# EXPRESSION OF TOLL LIKE RECEPTOR 2

# GENE IN RABBIT

**BY**

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**DEPARTMENT OF ANIMAL SCIENCE**
**FACULTY OF AGRICULTURE**
**UNIVERSITY OF BENIN**
**BENIN CITY**

**JANUARY, 2023**

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**A PROJECT SUBMITTED TO THE DEPARTMENT OF ANIMAL SCIENCE, FACULTY OF AGRICULTURE, UNIVERSITY OF BENIN, BENIN CITY**

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**JANUARY, 2023**

## CERTIFICATION

This is to certify that Emmanuel Ikechukwu MADUMERE from the Department of Animal Science carried out this experiment under my supervision and is hereby approved.

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| **Mr. G.O BELLO** | **PROF J.A. IMASUEN** |
| (Supervisor) | (Head of Department) |
| _____ | _____ |
| **DATE** | **DATE** |

## DEDICATION

This work is dedicated to Almighty God for his love and care and enablement towards me, to my parents and siblings for their support, love and encouragement.

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I want to firstly thank God almighty for his grace and strength throughout the period of my project, this process wouldn't be possible without him. I want to say thanks to Prof. A. M. Orheruata for support and role during this process, you were really patient, understanding and a motivation to me. I want to say a special thanks to Mr. G.O. Bello for his time, effort, support and concern he showed, I am truly grateful Sir. I want to say thanks to Mr. P. Aduba for his advice and support.

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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 inborn host defense is preserved in animals. Both adaptive and innate immune systems are inter-related in that the adaptive immune system also depends on an intact innate recognition and response system. This study was carried out at University of Benin farm project, to investigate the expression of toll like receptor 2 gene in rabbit. A total of 40 adult rabbits, 5 months and above of both sex (male and female) consisting of 3 breeds (Hyla, New Zealand and Dutch) were used for this experiment. The animals were fed concentrate, forage and a mixture of concentrates and forage. Routine management was carried out. Blood samples were collected and sent to Inqaba Biotechnology West Africa Ltd. Ibadan, Oyo State for DNA extraction and identification of RNA expression. Results are being awaited.

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background of the study

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 pathogens. 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). Toll-like receptor 2 also known as TLR2 is a protein that in humans is encoded by the TLR2 gene. TLR2 has also been designated as CD282 (cluster of differentiation 282). TLR2 is one of the toll-like receptors and plays a role in the immune system. TLR2 is a membrane protein, a receptor, which is expressed on the surface of certain cells and recognizes foreign substances and passes on appropriate signals to the cells of the immune system.

Toll-like receptors (TLRs) are the members of Pattern Recognition Receptors (PRR) superfamily (Medzhitov and Janeway, 1997; Akira and Takeda, 2004),

which detect invading pathogens by virtue of a series of conserved molecular structure known as pathogens associated molecular patterns (PAMPs).TLRs were firstly found in drosophila and characterized by a transmembrane region followed by a cytoplasmic Toll/Interleukin-1 receptor (IL-1R) homology (TIR) domain and N-terminal leucine-rich repeats (LRRs) (Takeuchi and Akira, 2010), and they are vital for innate immunoresponse (Takeda *et al.,* 2003). It has been known that the TLR family includes 13 genes in mammals, of which 10 have been identified in pig, cattle and human (Chang *et al.,* 2006; Werling and Coffey, 2007; Chuang and Ulevitch, 2001; McGuire *et al.,* 2006) and 12 in mouse (u) and TLR1-like, 2, 3, 4, 5, 6, 8 and10 in rabbit (Kajikawa *et al.,* 2005; Astakhova *et al.,* 2009; Abrantes *et al.,* 2013). MoreoverTLR2, 5,6and10werefoundonthemembrane of cellular surface, while TLR3 and 8 are located in cytoplasm (Ohnishi *et al.,* 2003; Schumann and Tapping, 2007). Various TLRs recognize the different PAMPs. Specifically, triacyl lipopeptides can be recognized by TLR1 or 2. Peptidoglycan, lipoarabinomannan, hemagglutinin, phospholipomannan, glycosylphosphophatidyl inositol and mucin can be recognized by TLR2. Lipopolysaccharide from Gram-negative bacteria, mannan, glycoinositolphos0810 275 8250holipids and envelop protein can be recognized by TLR4. Flagellin from bacteria can be recognized by TLR5. Diacyl lipopeptide, lipoteichoic acid and zymosan can be recognized byTLR2or6. The TLRs on cell surface mainly recognize the component from bacteria, mycobacteria, candida,

trypanosome, saccharomyces or streptococcus. Intracellular TLR 3, 7 and 8 can recognize ssRNA or dsRNA from virus and dsDNA from virus, CpG motifs from bacteria and plasmodium can be recognized by TLR 9 (Tirumurugaan *et al.,* 2010).

Therefore, TLRs are vital for innate immunity. TLRs are expressed predominantly in antigen processing and presentation cells such as macrophages, neutrophils and dendritic cells, but not restricted to these cell types. TLRs expressions have been detected in a wide range of tissue and their distribution of mammalian (Vahanan *et al.,* 2008), and their expression levels have been associated with altered immune responsiveness (Menzies *et al.,* 2006). For example, chicken TLRs have revealed that there was characteristic pattern of expression for each TLRs (Zhang *et al.,* 2012; Zarember and Godowski, 2002; Kogut *et al.,* 2005). TLR2, 3, 4 and 9 are expressed differently in cervix, placenta and uterus of mice (Gonzalez et al. 2007). The expression pattern of TLRs in various cells, tissues or organs have been determined In goat (Tirumurugaan *et al.,* 2010), water buffalo (Vahanan *et al.,* 2008), mice (Pruett *et al.,* 2004; Kurt-Jones *et al.,* 2004; Kokkinopoulos *et al.,* 2005) human (Zarember and Godowski, 2002; Hornung *et al.,* 2002), sheep and bovine (Menzies and Ingham, 2006). However, the studies on the expression profiles of TLRs of female rabbit still scarce, and the expression patterns of TLRs in different organs of female rabbit also have not been reported. Lipopolysaccharide (LPS) a cell coat component of Gram-negative bacteria, can

be recognized by TLR2 and 4. The opening reproductive system of female rabbit was easy to suffer from Gram-negative bacteria infection, such as Pasteurella, Brucella and Salmonella (Abrahams *et al.,* 2004). Despite the crucial function of innate immunity mediate by TLR2 and 4 for host resisting Gram-negative bacteria, little known about the expression of TLR2 and 4 in different organs of female rabbit, and whether LPS affected the expression of TLR2 and TLR4 in genital tract of female rabbit.

Therefore, the aim of the present study was to determine the expression of TLR 2 using RT-PCR method, and analyze the expression pattern of TLR 2 of rabbit by qRT-PCR.

## 1.2 Justification of the study

The application of selection for resistance to disease in livestock is in their exposure to pathogens. Traditional animal breeding alone cannot guarantee animal's immunity. Therefore, identification of genetic markers for disease resistance is necessary in identifying animals with potentials for disease resistance in farms. This study addresses the knowledge gap in the use of genetic markers to select rabbits that are resistant to bacteria using the expression of Toll like receptor 2 as a tool.

## 1.3 Aims and objectives

The aim of this work was to study the expression of toll like receptor 2 in rabbits raised in the University of Benin Teaching and Research Farm

## 1.4 Objective of the study

**The specific objectives are:**

1. to extract RNA from rabbit blood samples;
2. to determine the expression of TLR2 in rabbits using RT-PCR method;

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Toll-like Receptor 2

Toll like receptor 2 (TLR2) identification, molecular characterization, and cloning were first published in 1998, together with TLR1, TLR3, TLR4, and TLR5 (Rock et al., 1998). More than a decade of extensive research has demonstrated the importance of TLR2 in the vertebrate immunity. This receptor is the only TLR described so far to form functional heterodimers with more than two other types of TLRs. TLR2 also interacts with a large number of non-TLR molecules, allowing for recognition of a great number and variety of PAMPs (Zähringer et al., 2008). This diversity comprises different types of molecules from all microbial phyla including viruses, fungus, bacteria, and parasites. TLR2 expression has been detected in immune cells, endothelial, and epithelial cells (Flo et al., 2001; Brzezi´nska-Blaszczyk and Wierzbicki, 2010). This ubiquity is consistent with the wide range of roles and function of TLR2. TLR2 recognises PAMPs which are specific to microbes (Akira and Takeda, 2004). TLR2 could identify mycobacterial lipoglycan and other bacterial cell wall macroamphiphiles (Ray *et al.,* 2013). As a result, it is reasonable to consider that TLR2 is a critical candidate gene for studies of resistance or susceptibility to bacterial infection in rabbits. It is well known that nucleotide change of the TLR genes may affect their ability to

recognize PAMPs (Uenishi and Shinkai, 2009). Previous studies have demonstrated that the TLR genes could recognise slight differences among PAMPs (Janeway and Medzhitov, 2002). It has been hypothesise that the sensitive recognition ability is due to polymorphisms in TLRs. Furthermore, many studies of TLR genes have revealed an association between polymorphisms in the TLR genes and diseasere resistance (Bochud *et al.,* 2007; He *et al.,* 2007; Abu-Amero *et al.,* 2013).

