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REVIEW

Toll-like receptors in immune response to the viral infections

I. MIKULA Jr.[1,2*], S. PASTOREKOVÁ[3], I. MIKULA Sr.[2]

[1]Laboratory of Biomedical Microbiology and Immunology, University of Veterinary Medicine and Pharmacy, Komenského 73, Košice, Slovak Republic; [2]Institute of Neuroimmunology, Slovak Academy of Sciences, 842 45 Bratislava, Slovak Republic; [3]Institute of Virology, Slovak Academy of Sciences, 842 45 Bratislava, Slovak Republic

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Summary. – Toll-like receptors (TLRs) are members of the innate immunity system. They are responsible for the recognition of various antigens and take part in the modulation of immunity responses. In general, they are divided into "bacterial" and "viral" TLRs, even though this classification overlaps in some cases. Genetic similarity of TLRs gives them the status of highly conservative proteins throughout the animal kingdom**.** However, there is a certain level of variation between different species that can result in semi-disparate recognition ability. Furthermore, their universal signaling pathways predispose them not only as a target for vaccination trials in humans, but also for the genetic selection in veterinary medicine. Moreover, the selection pressure and their conservative properties make them a suitable system for the evolutionary studies, since each separate genetic system has its own unique ortholog/paralog. TLRs 2, 3, 4, 7, 8, and 9 play a crucial role in the recognition and modulation of the innate immunity in response to the viral infection due to their predominant localization on the white blood cells and endothelial cells, while intracellularly localized TLRs lead the way.

Keywords: TLR; virus; mutation; diversity; immunity; substitution

Contents:

*Corresponding author. E-mail: mvdr_ivanmikula@yahoo.com; fax: +421051-2700094.

Abbreviations: cDCs = conventional dendritic cells; CpG = cytidine-phosphate-guanosine; DCs = dendritic cells; ECD = extracellular domain; HSV-1, 2 = herpes simplex virus type 1 and 2; IFN = interferon; IL = interleukin; IRF 1, 7 = IFN regulatory factor 1 and 7; LBP = LPS-binding protein; LPS = lipopolysaccharide; LRR = leucine-rich repeat; NOD = nucleotide-binding oligomerization domain; ODNs = oligodeoxynucleotides; PAMP(s) = pathogen-associated molecular pattern(s); pDCs = plasmacytoid dendritic cells; RIG = retinoic acid-inducible gene I; TIR = Toll/IL-1 receptor; TLR(s) = Toll-like receptor(s); TM = transmembrane

1. Immune system and infection

Several distinct immunity recognition systems have evolved in the multicellular organisms in response to the infection challenge. These systems can be broadly categorized in the vertebrates as innate and adaptive immune systems. The first one was described by Elie Metchnikoff over a century ago, but the significance of his research was largely overshadowed by fascinating intricacies of the adaptive immunity.

A "quantitative outcome" of the specific immunity stimulation is production of antibodies through the repertoire of randomly generalized and highly diverse receptors expressed by T and B cells. A "qualitative fitness" is the direct result of clonal selection and expansion of the receptors with the relevant specificity and generation of the immunological memory. However, two major limitations appear in these defense mechanisms: (i) randomly generated antigen receptors are unable to recognize origin of the presenting antigen and (ii) clonal expansion and differentiation into the effector cells delays considerably the immunity response (Medzhitov, 2001).

A solution for these limitations is the nonspecific immunity system that is capable to destroy foreign antigens prior to the antibody production. Based on the evolutionary conservative patterns present only/mostly in the microorganisms, the nonspecific immunity is able to detect and recognize the nature of an antigen and in this way to direct the immunity response. Receptors that are responsible for the recognition of these pathogen-associated molecular patterns (PAMPs) are denoted as TLRs and are responsible for bypassing of the specific and nonspecific immunity system.

1.1 Immune system and viral infection

Since the viruses are intracellular parasites composed mainly of nucleic acid and supplementary protein complexes, their interaction with the cell and subsequent invasion into intracellular compartments is necessary for the replication initiation. Diverse mechanisms are participating in the defense against viral infections, where quick and proper recognition of the invading agent is necessary to overcome the rapidly replicating viral progeny. One of the most rapid mechanisms specifying organism response to the viral antigens is the production of interferons (IFNs) type I. At the point of IFN type I recognition, the cells modify their metabolism to lower their susceptibility to the viral infection and upregulate the expression of MHC I and II molecules to increase the presentation of viral peptides to T cells (Stark *et al.*, 1998; Bonjardim *et al.*, 2009). The production of "second wave" cytokines as IFN-γ, interleukin (IL)-6, IL-12, or IL-18 already possesses an immunomodulatory properties that stimulate the differentiation of Th naive cells in the direction of cell immunity (Th1 cells) (Akira *et al.*, 2001; Trinchieri, 2003). Upon ligand recognition, TLRs stimulate a strong production of a wide variety of cytokines (Vaidya and Cheng, 2003). The type of the produced cytokine depends on the recognized pattern and TLR by which it is recognized and in this way they play a major role in the process of immunostimulation and immunomodulation.

Even though TLRs were initially found to recognize bacterial molecular patterns, Kurt-Jones *et al.* (2000) introduced a viral recognition to the TLR family defense mechanisms demonstrating that TLR4 was responsible for the response to respiratory syncytial virus. Importance of the defense mechanisms depending on the particular TLR was proven besides TLR4 and the respiratory syncytial virus also for TLR3 and West Nile virus, TLR7/8 and influenza virus and human immunodeficiency virus 1, or TLR9 and mouse cytomegalovirus and herpes simplex virus type 1, 2 (HSV-1, 2) (Kurt-Jones *et al.*, 2000; Haynes *et al.*, 2001; Lund *et al.*, 2003; Diebold *et al.*, 2004; Heil *et al.*, 2004; Krug *et al.*, 2004a, b; Wang *et al.*, 2004). However, throughout the millennia, various viral infections developed specific strategies to overcome this recognition complex. So far, 3 different ways are presented for such an interaction: (i) TLR-mediated immunosuppression, (ii) prevention of recognition by TLR, and (iii) TLR-mediated induction of the viral replication (Netea *et al.*, 2004). Mechanisms of TLR interactions with microorganisms and the resulting expression of cytokines are further complicated by the interspecies differences of TLRs and prevalent pathogens that are present within the particular species. Many distinct features are introduced for these separate systems with certain amount of similarities connected mostly to the evolutionary closeness. Hence, we should avoid generalization of the concluding results.

