Aus der Medizinischen Klinik und Poliklinik IV
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Martin Reincke

ACTIVATION OF INNATE IMMUNE DEFENSE MECHANISMS CONTRIBUTES TO POLYOMAVIRUS BK-ASSOCIATED NEPHROPATHY

Dissertation zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Andrea Ribeiro
Arcos de Valdevez, Portugal
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Declaration

I here by declare that the present work embodied in this thesis was carried out by me under the supervision of PD Dr. med. Markus Wörnle, Internist-Nephrology, Medizinische Klinik und Poliklinik IV der Universität München. This work has not been submitted in part or fully to any other university or institute for any degree or diploma.

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Dedicated to …

My mother Ilda, my father Henrique, my brother Miguel and my husband Luis

…because you are my World

Contents

Zusammenfassung

Die Polyomavirus-assoziierte Nephropathie (PVAN) ist eine bedeutsame Ursache des Organsversagens nach Nierentransplantation und betrifft bis zu 10% der Patienten nach Nierentransplantation. Die meisten Fälle werden durch das BK Virus (BKV) im Rahmen der immunsuppresiven Therapie ausgelöst.

Das angeborene Immunsystem spielt eine wichtige Rolle bei der Erkennung viraler Infektionen und der Initiierung antiviraler Immunantworten. Nukleotidsequenzen viraler RNA werden pathogen- und zelltypspezifisch durch eine Vielzahl von molekularen Mustererkennungsrezeptoren, den Pattern-Recognition Receptors (PRPs) detektiert. Die Erkennung viraler RNA führt zu einer Aktivierung einer Signaltransduktionskaskade mit Aktivierung von NF-κB und der Synthese von antiviralen Mediatoren wie Typ I Interferonen und inflammatorischen Zytokinen.

Um die antiviralen Antworten des renalen tubulären Epithels zu untersuchen, analysierten wir die mögliche Beteiligung der viralen dsRNA Rezeptoren Toll-like Rezeptor 3 (TLR3) und retinoic acid inducible gene-I (RIG-I) in Transplantnierenbiopsien von Patienten mit einer PVAN, in humanen Sammelrohrepithelien (HCDCs) in Kultur nach Stimulation mit polyriboinosinic: polyribocytidylic acid (poly(I:C)), einem synthetischen Analogon viraler RNA sowie in einem Polyomavirus BK (BKV) Infektionsmodell. Die Immunfluoreszenzdoppelfärbung für BKV und TLR3 zeigte ein starkes Signal in Epithelien der distalen kortikalen Tubuli und Sammelrohre. In mikrodisseziierten tubulointerstitiellen Kompartimenten von Patienten mit PVAN war die mRNA Expression von TLR3 erhöht, aber nicht die Expression von RIG-I. Zusätzlich zeigten HCDCs eine intrazelluläre Expression von TLR3; eine Aktivierung von TLR3 und RIG-I durch poly(I:C)

führte zu einer erhöhten mRNA Expression von Zytokinen, Chemokinen und IFN-β. Diese Immunantwort konnte spezifisch durch siRNA für TLR3 geblockt werden.

Zusammenfassend führt eine Infektion von HCDCs mit BKV zu einer gesteigerten Expression von Zytokinen und Chemokinen, was schließlich zu einer effizienten antiviralen Immunantwort mit Hochregulation von TLR3 und RIG-I ohne Aktivierung von IL-1β oder von Komponenten des Inflammasom-Weges führt.

Die PVAN führt somit zu einer antiviralen Immunantwort mit Aktivierung des angeborenen Immunsystems und einer vermehrten Expression von inflammatorischen Zytokinen, Chemokinen und Typ I Interferonen.

Summary

Polyomavirus-associated nephropathy (PVAN) is an emerging cause of significant kidney transplant dysfunction affecting up to 10% of patients, often leading to graft loss. Most cases of PVAN are elicited by BK virus (BKV) in the context of intense immunosuppression.

The innate immune system plays a critical role in recognizing viral infections and evoking initial antiviral responses. Nucleotides from viral RNA are recognized in a pathogen-type- and cell-type-specific manner by a variety of pattern recognition receptors (PRRs). Recognition of viral RNA triggers downstream signaling cascades leading to activation of NF-κB and induces antiviral mediators such as type I interferons (IFNs) and proinflammatory cytokines.