Polymorphisms in the human TLR2 gene protect against malaria (Greene *et al.,* 2012). The Arg753Gln mutation in the human TLR2 gene is associated with vitiligo susceptibility and urinary tract infection (Tabel *et al.,* 2007; Karaca *et al.,* 2013). A16934T polymorphism in the human TLR2 gene is associated with severity of atopic dermatitis (Potaczek *et al.,* 2011).

The recent studies have demonstrated that the TLR genes could recognize slight differences among PAMPs (Janeway Jr and Medzhitov, 2002). It is well known that nucleotide change of the TLR genes may affect their capability to recognize PAMPs. Many studies of TLR genes have revealed an association between polymorphisms in the TLR genes and disease (Bochud *et al.,* 2007; Hawn *et al.,* 2007; He *et al.,* 2007). For example, the Asp299Gly mutation in the human TLR4 gene increases risk of infection (Barber *et al.,* 2004). Mutation at Arg392stop in

the human TLR5 gene is associated with decreased risk of Crohn's disease (Gewirtz *et al.,* 2006).

*Oryctolagus cuniculus* TLR2 sequences are highly homologous to sequences from other mammalian species (Zhang *et al.,* 2003). Through comparative prediction of TLR2 protein domain architectures via SMART for *O. cuniculus, B. Taurus, O. aries, C. familiaris, M. musculus, H. sapiens, R. norvegicus* and *S. scrofa*, we revealed five clusters of LRR patterning that were conserved among all species investigated. Moreover, comparative protein domain analysis for TLR2 showed that C. familiaris and H. sapiens were the species for which low-complexity regions were not confidently predicted by SMART. Furthermore, predicted signal peptides and Toll-interleukin-1 resistance (TIR) domains for TLR2 of all species were examined. Notably, one predicted amino acid replacement (R315W) encoded by a nsSNP could regulate the presence or absence of a LRR protein domain for *O. cuniculus* during sequential SMART analyses. The LRR protein domain did not occur for the rabbit TLR2 reference sequence, which encoded R at amino acid position 315. The predicted protein domain architectures for TLR2 were similar for all species investigated, implying that TLR2 in mammals are similar in function (Zhang *et al.,* 2003). Zhang *et al.,* 2012, has also shown that there are some nonsynonymous SNPs and synonymous SNPs within the coding sequences of the rabbit TLR2 gene. The SNPs may be useful for marker-assisted

selection for disease-resistance breeding. By summing up the distribution of SNPs in the coding sequence of TLRs in pigs, cattle, rabbit, and humans, we found that nonsynonymous SNPs were mainly located in the sequences encoding ectodomains. A study of crystal structures of mouse TLR2 associated with lipopeptide demonstrated that LRR of TLR2 are direct binding regions for ligand recognition (Jin *et al.,* 2007). Two substitutes that change the charges on the amino acids were detected at bases 259 and 319 in the TLR2 gene. Moreover, polar changes of substitutions in amino acids were detected at bases 997 in TLR2. It is possible that these replacements in amino acids in TLR2, which change the amino acid characteristics, will change their extracellular pathogen recognition capabilities. Furthermore, amino acid replacements that induce a subtle change of the amino acid characteristics might influence on the resistance to diseases. For example, A nonsynonymous SNP at A1775G (amino acid N592S) in human TLR5 is significantly associated with resistance to Legionnaires'disease (Hawn *et al.,* 2003).

## 2.2 TLR2 ligands for pathogen recognition

Toll-like receptor receptors are type I integral transmembrane glycoproteins composed of a conserved intracellular toll–interleukin1 receptor (TIR) homology domain, a single transmembrane helix domain and a solenoid ectodomain (Kumar *et al.,* 2009). The ectodomain is responsible for pathogen recognition and is

composed of 16–28 diverse leucine-rich-repeat (LRR) modules (Akira *et al.,* 2006). TLRs can be divided into six major families, according to the repeat number of LRRs and the motifs of two cysteine clusters flanking the LRRs. TLR2, together with TLR1, TLR6, and TLR10, are members of the TLR1 subfamily (Matsushima *et al.,* 2007).

Ligand specific recognition and signaling through TLR2 occurs via heterodimerization with TLR1 or TLR6. TLR2/TLR1 and TLR2/TLR6 heterodimers are thought to be pre-formed on the cell surface. In the absence of TLR1 and TLR6, TLR2 homodimerization was proposed but it has not been observed with current techniques (Jin *et al.,* 2007). The crystal structure of both heterodimers where determined, showing that LRR modules confers a horse-shoe shaped structure to the ectodomain and each heterodimer forms an "m" shaped complex with the ligand, stabilizing the two receptors. Without the ligand, the pre-formed heterodimers most likely have no interaction between their intracellular moieties and therefore there is no downstream signaling (Jin *et al.,* 2007; Kang *et al.,* 2009). New studies also describe the existence of TLR2/TLR10 pre-formed dimers, although their function is unclear (Guan *et al.,* 2010). TLR1 and TLR10 were described to form dimers with TLRs from the same subfamily (Hasan *et al.,* 2005), while TLR6 can form a heterodimer with TLR4 in response to endogenous ligands, promoting sterile inflammation (Stewart *et al.,* 2010).

The wide array of TLR2 ligands includes molecules with diacyl and triacylglycerol moieties, proteins and polysaccharides.The biochemical diversity of such ligand structures consequently raises questions about receptor specificity. For a long time the identity of most putative TLR ligands was doubted, due to potential lipoprotein contamination. A recent study claims that only lipoproteins/lipopeptides (LPs) are "true" TLR2 ligands, sensed at physiological concentrations by this receptor (Zähringer *et al.,* 2008). To assess such potential contamination, treatment with lipoprotein lipase (Hashimoto *et al.,* 2006, 2007), high temperatures (Liang *et al.,* 2007a), and solvent washes (Ikeda *et al.,* 2008) have been used, according to the molecular structure and physicochemical properties of the hypothetical TLR2 ligand. However, some of these methods were applied in the absence of proper ligand molecular structure analysis, leading to mistaken conclusions. An example is the use of lipoprotein lipase to assess lipoprotein contamination of diacylglycerol preparations, such as Lipoteichoic Acid (LTA), which are also cleaved by the lipase (Seo and Nahm, 2009). Endogenous ligands have also been described for TLR2, known as "alarmins" and indicating tissue damage, necrosis, or potential tumor cells. For example, human β-defensin-3, hyaluronan fragments, heat shock proteins, and high mobility group box 1 protein were identified as TLR2 endogenous ligands (Scheibner *et al.,* 2006; Funderburg *et al.,* 2007; Curtin *et al.,* 2009; Erridge, 2010). Although it seems plausible that these molecules contribute to host responses to infections leading to

this type of damage,there is no evidence, thus far, that endogenous ligands that engage TLR2 can modulate the course of microbial infections. Another complicating factor regarding studies with these TLR2 “endogenous ligands”is that contamination of the preparations with other non-endogenous TLR2 ligands was not always ruled out (Tsan and Gao, 2007).

The major TLR2 ligand characterized thus far are lipoproteins, ubiquitous to all bacteria and highly expressed in the outer membrane of Gram-positive bacteria. They present a unique NH3-terminal lipo-amino acid, N-acyl-S-diacylglycerol cysteine and usually three lipid chains (triacyl), except for those found in mycobacteria that can have two lipid chains (diacyl). These molecules have a variety of functions and are described in an extensive database (Babu *et al.,* 2006). Triacyl LPs are recognized by TLR2/TLR1 while diacyl LPs are recognized by TLR2/TLR6 (Beutler *et al.,* 2006). This has been described in detail by crystallographic techniques, demonstrating that the TLR1 ectodomain has a channel that binds the amide-bound lipid chain of the triacylated LP (Jin *et al.,* 2007), while the same channel in TLR6 is obstructed by amino acid residues (Kang *et al.,* 2009). TLR2 has a hydrophobic pocket that interacts with the remaining lipid chains in a less specific manner, allowing slight variations in length and chemical structure of lipid or hydrophobic moieties of its ligands (Kang *et al.,* 2009). No matter which of the two dimers is activated, the classical

signaling cascade triggered was found to be the same (Farhat *et al.,* 2008), although the kinetics could be different depending on the ligands, and lead to different physiological outcomes (Depaolo *et al.,* 2008; Long *et al.,* 2009). TLR10 appears to recognize the same ligands as TLR1 and requires the same intracellular adaptor myeloid differentiation primary-response gene 88 (MyD88). However, the activated TLR2/TLR10 dimer could not trigger the common signaling cascade, suggesting a different role for this dimer yet to be defined (Guan *et al.,* 2010). Recognition of lipopeptides through TLR2 in the absence of TLR1 or TLR6 participation was observed using either TLR-knockout mice or modified ligands (Buwitt-Beckmann, 2005). However, there is no demonstration that ligand recognition by TLR2 alone actually occurs at physiological concentrations in the course of an infection (Buwitt-Beckmann, 2005).