2. Innate immune recognition

Receptors of the innate immune system that recognize PAMPs, e.g. TLRs are called pattern-recognition receptors or pathogen pattern receptors (Janeway, 1989; Krejsek and Kopecký, 2004). Denotation PAMP is derived from the fact that certain gene products/patterns are present strictly in the microorganisms and are connected with their pathogenesis. PAMPs are usually coupled with "housekeeping" functions and are not able to be eliminated by pathogens. Some of these patterns are specific for the bacterial cells and distinct from the eukaryotic cells and can be "easily" recognized by the TLRs such as lipopolysaccharides (LPS), lipoproteins, peptidoglycans, and lipoteichoic acid.

Some other PAMPs though represent a fairly greater challenge. These are mostly PAMPs associated with the viral recognition, because motifs like viral DNA or RNA are to some extent similar to those present in the eukaryotic cells (Janeway, 1989, 1992; Pichlmair and Reis e Sousa, 2007; Bow-

ie and Unterholzner, 2008). However, nucleic acid PAMPs clearly have a feature that allows the distinction of potential pathogen from the host (Janeway, 1989). These features can be for example: (i) modifications of nucleic acids used in the replication cycle like the presence of 5'-triphosphate on many single-stranded RNAs of viral origin, which is absent in the cellular mRNA that bears a 5'-methylguanosine cap, (ii) heavily modified structural RNAs by conversion of uridine to pseudouridine or (iii) structural elements prevalent in viral RNAs, e.g. 5'-ends of influenza genomic RNA segment or untranslated portions of the hepatitis C virus genome (Alberts *et al.*, 2002; Hornung *et al.*, 2006; Knipe and Howley, 2007; Saito *et al.*, 2007). Moreover, quantitative differences in the prevalence of certain patterns, e.g. high abundance of unmodified uridine in the viral genome or its abnormal localization seem to aid in the distinction as well (Pichlmair and Reis e Sousa, 2007).

There is also another view on the innate immune system recognition. Quote: "The sensors that are responsible for innate immune recognition detect neither "patterns" nor "danger", but molecules." According to this view, there are no PAMPs or "danger signals". It is just convenient to lump many disparate molecules together by single phrase (Beutler *et al.*, 2004). However, a single phrase definition helps us to refer to these molecules with generalized approach but with the specific view.

3. TLRs

The name "toll", German slang for "fantastic", was given in reference to the retrieval of both dorsal and ventral alleles of protein (Rich, 2005). The Toll protein was originally identified in fruit flies (*Drosophila melanogaster*) and was involved in dorsoventral polarity during the embryonic development, what means that the gene and protein gradients established in the mother's cell trigger a signal, which is transmitted by Toll into the embryo (Lemaitre *et al.*, 1996; Rich, 2005). Further studies have shown that the Toll protein plays an essential role in the mounting of an effective immune response against the fungus *Aspergillus fumigatus* (Lemaitre *et al.*, 1996). These studies led subsequently to the identification of Toll homologues in humans and mice by means of the database search, which are referred to as TLRs (Medzhitov *et al.*, 1997).

3.1 Evolution of TLRs

Evolutionary conservative group of the immune receptors identified in fruit flies and mammals set up the motion for comparative evolutionary genomic investigation (Rich, 2005). Analyzing the diverse character of TLRs between these groups and within, we can now understand the necessity of different TLRs affinities present in various species and the selective pressure conditions forming this diversity. Moreover, gradual adaptation process to the external stimuli recognition enhanced by the continuous development of pathogenic microorganisms, can result in TLR orthologs and paralogs transformation beyond the recognition in the future (Rich, 2005). The highest mutation rate is associated with extracellular domain, which is a direct result of its involvement in PAMP recognition (Hajjar *et al.*, 2002; Zhou *et al.*, 2007) and therefore, it plays a major role in the defense mechanisms. By contrast, the intracellular Toll/IL-1 receptor (TIR) domain, responsible for the activation of signaling cascade is highly conserved between different TLRs and species (Werling *et al.*, 2009) with only mild pressure on its development, because of the relatively stable adaptor molecules. The conservative nature of TIR domain is further supported by the fact that genome searches have uncovered its orthologs not only within the animals, but also across the plant kingdom (Rich, 2005).

3.2 Structure of TLRs

The TLRs are type I transmembrane glycoproteins that consist of an extracellular domain (ECD), a transmembrane (TM) domain and a cytoplasmic TIR domain (Fig. 1) (Akira and Takeda, 2004; Gay and Gangloff, 2007).

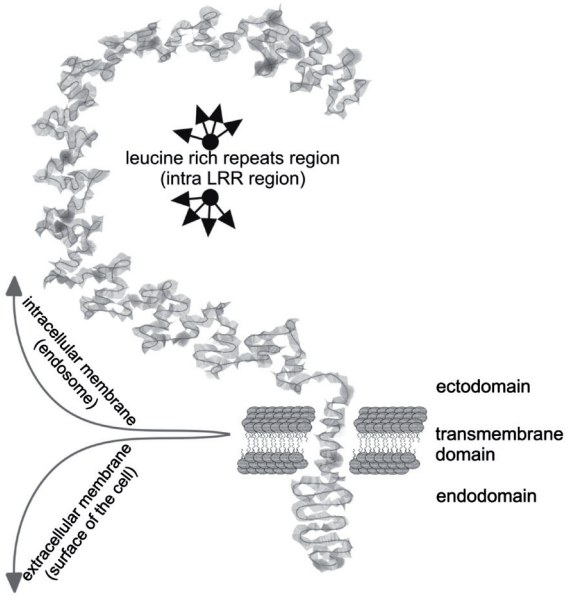

Fig. 1

TLR structure

LRR – leucine-rich repeat.

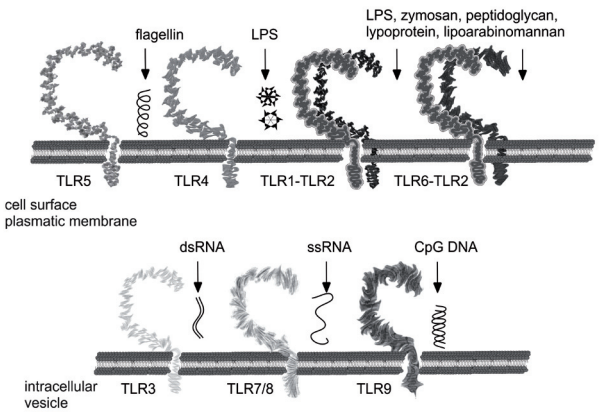

Fig. 2

Ligand specificities of TLRs

TLR – Toll-like receptor; LPS – lipopolysaccharide; CpG – cytidine-phosphate-guanosine.