In order to identify antiviral responses of the renal tubular epithelium, we analysed the potential activation of the viral dsRNA recognition receptors Toll-like receptor 3 (TLR3) and retinoic acid inducible gene-I (RIG-I) in allograft biopsy samples of patients with PVAN, in cultured human collecting duct cells (HCDCs) after stimulation by the dsRNA mimic polyriboinosinic:polyribocytidylic acid (poly(I:C)) and in a model of polyomavirus BK (BKV) infection. Immunofluorescence doublestaining for BKV and TLR3 revealed strong signals in epithelial cells of distal cortical tubules and the collecting duct. In microdissected tubulointerstitial specimen, TLR3, but not RIG-I mRNA expression, was increased in PVAN. In addition, HCDCs expressed TLR3 intracellularly and activation of TLR3 and RIG-I by poly(I:C) enhanced the mRNA expression of cytokines, chemokines and IFN-β. This inflammatory response could be specifically blocked by siRNA to TLR3. Finally, the infection of HCDCs with BKV enhanced the expression of cytokines

and chemokines, leading to an efficient antiviral immune response with TLR3 and RIG-I upregulation without activation of IL-1β or components of the inflammasome pathway.

Thus, we concluded that in PVAN, activation of innate immune defense mechanisms is involved in the antiviral and anti-inflammatory response leading to the expression of proinflammatory cytokines, chemokines and type I IFNs.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Innate immune system

The immune system in mammals can be divided in two branches: innate immunity which is phylogenetically conserved and is present in almost all multicellular organisms (1) and adaptive immunity which detects non-self through recognition of peptide antigens using antigen receptors expressed on the surface of B and T cells and also affords protection against re-exposure to the same pathogen (2). The response initially taking place is the innate immune response, which is responsible for initiating inflammatory and immune processes to resolve infections and repair injured tissues.

The innate immune system is an evolutionally conserved mechanism that plays a critical role in recognizing and eliminating invading pathogenic microorganisms by discriminating between self and non-self. It can be divided in three classes: (i) local recognition of infection and injury, which potentiates inflammatory responses and the initiation of innate immunity; (ii) systemic activation of innate immunity by infection or injury and release of inflammatory mediators; (iii) homeostatic interactions between innate immune system and environmental stimuli (3).

Due to their molecular heterogeneity and rapid evolution, the reliable detection of pathogens is a hard task.

1.1.1. Cells of the innate immune system

The cells involved in the innate immune system include natural killers (NKs), dendritic cells (DCs), macrophages, γ/δT lymphocytes, neutrophils, basophils, eosinophils, and B-1 cells (4, 5). These cells express a set of germline encoded pattern recognition receptors (PRRs) that are able to recognize conserved molecular patterns associated with microbial pathogens (6). These receptors sense the pathogen and induce a rapid inflammatory response by the production of proinflammatory cytokines and chemokines, stimulating the killing of infected or transformed cells, or stimulating phagocytosis or apoptosis. They can also upregulate expression of co-stimulatory molecules, subsequently initiating the adaptive immunity.

DCs and macrophages, in addition to their role in innate immunity, also act as a link to the adaptive immune system. The activation of these cells is triggered by the PRR signals that convert them in potent antigen-presenting cells (APCs) capable of promoting the expansion and effector differentiation of T cells by the presentation of antigens (5, 7).

However, specialized immune cells are often not the first cells to encounter pathogens. The epithelium, being at the interface between the internal and external environment, constitutes the primary cellular barrier. Epithelial cells (ECs) possess pathogen-recognition mechanisms that enable them to respond to microorganisms by the expression of PRRs, thereby initiating the first steps in the host-pathogen interaction (8, 9). By the expression of these receptors, ECs are able to induce the production of antimicrobial and proinflammatory molecules, playing a role in the first responses against the pathogens as well as in the initiation of adaptive immune responses.

This communication between innate and adaptive immune systems, which involves cell-cell interactions, enables the effective recognition and elimination of invading pathogens,

with minimal damage to the host, and provides protection from re-infection with the same pathogen.

1.1.2. Pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs)

The innate immune system senses microbial infection and recognizes microorganisms via a variety of germline-encoded recognition receptors. PRRs recognize conserved features from a broad spectrum of microbial components, known as PAMPs, and initiate specific downstream signaling pathways leading to the activation of NF-κB and production of cytokines, including inflammatory cytokines, chemokines and type I interferons (IFNs) (6, 10, 11).

PAMPs are appropriated for innate immune recognition for three main reasons: i) they are invariant among microorganisms of a given class; ii) they are products of pathways that are unique to microorganisms, allowing discrimination between self and non-self molecules; iii) they have essential roles in microbial physiology limiting the ability of the microorganisms to evade innate immune recognition through adaptive evolution of these molecules (5, 12). PAMPs can include bacterial components, fungus components, parasite components and virus components (**Table 1**).

The Toll-like receptor (TLR) family is the best characterized class of PRRs that participates in the recognition of microbial components, but more recently it has become apparent that another series of PRRs, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), also play an important role in the innate immune response (6) (**Table 1**).

Table 1. The roles of TLRs, RLRs and NLRs in pathogen recognition.