## 2.3 TLR2 receptor complexes and accessory molecules

TLR2 forms heterodimers with its co-receptors, which increases the diversity of molecules recognized by the receptor. Recently, a number of accessory molecules and co-receptors have been described to concentrate microbial products on the cell surface or inside phagosomes to facilitate TLR2 responses. A new ligand complex was just proposed for activation by diacylglycerol ligands, including lipopeptides: CD36 may bind ligands and transfer them to the accessory molecule

CD14, which, in turn, loads the ligand onto TLR2/TLR6 heterodimers (FSL-1, MALP-2, and LTA) or on TLR2/TLR1 (lipomannan) heterodimers (Jimenez-Dalmaroni *et al.,* 2009).

The ligand delivery occurs within lipid rafts, where CD14 and CD36 molecules are anchored, resulting in complex internalization to the Golgi apparatus, and this trafficking may be dependent on the TLR2 ligand. Despite this finding, activation does appear to occur at the cell surface and is independent from internalization (Triantafilou *et al.,* 2006; Jimenez-Dalmaroni *et al.,* 2009). The ectodomain of CD14 and CD36 is the active receptor moiety for ligand delivery (Jimenez-Dalmaroni *et al.,* 2009). It is notable that, even though CD14 and CD36 are not an absolute requirement (Hoebe *et al.,* 2005; Nakata *et al.,* 2006) for TLR2 signaling, the role of these molecules is to enhance responses, lower the threshold of the concentrations needed for receptor recognition and signaling. A similar mechanism likely occurs with GPI anchors from some protozoan parasites, since they also contain the diacylglycerol moiety and are known to be TLR2 ligands. Accordingly, CD36−/− macrophages have an impaired cytokine response compared to wild-type macrophages when stimulated with Plasmodium falciparum GPI (Patel *et al.,* 2007). Trypanosoma cruzi GPI needs CD14 to fully activate TLR2 (Almeida and Gazzinelli, 2001). However, this function of CD14 cannot be extended to all GPIs, since CD14 does not participate in Toxoplasma

gondii GPI stimulation of TLR2 and TLR4. Instead, galectin-3 seems to deliver T. gondii GPIs for these TLRs (Debierre-Grockiego *et al.,* 2010).

Interestingly, vitronectin, an extracellular matrix glycoprotein also present in the blood, has been reported as essential for triacyl LP engagement of TLR2. This protein, in its extended conformation, binds to triacyl LPs and is recognized by the integrin β3 receptor, which is part of the pre-formed TLR2/TLR1 signaling complex in resting monocytes (Gerold *et al.,* 2008). CD14 (but not CD36) also concentrates and delivers triacyl LPs to

TLR2/TLR1, without directly binding to the dimer (Hoebe *et al.,* 2005; Nakata *et al.,* 2006), and can contribute to the inflammatory response in phagocytes (Drage *et al.,* 2009). Other researchers identified radioprotective 105 kDa (RP105) as a receptor able to bind mycobacterial lipoproteins, mostly TLR2/TLR1 agonists, acting as an accessory molecule for the TLR2 receptor complex in macrophages and improving the response against this pathogen.

RP105 has an ectodomain related to the TLRs, but no intracellular moiety (Blumenthal *et al.,* 2009). Further research is needed to define if the accessory mechanisms involved with triacyl LPs are complementary, non-concomitant, or overlapping.

So far, only the ganglioside GD1a has been shown to potentially have an accessory function in recognition of non-acetylated TLR2 ligands. It binds the β subunit of type IIb heat-labile enterotoxin of

Escherichia coli, enabling this ligand to induce TLR2/TLR1 signaling within lipid rafts. GD1a does not appear to interact with triacyl molecules (Liang *et al.,* 2007b). Since bacterial porins, which have been shown to be TLR2 ligands (Massari *et al.,* 2002; Liu *et al.,* 2008), are also oligomeric pore forming proteins that bind to the same dimer, there is a possibility that GD1a may also affect or enhance their signaling, but there is no experimental evidence supporting this hypothesis (Massari *et al.,* 2006).

In regards to innate immune recognition of whole bacteria by TLRs, phagocytosis is an important step, forming phagosomes that could recruit TLRs and form different receptor complexes. The soluble molecule mannose binding lectin (MBL) was found to bind Staphylococcus aureus through membrane LTA and to synergize with TLR2/6, drastically increasing inflammatory responses upon complex internalization (Ip *et al.,* 2008). The same complex is likely to occur with peptidoglycan, lipoarabinomannan, and lipophosphoglycan, since they were described to bind to MBL (Ip *et al.,* 2009). CD36 may possibly maintain its ligand delivery role inside the phagosomes, because it is required for phagocytosis of *S. aureus* (Stuart *et al.,* 2005). Lack of integrin α3β1 impairs release of IL-6 after

Borrelia burgdorferi phagocytosis, due to weak activation of endosomal TLR2 (Marre *et al.,* 2010). In addition, the β3 integrin was reported to facilitate host cell invasion by several bacterial pathogens and could also be linked to TLR2 triggering inside phagosomes (Gerold *et al.,* 2008).

The only non-TLR molecule found to physically interact with TLR2 and induce cross-talk signaling was Dectin-1, the main receptor for β-glucans found on most fungi. Dectin-1 dependent signaling synergizes with both TLR2 and TLR4 for induction of tumor necrosis factor-α (TNF-α) in human primary peripheral blood mononuclear cells (PBMCs), when all three receptors are engaged and stimulated via their respective ligands (Ferwerda *et al.,* 2008).

## 2.4 TLR2 intracellular signaling network

Following ligand stimulation, TLR2 heterodimers generally initiate a MyD88-dependent intracellular signaling pathway, common to all TLRs except TLR3. This pathway induces nuclear translocation of nuclear factor-B (NF-B) to modulate gene transcription and consequent inflammatory cytokine production.

The cascade also triggers serine/threonine-specific protein kinases (MAPKs) that can influence both transcription of inflammatory genes and mRNA stability of those transcripts, by activation protein 1 (AP-1) induction (Watters *et al.,* 2007). Although the general signaling pathway is not unique, the phosphorylation and

ubiquitination processes crucial for the cascade are not completely elucidated, especially the feedback loops. For example, IL-1 receptor associated kinase 1/4 (IRAK 1/4) were found to phosphorylate, ubiquitinate, and increase degradationof TIR domain containing adaptor protein (TIRAP also termed

MyD88 associated ligand, MAL), indicating a possible downregulation mechanism for TLR2 and TLR4 activation, in addition to their role in activating these pathways (Dunne *et al.,* 2010).

Sub-activating doses of TLR2 ligands may tolerize dendritic cells for TNF-α production, by a mechanism that involves disruption of signal transduction at the IRAK1 level (Albrecht *et al.,* 2008). Bruton's tyrosine kinase (Btk) and PI3 Kinase are likewise able to modulate TLR2 production of cytokines stimulated by LTA, but their place in the cascade is not clear (Liljeroos *et al.,* 2007).

Since TLRs, nucleotide binding oligomerization domains (NODs), and Dectin-1 can induce NF-B translocation and MAPK p38 activation (O'Neill, 2008), intracellular cross talk for cytokine production synergy is likely to occur. In this context, Dectin-1 can synergize with TLR2 and TLR4 through an NF-B non-canonical pathway and augment cytokine expression induced by the cited TLRs (Gringhuis *et al.,* 2009). Human PBMCs stimulated with heat killed Listeria monocytogenes (recognized through TLR2), plus a TLR7/8 ligand, demonstrated synergistic up-regulation of IFNγ production and, to a minor extent, IFN-α

production (Ghosh *et al.,* 2007). Although with a different TLR2 ligand (Pam3CSK4), the opposite outcome of IFN-α was reported with human dendritic cells. In this case, TLR2 activation restrained the type I IFN (IFN-α/β) amplification loop used by TLR4 and TLR7/8 ligands (Wenink *et al.,* 2009). The controversy can be partially explained by the fact that TLR2 depletes early IFN-α/β release induced by TLR9/TLR7, due to transient degradation of IRAK 1, but not later induction of type I IFN (Liu *et al.,* 2012). The urokinase-type plasminogen activator receptor was recently described as important for optimal MAPK p38 activation and consequent inflammation induced by lipopeptides recognized by TLR2 (Liu *et al.,* 2011). Mutations of the NOD2 gene results in defective release of Interleukin-10 (IL-10) from PBMC stimulated with TLR2 ligands, suggesting another avenue of cross-signaling (Netea *et al.,* 2004a).