3.2.1 Extracellular domain (ectodomain)

The extracellular component of TLRs is characterized by the presence of a leucine-rich repeat (LRR) motif and is responsible for the binding to PAMPs. The LRR domain of TLRs consists of 16–28 tandem repeats of the LRR motif (Matsushima *et al.*, 2007) that sculpt the structure of ECD. It is involved in the recognition of ligands such as proteins (e.g. flagellin and porin from bacteria), sugars (e.g. zymozan from fungi), lipids (LPS, lipid A, and lipoteichoic acid from bacteria), cytidine-phosphate-guanosine (CpG)-containing DNA from bacteria and viruses and viral RNA and DNA, derivatives of protein or peptide (lipoprotein and lipopeptides from various pathogens), derivatives of lipid (lipoarabinomannan from mycobacteria) and a complex derivative of proteins or peptides, sugars, and lipids (diacyl lipopeptides from mycoplasma) (Fig. 2) (Akira *et al.*, 2006).

Considering the structural pattern and the sequence of LRR motifs, proteins of LRR family can be classified into seven subfamilies: typical, RI-like, cysteine-containing, plant specific, SDS22+ protein-like, bacterial, and *Treponema pallidum* LRR, with TLRs belonging to the typical subfamily (Ohyanagi and Matsushima, 1997). The successful utilization of the domains containing LRRs is demonstrated by its presence in cca 4,700 proteins (Matsushima *et al.*, 2005).

LRR motifs form a loop structure and the juxtaposition of several loops produces a solenoid-like shape of ECD. The LRR consensus motif forms the inner core of horseshoe shaped ECD, while extra LRR region forms the convex surface (Bell *et al.*, 2003). Besides, LRR domains in all known structures adopt an arc shape (Matsushima *et al.*, 2005). Tandem arrays of LRR motifs span the entire ECD interrupted only by internal cysteine-rich regions (Rich, 2005). Moreover, LRR blocks are often flanked by the cysteine-rich domains called LRR-NT and LRR-CT located at the N- and C-terminal ends of LRR regions, respectively (Buchanan and Gay, 1996; Matsushima *et al.*, 2005). These crucial capping structures bury the otherwise exposed hydrophobic residues at the end of LRR superhelices of extracellular proteins and ECDs and engage in the disulfide bridges of TLRs (Rich, 2005). The number and position of the present disulfide bridges plays a crucial role in the correct folding of a protein, what is vital for its function.

The defining feature of the LRR repeat is a highly conserved 11-residue hallmark sequence LxxLxLxxNxL. Any amino acid can be substituted in the position of 'x', while consensus leucine position 'L' can be substituted only by hydrophobic residues as isoleucine, valine or phenylalanine (Matsushima *et al.*, 2007). Each repeat is a structural unit, rolled up as a spiral in which the conserved residues are stacked. The hydrophobic consensus residues form the core of the protein and make intra- and inter-repeat interactions. The asparagine residue at position 9 has a central role in the LRR structure and is therefore highly conserved. Side chains of the asparagines present at the position 9 of respective LRRs form a hydrogen bond with the peptide backbone from the previous repeat and within the same repeat (Yoder and Jurnak, 1995).

In other words, asparagines in the motif create continuous hydrogen bonds with backbone carbonyls of neighboring strands throughout the entire protein and the resulting structure is referred to as 'asparagine ladder', which is crucial for the stabilization of turn. Similar to the position 'L', the conserved asparagine position 'N' can be replaced by other residues such as cysteine, threonine, or serine that are capable of hydrogen bond formation. The variable 'x' residues in the LRR motifs are hydrophilic and are exposed to the concave surfaces of the horseshoe-like structure. They are not involved in the structural stabilization and can be replaced by other hydrophilic residues without deterioration of the protein stability. Therefore, within each LRR repeat, the conserved residues provide a rigid structural framework, whereas variable residues are available for the interaction with ligands. These interactions are further supported by insertions into the highly conserved 11 amino acid segment (Kajava *et al.*, 1995; Kobe and Deisenhofer, 1995; Bell *et al.*, 2003; Werling *et al.*, 2009).

The production and crystallization of some LRRs including those from TLRs have proven to be extremely difficult. Only recently developed LRR hybrid technique has enabled to overcome this difficulty and allowed the first TLR crystal structure namely TLR3 to be assembled (Choe *et al.*, 2005; Jin and Lee, 2008). However, it did lack the bound ligand. Using this technique, three TLR-ligand structure complexes have been recently published allowing insights into the ligand-recognition and receptor dimerization (Jin *et al.*,

2007; Kim *et al.*, 2007; Liu *et al.*, 2008). The knowledge founded on these anatomic models can now be used for the prediction and modeling of the structure of different TLRs (Werling *et al.*, 2009). Moreover, it could be used for the prediction of altered protein structure caused by mutations and hence, their possible effect on the PAMP binding and recognition ability.

3.2.2 Transmembrane domain

The main function of TM domain is anchoring TLRs in the plasmatic membrane. Depending on the type of the TLR, it also possesses other functions, such as the localization of TLRs within the cell or its interaction with the accessory molecules within the ER (Nishiya *et al.*, 2005; Brinkmann *et al.*, 2007). The intracellular localization of TLR7 and TLR9 is defined by TM domain while, for example, TLR3 is directed by a cytosolic linker region that is connecting TM and TIR domain (Nishiya *et al.*, 2005; Kajita *et al.*, 2006). Since TLR8 has TM domain that is similar to that of TLR7 and TLR9, it might also be targeted to an intracellular compartment by TM domain (Nishiya and DeFranco, 2004; Nishiya *et al.*, 2005). However, it seems that there are some interspecies differences that determine their localization *in vivo pro rata temporis*.

It was proven using the chimeric TLRs that the TM domains of TLR3 and TLR9 are responsible for the binding to a functional polytopic ER-resident membrane protein denoted as UNC93B, which is necessary for the proper TLR signaling (Brinkmann *et al.*, 2007). Furthermore, as stated for the bovine TLR8, multiple regions including ECD, TM domain, TIR domain are involved in the determination of its intracellular localization within the ER before and after cell activation (Zhu *et al.*, 2009). It is clear that except for the already known information there are more functions that need to be elucidated for the different TLRs and different species.