PRRs (structure)	Adapters (structure)	PAMPs/ Activators	Species
TLR			
TLR1–TLR2 (LRR–TIR)	MyD88 (TIR–DD), TIRAP (TIR)	Triacyl lipopeptides	Bacteria
TLR2–TLR6 (LRR–TIR)	MyD88, TIRAP	Diacyl lipopeptides LTA Zymosan	Mycoplasma Bacteria Fungus
TLR2 (LRR–TIR)	MyD88, TIRAP	PGN Lipoarabinomannan Porins tGPI-mucin HA protein	Bacteria Mycobacteria Bacteria (*Neisseria*) Parasites (*Trypanosoma*) Virus (Measles virus)
TLR3 (LRR–TIR)	TRIF (TIR)	dsRNA	Virus
TLR4 (LRR–TIR)	MyD88, TIRAP, TRIF, TRAM (TIR)	LPS Envelope proteins	Bacteria Virus (RSV, MMTV)
TLR5 (LRR–TIR)	MyD88	Flagellin	Bacteria
TLR7 (LRR–TIR)	MyD88	ssRNA	RNA virus
hTLR8 (LRR–TIR)	MyD88	ssRNA	RNA virus
TLR9 (LRR–TIR)	MyD88	CpG DNA DNA Malaria hemozoin	Bacteria DNA virus Parasites
mTLR11 (LRR–TIR)	MyD88	Not determined Profilin-like molecule	Bacteria (uropathogenic bacteria) Parasites (*Toxoplasma gondii*)
RLR			
RIG-I (CARDx2–helicase)	IPS-1 (CARD)	RNA (5´-PPP ssRNA, short dsRNA)	Virus
MDA5 (CARDx2–helicase)	IPS-1	RNA (poly IC, long dsRNA)	Virus
LGP2 (helicase)		RNA	Virus
NLR			
NOD1/NLRC1 (CARD–NBD–LRR)	RICK (CARD), CARD9 (CARD)	iE-DAP	Bacteria
NOD2/NLRC2 (CARDx2–NBD–LRR)	RICK, CARD9	MDP	Bacteria

NALP3/NLRP3 (PYD–NBD–LRR)	ASC (PYD–CARD) CARDINAL (PYD–FIND)	MDP	Bacteria
		RNA	Bacteria, Virus
		ATP	Bacteria? Host?
		Toxins	Bacteria
		Uric acid, CPPD, amyloid-β	Host
NALP1/NLRP1 (CARD–FIND–NBD–LRR-PYD)	ASC	Anthrax lethal toxin	Bacteria
IPAF/NLRC4 (CARD–NBD–LRR)		Flagellin	Bacteria
NAIP5 (BIRx3–NBD–LRR)		Flagellin	Bacteria

Adapted from: *Int. Immunol.* **21**, 317–337 (2009).

1.1.3. Innate immune recognition of viral infection / Recognition of viral infection by PRRs

The innate immune system detects viral infections and senses the virus via their nucleic acid genome or as a result of their replicative or transcriptional activity, and evokes initial antiviral responses (13). These responses depend on different sensor systems that detect PAMPs and initiate specific downstream signaling pathways leading to activation of NF-κB and IRF and induce antiviral mediators such as type I IFNs that play a central role in the elimination of the viruses, and proinflammatory cytokines such as IL-6, TNF-α and IL-12 (14, 15). PAMPs are detected by PRRs that signal the presence of infection sensing critical viral components such as double-stranded (ds)RNA, single-stranded (ss)RNA, and ds or ssDNA

PRRs implicated in nucleic acid recognition can be divided in two groups based on cellular localization: PRRs localized in the endosome and PRRs localized in the cytoplasm of the cell.

Three classes of PRRs have been shown to sense viral components in innate immune cells, playing an essential role in the production of type I IFNs and proinflammatory cytokines: TLRs, RLRs and NLRs (13).

TLR3, TLR7, TLR8 and TLR9, which are localized to endosomes, represent a TLR sub-family that recognizes nucleic acids (16). TLR3 recognizes dsRNA, which is produced by many viruses during replication. TLR7 recognizes ribonucleic acid homologues such as imiquimod, resiquimod (R848) and loxoribine, and ssRNA while TLR9 recognizes unmethylated 2´-deoxyribo (cytidine-phosphate-guanosine)(CpG) DNA motifs present in bacterial and viral DNA (17, 18, 19). TLR8 is phylogenetically close to TLR7 mediating recognition of ssRNA and R-848 (20).

RLRs comprise a family of cytoplasmic proteins that function as alternative PRRs through recognition of dsRNA produced during viral replication (21).

NLRs constitute a large family of intracellular PRRs, such as NLRP-3, which detects the presence of dsRNA and induces the catalytic activity of caspase-1, which is essential for cleavage of pro-interleukin-1β (IL-1β) to the mature form (22, 23).

