Targeting Primary Cilia Immune Receptor Proteins for the Treatment of Polycystic Kidney Disease Mechanisms

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Targeting primary cilia immune receptor proteins for the treatment of polycystic kidney disease mechanisms

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April 2019
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ABSTRACT

Targeting primary cilia immune receptor proteins for the treatment of polycystic kidney disease mechanisms

by Nedaa S. Alomari

Background: Primary cilia are cellular organelles project from the cell surface of mammalian cell and play important roles in vertebrate development, organogenesis, health, and others genetic diseases. Primary cilium functions as a mechano-sensor and chemo-sensor. Defect in primary cilia causes the progression of polycystic kidney disease (PKD) which further leads to the inflammatory responses. We, therefore, investigated the role of Toll-like receptors 4 and 9 (TLR) in primary cilia towards PKD.

Purpose: The main purpose of the proposed study is to identify and target the immune reactive proteins i.e. TLRs in the primary cilia. By targeting those primary cilia immune reactive proteins using suitable agonist and antagonists to study the control of cystic formation and their progression mechanisms.

Methods: To target the ciliary immune TLR proteins (TLR4 and TLR9), we did immunostaining to evaluate their localization on primary cilia. Cilia lengths were measured and compared using differential interference contrast (DIC) and fluorescent imaging techniques. The in vitro 3D cyst progression was monitored by adding agonists lipopolysaccharide (LPS) and oligodeoxynucleotides (ODN) and antagonist 4-hydroxy chloroquine (HCQ).

Results: From our results we found that the TLR antagonist HCQ increases ciliary length in treated scrambled control, Pdk2 knockout (KO) and TLR4 KO cells as an immune response, whereas opposite results were observed with TLR9 KO. However, the selected agonists for TLRs (LPS/ODN) increases cilia length in TLR9 KO cells and decreases scrambled control, Pdk2 KO
and TLR4 KO. In our 3D cyst cultures, we used agonists and antagonist for both the TLRs and observed that the cyst formations and progressions were inversely related to the cilia lengths. From these observations, we speculated that the new ciliary TLR proteins have a role in cystic progression. In conclusion, we found that the TLRs agonists/antagonist can modulate cilia length and TLRs role in inflammatory actions. The primary cilium already has central roles throughout cell biology, but here we propose, for the first time, that the cilium and the regulation of its structural importance in inflammation of PKD.
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CHAPTER 1
INTRODUCTION

1.1 Cilia

Cilia are microtubule-based organelles that extend from the apical surface of many eukaryotic cells. Around 800 individual proteins have been identified in the cilia (Gherman, Davis, & Katsanis, 2006) (Ishikawa, Thompson, Yates, & Marshall, 2012). Cilia can be motile or non-motile (primary cilia). Motile cilia are found on the epithelial surfaces of ear, respiratory tracts, fallopian tubes and the ventricles of the central nervous system with different length in each organ. Motile cilia play critical roles in organ development, mucous clearance and sperm motility (O'Callaghan, Sikand, & Rutman, 1999).

Primary cilia are found on cells of the olfactory epithelium and act as sensory antennae responding to the intracellular signaling (Yoder, 2007). Defect in primary cilia structure or function known as ciliopathies. Ciliopathies include a group of diseases such as obesity, hypertension, cancer and PKD (Li et al., 2015) (Aboualaiwi et al., 2014) (Hildebrandt, Benzing, & Katsanis, 2011). The first “ciliopathic” disorder was attributed to Bardet-Biedl syndrome (Ansley et al., 2003).

Based on the cilia motility and structure, nodal cilia with unique hybrid type have been identified. Nodal cilia are solitary motile organelles which play role in embryonic development. Defect in nodal cilia could potentially result in internal thoracic and abdominal organ configuration from normal (situs solitus) to mirror-image configuration (situs inversus), randomized configuration (situs ambiguous), or configuration with duplication (situs isomerism).

1.2 Cilia structure

The structure of primary cilium contains of nine parallel pairs of microtubules called the axoneme to support the long cilia structure. In primary cilia the microtubules are without central pair. In the nodal cilia, the axoneme also lacks the central pair of microtubules. However, it requires for
motility dynein arms and radial spokes (Shinohara et al., 2015) (Figure 1). The discoveries of a “3+0” axonemal in a protozoan and “9+4” axoneme in Hensen’s node of a rabbit have made the classification of cilia structure more complicated (Feistel & Blum, 2006).

**Figure 1.** Structure of primary cilium. A cilium is a membrane-bound structure and composed of multiple central pairs of microtubules (axoneme) originating from the basal body. A basal body is a microtubule-based structure composed of mother and daughter centrioles. The ciliary membrane and axoneme contribute to the upper part of the cilium. The ciliary membrane is continuous with the cell membrane, but cilia have their own proteins, ion channels and/or receptors. The ciliary skeleton may have 9 + 0 or 9 + 2 axoneme compositions. Most 9 + 0 cilia lack inner and outer dynein arms, radial spokes, and central sheath and are commonly referred as non-motile primary cilia. Some 9 + 0 cilia lack the central microtubule only and are motile. Between the cell membrane and cilium, there is a transition-membrane at the junction of the basal body acting as a barrier for molecules to enter or exit from the primary cilium.


The ciliary membrane composed of many receptors. Most of the ciliary protein functions are not yet completely established. Some of the receptors located in the cilia are ion channel, sensory
proteins and protein transporters to provide support and function of the cilia. Cilioplasm is constituted within the soluble compartment of the cilia (Nauli, Jin, & Hierck, 2011) (X. Jin et al., 2014). The studies have shown that cilioplasm involved with the calcium signaling compartment in order to response to chemical and mechanical stimuli (X. Jin et al., 2014), (Masyuk, Gradilone, & LaRusso, 2014) (Lee et al., 2015) (Atkinson et al., 2015). Cilioplasm also contains other many more signaling proteins. However, ciliary dynamic compartment includes signaling and transport proteins for example, intraflagellar transport (IFT) proteins. Moreover, transport proteins and signaling moieties are essential for cilia structure and function. The basal body composed of the mother centriole to which the ciliary axoneme is rooted. In addition to its important structural role, the basal body contains different signaling proteins that provide several functions. The transition zone composed of transition fibers and transition zone. The transition zone connects ciliary axoneme and basal body which plays an important role in ciliary access and ciliogenesis (Garcia-Gonzalo & Reiter, 2012).