3.2.3 Intracellular domain (endodomain)

Ligand-induced dimerization of TLRs is believed to trigger recruitment of the adaptor proteins to their intracellular TIR domain to initiate the signaling (O'Neill and Bowie, 2007). This domain was first time described in the human IL-1 and *Drosophila* Toll receptor (Gay and Keith, 1991). Since that time, it has been reported in a broad range of organisms, e.g. plants, insects, or *Xenopus* (Rich, 2005). It has also been reported in the bacteria and viruses, where they may act as decoys that allow them to evade a host defense (Bowie *et al.*, 2000; Harte *et al.*, 2003).

According to the associated functional domains, three distinct subgroups of TIR domain superfamily exist. The first group comprises the TIR domains that are linked to three immunoglobulin-like ECDs (Vigers *et al.*, 1994). The second group is composed of the TIR domains linked to the extra and intracellular LRRs, and the third sub-group is comprised principally of the cytosolic adaptor molecules with solo TIRs or TIRs linked to other functional domains. Their function involves mediating protein-protein interactions and signal regulation, where TLRs belong (Rich, 2005).

Regardless of the way of TLR stimulation, the outcome of TIR domain activation is always the activation of the nuclear transcription factor (NFkB, IRF), which induces the expression of cytokines after the translocation to the cell core.

3.3 Localization of TLRs

Since all TLRs are typical type I TM proteins, it was initially assumed that all TLRs would be expressed on the cell surface (Nishiya *et al.*, 2005). However, studies using chimeric receptor, fluorescein-labeled TLRs, and anti-TLR antibodies have indicated that TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface. In contrast, TLR3, TLR7, and TLR9 are completely localized in the intracellular acidic compartments (Fig. 3) (Zhang *et al.*, 2002; Nishiya and DeFranco, 2004). Nevertheless, the intracellular localization of these TLRs is not associated with all cells. TLR3 has been shown to be present on the surface or in the cytoplasm of dendritic cells (DCs), macrophages, epithelial cells, fibroblasts and NK cells (Cario and Podolsky, 2000; Muzio *et al.*, 2000; Visintin *et al.*, 2001; Matsumoto *et al.*, 2002; Matsumoto *et al.*, 2003; Schmidt *et al.*, 2004; Sha *et al.*, 2004). Similarly, TLR8 appears to be localized primarily

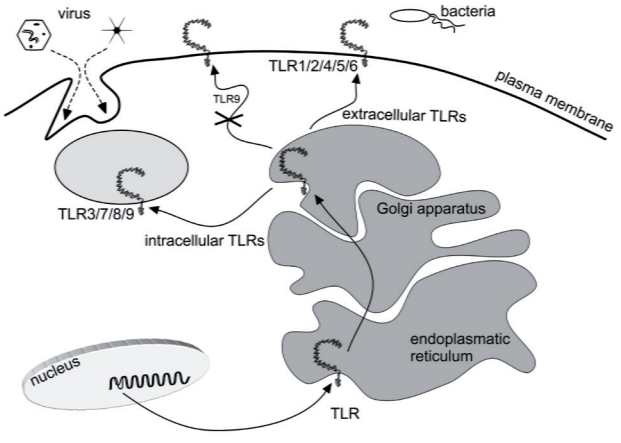

Fig. 3

Trafficking and processing of TLRs

For explanations see Fig. 2.

intracellularly, but a small fraction is present on the cell surface (Nishiya and DeFranco, 2004).

As stated above, TLRs are driven to the intracellular compartments by different protein regions as TM domain or cytosolic linker region. The reason for this may be the specific trafficking regulation, which diverts the particular TLRs to the endosomal compartments depending on the cell type or species. TLR3 and TLR7 are co-localized within the endosome in bone marrow-derived macrophages (Nishiya et al., 2005) as are TLR3 and TLR9 (Kajita *et al.*, 2006), since particular TLR combinations (homo- or hetero-dimers) are necessary for the signaling activation and/or potential signal depression for various antigens.

TLRs present within the ER seem to be in the inactive form. They are delivered to the endosomes after the uptake of virus particles, since the TLR-ligand interaction seems to be a prerequisite for receptor activation (Nishiya *et al.*, 2005; Barton and Kagan, 2009).

This means that the process of TLR activation follows several steps. Upon viral infection, phagocytes such as macrophages and DCs cells take up virus-infected cells in their intracellular compartments, such as phagosomes and activate the digestion process by the action of lysosomal enzymes (Nishiya *et al.*, 2005). During this time, TLRs are delivered to these compartments to engage in the TLR-ligand interaction (Barton and Kagan, 2009) and in this way enable the recognition process, which is indispensable for the mounting of anti-viral innate immune responses. Moreover, the strength of cytokine production seems to be coupled with the time of PAMP exposure to the TLR. It was proven that retention of CpG motif in the endosomes where it interacts with TLR9 is responsible for the induction of a robust IFN production (Honda *et al.*, 2005).

3.4 Interactions of TLRs

TLRs assemble as heteromeric or homomeric complexes and these combinations define the microbial repertoire that can be recognized (Ozinsky *et al.*, 2000). It means that the combination of more than one TLR type is required for the recognition of some ligands. For example, *in vitro* studies using co-transfection of the different TLRs into the cells that normally do not express TLRs have confirmed that heterotypic interactions between TLR1 x TLR2, TLR2 x TLR6, and TLR4 x TLR6 are required for the recognition and activation of signaling in response to the triacyl lipopetides, diacyl lipopeptides and other bacterial cell wall components (Ozinsky *et al.*, 2000; Akira *et al.*, 2001; Hajjar *et al.*, 2001; Takeuchi *et al.*, 2002; Sandor *et al.*, 2003; Latz *et al.*, 2004).

However, TLR x TLR heterotypic interactions are not always activating. The heterotypic TLR1 x TLR4 interaction was shown to be inhibitory to the TLR4 signaling in response to LPS (Spitzer *et al.*, 2002). Functionally active TLR8 or TLR9 in humans also plays a secondary role in the inhibition (regulation) of TLR functions. It was demonstrated that TLR8 has inhibitory effect on TLR7 and TLR9, while TLR9 has inhibitory effect on TLR7, *ex vivo*. The significance of these inhibitory effects in the regulation of the innate immune system response to a given PAMP is not clear so far. Nevertheless, the indirect evidence suggests that the inhibitory interactions among these three TLRs may contribute to the regulation of inflammation process (Wang *et al.*, 2006).