Type I interferon (IFN-β/α) production was recently found to be induced through TLR2 engagement. It was then necessary to postulate an additional signaling pathway to account for this phenomenon, as type I IFN synthesis requires activation of IFN regulatory factors (IRF), but not NF-B or AP-1, traditionally seen with TLR2 signaling. The stimulation occurs in the endosomal environment, which requires microbial or ligand endocytosis. In vitro stimulation of phagocytes with vaccinia virus (VV) and Lactobacillus acidophilus induced IFN-β production, while LTA from S. aureus stimulated IFN-α production in murine macrophages

(Liljeroos *et al.,* 2008; Dietrich *et al.,* 2010; Weiss *et al.,* 2010). It is possible that L. acidophilus also stimulates IFN-α due to LTA presence. Surprisingly, bacteria but not viruses induced TLR2-dependent production of IFN-β in dendritic cells, suggesting cell specificity related to the type of microbe. Although not yet elucidated, the newly discovered pathway was described asMyD88-dependent and requires IRF1/7 for IFN-β and IRF1/2 for IFN-α release (Figure 1). Activators of transcription 1 (STAT1), STAT 3, PI3, and Btk were also reported as part of the signaling cascade required for IFN-α release induced by LTA. TRIF adapter is not required for TLR2 activation of interferon production, as it is for TLR4 and TLR3 (Barbalat *et al.,* 2009).

The intracellular cross-talk scenarios are essential for understanding host–microbe interactions, since most invaders have multiple molecules recognized by multiple innate immune receptors. Investigators have still not been able to relate most of what has been learned about intracellular regulation of pathogens with infection outcomes in the host, and this must be the focus for future studies.

**Infectious diseases and the role of TLR2**

In order to assess the rolefor TLR2 in immune protection or pathogenesis of infections, a number of techniques have been developed. The vast majority of researchers have used inbred mice as the in vivo model, limiting individual gene variability and allowing for examination of knockout or transgenic mice.

However, limiting genetic variability also limits immune response variability. In addition, another important consideration in interpreting results is the phenotypic divergence between mice and humans, including the level of TLR2 expression and which cells express TLR2. Comparison of murine and human TLR2 sequences has revealed higher correlation between intracellular domains (84%) than between extracellular domains (65%; Grabiec *et al.,* 2004), the latter being involved with ligand recognition. Regarding cellular expression, one example of the divergence between mice and humans is the fact that murine T lymphocytes express TLR2 in a constitutive manner while human T cells do not (Rehli, 2002). These discrepancies may contribute to the differential pathology and immune response observed in mouse models of infectious diseases as compared to humans. In addition, there is evidence of associations between human TLR2 polymorphisms with disease course and susceptibility, but other factors, such as ethnicity, location, environmental factors, and exposure to infectious agents contribute to the conflicting results regarding these associations (Corr and O'Neill, 2009).

Specific descriptions of TLR2 polymorphisms are included in the following sections. To our knowledge, it is unclear whether TLR1 or TLR6 can activate host immune response in the absence of TLR2 involvement. Nevertheless, we cannot assume that TLR1 or TLR6 polymorphisms that lead to alteration in receptor

function are impacting TLR2 recognition and activation. There is evidence that TLR1 and TLR6 polymorphism do exist in humans that affect responses to infectious diseases (especially mycobacteria; Kesh *et al.,* 2005; Bhide *et al.,* 2009; Shey *et al.,* 2010). However, for sake of clarity, this review will not address TLR1 or TLR6 polymorphisms and will only focus on TLR2 polymorphisms.

In vitro models are mostly based on the use of murine cells, human cells, or cell lines derived from both species, including dendritic cells, macrophages, and human PBMCs. The use of human PBMC is not free of criticism as well, since these cells might not replicate the responses of tissue resident cells in a specific infected organ (von-Herrath and Nepom, 2005).

All models have discrepancies from true biological responses, so the conclusions based on these models need to be examined closely to insure that there is no over-interpretation. We shall examine the role of TLR2 in infections by pathogen type: viruses, fungi, protozoan/helminthes, and bacteria.

**Viruses**

Viruses are biological organisms that only replicate inside host cells. Cell adhesion and entrance usually occurs without specific PAMP recognition. In fact, almost all viral PAMPs are nucleic acidbased, which are not exposed until after host cell entry, and TLRs that recognize these PAMPs are almost exclusively

intracellular. For this reason, host immune recognition of viruses relies mainly on a complex combination of endosomal TLRs and cytosolic non-TLR receptors, activated by viral nucleic acids uncommon to the host (Thompson and Iwasaki, 2008). Type I interferon (IFN) seems to be a key innate immune factor against viruses since IFN α/β receptor knockout mice are extremely vulnerable to multiple viral infections (Müller *et al.,* 1994). For some types of viruses, type II IFN (IFN-γ) is also essential to assure viral clearance (Zhang *et al.,* 2008). Type I IFN, mainly produced by dendritic cells, can prime natural killer (NK) cells, essential for early infection control (Szomolanyi-Tsuda, *et al.,* 2006), and induce maturation of T helper 1 (Th1) cells (Joffre *et al.,* 2009).

The production of neutralizing antibodies and activation of cytotoxic T lymphocytes (CTL) are likewise important for a specific and effective antiviral immune response (Koyama *et al.,* 2008). However, despite these observations, there is mounting evidence of TLR2 involvement in responses to viral infections. IFN-β production by murine monocytes can be triggered by TLR2 following stimulation with virus particles like VV or murine Cytomegalovirus (CMV; Barbalat, *et al.,* 2009). TLR2 is also involved in NK cell response, as demonstrated by impaired NK cell activity and increased murine CMV load in TLR2 knockout mice (Szomolanyi-Tsuda, *et al.,* 2006).

In humans, malfunction of TLR2 caused by homozygosis for R753Q polymorphism contributes to CMV infection after liver transplantation (Kang, *et al.,* 2012). VV was recently shown to directly stimulate NK cells via the TLR2–MyD88 signaling pathway (Martinez *et al.,* 2010), in addition to directly promoting CD8 T cell survival, allowing cloning expansion and memory cell establishment in mice (Quigley, *et al.,* 2009). TLR2/TLR6 were identified as important murine receptors to control murine respiratory syncytial virus (RSV; Murawski, *et al.,* 2009). Based on these studies, TLR2 seems to have an overall protective role in VV, CMV, and RSV infections. Lack of functional viral Hemagglutinin impairs TLR2 response to measles virus, resulting in diminished IL-6 secretion by murine monocytes and measles receptor CD150 (Bieback *et al.,* 2002).

Varicella zoster virus (VZV) virulent strain induces IL-6 production by human monocytes through TLR2 and CD14, but blocks TLR2 mediated production of IL-12 in human dendritic cells to evade the Th1 driven immune response. The attenuated Varicella strain used for the current vaccine cannot block IL-12 production, which suggests that Th1 driven immune responses are important for protection against VZV. However, the mechanisms of TLR2 blockade or IL-12 inhibition have not been clarified (Wang *et al.,* 2005; Gutzeit *et al.,* 2010). dUTPase from Epstein–Barr Virus also induces IL-6 production through TLR2 in

TLR2 over-expressing HEK293 cells, but CD14 does not act as the co-receptor (Ariza *et al.,* 2009). Yellow fever-attenuated virus, used as the current Yellow Fever vaccine, is capable of activating multiple TLRs and induces a balanced Th1/Th2 response in murine dendritic cells.

However, in TLR2 knockout mice, the Th1 responses are considerably intensified while IL-6 and IL-12 production is decreased (Querec *et al.,* 2006). Although hepatitis B virus capsid particleswas also found to prime human THP-1 macrophages for production of TNF-α, IL-6, and IL-12p40 via TLR2 (Cooper *et al.,* 2005), the lack of purity of this preparation makes these results controversial (Vanland schoot *et al.,* 2005).

Hepatitis C virus (HCV) can be recognized by the host by the TLR2/TLR6 heterodimer by recognition of the HCV core and NS3 proteins, along with recognition of viral genome by TLR3, 7, and 8 (Chang *et al.,* 2007; Moriyama *et al.,* 2007). HCV TLR2 recognition activates human monocytes and macrophages to secrete mainly IL-10 and TNF-α, but inhibits macrophage-dependent dendritic cell differentiation and function (Chang *et al.,* 2007). Accordingly, patients with chronic hepatitis were also found to have increased expression of TLR2 in PBMC, correlated with augmented circulating TNF-α and hepatic necroinflammatory activity (Riordan *et al.,* 2006; Wang *et al.,* 2010). Activation of PBMCs from chronic HCV patients induced by TLR2/TLR4 ligands resulted in decreased IL-6

production when compared to healthy subjects, probably caused by TLR2-induced tolerance (Chung *et al.,* 2011). Divergent results demonstrated that the TLR2 polymorphism R753Q impairs TLR2 recognition of HCV core and NS3 proteins and increases the risk of allograft failure after liver transplantation for chronic hepatitis C patients with this mutation (Brown *et al.,* 2010). This is an example of the contrasting roles TLR2 may play in infectious processes in humans. The ability of viruses to stimulate cells can differ from species or viral type, but also according to the murine strain, organ, or cell type infected. TLR2/TLR9 double-knockout mice infected intravaginally with herpes simplex (HSV) 2 had significant higher viral loads in the brain than single knockouts or wild-type mice. Viral loads in the vaginal mucosa or liver, on the other hand, did not differ between groups of mice.