1.3 Roles of primary cilia in cellular signaling

Receptors located in the primary cilia can serve a wide range of functions. Recent data sets about the ciliary genomics and proteomics have shown the cilium function engaged approximately 1,000 different polypeptides (Gherman et al., 2006). The fundamental role of cilia is to sense and conduct the signaling pathways from either chemical stimulus as ligand and growth factor or mechanical stimuli as pressure and flow. The ciliary pathways include sonic hedgehog, calcium, mTOR, MAPK, JAK/STAT and Wnt, and these signaling pathways play vital roles in cellular processes for instance, development, differentiation, cell cycle and apoptosis (Satir, Pedersen, & Christensen, 2010).
Primary cilia can also play an opposite role of chemosensory role by performing “chemosecretory” function, sending information to the extracellular environment (Wood, Huang, Diener, & Rosenbaum, 2013). The study has demonstrated that ciliary membrane protein polyductin undergoes proteolytic cleavage and release of extracellular domain into the lumen (Kaimori et al., 2007). Another studies, Barr and Sternberg showed the polycystin-1 and -2 (PC-1/-2) are involved in the PKD and autosomal dominant polycystic kidney disease (ADPKD) in human and the loss of these two proteins affect the cilia behavior and function (Barr & Sternberg, 1999). Moreover, genetic studies have demonstrated the link between the cilia; cyst formation and fibrosis. In case of the complete loss of cilia in mouse result in slow down the growth of cysts, while in case of cilia-polycystin mutations result in increased cysts proliferation ADPKD models (Ma, Gallagher, & Somlo, 2017). The idea of studying the ciliary protein functions is interesting and worth investigating further.

1.4 Polycystic Kidney disease

ADPKD is one of the most common inherited disease. ADPKD affects around 1 in 500 to 1000 person in the worldwide (Ali et al., 2015) (Pei & Watnick, 2010). In the United States of America, approximately 2,144 patients start renal replacement therapy annually. African Americans are less common to have end-stage renal disease (ESRD) as complication to ADPKD comparing to the white people. The rate of the ESRD caused by ADPKD is 7·8 and 6·0 cases per million in Europe from 1998 to 1999 and 8·7 and 6·9 cases per million in USA from 1998 to 2001 per year (Stengel et al., 2003).

ADPKD is characterized by fluid filled cysts that grow in the kidney and interfere with their ability kidney function (filtration) and eventually cause kidney failure (Figure 2). The cysts may grow in other organs like liver (Nadasdy et al., 1995). The gene involved in the disease is PKD1 discovered
in 1994. **PKD1** located on the short arm of the chromosome 16 and produces PC1. The **PKD2** was discovered in 1996. **PKD2** is located on the long arm of chromosome 4 and produces a smaller glycoprotein, PC2. PC2 protein involves in calcium transport. PKD1 mutation responsible for 85% of ADPKD cases while PKD2 accounts for 15% (Ali et al., 2015) (Braun, 2014) (Chapman et al., 2015). Although PKD1 and PKD2 mutations produce similar extrarenal and renal manifestations, PKD2 patients diagnosed later in life with less complications and longer renal survival compared with PKD1 patients (Hateboer et al., 1999).

![Figure 2](https://mosaiques-diagnostics.de/mosaiques-diagnostics/Polycystic_kidney_disease)

**Figure 2.** Kidney disease progression in autosomal polycystic kidney disease (ADPKD).

**Adapted from:**
https://mosaiques-diagnostics.de/mosaiques-diagnostics/Polycystic_kidney_disease
1.5 Role of inflammation in polycystic kidney disease

In general, ADPKD is not an inflammatory disease. However, subclinical inflammation has been linked with ADPKD. Studies have indicated that the inflammation occurs in early stage of the disease and an inflammatory component exist in ADPKD patients. For instance, in cyst fluid of PKD patients contained tumor necrosis factor-α (TNF-α), IL-2 (proinflammatory cytokines) and interleukin (IL)-1β (Gardner, Burnside, Elzinga, & Locksley, 1991) (D. Zheng et al., 2003). In addition, ADPKD patients’ urine had monocyte chemoattractant protein-1 (MCP-1). The level of MCP-1 was positively linked to the progression of ADPKD patients (Mrug et al., 2008).

Zhou and co-workers observed that in cystic mice, a non-orthologous mouse model of autosomal recessive polycystic kidney disease (ARPKD) was reported to upregulate genes that involved in the innate immune system. They also found a high level of expression of macrophages and monocytes (Mrug et al., 2008). Specifically, they found highly upregulated and activated CD14, which is a pattern recognition receptor involved in TLRs for their activation (Zhou et al., 2010).

Menon and co-workers found that in declining kidney function the inflammatory markers was increased such as C-reactive protein (CRP) and IL-6 (Menon et al., 2011). Thus, the data show a potential role of inflammation in the progression of ADPKD.

1.6 Toll-like receptors (TLRs)

The immune system can sense and eliminate any invading pathogen in our body by the ability to distinguish between self and non-self. The immune system is classified into “adaptive immunity” and “innate immunity”. The adaptive immunity protects the body in a specific manner from infectious disease using antigen on B and T cells surface. However, the innate immunity is the first line defense mechanism in all organisms and it is nonspecific (Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999). Toll receptor was first found in drosophila which is considered as
transmembrane receptor (Hashimoto, Hudson, & Anderson, 1988). A mammalian homologue of drosophila Toll was found one year after the discovery of the Toll receptor in the drosophila (Medzhitov, Preston-Hurlburt, & Janeway, 1997).