It appears that there are a few factors that play a major role in facilitating either homo- or heterotypic interactions among the evolutionary closely related TLR1, TLR2, TLR4, and TLR6. These involve a spatial distribution of TLRs on the plasma membrane, amino acid sequence homology based on the evolutionary closeness of TLRs, the biochemical nature of the PAMPs and the origin of the PAMPs (Beutler and Rehli, 2002; O'Neill, 2004; Wang *et al.*, 2006). Other highly homolog TLR group that consists of TLR7, TLR8, and TLR9 and is present in the same intracellular compartment in certain combinations (Chuang and Ulevitch, 2000; Du *et al.*, 2000; Latz *et al.*, 2004; O'Neill, 2004) may also involve a similar facilitating factors used during their distribution and interactions.

3.4.1 Interactions of TLRs with other proteins

The extent of the pathogens that are sensed by TLRs depends also on the TLR accessory molecules, which play a variety of roles in the regulation of TLR function (Akashi-Takamura and Miyake, 2008; Kaisho and Wagner, 2008). Most TLRs, such as TLR2 require no further adaptor protein interaction to recognize a PAMP. By contrast, TLR4 recognizes LPS after its interaction with MD2-binding protein or LBP (LPS-binding protein) before forming a heterodimer with CD14. Even though the role of these accessory molecules is not yet fully understood, they might enhance the elimination of the Gram negative bacteria by adjusting the response of TLR4 (Werling *et al.*, 2009).

Up to now, three major interactions seem to be rendered in response to LPS presence. First one consists of MD2 protein, which seems to function as an opsonin for Gram negative bacteria, since the formation of LPS-MD2 complex results in the increased phagocytosis (Jain *et al.*, 2008). Second one concerns a diverse LPS chemotypes recognition through the interaction of CD14 and TLR4, which seems to nullify their distinct features and result in the stimulation of alternative signaling pathways (Jiang *et al.*, 2005). Third one utilizes a soluble acute-phase LPS-binding protein that binds to LPS, whereupon, interaction of this complex with CD14 and/or TLR4 results in the immune response induction (Weiss, 2003) (Fig. 4).

There are also other molecules that were "tailored" with TLR signaling as PRATA4 or gp96. Although they seem to function as chaperones in the trafficking of some TLRs within ER, their interactions need to be further explored (Akashi-Takamura and Miyake, 2008). However, we could divide these molecules into two groups, where the first group comprises regulatory molecules for the cell surface TLRs (e.g. MD2) and the second group resides in the ER with TLRs trafficking function (e.g. PRATA4, gp96) (Akashi-Takamura and Miyake, 2008).

3.5 Expression of TLRs

As mentioned before, the inhibitory effect of TLRs within the same compartment seems to regulate the mounting level of immune responses and antigen-dependent proliferation. These reciprocal interactions can be explained on the model of TLR7 and TLR9 that are expressed mostly on the plasmacytoid dendritic cells (pDCs) and B cells in humans (Applequist *et al.*, 2002; Hornung *et al.*, 2002; Ito *et al.*, 2002; Edwards *et al.*, 2003). After a proper stimulation both receptors are inducing the production of IFNs type I in pDCs and the proliferation of B cells (Krieg and Yi, 2000; Viglianti *et al.*, 2003; Gorden *et al.*, 2005; Ito *et al.*, 2005; Lau *et al.*, 2005).

Even though the outcome of physical interaction of TLR7 and TLR9 is inhibitory for TLR7, it is still capable of active signaling that leads to the IFN type I production in pDCs or proliferation of B cells. To explain this situation, two hypotheses can be applied: (i) both these cells may express an excess level of TLR7 to overcome the inhibitory effect of TLR9, (ii) the inhibitory effect of TLR9 on TLR7 is more gradual as a function of increasing levels of TLR9 protein (dose-dependent inhibition) and it is possible that the relative expression of these TLRs is optimized to overcome the inhibitory effects of TLR9 (Wang *et al.*, 2006).

On the other hand, naive B cells express very low but comparable levels of both TLR7 and TLR9, but they proliferate in response to the TLR9 agonists only (Bernasconi *et al.*, 2003; Bekeredjian-Ding *et al.*, 2005). This appears to be consistent with the inhibitory effect of TLR9 on TLR7, but not *vice versa* (Wang *et al.*, 2006). IFN α selectively upregulates TLR7 in naive B cells, which results in the proliferation of these cells in response to TLR7 agonists. However, the TLR9-dependent proliferation activity remains unchanged, what suggests that the inhibition of TLR7 is TLR9 dose-dependent (Bekeredjian-Ding *et al.*, 2005; Wang *et al.*, 2006). Thus, it seems that there is a need for a yet more cautious regulation of TLR7 ligand recognition in comparison to the TLR9 and possibly TLR8.

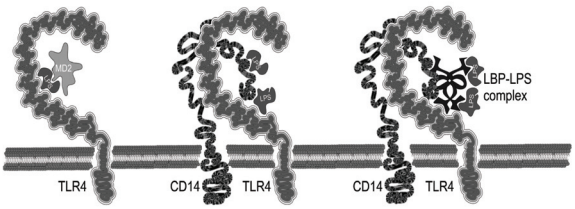

Fig. 4

Alternative ways of TLR4 ligand recognition

TLR – Toll-like receptor; LPS – lipopolysaccharide; LBP – LPS-binding protein; CD14 –cluster of differentiation 14 (co-receptor for LPS recognition).

3.6 Species differences in TLRs

The classification and the characterization of TLRs**,** as they are evaluated in different studies**,** are often specific for the mammal systems**,** usually humans and mice. However, there is a fairly high amount of variations between different animal classes including closely related organisms that are connected to the PAMP recognition.

In mammalian system, specific heterodimers like TLR2/1 and TLR2/6 are responsible for the detection of the molecules like tri-acylated peptide Pam_3CSK_4 and the di-acylated peptide FSL-1, respectively (Farhat *et al.*, 2008). On the contrary, the combination of bovine TLR2 and bovine TLR1 or chicken TLR2 type 2 and chicken TLR16 are responsible for the recognition of these respective complexes (Keestra *et al.*, 2007; Higuchi *et al.*, 2008). It remains to be seen whether these distinctions are based on the structural differences or differences in surface charge distribution in the ECDs as was identified between ECDs of human and bovine TLR2 and between human, bovine, and ovine TLR9 (Mikula Jr. and Mikula Sr., 2011; Werling *et al.*, 2009).

