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Toll-like receptors 4, 5, 6 and 7 are constitutively expressed in non-human primate retinal neurons

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ABSTRACT

The purpose of this study was to characterize cell-specific expression patterns of Toll-like receptors (TLR) in non-human primate (NHP) neural retina tissue. TLR 4, 5, 6, and 7 proteins were detected by immunblotting of macaque retina tissue lysates and quantitative PCR (qPCR) demonstrated TLRs 4–7 mRNA expression. Immunofluorescence (IF) microscopy detected TLRs 4–7 in multiple cell types in macaque neural retina including Muller, retinal ganglion cells (RGC), amacrine, and bipolar cells. These results demonstrate that TLRs 4–7 are constitutively expressed by neurons in the NHP retina raising the possibility that these cells could be involved in retinal innate inflammatory responses.

1. Introduction

Although the eye is considered an immunoprivileged site, this privilege can clearly be broken (Chang et al., 2004; Chang et al., 2006). Ocular inflammation can be the consequence of an immune response to an infectious agent, result from allergic or autoimmune responses, or be idiopathic (Chang et al., 2004; Chang et al., 2006). The resulting inflammation can affect any part of the eye and may threaten sight. Microbial infections of the eye, including herpes simplex virus keratitis, cytomegalovirus retinitis, bacterial keratitis, and bacterial endophthalmitis are quite common (Chang et al., 2004; Chang et al., 2006). In addition, viral gene delivery vectors can trigger ocular inflammation which may compromise transgene expression and preclude further vector administration (Bennett, 2003). Posterior uveitis, the inflammation of the posterior uveal tract (retina and choroid), can have infectious origins, which is more common in developing countries, non-infectious origins, or be part of a masquerade syndrome (Lee et al., 2017; Tsirouki et al., 2016). Acute retinal necrosis can be caused by herpes simplex virus or varicella zoster virus and viral retinitis can also occur as a consequence of intraocular corticosteroid injections (Lee et al., 2017).

Innate immune responses, which occur very early in inflammation, play an important role in direct anti-microbial responses, inducing inflammation, and modulating the adaptive immune response (Chang et al., 2004; Chang et al., 2006). One of the key groups of innate immune receptors that recognize and respond to microbes are the Toll-like receptors (TLRs), which are constitutively expressed in multiple cells types and up-regulated in inflammatory conditions (Akira et al., 2001). There are currently ten known mammalian TLRs which recognize conserved structural moieties called pathogen-associated molecular patterns (PAMPs) (Bowie and Haga, 2005; Pandey et al., 2013). PAMPs recognized by TLRs include microbial components such as lipoproteins (TLR1, TLR2, TLR6), glycoproteins (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5), zymosan (TLR6), and viral and bacterial nucleic acids (TLR3, TLR7, TLR8, TLR9) (Pandey et al., 2013). The ligand specificity and function of TLR10 is still unknown, but its main function may be modulation of the inflammatory response (Oosting et al., 2014).

In addition, molecules released from damaged cells, including damage associated molecular patterns (DAMPs), can activate pattern recognition receptors (PRRs), including TLRs, and exacerbate the inflammatory response (Wakefield et al., 2010). The recognition of PAMPs or DAMPs by TLRs initiates signal transduction pathways, involving the transcription factors NF-κB or IRF-3, leading to altered gene expression and the induction of inflammatory cytokines (Arancibia

Abbreviations: TLR, Toll-like receptor; NHP, Non-human primate; IF, immunofluorescence; qPCR, quantitative polymerase chain reaction; PAMPs, pathogen-associated molecular patterns; NF-κB, nuclear factor kappa-beta; IL, Interleukin; RPE, retina pigment epithelium; PBS, phosphate buffered saline; HRP, horseradish peroxidase; FBS, fetal bovine serum; RT, room temperature; ON, over night; INL, inner nuclear layer; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; PR, photoreceptors; RGC, retinal ganglion cells; GFAP, glial fibrillary acidic protein; RBPMS, RNA binding protein with multiple splicing; PKC-α, protein kinase C alpha; mAbs, monoclonal antibodies; RNA, ribonucleic acid; cDNA, complementary DNA; EDTA, Ethylenediaminetetraacetic acid

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et al., 2007). Alterations in cytokine expression, including IL-1, IL-6, IL-17, and TNF have been associated with uveitis, which suggests the innate immune response may trigger the signaling events leading to ocular inflammation (Schwartzman, 2016).

Previous studies, using human retinal pigment epithelial (RPE) cell cultures, have detected expression of TLRs 1–7, 9, and 10 (Kumar et al., 2004), linked TLR3 signaling to an increase in inflammatory cytokine production (Brosig et al., 2015), and demonstrated that exposure to pro-inflammatory cytokines decreased the expression of genes critical for RPE function (Kutty et al., 2016). Recently, the expression pattern of TLRs in human retinal and choroidal vascular endothelial cells has been studied (Stewart et al., 2015). Muller glial cells have received the most attention to date, and have been shown to play a role in inflammatory responses during autoimmune uveitis (Hauck et al., 2007) and bacterial infection (Kumar et al., 2013; Kumar et al., 2010; Shamsuddin and Kumar, 2011; Singh et al., 2014). The human retinal Muller cell line MIO-M1 expresses TLRs 1–10 and TLR agonist or live pathogen challenge produces an increase in inflammatory mediators (Kumar and Shamsuddin, 2012). Murine Muller cells (Kumar and Shamsuddin, 2012; Lin et al., 2013) and a murine photoreceptor cell line (Singh and Kumar, 2015) also express TLRs and initiate innate responses following TLR ligand challenge (Gao et al., 2017; Singh and Kumar, 2015).