Afterward, the family of TLRs was identified which consist of 10 receptors TLR1 to TLR10 (Medzhitov et al., 1997) (Du, Poltorak, Wei, & Beutler, 2000) and the microbial ligand for these receptors has been identified (Figure 3). Recently, an analysis of 10 human and 12 mice TLRs found that they can be grouped into two categories based on their localization or their activation by microbial membrane lipids, microbial nucleic acids, or bacterial proteins (De Nardo, 2015) (Lim & Staudt, 2013). The ligands found in microbes include bacterial cell wall. TLR4 responds to bacterial LPS found in Escherichia coli and TLR2 responds to peptidoglycan found in Staphylococcus aureus (Poltorak et al., 1998) (Yoshimura et al., 1999). Furthermore, TLR2 and TLR1 responses to tri-acylated lipoproteins and TLR2 and TLR6 respond to di-acylated lipoproteins (Brightbill et al., 1999) (Ozinsky et al., 2000) whereas TLR9 responds to unmethylated CpG DNA (ODN D-SL03; ODN) found in bacterial genomes (Hemmi et al., 2000).
Figure 3. Toll-like receptors (Human) display specificity in their recognition of pathogen-associated molecular patterns and/or synthetic compounds.


1.7 Roles of Toll-like receptors in kidney disease

TLRs are a group of innate immune receptors that detect pathogen and regulate inflammation and immune responses. The innate response works as first defense mechanism which is usually nonspecific. We can classify the innate immune interaction into three different classes. First, it is by recognizing the infection and start local activation of inflammatory and immunity response. Second, it is by activating innate immunity systemically which leads the kidney to release inflammatory mediators. Third, it is the homeostatic interactions between environmental stimuli and innate immune system. Toll-like receptors (TLRs), nucleotide-binding oligomerization domain receptors (NLRs) and PYD domains-containing protein 3 (NLRP3) monitor the kidneys repair mechanism (Smith, 2009). These receptors play an important role in immune-mediated responses and inflammatory diseases. It has been shown that TLR2, TLR4 and TLR9 are involved in different inflammatory kidney disorders, such as in diabetic nephropathy, renal transplant rejection, lupus nephritis and urinary tract infections. (Leemans, Kors, Anders, & Florquin, 2014, Smith 2009) (Patole et al., 2006).

There are no studies available on the role of localization of TLR4 and TLR9 in the primary cilia and they are cause/progression of PKD. Thus, we aimed to investigate the potential role of TLRs in the cyst formation and we also tried to identify their role of TLRs specifically by using cellular knockout of TLR4 and TLR9 and using genetic pharmacological approach.
1.8 Toll like receptors recognition of agonists

The structural studies on TLRs have shown how some of the agonists bind to the receptor and activate it. For instance, TLR1 and TLR2 bind to triacylated lipopeptide and TLR3 to viral double-stranded RNA. Unmethylated CpG motifs in viral DNA activates TLR9 and guanosine in virus RNA activates TLR7 and TLR8 (Pichlmair & Reis e Sousa, 2007). Moreover, LPS from Gram-negative bacteria, envelope protein from mouse mammary tumor virus (MMTV) and protein from respiratory syncytial virus (RSV) can stimulate TLR4 (Kurt-Jones et al., 2000) (Rassa, Meyers, Zhang, Kudaravalli, & Ross, 2002). The agonists bind specifically to the receptors and form a bridge which brings together the glycan-free surfaces of the TLRs (M. S. Jin et al., 2007) (Liu et al., 2008).

1.8.1 Lipopolysaccharide (LPS)

LPS is the principal component of Gram-negative bacteria that activates the innate immune system. LPS recognition is predominantly mediated by TLR4 (Poltorak et al., 1998). This recognition involves the binding of LPS with lipopolysaccharide-binding protein and subsequently with CD14 which physically associates with a complex including TLR4 and MD2 (Shimazu et al., 1999). Formation of the TLR4-centered LPS receptor complex induces the production of proinflammatory cytokines through the MyD88 pathway. LPS signaling also involves a MyD88-independent cascade that mediates the expression of IFN-inducible genes.

1.8.2 CpG ODNs (ODN)

CpG ODNs are short synthetic single-stranded DNA molecules containing unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs). CpG ODNs possess a partially or completely phosphorothioated (PS) backbone, as opposed to the natural phosphodiester (PO) backbone found in genomic bacterial DNA. Three major classes of stimulatory CpG ODNs have
been identified based on structural characteristics and activity in human peripheral blood mononuclear cells (PBMCs), in particular B cells and plasmacytoid dendritic cells (pDCs). These three classes are Class A (Type D), Class B (Type K) and Class C; CpG-A ODNs are characterized by a PO central CpG-containing palindromic motif and a PS-modified 3’ poly-G string. They induce high IFN-α production from pDCs but are weak stimulators of TLR-9-dependent NF-κB signaling and pro-inflammatory cytokine (e.g. IL-6) production. CpG-B ODNs contain a full PS backbone with one or more CpG dinucleotides. They strongly activate B cells and TLR9-dependent NF-κB signaling.

1.9 Toll like receptors recognition of antagonists

1.9.1 Hydroxychloroquine (HCQ)

The studies on TLRs have demonstrated an example of the agents that can inhibit or reduce the activity of the receptors. HCQ the antimalarial drug has been shown to modulate the intracellular TLR pathway by reducing TLR4 and TLR9 expression (Sperber, Kalb, Stecher, Banerjee, & Mayer, 1993). Recently, a new mechanistic model of this antagonist drug has been proposed that HCQ directly interacts with nucleic acids and consequently causes structural modifications of the TLR ligand to prevent the ligand from binding to TLR (Kuznik et al., 2011) (Venuturupalli, Gudsoorkar, & Wallace, 2012) (Wallace, Gudsoorkar, Weisman, & Venuturupalli, 2012).
CHAPTER 2
METHODOLOGY

2.1 Materials

LLCPK-1 (ATCC® CL-101.1TM) porcine renal epithelial cells from proximal tubule were obtained from American Type Culture Collection (ATCC; Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from VWR International (Brooklyn, NY); trypsin, penicillin-streptomycin solution and phosphate buffered saline (PBS) were ordered from Corning (Manassas, VA). Fetal bovine serum was obtained from Seradigm (Logan, UT). The pharmacological agents were purchased from different chemical companies. 4-Hydroxychloroquine was purchased from Cayman chemicals, and lipopolysaccharide/CpG oligodeoxynucleotides were purchased from InvivioGen (San Diego, CA). Most LPS preparations on the market are contaminated by other bacterial components, such as lipoproteins, thus activating TLR2 signaling as well as TLR4 signaling. High concentration (1 µg/ml) of the standard preparation of LPS can induce TLR2 activity, whereas LPS (InvivoGen) activates the TLR4 pathway specifically (0.1 nM).