Moreover, the comparative analysis of TLR5 recognition ability to flagellin of *Salmonella enterica* serovar Typhimurium between humans, mice, and chickens showed certain species specificity, where mice constantly yielded the strongest response to this antigen followed by chickens and humans (Andersen-Nissen *et al.*, 2007). Nevertheless, this reactivity was not observed with flagellin of *Salmonella enterica* serovar Enteritidis when comparing these species (Keestra *et al.*, 2008). These findings show a different susceptibility to the infection based on the flagellin sensing qualities by TLR5 in different organisms that can be based already on a single amino acid substitution in the bacterial flagellin (Werling *et al.*, 2009). Based on these facts and further supported by dissimilar susceptibility of sheep breeds to maedi visna infection (Hunter and Munro, 1983; Cutlip *et al.*, 1986; Sharp *et al.*, 1986) we could assume a similar presence of a "breed typical" amino acid composition, localized at ECD of maedi

visna sensing TLR that determines its recognition ability (Mikula Jr. and Mikula Sr., 2011).

Considering the group of endosomaly localized TLRs as TLR3, 7, 8, and 9, only a few studies have tried to identify species-specific ligands, but expression studies using chicken TLR3 and chicken TLR7 in mammalian cells revealed their response to poly (I:C), but not to the mammalian TLR7 ligand R848 (Schwarz *et al.*, 2007). Furthermore, experiments showed that the preferential recognition for TLR8 in humans is the single-stranded RNA derived from human immunodeficiency virus 1 and R-848, whereas TLR8-deficient mice responded normally to these molecules, which suggested a species-specific function (Heil *et al.*, 2004). Nowadays, we know that although both TLR7 and TLR8 are expressed in mice, TLR8 seems to be inactive or nonfunctional within this system (Chi and Flavell, 2008).

Similarly, different DNA motifs are required for the optimal stimulation of mouse and human immune cells by CpG oligodeoxynucleotides (ODNs). These ONDs are largely classified into three groups: (i) A/D type – ODNs that induce secretion of type I IFN by pDCs, but they have low ability to induce B cell activation and IL-12 production, (ii) B/K type – ODNs that stimulate B cell activation and IL-12 production, but the induction of IFN type I is poor, and (iii) C type – ODNs that possess the properties of the first two classes, so it can activate B cells and induce IFN type I production (Kawai and Akira, 2008).

CpG ODNs are recognized by TLR9 and different variants of ONDs have been used to determine the TLR9 ligand specificity in a variety of species (Mutwiri *et al.*, 2003). Analysis of these reactions confirmed that the GTCGTT motif, which has an "optimal" activity in humans is also active in the various domestic species, whereas GACGTT motif displaying the highest activity in the murine system shows little or no activity in any of these domestic species. Within ruminants, ODN 2007 works only in the ovine system, whereas ODN 2216 stimulates only the bovine peripheral blood mononuclear cells (Werling *et al.*, 2009). Furthermore, both ODN 2216 and ODN 2007 induced CpG-specific non-MHC-restricted cytotoxicity with the ovine but not bovine peripheral blood mononuclear cells. Even more interestingly, there was not a single assay in which the peripheral blood mononuclear cells from sheep or cattle responded at a detectable level in these studies (Mena *et al.*, 2003). These facts suggest that TLR9 from different species recognizes a species-specific "optimal" CpG ODN (Mena *et al.*, 2003). Interestingly, such marked differences in CpG-ODN induced innate responses exist between and within two closely related species (Werling *et al.*, 2009). Moreover, there is direct and indirect evidence that suggests a dissimilar recognition ability of certain TLRs between breeds, taking in account the impact of breeding programs, different environmental conditions, reported differences between breed susceptibilities or various substitutions found throughout the different breeds (Mikula *et al.*, 2010; Mikula Jr. and Mikula Sr., 2011; Hunter and Munro, 1983; Cutlip *et al.*, 1986; Sharp *et al.*, 1986; Werling *et al.*, 2009). Thus, we can conclude that various breeds possess a breed-specific recognition ability of PAMPs and hence, every breed is characterized by a "typical breed resistance" based on its gene pool/TLR genes as a result of the mentioned conditions.

3.7 Characterization of TLRs 3, 7, 8, and 9

TLR3 was originally identified as a receptor for double-stranded RNA on the basis of its ability to mediate the response to poly(I:C) and purified double-stranded RNA, but recently it has been found to be responsive also to the poly(I) (Alexopoulou *et al.*, 2001; Matsumoto *et al.*, 2002; Marshall-Clarke *et al.*, 2007). With the help of TLR3, antigen-presenting cells are capable of the recognition of double-stranded RNA present within a phagocytosed virus-infected cell (Schulz *et al.*, 2005).

Murine TLR7 and human TLR8 recognize synthetic antiviral imidazoquinolines, e.g. R848 (resiquimod) or Immiquimod, certain guanine nucleotide analogs e.g. loxoribine, and uridine-rich or uridine/guanosine-rich single-stranded RNA of both viral and host origin (Diebold *et al.*, 2004; Lund *et al.*, 2004). Moreover, TLR7 recognizes certain synthetic poly(U) RNA and certain siRNAs (Diebold *et al.*, 2004; Hornung *et al.*, 2005). TLR7 mediates the response toward the single-stranded RNA in viruses such as influenza, vesicular stomatitis virus, and Sendai virus (Diebold *et al.*, 2004; Lund *et al.*, 2004).

TLR9 was originally identified to recognize unmethylated CpG DNA motifs, which are frequently present in the bacteria, but not in vertebrates and to recognize also the viral DNA (Hemmi *et al.*, 2000; Lund *et al.*, 2003). In response to these PAMPs, TLR9 mount the response to the viruses e.g. adenovirus, HSV-1, 2 or mouse cytomegalovirus (Lund *et al.*, 2003; Krug *et al.*, 2004a, b).

3.7.1 Recognition of PAMPs by DCs in diverse systems

DCs are professional antigen-presenting cells that link innate and acquired immune responses. On the other hand, B cells perform the role of antigen-presenting cells and antibody producing cells that finally develop into the memory cells. TLR7 and TLR9 (but not TLR3) are selectively expressed by these cells (Colonna *et al.*, 2004; Liu, 2005). TLR9 is highly expressed within the DCs and B cells; however, a different pattern of expression exists between human and mouse cells. In mice, TLR9 is broadly expressed by the different subsets of DCs, while in humans it is predominantly

expressed by pDCs. Activation of TLR9 in pDCs leads to a high production of IFNs type I, especially IFNα (Ishii and Akira, 2006). On the contrary, mouse conventional dendritic cells (cDCs) produce IFNβ, but not IFNα in response to the TLR9 stimulation (Negishi *et al.*, 2006; Schmitz *et al.*, 2007). This means that pDCs link TLR9 activation to IRF7 (interferon regulatory factor 7), whereas cDCs link the same receptor to IRF1 (Hoshino *et al.*, 2006). Moreover, even though pathways in pDCs lead preferentially to the IFN production, these pathways are not operative in other cell types like cDCs or macrophages in humans. TLR9 (or TLR7) stimulation leads to the production of cytokines such as IL-6 or IL-12 (Pichlmair and Reis e Sousa, 2007). However, TLR3 is expressed more widely including non-hematopoetic cells with preferential expression in non-plasmacytoid cDCs (Iwasaki and Medzhitov, 2004; Reis e Sousa, 2004).