Besides these three families of receptors, more recently it has also been described a member of the PYHIN (pyrin and HIN domain-containing protein) family, named absent in melanoma 2 (AIM2), which senses foreign cytoplasmic dsDNA (24).

This work focuses on two main dsRNA receptors, TLR3 and RIG-I, with a short overview about NLRP3 and AIM2 receptors.

1.1.3.1. Toll-like Receptors

So far, 13 murine TLRs and 10 human TLRs have been identified (25). TLRs comprise a subfamily within the larger superfamily of interleukin (IL) receptors showing a similarity within their cytoplasmic regions and termed a Toll/IL-1 receptor (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions known as boxes 1, 2 and 3. In contrast, the extracellular portions of both types of receptors are quite distinct. The IL-1 receptors possess three immunoglobulin-like domains, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain (26, 27) (**Figure 1**).

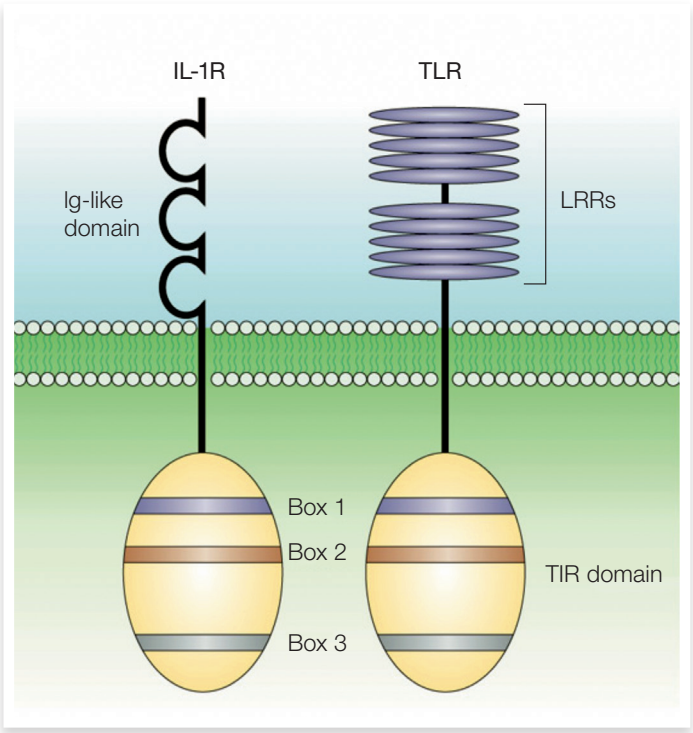


Figure 1. TLR structure.
Taken from: *Nat. Rev. Immunol.* 4, 499–511 (2004).

The TIR domain, located in the cytosol in all TLRs, associates with TIR-domain containing adaptors molecules, such as MyD88 (myeloid differentiation primary response gene 88), TRIF (TIR-domain-containing adaptor-inducing interferon-β), TIRAP (TIR-containing

adaptor protein), and TRAM (TRIF-related adaptor molecule) (28, 29, 30). Except for TLR3, MyD88 is involved in all TLR-mediated signaling (31, 32) whereas TRIF is involved in TLR3 and MyD88-independent TLR4 signaling (28). TIRAP is involved in the Myd88-dependent signaling pathway through TLR2 and TLR4 (29), and TRAM is specifically involved in the Toll-like receptor-4 mediated MyD88-independent signaling pathway (30).

Unlike the other TLRs, which are expressed mainly on cell surface, TLR3, 7, 8 and 9, involved in the recognition of nucleic acids, are localized within endolysosomal compartments (**Figure 2**).

This work mainly focuses on the dsRNA recognition receptors. Therefore, TLRs that recognize non-nucleic acid molecules will not be described here.