Western blot visualization kit was obtained from ThermoScientific (Rockford, IL). Nonfat dry milk was purchased from LabScientific (Livingston, NJ). Protein lysis (2X) sample buffer was obtained from Bio-Rad (Hercules, CA). Primary antibodies, acetylated-α-tubulin and anti-TLR9 were ordered from Sigma (St. Louis, MO); anti-TLR4, anti-PC2 and anti-GAPDH were purchased from Santa Cruz Biotechnology.

The secondary antibody fluorescein anti-mouse and mounting media with DAPI were purchased from Vector Laboratories (Burlingame, AL). For immunofluorescence, paraformaldehyde (PFA) from EMS (Hatfield, PA) was used. Sucrose and Triton-X were purchased from Fisher Scientific (Fair Lawn, NJ). For 3D cysts culture, Corning Matrigel matrix was purchased from CORNING
(Corning, NY). For Crisper Cas9, Crisper/Cas9 Knockout Kits were obtained from ORIGENE (Rockville, MD).

2.2. Cell Culture

Porcine renal epithelial cells from proximal tubule (LLCPK-1), *Pkd2*, *TLR4* and *TLR9* knockout cells were cultured as monolayer in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 C in 5% CO₂ incubator. Cells were trypsinized with 0.05% solution of trypsin for passaging. Once differentiated, HCQ, LPS and ODN were added onto culture plates to with final concentrations of 1.0 μM, 1000 nM and 4 μM, respectively. For control experiments, PBS was added to cells in the same manner and volume.

2.3. Cilia length analysis/ Immunofluorescence staining

First, cells were fixed using 4% paraformaldehyde and 2% sucrose in PBS for 10 minutes then wash it with PBS three times for 5 minutes. Second, cells were permeabilized using 10% triton X-100 for 5 minutes and washed with PBS three times for 5 minutes. Third, cells were incubated with primary antibody acetylated-α-tubulin (1:10,000 dilution, Sigma Aldrich, St. Louis, MO) for 24 hours at 37°C. Then wash it with PBS three times for 5 minutes. Forth, cells were incubated with secondary antibody using fluorescein isothiocyanate (TexRed/FITC)-conjugated (1:1000 dilution, Vector Labs Burlingame, CA) for 1 hour at 37°C. Slides were then mounted with DAPI (Southern Biotech, Birmingham, AL). Nikon Eclipse Ti-E inverted microscope with NIS-Elements imaging software (version 4.30) was used to capture images of single cell with their single primary cilium with 100× magnification fields.
2.4 3D cyst culture *in vitro*

This protocol was done accordingly with *Corning* instructions. First, *Corning® Matrigel®* basement membrane matrix was thawed overnight by submerging the vial in ice in a 4°C refrigerator before use. Once Matrigel matrix is thawed, vial was swirled to ensure that the material was dispersed. On Day 0, the Matrigel matrix was diluted to 5 mg/mL with ice-cold complete cell culture medium (DMEM + 10% FBS). Using pre-chilled tips, the pre-chilled 24-well plate was coated by adding 100μL of Matrigel matrix (5mg/mL) into each well, spread evenly with a pipet tip, and incubated at 37°C for 30 min to form gel. Next, the cells were trypsinized from a monolayer to make a single-cell suspension and then pelleted through centrifugation at 125 x g for 5 min at room temperature. After, re-suspend the cells with DMEM medium and adjust the cell density to $5 \times 10^6$ cells/mL. 30 μL prepared cell suspension was added to 270 μL Matrigel matrix solution (5 mg/mL) which was kept on ice for a final cell density is $5 \times 10^5$ cells/mL. The plate was incubated at 37°C for 30 to 45 min. 500 μL DMEM complete media was gently added to glass bottomed 8 well plate. Culture was kept for 12 days and medium was changed every 2 days.

2.5 3D cysts measurements

The cyst growth was observed by using Nikon live cell microscope. The DIC images were captured for every 3 days. Finally, cysts were fixed and immunostained. The fluorescence imaging was done with Nikon confocal microscope to see the cyst formation or morphology.

2.6 Pharmacological agonist/antagonist

Cilia length measurement studies and 3D cysts culture experiments were conducted separately. All the pharmacological agents used for 24 hour treatment. HCQ, LPS and ODN were diluted and prepared for desired concentrations and stored under 4°C until use. table below shows the concentrations and modes of action of the agents (Table 1).
Table 1. The pharmacological agents used in our study along with their final concentrations used. The modes of action for the same are also presented.

<table>
<thead>
<tr>
<th>Agonist/antagonist</th>
<th>Concentration</th>
<th>Mode of actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxychloroquine</td>
<td>1 µM</td>
<td>Inhibit TLR9 and TLR4</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>1 µM</td>
<td>Activate TLR4</td>
</tr>
<tr>
<td>CpG oligodeoxynucleotides</td>
<td>4 µM</td>
<td>Activate TLR9</td>
</tr>
</tbody>
</table>

2.7 Immunoprecipitation

The cell protein lysates were first pre-cleared with the protein A or G agarose beads. Pre-clearing the lysate can help reduce non-specific binding by incubating 20 µl of protein A or G agarose beads (20 µl of 50% bead slurry) for 1 hour. The lysates were incubated with 10 µg of primary antibody for gentle rocking overnight at 4°C, following which 20 µl of protein A or G agarose beads will be added for 1 hour. Beads were collected by centrifugation and the supernatant was be removed. Agarose beads were washed five times in 500 µl of 1X cell lysis buffer supplemented with protease inhibitor to remove any non-specific bonds to protein of interest. The collected beads were resuspended in 20 µl of 2X SDS-PAGE sample buffer and the pelleted protein were stored at -80°C.
Figure 4. A schematic image showing the total immunoprecipitation process for the western blot sampling.