The observations lead to a synergistic protective role for both TLRs in the brain, benefic for encephalitis prevention, but not effective for general decrease of HSV2 viral loads (Sorensen *et al.,* 2008). TLR2/TLR9 were shown to have a more prominent protective role for HSV1, as TLR2/TLR9 doubleknockout mice all died after intranasal infection with HSV1 EK strain, as compared to 90% survival of infected wild-type mice or TLR2 knockout mice and 40% survival of infected TLR9 knockout mice. Another experiment pointed out a deleterious role

for TLR2 in mice infected with HSV1 KOS strain, in which TLR2 knockout mice were more resistance to infection as compared to WT mice.

Nevertheless, these apparently contradictory results were observed using different HSV1 strains and viral loads, inoculated through different routes (Krug *et al.,* 2004; Lima *et al.,* 2010).

More work is obviously needed to truly dissect the dual property of TLR2 to be involved in both HSV disease protection and disease induction. TLR2 may also be involved in inflammation related to morbidity and mortality caused by lymphocytic choriomeningitis virus (LCMV); TLR2 is essential for induced monocyte chemotactic protein 1 (MCP-1), RANTES, and TNF-α, in mouse central nervous system glial cells (Zhou, *et al.,* 2008; Lee *et al.,* 2012).

The common immune response measured by most studies when examining TLR2 function has been increase in IL-6 and TNF-α production during acute processes, while IL-10 was consistently found in chronic infections. IL-6 secreted by dendritic cells drives Th2/Th17 T cell differentiation (Wenink, *et al.,* 2009).

Considering that type I IFN is released upon virus stimulation and constrains Th17 differentiation in mice (Guo *et al.,* 2008), IL-6 in the context of viral infection most likely drives Th2 responses. When the Th1 phenotype is dominant,

TNF-α act as an enhancer of IFN-γ killing through activation of cellular immune responses.

In contrast, when Th2 or Th0 is dominant, TNF-α causes tissue damage (Hernandez-Pando and Rook, 1994; Rizzardi, *et al.,* 1998). In this context, TLR2 activation may improve viral clearance when the Th1 response is fully functional and dominant during the initial stage of infection. TLR2 can also promote Th2 responses, which are important for adequate antibody production and protect the host by inhibition of exacerbated Th1 response.

To our knowledge, none of the viral particles were tested for TLR2 induction of regulatory T (Treg) cells or Th17 cell function or activation, which could help to elucidate outcomes of TLR2 receptor activation and reveal new information regarding the Th1/Th2 bias studied so far. It is clear that TLR2 function and its role in viral immunity are co-dependent on other PRRs and the subsequent adaptive immune response. Therefore, isolated analyses can be insufficient to determine the true role of TLR2 in each infection type.

In relation to recognition, the examples of viruses mentioned above are variable in relation to species, genetic material and disease outcomes, but all viruses assumed to be recognized by TLR2 are enveloped virus. Some viruses do not have their TLR2 ligands defined. TLR2/6 was the only TLR2 heterodimer described for viral ligands, but the heterodimer needed for TLR2 recognition of many other

viral ligands has not been discerned. The involvement of the TLR2/6 heterodimer is consistent with the cytokine profile described above, since the same heterodimer has been found to drive IL-10 production in dendritic cells (Depaolo, *et al.,* 2008).

Studies on TLR2 accessory molecules involved with viral recognition has also been minimal and only revealed a role for CD14.

**FUNGI**

As opposed to viruses, pathogenic fungi are facultative intracellular or extracellular eukaryotic organisms and therefore do not depend on the host cell to replicate. Some fungal species are dimorphic and change between different forms, for example, yeast or conidia to hyphae, and consequently modify the way they are recognized by the host. In addition, cell wall composition varies among species and serotypes, which can also change the way these cells are processed by the immune system (McKenzie, *et al.,* 2010). Fungal infections remain generally localized when controlled by a regular immune response, but systemic dissemination more often occurs when the host is immunocompromised, has its physical barriers disrupted or has its microbiota altered (Chai *et al.,* 2009a). Dendritic cells usually trigger adaptive immune response against fungal invasion, while direct clearance is performed by neutrophils, monocytes, and macrophages. All of these cells express dectin-1, TLR2, and TLR4, receptors associated with

fungal recognition (Hohl *et al.,* 2006). Most yeast can be phagocytized by macrophages through Dectin-1 receptor recognition, whereas TLR2 helps initiate the concomitant inflammatory state (Zak and Aderem, 2009). The two receptors, when stimulated, mutually synergize their cytokine and immune responses to promote a consistent pathogen clearance (Zak and Aderem, 2009). In contrast, phagocytosis of hyphae is believed to occur without independent PRR recognition and leads to Th2/Treg cells bias (Romani, 2004).

The role of TLR2 in response to one of the most common fungi involved in human disease, Candida albicans, is unclear and results have been somewhat contradictory. The TLR2 ligand phospholipomannan is well defined and present in hyphae and yeast forms (Li *et al.,* 2009), while the outcomes triggered by it are not quite clearly understood. Human keratinocytes stimulated with phospholipomannan produce IL-6 and IL-8, necessary for activation and recruitment of neutrophils and macrophages in situ (Li *et al.,* 2009). Studies with TLR2 knockout mice showed both resistance (Netea *et al.,* 2004b) or susceptibility to primary or secondary systemic C. albicans infection (Gil *et al.,* 2005; Hise *et al.,* 2009), while TLR2 knockout macrophages showed impaired fungal clearance (Blasi *et al.,* 2005), with both impairment or enhancement of pro-inflammatory cytokine production (especially TNF-α; Blasi *et al.,* 2005; Gil and Gozalbo, 2006). The absence of TLR2 also decreased chemotaxis rate of

murine neutrophils, together with decreased fungal killing mechanisms and survival of these cells (Tessarolli *et al.,* 2009). TLR2 is probably not directly involved with humoral response after secondary C. albicans systemic infection, since this parameter was not altered in TLR2 knockout mice (Villamón *et al.,* 2004). On the other hand, Dectin-1 and TLR2 were directly related with protection against oral candidiasis through an effect on IL-1 β release, but only when both receptors are engaged (Hise *et al.,* 2009). Other factors can influence TLR2 mediated effects and contribute to fluctuations in cytokine levels in candidiasis, for example high concentrations of vitamin D3 in mice can decrease TLR2 surface expression (Khoo *et al.,* 2011).

Similar to C. albicans, Aspergillus fumigatus recognition varies with morphology. Both conidium and hyphae are recognized by TLR2 (Chai *et al.,* 2009b), but hyphae are not recognized by TLR4 and result in IL-10 production through TLR2 activation (Netea *et al.,* 2003). Murine neutrophils, on the other hand, have increased fungicidal activity and release pro-inflammatory cytokines when activated by TLR2. It has been shown that TLR2 is essential for protection in immunocompromised mice but not immunocompetent mice (Chignard *et al.,* 2007).

Paracoccidioides brasiliensis is recognized by TLR2, and pulmonary infection of TLR2 knockout mice leads to Th17 skewed immunity and increased (Loures *et al.,*

2009) or similar (Calich *et al.,* 2008) lung inflammation as compared to wild-type mice.

Even so, fungal burden is either diminished or the same as wildtype mice (Calich *et al.,* 2008; Loures *et al.,* 2009), with equal mortality rates demonstrating that overall outcomes are not necessarily affected by TLR2 signaling.

Cryptococcal recognition by TLR2 occurs through the polysaccharide glucuronoxylomannan, and the level of NF-κB activation differs based on polysaccharide effective diameters of the varying cryptococcal species (Fonseca *et al.,* 2010). Although TLR2 was found to be important for control of Cryptococcus neoformans murine infection (Yauch *et al.,* 2004), this is still quite controversial (Nakamura *et al.,* 2006).