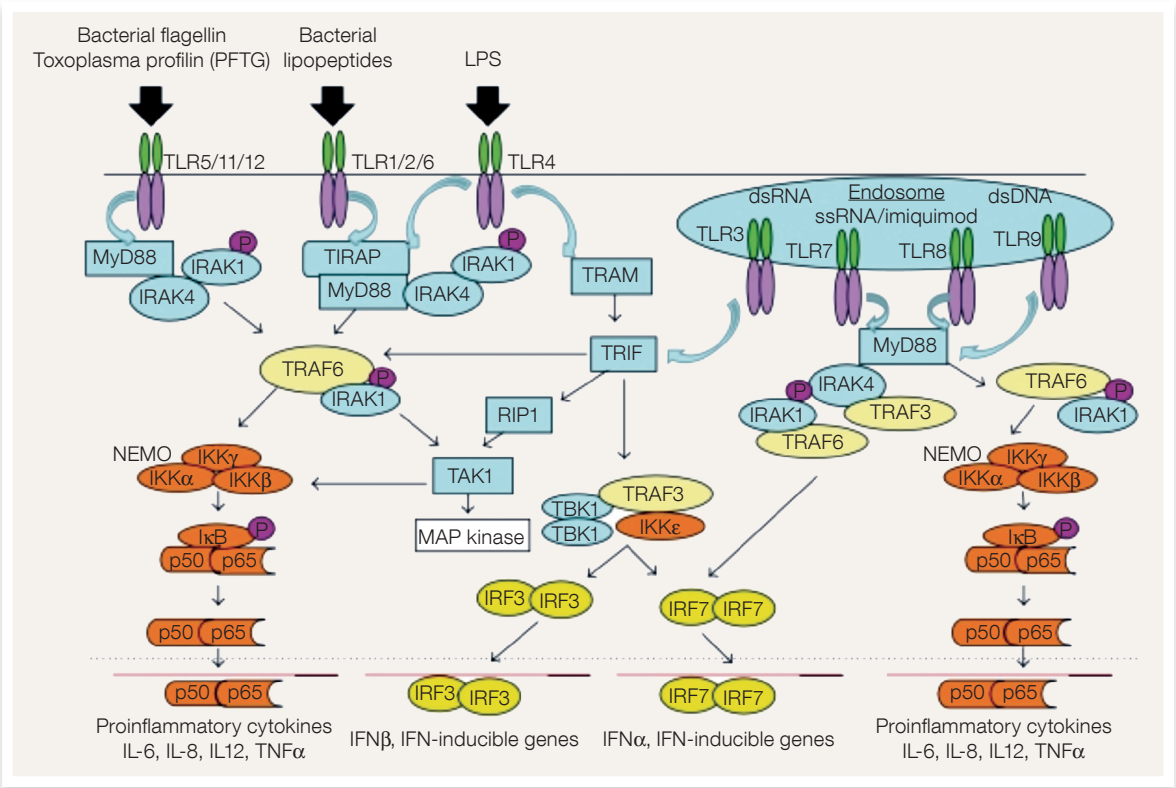


Figure 2. TLR signaling pathway.
Taken from: *Mediators Inflamm.* **2010**, 781235 (2010).

1.1.3.1.1. TLR signaling pathways

1.1.3.1.1. a) MyD88-dependent pathway

Stimulation of TLRs by microbial components triggers expression of several genes that are involved in immune responses. Many TLRs induce inflammatory responses activating a common signaling pathway mediated by the adaptor molecule MyD88 which binds to the cytoplasmic domain of the TLRs through their respective TIR domains. When associated with a TLR, MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family such as IRAK4, IRAK2 and IRAK1, through a DD-DD interaction (33, 34). Activated IRAKs form a complex with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) interacting with Ubc13 and Uev1A (35), leading to the activation of two distinct pathways: (i) activation of AP-1 transcription factors through activation of MAP kinases; (ii) activation of TAK1/TAB complex, which activates the IκB (IKK) complex consisting of IKKα, IKKβ and NEMO/IKKγ. IKK complex phosphorylates IκB resulting in nuclear translocation of NF-κB and activation of NF-κB-dependent genes, such as TNF-α, IL-1 and IL-6 (27, 36) (**Figure 2**). This pathway is used by all TLRs except TLR3.

1.1.3.1.1. b) MyD88-independent/TRIF-dependent pathway

The Myd88-independent pathway exists downstream of LPS/TLR4 and dsRNA/TLR3. TLR4 and TLR3 have the ability to induce type I IFNs and IFN-inducible genes via this pathway. TLR4 triggers the MyD88-independent pathway/TRIF-dependent pathway via another TIR-domain-containing adaptor named TRAM, which associates with TRIF and TLR4 (but not with TLR3) working as a bridge between these two molecules (28, 37, 38). Activated TRIF transmits a signal to the receptor-interacting protein 1 (RIP-1)

which is responsible for the activation of NF-κB (39). In response to TLR4 and TLR3 ligands, TRIF can also activates IKK-related kinases TBK1 and IKK-ε via TRAF3 and these kinases directly phosphorylate IRF3 and IRF7 (40, 41), contributing to the activation of type I IFNs and IFN-inducible genes (**Figure 2**).