Adapted from: https://www.expedeon.com/resources/applications/immunoprecipitation/

2.8. Immunoblotting

Scrambled, TLR4 KO, TLR9 KO and Pkd2 KO cells were collected by scraping then lysed in lysis buffer and sonicated to isolate the proteins. After centrifugation protein concentrations were measured using Nanodrop instrument. Then protein normalization was done and each sample contain 30 μg of protein within loading buffer. After sample preparation, proteins were loaded on 10% SDS-polyacrylamide gel and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk in TBST buffer and probed overnight at 4°C with primary antibodies with anti-TLR4 (1:200 dilution), anti-TLR9 (0.5 μg/mL), anti-PKD2 (1:400 dilution) and anti-GAPDH (1:400 dilution). For visualization, HRP-conjugated anti-rabbit (1:1000 dilution) used as secondary antibody. The protein bands were visualized and quantified by using Bio-Rad imaging system.
2.9. CRISPR knockout for *TLR4* and *TLR9*

One day prior to transfection, seed approximately $1.0 \times 10^5$-3.0 x $10^5$ cells in each well of a 6-well plate containing 2 mL of complete growth medium. Cells were grown overnight to approximately 90%-95% confluence. To transfect, initially equilibrate DNA with Turbofectin reagent and Opti-MEM® I reduced serum medium to room temperature. 2.5 μg plasmid DNA was diluted with 125 μL Opti-MEM® I. Later, the mixture for 5 minutes was incubated at room temperature. Once the transfection reagent was diluted, it was combined with the DNA within 30 minutes. The diluted DNA was combined with the diluted transfection reagent, which was incubated at room temperature for 5 to 20 minutes to allow DNA-transfection reagent complexes to form. The DNA-transfection reagent complexes were added directly to the well and mix gently by rocking the plate back and forth. The cells were incubated at 37°C in a CO₂ incubator for a total of 24-48 hours.

![Figure 5. Schematic of CRISPR-Cas9 mechanism in gene knockout process.](https://labiotech.eu/features/crispr-cas9-review-gene-editing-tool/)
2.10. Statistical Analysis

All quantifiable data are reported as the mean ± standard error of the mean (SEM). Statistical analysis was performed using ANOVA followed by Bonferroni post hoc test. The statistical analyses were performed with GraphPad Prism (version 7.0 Sample sizes are included in figure legends. “∗” represent statistically significant differences at various probability levels (P). Significance was designated as follows: *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.
CHAPTER 3
RESULTS

3.1. Immunolocalization of Toll-like receptors in primary cilia

We used immunostaining technique to look for functional receptor proteins TLR4 and TLR9 in primary cilia. TLR4 and TLR9 proteins were detected by immunofluorescence analysis of primary cilia in LLCPK-1 cells with monoclonal antibodies against human TLR4 and TLR9 after 24 hours serum starvation. Figure 6 shows the single cell single cilium from the side view and strong staining was observed on the primary cilia for both TLR4 and TLR9 (red). The DIC (brightfield) and nuclear staining (blue) was also observed. In contrast, no staining was found in these cells when tested with the irrelevant (nonspecific) antibody.

![DIC, TLR-4, Merged](image1)

![DIC, TLR-9, Merged](image2)

**Figure 6.** Immunostaining for TLR4 and 9 is shown in middle panel (red), left panel showing the DIC images. Right panel showing the merged image with nuclear stain DAPI (Blue). No counterstain was done for cilia. N=3 for all experiments.
3.2. *TLR4, TLR9* and *PKD2* knockout

To test the effects of TLR on primary cilia structure, we further KO the TLR4 and TLR9 using CRISPR-Cas9. The absence of TLR4 and TLR9 protein expression in LLCPK-1 cells was confirmed by Western blot analysis using anti-TLR4 and TLR9 antibodies (Figure 7). We further knockout the *PKD2* and confirmed by Western blot (Figure 7).

**Figure 7.** Representative Western blot data shows that TLR4 and TLR9 expressions are reduced in our TLR KO cells compared to control and *Pkd2* KO cells. In the third row of image PC2 knockout was also confirmed. GAPDH used as a Western blot validation control. N=3 samples per group in each study.

3.3. Effects of hydroxychloroquine on cilia length

Figure 8 shows the increase in cilia length when we used different concentrations of HCQ in different cells. For this experiment we used different cell lines, i.e., control cells (vehicle), TLR4 KO, TLR9 KO and Pkd2 KO cells. From the results it is observed that the very long cilia (10-15 µm) were grown when scrambled and *Pkd2* KO cells treated with different concentrations.
However, little increase in cilia length (7 µm) was observed when the TLR4 KO cells treated with HCQ. The TLR9 KO cells treatment with HCQ showing the decrease in cilia length (3 µm) when increasing the HCQ concentrations. In 2003 a novel mechanism was described that HCQ inhibits stimulation of the TLR family receptors (Takeda, Kaisho, & Akira, 2003). Our results suggest that this inhibition may cause the decrease in cilia length which has never been studied before. Statistical analysis showed significant differences in cilia length between control and HCQ-treatment in both normal and KO cells. All independent cilia length measurement data were also shown (Figure 9); this distribution indicates the variability between cells and their cilia length.
**Figure 8.** Representative immunofluorescence images show that effects of 4-hydroxychloroquine at different concentrations (0 to 1 µM) on the cilia length. Green indicates cilia labeled to acetylated-α-tubulin (FITC) and blue indicates cell nucleus (DAPI). Representative bar graphs indicate the measurements of 50 cell on cilia lengths. The significant differences between no treatment and each concentration of 4-hydroxychloroquine treatment were shown. Acquired from 5 preparations in each group; a minimum of 10 cilia were randomly selected from each preparation. ***, p<0.01; ****, p<0.001; and *****, p<0.0001 compared with the control group.