TLR2 was also shown to play a major role in protection against Penicillium marneffei, due to stimulation of murine bone marrow-derived dendritic cells to produce IL-12p40 upon fungal recognition (Nakamura *et al.,* 2008). Histoplasma capsulatum is recognized by mice TLR2 through the cell wall protein Yps3p, resulting in activation of NF-κB (Aravalli *et al.,* 2008). Regardless, TLR2 knockout mice had no alteration in inflammatory cytokine production induced by this pathogen (Lin *et al.,* 2010). Generation of a dominant TH1-cell response is required for most protective immune responses to fungi (Romani, 2004). The infection models mentioned above did not evaluate fungal morphology during the

course of infection, which can account for the varied immune responses and survival rates described. Other TLRs and Dectin-1 expression were also not measured in parallel, and these parameters are able to alter the profile of TLR2 responses. The shielding of β-glucans can impair Dectin-1 recognition and consequent loss of synergy with TLR2, which allows for a preferential induction of an anti-inflammatory response when stimulated by fungal particles (Netea *et al.,* 2008). In addition, it has been shown that different murine strains have different dectin-1 isoforms, which may impact on TLR2 function (Heinsbroek *et al.,* 2008). However, it is safe to say that the absence of

TLR2 influences macrophage and neutrophil anti-fungal activity, mainly through an effect on TNF-α production. This cytokine is important for induction of fungicidal activity (Chignard *et al.,* 2007). The relevance of this effect varies according to the infectious microenvironment and with Dectin-1 function. When TLR2 responses were found to be detrimental to the host, it was due to an overwhelming suppression of the early inflammatory response and consequent increased fungal burden. Since almost all fungi are recognized to some extent by TLR2 and are recognized by TLR4 as well, it would be meaningful to evaluate the synergism between these two TLRs in the context of fungal infections, as bacterial ligands have been shown to synergistically induce IL-10 when both TLRs are engaged (Hirata *et al.,* 2008). To date, TLR2 polymorphisms have not

been shown to be related to susceptibility or protection to fungal infections, but it is also true that these studies are scarce in the literature.

**Protozoan/Helminths**

Protozoa are a group of intracellular or extracellular eukaryotic organisms that can cause human disease and depending on its life cycle may mainly cause chronic infections (Sacks and Sher, 2002). TLR2 is activated by glycosylphosphatidylinositols (GPIs) present on some of these protozoa. The level of the inflammatory response induced is directly linked to GPI lipid and carbohydrate content (Almeida and Gazzinelli, 2001). *P. falciparum*, one of the plasmodia that cause malaria, can trigger TNF-α and IL6 production in murine macrophages by means of TLR2/TLR1 or TLR2/TLR6 activation with GPI. This GPI is only recognized in merozoites, the erythrocyte infective form of the protozoan (Wong-Baeza *et al.,* 2010; Zhu *et al.,* 2011).

GPI can also modulate internalization of malaria-parasitized erythrocytes. Still, when inside the erythrocyte, the parasite does not allow for display of its TLR ligands to murine and human macrophages, resulting in a non-inflammatory phagocytosis of these erythrocytes and delayed cytokine responses (Erdman *et al.,* 2009). No TLR2 polymorphisms are related with P. falciparum infections (Greene *et al.,* 2009). However, the presence of human heterozygotes for a TIRAP polymorphism (TIRAP S180L) has been related to protection of malaria. This

adaptor protein mediates TLR2 and TLR4 signaling whereas its polymorphic variant severely decreases IL6 production triggered by these TLRs, suggesting that a normal TLR2 response would be detrimental to the infected human host (Khor *et al.,* 2007).

Toxoplasma gondii GPIs from the highly dividing tachyzoite form, also trigger TLR2 for TNF-α and MCP-1 production. TLR2 knockout mice have increased susceptibility to infection caused by high doses of T. gondii (Mun *et al.,* 2003). However, the use of lower doses leads to an increase parasite burden only in double TLR2/4 knockout mice (Debierre-Grockiego *et al.,* 2007), suggesting a protective role for TLR2 in synergy with TLR4. Strong IL-12 induction through TLR11 is protective and part of the murine immune response, but since this gene is not functional in humans, other TLRs can have a different role in this host (Yarovinsky *et al.,* 2005).

A human embryonic intestinal epithelial cell line recognizes the parasite through TLR2, resulting in production of IL-8 and other chemokines important for neutrophil and phagocyte recruitment (Ju *et al.,* 2009). TLR2 mediated signaling by GPIs from T. cruzi infective trypomastigotes results in IL-12, TNF-α, and nitric oxide production by murine macrophages (Campos *et al.,* 2004). The T. cruzi protein 52 (Tc52) can induce IL-6 and IL-8 in a TLR2-dependent manner (Wong-Baeza *et al.,* 2010).

TLR2 stimulation leads to IL-12 and TNF-α production in T. cruzi infections, in addition to cooperating with TLR9 to enhance this production. T. gondii infection of TLR2/TLR9 double-knockout mice had increased susceptibility to T. cruzi infection. The higher parasite burden of double knockouts was related to low IL-12 and IFN-γ levels (Bafica *et al.,* 2006). Surprisingly T. cruzi IL-10 modulation of murine bone marrow-derived dendritic cells was found to be independent of TLR2 (Poncini *et al.,* 2010). An interesting cooperation between TLR2 and the bradykinin receptor in T. cruzi murine subcutaneous infection has been reported, where TLR2 induces leak of plasma that leads to a kininogen accumulation.

This molecule is degraded by T. cruzi proteases to kinins recognizable by the bradykinin receptor, with consequent induction of IL-12 production by murine dendritic cells (Monteiro *et al.,* 2006). Entamoeba and Leishmania are protozoa genera that have lipopeptidophosphoglycans (LPPG) and lipophosphoglycans (LPG), respectively, which have been identified as TLR2 ligands. Entamoeba LPPG, also recognized by TLR4, leads to TNF-α, IL-12, IL-10, and nitric oxide release in phagocytes (Wong-Baeza *et al.,* 2010). It also stimulates murine NK Cells for production of IFNγ through TLR2, which is directly related with protection from amebic liver abscess (Lotter *et al.,* 2009).

Membrane LPG from Leishmania up-regulates the same cytokine milieu as LPPG in J774 murine macrophage cell line (Kavoosi *et al.,* 2010). However, Leishmania

brasiliensis infection of TLR2 knockout mice presented higher levels of IFN-γ and resistance to infection in vivo when compared to wild-type mice, while DCs from knockout mice had up-regulated IL-12 secretion in vitro (Vargas-Inchaustegui *et al.,* 2009). This is likely associated with a diminished Th2 environment that may be normally induced by TLR2 stimulation, as this has been shown to be associated with increased protection from leishmaniasis.

Human parasitic helminths are complex eukaryotic organisms that mostly live asymptomatically inside the host, with infection usually associated with tolerogenic responses (Maizels and Yazdanbakhsh, 2003). TLR2 is known to be activated by lysophosphatidylserine of two genera of this group: Schistosoma and Ascaris. There is no information regarding the relevance of TLR2 in Ascaris infection. *S. mansoni* eggs fraction containing lysophosphatidylserine activate DCs via TLR2 to induce a Th2 response and regulatory T-cell development (van-der-Kleij *et al.,* 2002).

Conversely, *S. mansoni* infection of MyD88−/− mice had a normal Th2 response while other research has shown that SEA induces a Th2 bias in a TLR2 independent manner. It is likely that the SEA used in the latter experiments lacked lysophosphatidylserine to explain such contradictory results (Kane *et al.,* 2008).

As expected, the effect of TLR2 mediated stimulation is dependent on the cellular environment in which activation occurs. Parasite clearance is related to early IFN-

γ synthesis by NK cells, which, in turn, is mostly induced by IL-12 produced by dendritic cells (Campos *et al.,* 2004; Patel *et al.,* 2007). TLR2 ligands derived from parasites induce IFN-γ directly in NK cells and IL12 in dendritic cells, but production of the latter is more efficient with concomitant TLR4 or TLR9 stimulation. Nevertheless, lack of TLR2 does not increase parasite burden or decrease Th1 responses, suggesting a redundant role in early infection. TNF-α induced via TLR2, on the other hand, may be involved with pathology seen with these infections, when induced at later time points during the course of disease. This may explain why TLR2 knockout mice or human TLR2 polymorphism can lead to an attenuated or resolved infection.

Another possibility is that TLR2-driven Th2 responses are involved with a more severe disease state, as commonly seen in leishmaniasis (Deak *et al.,* 2010). It is likely that a defective early IFN-γ response allows for TLR2-induced Th2 skewing and consequent suppression of IL-12 production. Parasites can infect cells and replicate inside them without TLR recognition, whereas the infected cell is frequently phagocytized in a non-inflammatory process, which may allow for delayed recognition and TLR2 mediated responses to these pathogens. Curiously,

TLR2 mediated regulation of IL-10 during parasitic infections has only been to demonstrated for schistosomiasis, showing that the Th2 skewing outcome was

more relevant than the alteration of the Th1/Th2 profiles in TLR2 context in this disease.