Studies with human ocular tissue identified TLR4 expression in iris/ciliary body, choroid, retina, sclera, and conjunctiva, however, TLR4 protein was only detected in uveal antigen presenting cells (Chang et al., 2004). Corneas from healthy patients expressed TLRs 1–10 mRNA and, following herpes simplex virus infection, the expression of all TLRs was upregulated (Jin et al., 2007). A direct correlation between TLR activation, cytokine production, and anterior uveitis has been established in mice (Allensworth et al., 2011). In addition, TLR3 activation has been linked to inflammation and photoreceptor cell death in the mouse model of dry age-related macular degeneration (Gao et al., 2017). There is a lack of data, however, on TLR expression in the non-human primate neural retina.

The importance of TLR signaling in viral gene delivery vector induced ocular inflammation cannot be overlooked. Secretion of IL-6 was detected following herpes simplex virus vector transduction of RPE cells (Cai and Brandt, 2008). Recent studies demonstrated that cytokines, including IL-6 and IL-10, as well as TLRs 6 and 7, were upregulated, following herpes simplex vector challenge of non-human primate neural retina tissue (Sauter and Brandt, 2016). To date, a detailed examination of which TLRs are expressed in NHP neural retina, and the cell types expressing them, has not been done. This study includes data only for TLRs 4, 5, 6, and 7, because we were able to source antibodies which recognized single bands of the appropriate size by immunoblotting of macaque retina lysates for these receptors.

2. Materials and methods

2.1. Macaque retina tissue

Eyes from euthanized rhesus macaques (*Macaca mulatta),* or cynomolgus macaques (*Macaca fascicularis*), were obtained as they became available from the Wisconsin National Primate Research Center of the University of Wisconsin-Madison or Covance (Madison, WI). Animals were free of infectious agents at the time of sacrifice. No animals were deliberately sacrificed for these studies. Macaque eyes were kept on ice and dissected within one hour of sacrifice. Posterior eye cups were incubated with PBS/1 mM EDTA for 30 min at 37 °C to loosen neural retina tissue and separate the retina from RPE cells. Neural retina tissue was rinsed in PBS before proceeding with further studies. All experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Table 1
Quantitative PCR primers for macaque Toll-like receptors.

Gene	Primer Sequence	Product Size
TLR4[a]	5′ TGGATACGTTTCCTTATAAG 3′ 5′ GAAATGGAGGCACCCCTTC 3′	507 bp
TLR5	5′ ATTGCGTGTACCCTGACTCG 3′ 5′ TTGAACACCAGTCTCTGGGC 3′	214 bp
TLR6	Qiagen RT^2 qPCR primer assay PPQ00238B	92 bp
TLR7[a]	5′ TCTACCTGGGCCAAAACTGTT 3′ 5′ GGCACATGCTGAAGAGAGTTA 3′	388 bp

[a] Indicates that primer sequence was taken from human TLR primers designed by Kumar and Shamsuddin (Kumar and Shamsuddin, 2012).

2.2. RNA isolation

Macaque neural retina tissue was rinsed in PBS prior to homogenization and RNA isolation using the TRIzol Reagent protocol (Ambion/Life Technologies, Grand Island, NY, #15596-026). DNase digestion (Qiagen, Valencia, CA, RNase-Free DNase Set, #79254) was completed prior to RNA cleanup on RNeasy spin columns (Qiagen, RNeasy Mini Kit, #74104). RNA was eluted in RNase-free H_2O and quantitated on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, #ND-1000).

2.3. Quantitative PCR

PCR primers were designed for macaque TLR5 (Table 1). TLRs 4 and 7 primers were synthesized based on previously published human TLR primers (Table 1) (Kumar and Shamsuddin, 2012). A commercial primer pair was utilized for TLR6 (RT^2 qPCR primer assay, Qiagen, PPQ002388). cDNA from cynomolgus and rhesus macaques was amplified with primers to TLRs 4, 5, 6 or 7 following the standard Qiagen RT^2 qPCR primer assay protocol using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, #330520) and an ABI 7300 cycler. The annealing temperature was reduced to 55 °C for the TLR4 and TLR7 reactions. qPCR reactions were performed in triplicate and mean C_T values were plotted versus the negative control (no primers) and positive control (β-actin primers, Qiagen, PPQ00182A).

2.4. Immunoblotting

Lysates were prepared from macaque neural retina tissue immediately post-sacrifice following the method of Gerhardinger et al. (2001). The protein concentration was determined by Pierce BCA assay (Thermo Scientific, Rockford, IL #23225). Lysates were run on 4–15% Mini-PROTEAN TGX precast gels (BIORAD, Hercules, CA 4568084S) with BenchMark Pre-stained Protein Standard (Invitrogen, Carlsbad, CA 10748-010) as a molecular weight marker (MWM). Proteins were electrophorectically transferred to nitrocellulose prior to blocking with 5% non-fat dry milk in Genius Buffer I (100 mM maleic acid, 150 mM NaCl, pH 7.5) containing 0.3% v/v Tween-20. The primary antibodies were diluted in 5% non-fat dry milk in Genius Buffer I and incubated as noted in Table 2, followed by washing in Genius Buffer I containing 0.3% v/v Tween-20. HRP-conjugated secondary antibodies were diluted 1:5000 and applied for 1 h at RT. After washing, the blots were developed using WesternSure PREMIUM chemilluminescent Substrate (LI-COR, Lincoln, NE, 926-95000).

2.5. Immunofluorescence

Neural retina tissue obtained from euthanized macaques was rinsed in PBS and fixed in 10% neutral buffered formalin (Thermo Fisher, Kalamazoo, MI, #305-510) for 24 h before paraffin embedding and sectioning. Tissue sections were de-paraffinized and antigen retrieval