**Figure 9.** Length of primary cilia is altered after 4-hydroxychloroquine treatment. After treatment with increasing concentrations of 4-hydroxychloroquine the length of primary cilia was measured from immunofluorescence slides. Length measurements were made from images taken at one single plane in triplicate. Cilia length was grouped in a discrete range, and the % distribution was represented in histograms for each 4-hydroxychloroquine concentration. The cilia length
measurements were shown on X-axis and the % distribution of cilia lengths was shown on Y-axis. N=50 cilia in each study.

3.4. Treatment of lipopolysaccharide and cilia length effect

Figure 10 shows the immunofluorescence images of cilia lengths with the treatments of different concentrations (0 to 1000 nM) of LPS. LPS decreased primary cilia length in control, TLR4 KO and Pkd2 KO cells, but had an opposite effect in TLR9 KO cells. Unfortunately, there were no previous reports available on the localization of TLR4 or TLR9 receptors on primary cilia and their effects on cilia (structural effects). Figure 11 shows the histograms for cilia lengths when different cells were treated with different concentrations of LPS. A significant increase in cilia length was observed when the TLR9 KO cells treated with LPS, whereas decreasing cilia length was observed when the control, TLR4 KO and Pkd2 KO cells treated with LPS in a similar fashion. Our results are in consistent with the previous results (Baek et al., 2017). Statistical analysis showed significant differences in cilia length between control vs. HCQ-treatment in both normal and KO cells.
Figure 10. Representative immunofluorescence images show that different concentrations (0 to 1000 nM) of lipopolysaccharide (LPS) effects on the cilia length. Green indicates cilia labeled to acetylated-α-tubulin (FITC) and blue indicates cell nucleus (DAPI). Representative bar graphs indicate the measurements of 50 cell cilia lengths. The significant differences between no treatment and LPS treatment at different concentrations were shown. Acquired from 5 preparations in each group; a minimum of 10 cilia were randomly selected from each preparation. *, p<0.05; **, p<0.01; ***, p<0.001; and ****, p<0.0001 compared with the control group.

Figure 11. Length of primary cilia is altered after treatment with lipopolysaccharide (LPS) treatment. After treatment with increasing concentrations of LPS the length of primary cilia was measured from immunofluorescence slides. Length measurements were made from images taken at one single plane in triplicate. Cilia length was grouped in a discrete range, and % distribution was represented in histograms for each LPS concentration. The cilia length measurements were shown on X-axis and the % distribution of cilia lengths was shown on Y-axis. N=50 cilia in each study.
3.5 Treatment of CpG oligodeoxynucleotides and cilia length effect

After treatment with 0 to 5 μM ODN, ciliary length was measured through immunofluorescence staining (Figure 12). Compared to control, TLR4 KO and Pkd2 KO cell treatment with ODN, cilia length was increased in TLR9 KO cells. In TLR9 KO cells, the cilia length was increased up to 10 μm compared with control, TLR4 KO and Pkd2 KO cells (3 μm). Our knockout experiments suggest that a significant difference in cilia length was observed in TLR9 KO cells compared with control scrambled cells when treated with ODN. Figure 13 shows the histograms of cilia lengths when different cells treated with different concentrations of ODN. Statistical analysis showed significant differences in cilia length between control vs. ODN-treatment in both normal and KO cells.
**Figure 12.** Representative immunofluorescence images show that effects of ODN D-SL03 (ODN) at different concentrations (0 to 5 μM) on the cilia length. Green indicates cilia labeled to acetylated-α-tubulin (FITC) and blue indicates cell nucleus (DAPI). Representative bar graphs indicate the measurements of 50 cell cilia lengths. The significant differences between no treatment and ODN treatment at different concentrations were shown. Acquired from 5 preparations in each group; a minimum of 10 cilia were randomly selected from each preparation. *, p<0.05; **, p<0.01; ***, p<0.001; and ****, p<0.0001 compared with the control group.

**Figure 13.** Length of primary cilia is altered after treatment with ODN D-SL03 (ODN) treatment. After treatment with increasing concentrations of ODN the length of primary cilia was measured from immunofluorescence slides. Length measurements were made from images taken at one single plane in triplicate. Cilia length was grouped in a discrete range, and percent distribution was
represented in histograms for each ODN concentration. The cilia length measurements were shown on X-axis and the % distribution of cilia lengths were shown on Y-axis. N=50 cilia in each study.

3.6 Effects of TLR4, TLR9 and PKD2 KO on the formation of cyst structure in 3D culture
The LLCPK-1 cell line has not generally been used in 3D tissue models due to its inability to reliably form large numbers of cysts over long periods of time. In one case, isolation of a specific clone was required to facilitate cyst formation in collagen gels (Wohlwend, Montesano, Vassalli, & Orci, 1985). Therefore, to use the LLCPK-1 and their KO model cell lines to consistently induce cyst growth in 3D, we used hormonal media composition previously shown to produce structural growth of immortalized human renal proximal tubule cells and primary human cortical cells in 3D tissues (DesRochers, Suter, Roth, & Kaplan, 2013) (Neufeld et al., 1992).
To observe if the scrambled, TLR4 or TLR9 KO and their antagonists/agonists can play any role in the cyst formation, cyst growth/progression was evaluated with 3D cyst culture assays. Figure 14 shows that the representative DIC images of cyst culture with different cell types for 3, 6, 9 and 12 days. From the images we can clearly observe that the scrambled/control cells treated with scrambled shown no cyst formation. On the other hand, with the PKD2, TLR4 and TLR9 KO cells a clear cyst formation and progression was observed in a time dependent manner. In Another case, the scrambled cyst cultures treated with PBS did not show any sign of cyst formation. The immunofluorescence results indicate a clear lumen formation in consistent with the brightfield imaging results.
Figure 14. Representative 3D cyst DIC images showing cyst formation in Matrigel in basal medium at different days of 3, 6, 9 and 12 days in different cell systems (scrambled, TLR4 KO, TLR9 KO and Pkd2 KO) when treated with PBS. The representative immunofluorescence images showing that the lumen formation as cyst growth increasing. Green indicates both apical and basolateral membranes. Blue -nucleus (DAPI).