## BACTERIA

Bacteria are prokaryotic unicellular organisms with varying strategies for energy production, survival, infectivity, and self-protection. Bacterial cells are limited by a common phospholipid bilayer membrane with inserted functional proteins, covered by a peptidoglycan cell wall. Some species have an outer membrane while others have a capsule or are able to sporulate, which alters the way they are recognized by the host (Silhavy *et al.,* 2010). Lipoproteins, important TLR2 ligands, are produced by all bacteria. Due to the high number of bacterial pathogenic species, they are analyzed in the present review by their genera and Gram staining groups. The genera to be explored are the ones that include the main human pathogens.

### Gram-positive bacteria

The Gram-positive bacterium cell wall contains a thick peptidoglycan layer, combined with teichoic acids and extracellular proteins.

Lipoteichoic acid (LTA), as well as lipoproteins and peptidoglycan, can differentially activate TLR2 dependent on the bacteria of origin (Ryu *et al.,* 2009;

Müller-Anstett *et al.,* 2010). The pathogenic bacteria considered here are from the genera Corynebacterium,

Nocardia, Bacillus, Listeria, Staphylococcus, Clostridium, Enterococus, Streptococus, Mycobacterium, and Mycoplasma. The last two genera do not stain as Gram-positive because they have different cell wall structure, but are regularly grouped with Gram-positive bacteria due to the genetic background (Silhavy *et al.,* 2010). So far, all, Gram-positive bacteria activate TLR2 to some extent. Murine phagocytes with impaired TLR2 function, either by using blocking antibodies or gene knockouts, when exposed to the above bacteria or their cell wall components have diminished or absent production of TNF-α or/and IL-6, as opposed to normal cells (Takeuchi *et al.,* 1999, 2000; Torres *et al.,* 2004; Bafica *et al.,* 2005; Leendertse *et al.,* 2008; Love *et al.,* 2010).

Various infection models have helped to characterize some functions of TLR2 in recognition of Gram-positive bacteria. Respiratory infection models of TLR2 knockout mice with Mycoplasma and Mycobacterium showed increased bacterial burden, tissue damage, and death. This potentially protective role for TLR2 in mycobacterial infection was confirmed in humans, where TLR2 polymorphisms that decrease TLR2 expression were found to predispose people to tuberculosis (Yim *et al.,* 2006) and non tuberculous mycobacterial lung disease (Yim *et al.,* 2008). Conversely, protection from respiratory infection with Streptococcus

pneumoniae only requires TLR2 when the TLR4 ligand pneumolysin is not expressed, indicating redundancy in recognition and protective responses to these bacteria (Dessing *et al.,* 2008).

Interestingly, middle ear infections (Han *et al.,* 2009) and meningitis (Letiembre *et al.,* 2005) caused by Streptococcus are exacerbated when TLR2 is absent, which may indicate that pneumolysin may be differentially expressed depending on the environment and strain variant. Intraperitoneal inoculation of Enterococcus (Leendertse *et al.,* 2008), Listeria (Torres *et al.,* 2004), and Staphylococcus (Mullaly and Kubes, 2006) caused similar disease and inflammatory states in TLR2 knockout as compared to wild-type mice, while, in contrast, intravenous inoculation of these TLR2 KO mice with Listeria (Torres *et al.,* 2004) or Staphylococcus (Takeuchi *et al.,* 2000) resulted in death and bacterial burden as compared to wild-type mice. This suggests that the presence of TLR2, for these organisms at least, triggers an immune response that prevents dissemination. However, once dissemination occurs, TLR2 stimulation is no longer occurs or is protective. Regardless of strain variations, the route of infection seems quite important in regards to protection induced by TLR2 toward Gram-positive bacterial infections, at least in mice.

Human polymorphism studies found no relationship between TLR2 gene polymorphism R753Q and serious pneumococcal (Moens *et al.,* 2007; Yuan *et al.,*

2008), streptococcal (Liadaki *et al.,* 2011), staphylococcal infection (Moore *et al.,* 2004), or altered cytokine patterns in Gram-positive septic patients (Woehrle *et al.,* 2008), even though this polymorphism prevents TLR2 from responding to LTA. However, one functional allele is enough to compensate for this deficiency, since heterozygous subjects respond to infections the same way as the ones without the polymorphism (von Aulock *et al.,* 2004). A TLR2-16933AA promoter human polymorphism has been associated with increased prevalence of Gram-positive bacterial sepsis but not an increase in true shock or mortality (Sutherland *et al.,* 2005). Accordingly, blood monocytes from septic patients had higher TLR2 and CD14 expression than healthy individuals, but mortality was associated with down-regulation of the same receptors and consequent cytokine induction (Schaaf *et al.,* 2009). These studies demonstrate that TLR2 is important, if not essential, for control of Gram-positive infections in general, but its absence can be compensated by activation of other receptors by some genera.

**Gram-negative bacteria**

The Gram-negative bacterium cell wall contains a thin peptidoglycan layer with pore forming proteins, covered by an extra layer of lipopolysaccharides (LPS). However, unlike Gram-positive bacteria, they do not produce the TLR2 ligand lipoteichoic acid. LPS is an important TLR ligand present in all Gram-negative bacteria, with its molecular composition varying among the genera. These

variation leads to different receptor activation and subsequent inflammation (Munford and Varley, 2006).

Campylobacter, Bordetella, Shigella, Escherichia, Haemophilus, Salmonella, Neisseria, Klebsiella, and Leptospira produce the typical LPS, a hexaacylated form capable of activate inflammation through TLR4 (Munford andVarley, 2006). All of the above genera can stimulate cells via TLR2. Escherichia, Haemophilus, Salmonella, Neisseria, Klebsiella, and Leptospira have all been examined in animal models of infection to determine the role of TLR2 in protection or pathology. In this context, TLR2 was not essential for resolution of infection for these bacteria, while TLR4 played a much greater role (Lorenz *et al.,* 2005; Spiller *et al.,* 2007; Sjölinder *et al.,* 2008; Fedele *et al.,* 2010; Moranta *et al.,* 2010; Pore *et al.,* 2010; Seibert *et al.,* 2010). Nevertheless, TLR2 knockout mice had higher bacterial burdens during the course of infections with Klebsiella and Salmonella, but with mortality or antibody responses were not altered as compared to wild-type mice (Spiller *et al.,* 2007; Seibert *et al.,* 2010). TLR2/TLR4 double-knockout mice were even more susceptible to infection with Leptospira and Klebsiella, as compared to TLR4 single knockout mice, demonstrating an important role for TLR2 recognition in control of these infections and a potential synergistic function with TLR4, demonstrating another

potential redundancy of immune system activation (Spiller *et al.,* 2007; Chassin *et al.,* 2009).

Regarding animal models of sepsis, Escherichia and Salmonella trigger excessive inflammation and subsequent mice death via TLR4 and TLR2. IFN-γ induced by TLR4 stimulation increases TLR2 up-regulation and affects the immunopathology, a probable reason why TLR4/TLR2 double-knockout mice all survived infection with these organisms after antibiotic therapy as opposed to single knockouts or wild-type mice, where antibiotics could not rescue these animals (Spiller *et al.,* 2008). This demonstrates a pathologic effect of the inflammatory response induced by bacteria through these TLRs.

The under-acylated forms of LPS are weak inducers of inflammation, some of them capable of competing with typical LPS for TLR4 and therefore acting as anti-inflammatory agents (Munford and Varley, 2006). Gram-negative bacteria that produce these forms of LPS generally evade TLR4-driven immune response and the host relies on other receptors to recognize and react to the threat. Porphyromonas and Coxiella infection resolution are partially dependent on TLR4, but pro-inflammatory cytokine production is dependent on TLR2. The establishment of Porphyromonas chronic infection and inflammation appears to be affected by TLR2, where the absence or presence of TLR2 has been associated with both deleterious and protective effects (Shin *et al.,* 2010).

Chlamydia murine infections have TLR2 dependent immune pathology, although TLR2 human polymorphisms have not been associated with the disease (Karimi *et al.,* 2009). It has also been shown that TLR2 mediates morbidity and mortality in murine models of infection with Burkholderia species, as TLR2 knockout mice have decreased pathology and increased survival as compared to wild-type mice (Wiersinga *et al.,* 2007). TLR2 can mediate protection as well, i.e., in murine infection models of Legionella, Francisella, Citrobacter, and Borrelia. The resolution of these infections or consequent inflammation are TLR4 independent, while lack of TLR2 appears to be associated with higher bacterial burden (Archer and Roy, 2006; Abplanalp *et al.,* 2009; Dickinson *et al.,* 2010), tissue damage (Gibson *et al.,* 2008), or decreased survival (Fuse *et al.,* 2007; Gibson *et al.,* 2008;Abplanalp *et al.,* 2009; Dickinson *et al.,* 2010). TLR2 mice orally infected with Yersinia develop a gut barrier defect and diminished intestinal clearance, demonstrating a role for TLR2 in full protection from this infection, but this may not just be specific for this bowel pathogen (Dessein *et al.,* 2009).