1.1.3.1.2. TLR3

TLR3 was the first TLR implicated in the sensing of viral nucleic acids. It recognizes dsRNA derived from dsRNA virus such as reovirus and dsRNA produced during the course of replication of ssRNA virus such as respiratory syncytial virus (RSV), encephalomyocarditis virus (EMCV) and West Nile virus (WNV). Poly(I:C), a synthetic analogue of dsRNA, can also mediate responses through TLR3 (42) (**Table 1**). TLR3 is expressed in the endosomal compartments of the immune cells by conventional dendritic cells (cDCs) and macrophages, as well as nonimmune cells including fibroblasts, epithelial, mesangial and mesothelial cells (43, 44, 45). The stimulation of TLR3 by dsRNA induces the activation of the transcription factors nuclear factor (NF)-κB and interferon regulatory factor (IRF)/interferon-sensitive response element (ISRE) via MyD88-independent signaling pathways, which involves the adaptor molecule TRIF also called TICAM-1 (28, 46). TLR3 is involved in antiviral host immune responses by producing inflammatory cytokines and type I IFNs (**Figure 2**).

1.1.3.2. RIG-I-like receptors

RLRs comprise a family of cytoplasmic proteins consisting of three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP2). Once TLRs that recognize nucleic acids

are localized to endosomes and are unable to recognize virus in the cytoplasm, RLRs recognize viral RNAs in the cytoplasm and trigger signaling pathways that induce the production of type I IFNs and proinflammatory cytokines (16).

RIG-I and MDA5 consist of two N-terminal caspase-recruitment domains (CARDs), a C-terminal domain and a DExD/H box RNA helicase domain, whereas LGP2 lacks the CARD, which is required for triggering intracellular signaling cascades (47) (**Figure 3**).

1.1.3.2.1. RIG-I

RIG-I, a prototypical member of the RLR family, contains a DExD/H helicase domain, which has an ATP-binding motif, and two caspases recruiting-domain (CARD)-like domains at its N-terminus. These domains function in interaction with other CARD-containing proteins and are required for activating downstream signaling pathways. The C-terminal domain contains a repressor domain (RD), which inhibits downstream signals (48, 49).

In response to detection of viral RNAs, RIG-I recruits an adaptor protein designated IFN-β promoter stimulator-1 (IPS-1) [also known as mitochondrial antiviral signaling (MAVS), CARD adaptor inducing IFN-β (Cardif) or virus-induces signaling adaptor (VISA)], in a CARD-CARD-dependent manner (50). IPS-1 activates IRF3 and IRF7 via TBK1/IKKi although it does not directly bind to these protein kinases. TRAF3 binds IPS-1 and recruits and activates TBK1/IKKi, which phosphorylate IRF3 and IRF7 (40, 41). Phosphorylation of IRF3 and IRF7 induces the formation of homodimers and/or heterodimers which translocate into the nucleus and bind to IFN-sensitive response elements (ISREs), resulting in the activation of type I IFNs and IFN inducible genes (51) (**Figure 3**).

IPS-1 also interacts with FAS-associated death domain-containing protein (FADD), which forms a complex with caspase-8 and caspase-10 and this pathway is capable of activating NF-κB downstream of IPS-1 (52) (**Figure 3**).

A transmembrane domain at the C-terminal end of IPS-1 is required for mitochondrial targeting being essential for IRF and NF-κB activation, suggesting that mitochondria may somehow be important in antiviral immune responses (53) (**Figure 3**).