3.7 Effects of hydroxychloroquine on the formation of cyst structure in 3D culture

To observe if and how the HCQ (1 μM) affected cilia length in Pkd2, TLR4 or TLR9 KO cells and further leads to the cyst formation, cyst growth/progression was evaluated with 3D cyst culture assays. Figure 15 shows that the representative DIC images for cyst culture in different cell types for 3, 6, 9 and 12 days. From the images we can observe a surprising result that the all cyst cultures showing the formation of small to medium range cyst when treated with HCQ except TLR4 KO cultures. The immunofluorescence results are also in consistent with the brightfield imaging results.
Representative 3D cyst DIC images showing cyst formation in Matrigel in basal medium at different days of 3, 6, 9 and 12 days in different cell systems (Scrambled, TLR4 KO, TLR9 KO and Pkd2 KO) when treated with 4-hydroxychloroquine (1 μM). The representative immunofluorescence images showing that the lumen formation as cyst growth increasing. Green indicates both apical and basolateral membranes. Blue -nucleus (DAPI).

### Figure 16

3.8 Effects of lipopolysaccharide on the formation of cyst structure in 3D culture

To observe if the LPS (1 nM), affected cilia length in Pkd2, TLR4 or TLR9 KO cells and further leads to the cyst formation, cyst growth/progression was evaluated with 3D cyst culture assays.

Figure 16 shows that the representative DIC images of cyst culture with different cell types for 3, 6, 9 and 12 days. Figure 16 indicates that there was no cyst formation in the TLR4 cultures and treatment with LPS, whereas a clear cyst formation and progressions were observed in the scrambled and Pkd2 cyst cultures. This cyst formations were confirmed by immunostaining of the
3D cysts, where the larger cysts showed a clear lumen formation apparently by dead cells and partial hollowing out of the center of the cysts. The cyst formation results were consistent with our cilia length measurements, i.e. cilia lengths are inversely proportional to cyst sizes.

![Figure 16](image)

**Figure 16.** Representative 3D cyst DIC images showing cyst formation in Matrigel in basal medium at different days of 3, 6, 9 and 12 days in different cell systems (scrambled, TLR4 KO, TLR9 KO and Pkd2 KO) when treated with lipopolysaccharide LPS (1000 nM). The representative immunofluorescence images showing that the lumen formation as cyst growth increasing. Green indicates both apical and basolateral membranes. Blue -nucleus (DAPI).

### 3.9 Effects of CpG oligodeoxynucleotides on the formation of cyst structure in 3D culture

To observe if the ODN (4 µM) effect, TLR9 KO, TLR4 KO and Pkd2 KO cells can play any role in the cyst formation, cyst growth/progression was evaluated with 3D cyst culture assays. Figure
17 shows that the representative DIC images of cyst culture with different cell types for 3, 6, 9 and 12 days. Figure 17 indicates that there was no cyst formation in the TLR9 cultures and treatment with LPS, whereas a clear cyst formation and progressions were observed with the scrambled, TLR4 and Pkd2 cyst cultures. This cyst formations were also confirmed by immunostaining of the 3D cysts, where the larger cysts showed a clear lumen formation apparently by dead cells and partial hollowing out of the center of the cysts. The cyst formation results were consistent with our cilia length measurements with TLR4 KO and TLR9 KO cells.

![DIC images of cyst culture](image)

**Figure 17.** Representative 3D cyst DIC images showing cyst formation in Matrigel in basal medium at different days of 3, 6, 9 and 12 days in different cell systems (Scrambled, TLR4 KO, TLR9 KO and Pkd2 KO) when treated with ODN D-SL03 ODN (4 μM). The representative immunofluorescence images showing that the lumen formation as cyst growth increasing. Green indicates both apical and basolateral membranes. Blue -nucleus (DAPI).
In all our cyst culture experiments, a consistent 50 numbers of cysts were taken in to consideration for the cyst size and progression measurements. The representative dot plot data (Figure 18) shows that the measurements of 50 individual cysts for each independent group. We evaluated the cyst progression for 3, 6, 9 and 12 day’s time periods. The cysts of all three KO lines (TLR4, TLR9 and PKD2) remained larger than the scrambled control (LLCPK-1) for the remainder of the time course. This relative increase in cyst size was due to both an actual change in dead cell count/lumen formation for each knockdown cell line, and to the fact that the cysts of the scrambled control did not change in size after 12 days in 3D culture. The TLR4 and TLR9 KO cell lines showed reduced cyst growth from 3 to 12 days when treated with LPS and ODN, respectively.

**Figure 18.** The representative dot plots showing the mean values of cyst diameters at 3, 6, 9 and 12 days for different cell systems (Scrambled, TLR4 KO, TLR 9 KO and Pkd2 KO) and also with
different treatments. N=50 measurements of cysts in each study. *, p<0.05; **, p<0.01; ***, p<0.001; and ****, p<0.0001 compared with the control group.

Figure 19 shows the cyst progression over time period. Throughout the experiment, scrambled cells treated with agonists/antagonists had the most dramatic increase in both cyst size and rate of growth, whereas TLR4 and TLR9 KO cells treated with their corresponding agonists showed reduced cyst growth and number over time. Taken together, these data indicate that loss of TLR4, 9 or PC2 leads to increased cyst size over time and that loss of TLR9 has the most significant impact upon cyst size.