Overall, the role of TLR2 in Gram-negative infections seems to be directly related with the dominant PRR activated by the bacterium. Often, at least in animal models, an excessive bacterial burden increases TLR2 sensitivity and its role in pathology. It is important to note that infection models often use attenuated or heat killed bacteria, which can change the predominant roles of participating

PRRs. Accordingly, heat killed Treponema signals through TLR2, as opposed to live bacteria which suppresses activation by this receptor (Shin *et al.,* 2010). Bartonella quintana, Helicobacter pylori, Moraxella catarrhalis, Pasteurella multocida, Rickettsia akari, and Vibrio vulnificus are also recognized by TLR2 with subsequent induction of many inflammation related cytokines or chemokines: TNF-α, IL-6, IL-10 (phagocytes), or IL-8 (mucosal epithelial cells), depending on which cells were examined (Slevogt *et al.,* 2007; Matera *et al.,* 2008; Hildebrand *et al.,* 2009; Peek *et al.,* 2010; Lee *et al.,* 2010; QuevedoDiaz *et al.,* 2010). Surprisingly, no relationship to disease has been studied or confirmed (Oliveira *et al.,* 2008).

There are a large variety of microbial pathogens in various phyla that express components that are recognized by TLR2. These molecules then trigger various innate immune responses that are involved with protection from these organisms and are also involved with inflammatory sequelae that are associated with disease caused by these microbes. The outcomes of ligand recognition were shown to be dependent on the recognition dimer (TLR2/TLR1 or TLR2/TLR6), and co-receptors. It is important to remember, however, that the innate immune system does not recognize microbial TLR2 ligands in a vacuum, and many of these pathogens express ligands recognized by other PRRs, i.e., LPS and TLR4, RNA and TLR3, CpG DNA motifs and TLR9, etc. Therefore, the responses to these

organisms are complex and not easily predicted. It needs to be mentioned, though it is beyond the scope of this review, that many investigators have taken advantage of the ability of some of these microbial derived molecules to stimulate cells via TLR2, or other TLRs, to utilize this ability in at the development of vaccine adjuvants (Wetzler, 2010; Duthie *et al.,* 2011).

# CHAPTER THREE

## 3.0 MATERIALS AND METHOD

### 3.1 Experimental location

The experiment was conducted in University of Benin Teaching and Research Farm EDO State. It is located at latitude 60°E and longitude 40°N in the forest zone with a temperature 27.6° c. Annual rain fall ranges from 1498 to 3574mm with the mean value 2162mm. Relative humidity ranges between 63.3 and 81.71%, and daily sunshine is between 5.85 and 7.5w/m² hours with mean value of 7.25 and 6.8hours respectively (NAA, 2015).

## 3.2 Experimental Animal

A total of 40 adult rabbits 5months and above were raised in the University of Benin teaching and research farm. From this flock, 9 rabbits consist of New Zealand, Hyla and Dutch breeds were selected for the experiment.

## 3.3 Experimental Material

Other experimental Materials used are steel hutch, metabolic feeders and drinkers ,steel cages, feacal Tray , sensitive scale and weigh balance.

## 3.4. Management

Steel hutches With several compartments were constructed with steel material and wire mesh specially for housing the experimental animals. The cages were constructed in way that the feeders and drinkers are tightly fixed and detachable when necessary. Prior to Kindling the entire hutches were repaired, cleaned as well as the equipment. The outside surroundings were also cleaned.

Metabolic Feeders and drinkers were specially designed to ensure zero wastage of feed and water. This enables taking account of remnant of feed per day.

A sensitive scale and weigh balance of 5kg was used to measure the feed, fed to the animals daily and their weekly body weight gain, birth weight, average birth weight, weaning weight and Average weaning weight.

The animals were housed in hutches (60cm x 60cm x 80cm) placed in a normal dwarf wall building. The hutches were constructed with wood and wire mesh and some others made of metal to allow for proper ventilation and for proper waste disposal.

The rabbit were administered coccidiostat to treat coccidiosis. Animals were treated of mange using ivermectin, Multi-vitamin were given and antibiotics were also administered.

Rabbits were fed on a commercial grower's mash of about (16% cp and 2,906kcal/kg ME) in the morning and forages such as Panicum maximum offered ad libitum. The forage was also given to prevent bloating. The drinkers and Feeders were emptied and washed daily before cool Fresh water and feeds were replaced.

### 3.5 Blood sample collection

A shield™ preparation or blood collection 50mil DNA/RNA shield™ was purchased from Inqaba indotec (*Inqaba bristec* West Africa Ltd) No 17949735__001RNA shied™ for protecting the RNA component of the blood.

Blood sample collection 2ml of blood were collected from the ear vein of the rabbit using sterile needle and placed in a plain bottle already containing 6ml of RNA shield. Nine (9) samples were collected.

### 3.6 Laboratory analysis

Laboratory analyses were performed using laboratory facilities and equipment at Inqaba biotech laboratory Ibadan, Oyo State.

### 3.7 RNA Extraction

Total RNA was extracted from the liver and the thymus using ISOLATE II RNA Mini kit (BIOLINE, USA). The kit is highly efficient for purification of total RNA from RNALater treated tissues (and other samples) in as little as 30 minutes. Single Nucleotide Polymorphism (SNP) identification and estimation of genetic diversity indices. The identification of single nucleotide polymorphisms (SNPs) of the rTLR2 gene in the different breeds of rabbits (New Zealand, Dutch, Hyla) was done using DNAsp version 5.10 software.

Genetic diversity indices were estimated using MEGA 7.0 and Asp version 5.10. Genetic diversity indices estimated were nucleotide diversity (Pi), number of haplotypes (H), and number of polymorphic sites, parsimony informative sites and gleton variable sites. Other genetic diversity indices estimated were haplotype diversi and distribution, gene flow (Fst) and nearest-neighbour statistics (Snn).

## 3.8 Complementary DNA (eDNA) synthesis

Complementary DNA (cDNA) was synthesized using SensiFAST™ cDNA synthesis kit (BIOLINE, USA), following manufacturer's instructions. The kit provides a rapid and very sensitive method for first strand cDNA synthesis for use in real-time PCR studies. All RNA samples were normalized to 0.57 ug for cDNA synthesis due to differences in the concentration of RNA samples. After normalization of RNA samples, 4 ul 5x TransAmp Buffer and I ul reverse transcriptase were added to each sample in a PCR tube and mixed gently by pipetting. The PCR conditions were 25°C for 10 minutes (primer annealing), 42°C for 15 minutes (reverse transcription), 48°C for 15 minutes (optional step). 85°C for 5 minutes (inactivation) and 4°C hold. After polymerase chain reaction, CDNA samples were stored at -20°C in refrigerator before real-time PCR.

## 3.9 Real-Time Polymerase Chain Reaction (qPCR)

The expression pattern of rTLR2 RNA in the liver and thymus was determined by qPCR assay. The EVA Green dye chemistry was used in detection. The PCR primers were designed using Primer 3 and blast option at the NCBI website. The information on primers used is presented in Table 3.3. House-keeping gene B-

actin was used as the reference gene. Real-Time PCR reactions were performed in duplicates in 25 µl reaction volume comprising 5 ul PCR Master mix (FIREPol", Solis BioDyne, Estonia), 0.6 µl each of forward and reverse primers, 4 µl of template cDNA and 14.8 µl of nuclease-free water in a smart cycler tube. A 25 µl non-template reaction mixture was also run alongside negative control. The qPCR conditions were as follows: 40 cycles each of initial denaturation at 95°C for 15 minutes; denaturation at 95°C for 15 s; annealing at 65°C for 20 s and extension at 72°C for 20 s.

**Table 1: Primer pairs for PCR**

| Target molecules | | Primer sequences (5' TO 3') | Product size (bp) | Annealing temperatures($^0$C) |
|---|---|---|---|---|
| TLR2 | Sense | TGTCTGTCACCGAACCGAATCCAC | 134 | 54 |
| | Antisense | TCAGGTTTTTCAGCGTCAGCAGGG | | |

### 3.10 Assembling of qPCR data and statistical analysis

Data on qPCR of rTLR RNA were assembled using Microsoft Excel (2010). The average Cr values of technical replicates and change in C values (ACT Cr target gene - Cf reference gene) were also inferred using Microsoft Excel (2010). Fold changes in expression levels of TLR2 gene in rabbits. Fold changes (2-4) in gene expression were determined using the comparative AACT method as described by Livak and Schmittgen (2001). Data on fold changes in gene expression were

subjected to One-way Analysis of Variance (ANOVA) and means with significant differences were separated using Duncan's multiple range tests. ANOVA and mean separation were performed using Statistical Package for the Social Sciences (SPSS) software version 21.0.

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