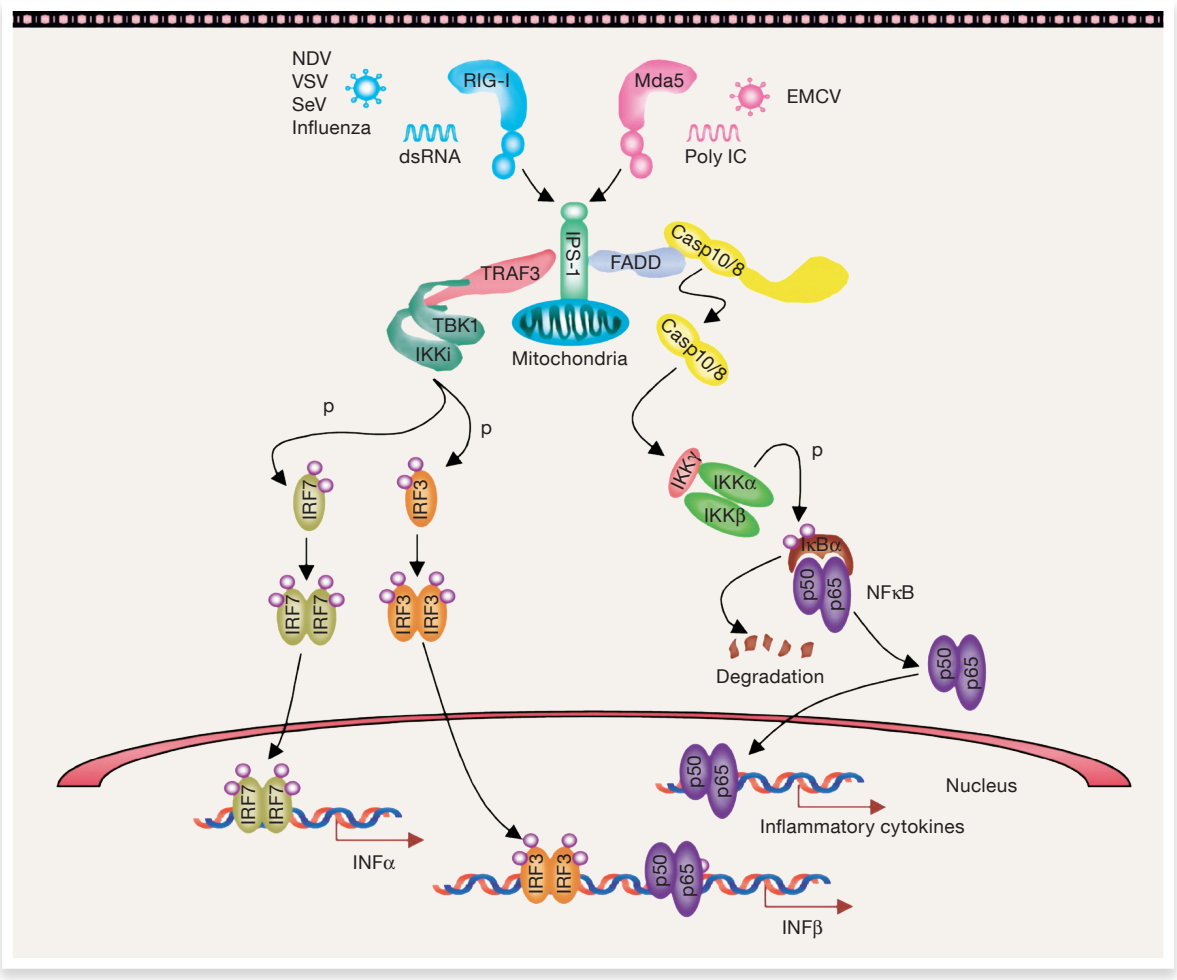


Figure 3. RLR signaling pathway.
Taken from: *J. Biochem.* **141**, 137–145 (2007).

RIG-I-activated signaling pathway has been implicated in antiviral responses to Sendai virus (SeV), vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), as well as different flaviviruses and Kaposi's sarcoma-associated herpes virus (KSHV) (54, 55, 56). In addition, Chiu and coworkers recently demonstrated that a cytosolic B form of dsDNA, poly(dA-dT) · poly(dA-dT) is able to activate RIG-I and to induce IFN-β production via the cytosolic DNA-dependent RNA polymerase III (PolIII) (57).

1.1.3.3. NOD-like Receptors

NOD-like receptors are a family of molecules that detect the presence of PAMPs and endogenous molecules within the cytoplasm of cells (24, 58). So far, 23 family members have been identified in humans and approximately 34 in mice. These sensors consist of three domains: a c-terminal leucine-rich repeat which is thought to be involved in the recognition of microbial PAMPs or endogenous host molecules; an N-terminal signaling domain that contains a dead effector domain (DED), a Pyrin domain (PyD), a CARD, baculovirus inhibitor repeats (BIRs) and an acidic domain which is required for homotypic interactions with downstream signaling proteins; a central nucleotide-binding NACHT domain composed of a CARD, which is required for ligand-induced, ATP-dependent oligomerization of the sensors and formation of active receptor complexes for activation of downstream signaling (59, 60).

Members of NLR includes: NODs (nucleotide-binding oligomerization domain) which recognize peptidoglycans, a component of the cell wall of bacteria; NLRC (NLR family, CARD domain); NLRPs (contains a Pyrin domain); NLRX1 (NLR family member X1, contain an unknown domain); CIITA (class II, major histocompatibility complex, transactivator); NAIP (NLR family, apoptosis inhibitory protein) (12) (**Table 1**).

1.1.3.4. Inflammasome

The inflammasome is a large multiprotein complex that mediates the activation of inflammatory caspases, including caspase-1, which in turn, promotes the proteolytic maturation of the zymogen form of IL-1 family cytokines such as IL-1β and IL-18 (60).

It is assumed that the adaptor protein apoptosis speck-like protein containing a CARD (ASC) is required in all inflammasome complexes for procaspase-1 activation. It binds to the CARD-containing pro-domain of caspase-1 to mediate its oligomerization and subsequent activation (61, 62, 63).

Many viruses are able to activate caspase-1 leading to the production of IL-1β and IL-18. Two distinct inflammasome complexes have been described as being involved in antiviral immunity: the NLRP3 inflammasome and the AIM2 inflammasome (64, 65, 24) (**Figure 4**).