The viruses are likely to be taken up by endocytosis to the endosomal compartments, where they are subsequently degraded allowing viral nucleic acids to contact TLRs (Kawai and Akira, 2008). Replication of the enveloped viruses is not necessary for the induction of IFN type I response by pDCs (Kawai and Akira, 2008). On the contrary, recent report has demonstrated that TLR7 senses vesicular stomatitis virus that enters the cytoplasm (Lee *et al.*, 2007). This process is independent of the active replication and hence, it allows the recognition of viruses that do not normally replicate in pDCs (Pichlmair and Reis e Sousa, 2007). This is particularly relevant during infections when viruses neutralized by antibody or complement can be taken up via Fc or complement receptors, what leads to the TLR stimulation (Wang *et al.*, 2007).

3.8 Importance of TLRs

There is a question as to what extent is TLR3 redundant for the protection. The importance of TLR3 was determined in an experiment with knock-out mice that did not show any increased susceptibility to the infection with vesicular stomatitis virus, lymphocytic choriomeningitis virus or reovirus (Edelmann *et al.*, 2004; Johansson *et al.*, 2007). Paradoxically, the presence of TLR3 was contraindicated for West Nile virus infection. While TLR3 -/- mice were fairly resistant to infection with its lethal doses, in TLR3 +/+ mice it lead to the leakiness of the blood-brain barrier as a result of inflammatory response to the virus recognized by TLR3, allowing the infection to enter the brain (Wang *et al.*, 2004). Similarly, lack of TLR3 promotes increased survival of mice with acute pneumonia induced by the influenza virus despite higher virus titers in the lungs (Le Goffic *et al.*, 2006).

The role of TLR9 in mouse cytomegalovirus infection is in the promotion of cytokine production, what leads to the activation of NK cells (Krug *et al.*, 2004a). TLR9 is also important for the protection against intravaginal HSV-2 infection, where it mediates an early production of IFNs by locally recruited pDCs and thereby limiting the virus spread (Lund *et al.*, 2006). However, it is not required for the protection against HSV-1 infection in experimental footpad inoculation or corneal scarification model (Krug *et al.*, 2004b). Thus, the importance of the TLR-dependent immune response depends on the type of the pathogen and its ability to subvert or exploit these pathways for its own benefit.

The importance of innate/TLR recognition pathway can be determined in the population naturally exposed to viruses (Pichlmair and Reis e Sousa, 2007). So far, patients lacking IL-1 receptor-associated kinase 4 have been found to possess a normal resistance to the viruses even though they cannot couple TLR7 or TLR9 necessary for the IFNs induction (Yang *et al.*, 2005). Thus, the TLR pathway might be dispensable for the response to many viruses in human population.

Anyway, it should be reminded that viruses have developed various escape mechanisms to invade the organism, such as NS3-4A protein of hepatitis C virus that can cleave both IPS-1 and TRIF and thus antagonize both retinoic acid-inducible gene I (RIG)-like receptor (RLR) and TLR3 (Foy *et al.*, 2005; Li *et al.*, 2005; Meylan *et al.*, 2005). Several other viruses belonging to the different classes have been reported to interfere with the activation of IRF3 suggesting either direct interaction with IRFs or inhibition of upstream kinases (Weber *et al.*, 2004). This suggests that even though viruses developed certain ways to suppress the immune recognition by particular receptors, there are other recognition pathways that are capable to partially/fully supplement the anti-viral response. However, the extent of these abilities needs to be further analyzed.

There are other TLRs besides TLR3, TLR7, TLR8, and TLR9 that have also been suggested to play a role in sensing the viruses such as TLR2 and TLR4. Their recognition involves surface proteins and is often restricted to few virus isolates (Sato *et al.*, 2006). TLR2 responds to the components of measles, hepatitis C virus, mouse cytomegalovirus, and HSV-1 (Bieback *et al.*, 2002; Duesberg *et al.*, 2002; Compton *et al.*, 2003; Kurt-Jones *et al.*, 2004). TLR4 can respond to the respiratory syncytial virus, retroviruses, and coxsackie B virus (Kurt-Jones *et al.*, 2000; Rassa *et al.*, 2002; Richer *et al.*, 2006). Surface proteins often mutate to escape the adaptive immune mechanism (Hangartner *et al.*, 2006). However, measles virus seems to preserve a certain form of hemagglutinin allowing to target TLR2 that upon the activation upregulates expression of the virus entry protein (Bieback *et al.*, 2002). Similarly, the analysis of the mouse mammary tumor virus infection in TLR4 deficient mice indicates that the virus targets TLR4 to replicate in B cells and to suppress CD8+ cytotoxic T-lymphocytes response (Rassa *et al.*, 2002; Jude *et al.*, 2003). This means that the effective TLR-mediated viral elimination depends also on the level of virus exploitability. Here belongs e.g. the function of

TLR as a viral entry mediator, TLR-mediated immunosuppression or TLR-mediated immunomodulation toward the "false pathogen type".

3.9 Mutations of Toll and TLRs

The first observation of the connection between mutation and changed phenotype was accidental, when mice of C3H/Hej strain were hypo-responsive to the lethal dose of LPS (Heppner and Weiss, 1965). However, this finding was fully understood many decades later, when all cellular responses to the LPS including the adjuvant effect of LPS on adaptive immune responses were impaired by a single mutation affecting a locus called *Lps* (Watson and Riblet, 1974; Skidmore *et al.*, 1975; Skidmore *et al.*, 1976; Skidmore *et al.*, 1977). The failure to sense LPS was associated with markedly enhanced susceptibility to the infection by Gram negative bacteria (Rosenstreich *et al.*, 1982; Hagberg *et al.*, 1984). The *Lps* locus was characterized by positional cloning, although at that time it was already known as TLR4 (Poltorak *et al.*, 1998a; Poltorak *et al.*, 1998b; Beutler *et al.*, 2004).

The first identification of mammalian TLR was based on the homology searches and TLR was thought to be active in the developmental functions (Nomura *et al.*, 1994; Taguchi *et al.*, 1996). Besides the developmental role, a mutation within Toll was responsible for hyper-susceptibility to the infection by fungal pathogens and Gram positive bacteria, what influenced the further studies of TLRs (Lemaitre *et al.*, 1996; Rutschmann *et al.*, 2002).