**Figure 19.** The representative line graphs showing the mean values of cyst diameters for 3 to 12 days when Scrambled, TLR4 KO, TLR9 KO and Pkd2 KO cells treated with phosphate buffered
saline, lipopolysaccharide, ODN D-SL03 or 4-hydroxychloroquine. N=50 measurements of cysts in each study. **, p<0.01; ***, p<0.001; and ****, p<0.0001 compared to the control group.
CHAPTER 4
DISCUSSION & CONCLUSIONS

Toll-like receptors (TLRs) constitute the body's primary defense system because they detect and rapidly respond to foreign substances or endogenous signals and initiate inflammatory cascades. This study, for the first time, was undertaken to further understand the potential role of TLRs localization in primary cilia and their role in PKD. The role of TLRs in innate immunity and their ability to recognize microbial products have been well characterized. TLRs are also able to recognize endogenous molecules which are released upon cell damage and necrosis and have been shown to be present in numerous autoimmune diseases. Therefore, the release of endogenous TLR ligands during inflammation and consequently the activation of TLR signaling pathways may be the mechanism initiating and driving autoimmune diseases (Drexler & Foxwell, 2010). The main function of TLRs is considered to be the recognition and response to microbial pathogens. More recently, however, they have also been reported to recognize endogenous ligands, so called “danger signals”, which are released during tissue damage, infections and cell necrosis (Anderson, Jurgens, & Nusslein-Volhard, 1985).

The primary cilium is a cellular organelle present in the majority of mammalian cell types where they function as coordinating centers for mechanotransduction, Wnt and hedgehog signaling. The length of the primary cilium is proposed to modulate cilia function, governed in part by the activity of intraflagellar transport. For example, in articular cartilage, primary cilia length is increased and hedgehog signaling activated in osteoarthritis (Wann & Knight, 2012). In this study, we present the effect of TLRs agonists/antagonist for the first time, on the potentially fundamental role of IFT-mediated primary cilia elongation in the progression of inflammation. Our immunostaining showed that, the two major TLRs, i.e. TLR4 and TLR9 were identified in the primary cilia;
however, TLR4 showed stronger expression than TLR9 when we did immunostaining and Western blotting. In accordance with our results, TLRs have been shown to be expressed throughout the cell including cilia. Here, we examine primary cilia length with exposure to TLRs inflammatory antagonists and agonist substances. Observations of renal epithelial control (vehicle) cells, TLR4 KO and Pkd2 KO cells when treated with HCQ the cilia length was increased except for TLR9 KO cells. We speculate that the HCQ mainly affect TLR9 KO cell inflammatory action than TLR4 KO. When we use specific agonists for TLR4 and TLR9 the cilia lengths were changed significantly. The use of LPS as an agonist for TLR4 KO cells the cilia length was decreased significantly, whereas when we used ODN as agonist for TLR 9 KO cells the cilia length was increased significantly. This indicates that TLR 9 plays a major role in cilia length control.

We show that this phenomenon of cilia elongation causes decreased cyst formation in response to the inflammatory action is clearly visible. Our results clearly disclose that cilia length is inversely proportional to the cyst formations. When we used pharmacological agents or genetic approach to increase cilia length the cyst formation or growth was significantly reduced, whereas shorter cilia promote cysts. TLRs and inflammations are taken into high consideration because, under normal circumstances inflammatory reaction is tightly controlled to restrict cell growth until infections are resolved and tissue recovery process completed. In contrast, chronic inflammation establishes an environment rich in inflammatory cells, which produce growth factors. In recent studies, specific inflammatory conditions have been associated with certain cancers (Balkwill & Coussens, 2004) (Balkwill & Mantovani, 2001) which is more dangerous. On the other hand, constant intake of anti-inflammatory drugs can reduce the risk of developing certain types of cancer. Numerous
reports have also described an association of certain TLRs with the risk of developing certain cancers (S. L. Zheng et al., 2004).

Several researchers hypothesized that TLR involvement in tissue repair as well as inflammation is supported by the increasing amount of evidence for endogenous TLR ligands (Rifkin, Leadbetter, Busconi, Viglianti, & Marshak-Rothstein, 2005) (Brentano, Kyburz, Schorr, Gay, & Gay, 2005). However, further studies are necessary to investigate possible endogenous targets on primary cilia for TLRs. The recognition of so called “danger signals”, released following injury and cell necrosis, would enable TLRs to survey their environment and induce an inflammatory response in case of infection as well as a proliferative response subsequent to injury (Jiang et al., 2005).

ADPKD is one of the hereditary and progressive systemic disorders that results in end stage renal disorder (Gabow, 1993). Due to its systemic and genetic pattern, many studies were conducted on the early stage of the disease to find measurements for halting the disease growth (Santoro et al., 2015) (Chapman, 2008). Through recent studies it is understood that the systemic inflammation is evident in early phase ADPKD patients and this inflammatory action may contribute to both heart and kidney function deterioration (Menon et al., 2011) (Kocyigit et al., 2012) (Swenson-Fields et al., 2013). It is thought that the activation of systemic and local immune responses contributes to renal dysfunction, but the mechanisms of activation are poorly understood.

Primary cilia have been proposed as a link between the development of kidney cysts and the polycystin proteins. To assess the downstream functionality of cilia elongation in an inflammatory context, we employed a CRISPR approach to knockout TLR4 and TLR9 whilst assessing agonists/antagonists stimulated cyst formation (3D cyst cultures) classic inflammatory results of PKD in many cell types. When we used LPS and ODN in TLR4 KO and TLR9 KO cells, respectively no cyst formation/progression was observed. This may explain the downstream
inflammatory signaling. This is also consistent with our cilia length increase and shows the potential proof between cilia length and cyst formation through downward inflammatory response. These results are in consistent with the previous results (Baek et al., 2017), in this TLR4 mediated inflammatory response in hippocampal neurons were reported. In contrast, the cilia length was decreased and surprisingly the cyst size also decreased in TLR4 KO cells with treatment of HCQ, it tells us another independent mechanism is responsible for this action.

4.1 Conclusions

In summary, our study provides new understanding about TLR on cilia length and cyst formation. TLR4 and TLR9 gene expression may be associated with rapid progression in our 3D cyst culture studies. We find that the TLRs agonists/antagonists can modulate cilia length and their role in inflammatory actions. The primary cilium already has central roles throughout cell biology, but here we propose, for the first time, that the cilium and the regulation of its structure are of fundamental importance in inflammation of ADPKD. Further studies are needed to investigate the potential role of ciliary TLRs via inflammation and immune pathways in ADPKD animal models.


