potentially causative genes for variations of health-related traits (Jann et al. 2009). These include susceptibility to clinical mastitis in dairy cattle, general disease resistance in sheep, cattle, human and mice, and tolerance to protozoan infection in cattle and mice. Four TLR-related genes (TLR1, 6, MyD88, IRF3) appear to be the most likely candidate genes underlying quantitative trait loci (QTL) which control the resistance to the same or similar pathogens in several species (Jann et al. 2008).

Fine examples of an association between infectious disease and mutations have been reported by Bhide, Mucha and collaborators. Novel NSSNP of cattle TLR were reported, some of which were found to be associated with facilitated infection with *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease (Mucha et al. 2009). In sheep, there was also an association between novel NSSNP in TLR1 and TLR2 and the severity of infection with *Mycobacterium avium* subsp. *paratuberculosis* (Bhide et al. 2009).

Pig

In the pig, 21, 11, 7 and 13 NSSNP were found in TLR 2, 4, 5 and 6, respectively, many of which are in the extracellular LRR (Shinkai et al. 2006b). The biased distribution suggests that these NSSNP are the result of coevolution of host and certain unknown pathogens, Despite intensive breeding, heterozygosity with regard to TLR is apparently an advantage. In another porcine study, TLR1, 6 and 10 genes have been completely (re)-sequenced (Shinkai et al. 2006a). An analysis of the cytoplasmatic portion (Fig. 1) suggested that the signal transduction pathway of TLR10 is different from that of the closely related TLR1 and 6 (Shinkai et al. 2006a). This is surprising, as in the mouse and in human, TLR1, 2, 6, and 10 all express a TIR domain interacting with MyD88 and are thought to mediate NF-κB activation. In the latter study, six polymorphic microsatellite markers within the genetic regions of TLR1, 6 and 10 were developed as they might be useful in association studies of TLR variants and resistance to disease. According to a more recent study, in the genes coding for porcine TLR1, 2 and 6, a total of 20, 26 and 27 NSSNP were found, respectively (Bergman et al. 2010).

Horse

In the horse, a total of 13 SNP were found in TLR3, 7 and 8 (Astakhova et al. 2009). Allelic frequencies were determined in 154 horses belonging to 5 different breeds. A comparison with TLR3, 7 and 8 from other mammals revealed several conserved regions within the variable LRR. This is in line with an earlier study specifically looking at TLR4 and showing a restricted degree of polymorphism in horse TLR4 (Vychodilova-Krenkova et al. 2005).

Concluding remarks

A lot of information has been generated from the study of either the murine or the human TLR system, since these species appear to be the most interesting and the most accessible. The tacit assumption that the TLR system in all higher vertebrates is alike roots in two facts. Firstly, not many tools exist for the study of the TLR system of domestic animal species. Although working in principle in several species, the use of the knockout technology, which was essential for progress, favors to study the TLR system in mice. Humans are more interesting than domestic animal species for researchers and their sponsors. Secondly, given the fact that the TLR system is locked by both MAMP recognition and interaction with signaling molecules, which have been conserved, this system could not deviate a lot in evolutionary terms, which provides a rationale for comparing chickens, fishes and mammals in order to learn more about evolutionary constraints. Nevertheless, since recognition by the TLR system is associated with adaptive immunity, by learning more about the TLR system in domestic animals, one might learn more about adjuvants in vaccines, as the TLR system provides optimal immunostimulation. In other words, if it is understood how to stimulate maximally the TLR system may be the key to immunize optimally. Therefore, to study domestic animal species with regard to the TLR system might not only tell us about the constraints imposed by pathogens of a given species; it also might provide an optimal vaccine regime in the species studied.

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