Mutations of receptor protein may alter its function and in this way to pervert the proper immune response. Many phenotypes created by mutagenesis that were identified diminished their proper function: (i) dominant phenotype PanR1, in which TNF production in response to all microbial inducers is markedly diminished, (ii) Achtung and Achtung2 phenotypes in mice that show an evident susceptibility to the spontaneous infection, (iii) Flake phenotype caused by mutation responsible for altered immunity to the Gram positive bacteria (Beutler *et al.*, 2004), and more.

Mutations in the TLRs may cause impaired pathogen recognition and limit the innate immune activation (Hawn *et al.*, 2007; Henckaerts *et al.*, 2007). Up to now, studies have shown the association between the mutations in TLR genes and host susceptibility to the diseases like tuberculosis, paratuberculosis, hepatitis caused by hepatitis C virus, AIDS, leprosy, urinary tract infections, and disease conditions like periodontitis, acute rheumatic fever, Crohn's disease, staphylococcal infection (Lorenz *et al.*, 2000; Bochud *et al.*, 2003; Berdeli *et al.*, 2005; Schroder and Schumann, 2005; Fukusaki *et al.*, 2007; Hong *et al.*, 2009; Tabel *et al.*, 2007; Thuong *et al.*, 2007; Oh *et al.*, 2008, 2009; Schott *et al.*, 2008; Mucha *et al.*, 2009) and many others.

3.10 Distribution of TLRs in the animal kingdom

TLRs have been found to be present in animals ranging from cnidarians to the mammals, though they appear to be absent in platyhelminthes (Zheng *et al.*, 2005). For example, *D. melanogaster* possesses 9 TLRs and *Anopheles gambiae* encodes 10 TLRs. At least one TLR was found in the insect's genome within the phylum *Arthropoda*, one TLR gene has been found in horseshoe crab *Tachypleus* and *Homarus americanus* and at least one TLR gene is present in *Euprymna*, a Hawaiian squid that belongs to the phylum *Mollusca* (Inamori *et al.*, 2004; Zheng *et al.*, 2005). Similarly, one TLR is encoded by each – *Caenorhabditis elegans* and *Caenorhabditis briggsea* (Zheng *et al.,* 2005). However, *Hydra magnipapillata* has at least 3 TLR genes. Interestingly, according to the functional study of TLR gene (CeTol-1) in *Caenorhabditis elegans*, it has no function in the innate immunity (Pujol *et al.*, 2001).

3.10.1 Selective pressure

TLR phylogeny is a result of species-specific co-evolution caused by the gene conversion between receptors that can form a heterodimer or gene duplication possibly followed by the deletions (Kruithof *et al.*, 2007; Temperley *et al.*, 2008). The process of protein evolution is further shaped by the selective pressure to the sites of functional relevance (Werling *et al.*, 2009). TLRs are constantly under the strong selection of both maintenance and adaptation of function (Roach *et al.*, 2005), what is reflected by the presence of numerous substitutions found therein. The maintenance process of the protein and the adaptation of protein function (due to the pathogen pressure) can be executed against occurring disadvantageous mutations by the purifying selection resulting in a high conservation of these sites or domains, or by the positive selection of advantageous mutations, respectively (Hughes *et al.*, 1990; White *et al.*, 2003). As we know, a quicker reproduction cycle allows for better adaptation of proteins through both positive and negative selection, what is important especially for the immune defense. Considering this view, animals with a shorter life spawn and increased reproduction represents an entity with superior/opportunistic defense mechanism.

As mentioned before, TIR domain is very conservative throughout the animal kingdom. By contrast, ECD of TLRs tends to possess a fair amount of substitutions depending on the species and TLR type. This fact is given by its involvement in PAMP recognition with different surface charge of ECD for various species (Kubarenko *et al.*, 2007; Walsh *et al.*, 2008; Werling *et al.*, 2009). Furthermore, the results of various polymorphism studies suggest a higher presence of substitutions in the ECDs of TLRs expressed on the surface of cells compared to the TLRs localized intracellularly (Mikula

et al., 2010; White *et al.*, 2003; Cargill and Womack, 2007; Seabury *et al.*, 2007; Jann *et al.*, 2008). This is possibly caused by the fact that PAMPs (as DNA or RNA) recognized by intracellular TLRs manifest mainly as a "functional property" of pathogens, in contrast to those that recognize PAMPs designated more as a "structural property" of pathogens.

Polymorphism in bovine TLR2 occurs rather between different breeds as between individuals within the same breed, possibly because of a different geographic and microbial environment (Werling *et al.*, 2009). However, polymorphism of ovine TLR9 of Tsigai sheep breed shows a presence of high amount of synonymous substitutions within the same breed, while differing by non-synonymous substitutions between the breeds (Mikula Jr. and Mikula Sr., 2011). Thus, extensive polymorphism studies of different breeds of various species could provide us with a valuable benchmark data, which would help us to further understand the potential effect of a genetic variation and the selective adaptation pressure factors. Moreover, these results can facilitate identification of the particular disease susceptibility in animals and provide a valuable tool for the breeding industry (Werling *et al.*, 2009).

4 Conclusions

Opening "Pandora box" called the non-specific immunity system brought us not only different TLRs and their intricacies, but recent studies confirmed the various recognition systems that co-exist within the different cells like RIG-like receptors that recognize viruses and NOD-like receptors recognizing bacteria (Creagh and O'Neill, 2006). However, TLRs are preferentially used by the cells of immunity system especially by the antigen-presenting cells that connect innate and adaptive immunity. Even though TLRs found within different species possess high similarity features, a big variation of responses throughout the recognition and synthesis of cytokines suggests a selective pathogen pressure on the particular systems. The mutations trigger many disease conditions and alter a predisposition to the many infections (Bochud *et al.*, 2003; Schroder and Schumann, 2005; Fukusaki *et al.*, 2007; Hong *et al.*, 2007; Tabel *et al.*, 2007; Thuong *et al.*, 2007; Mucha *et al.*, 2009). Screening for the presence of mutations is crucial for the human medicine especially for the assessment of familial genetic disorders. However, even though various studies already pinpoint several mutations that cause negative alteration of immune response toward different infections in TLRs within animals, caution is advised, since different breeds of some species could possess an alternative mutation hallmarks connected with the same infection. Furthermore, both "viral" and "bacterial" TLRs should be carefully analyzed for the possible positive selection pressure "changes" due to the rapid development and adaptation ability of the pathogens.

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