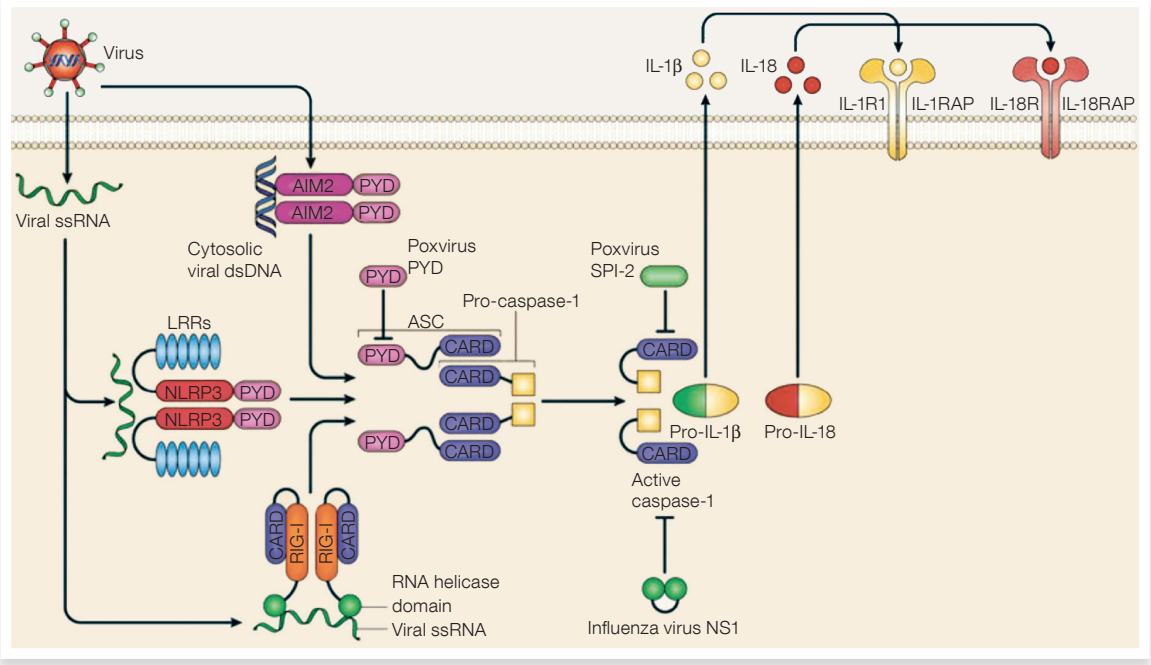


Figure 4. Inflammasome signaling pathway.
Taken from: *Nat. Rev. Immunol.* **10**, 688–698 (2010).

1.1.3.4.1. NLRP3 inflammasome

NLRP3 (also known as NALP3, cryopyrin, cold-induced autoinflammatory syndrome-1 [CIAS1], CLR1 and PYPAF1) is probably the most widely studied inflammasome so far. Its expression is mainly detected in the cytosol of granulocytes, dendritic cells, monocytes, T and B cells, epithelial cells and osteoblasts (66, 67), suggesting an essential role in the primary defense mechanism against pathogens.

NLRP3 inflammasome can be activated by numerous PAMPs (viruses, bacteria and fungi), DAMPs (danger-associated molecular patterns [heat shock proteins and BCL2]), and endogenous molecules such as urate crystals and ATP, bacterial poreforming toxins and particulate matter including silica and asbestos (68, 69, 70, 71) (**Table 1**).

NLRP3 inflammasome is composed by NLRP3, ASC and procaspase-1 and its activation by the appropriate ligands induces caspase-1 activation, which promotes processing of pro-IL-1β and the release of IL-1β after brief stimulation with high concentrations of ATP (**Figure 5**). The CARD of adaptor protein ASC interacts with the CARD of procaspase-1 inducing catalysis of procaspase-1 to yield caspase-1, which catalysis the proteolysis of pro-IL-1β (inactive) to IL-1β (active) (60).

Several events seem to be required for the activation of NLRP3. This includes: extracellular ATP which activates the purinergic ATP-gated P2X7 receptor which then helps to allow the pannexin-1 receptor to cause K+ efflux, being important for NLRP3 inflammasome activation; the generation of reactive oxygen species (ROS); lysosomal destabilization after phagocytosis of several crystalline and insoluble ligands, which leads to disruption of lysosomal membranes and the release of lysosomal proteins, activating NLRP3 inflammasome (72, 73, 74) (**Figure 5**).

Although virus recognition mechanisms by NLRP3 inflammasome are still not well understood, it was suggested by several groups that NLRP3 further senses infections by dsRNA, ssRNA and DNA including Sendai virus, and influenza virus (64, 75, 76).

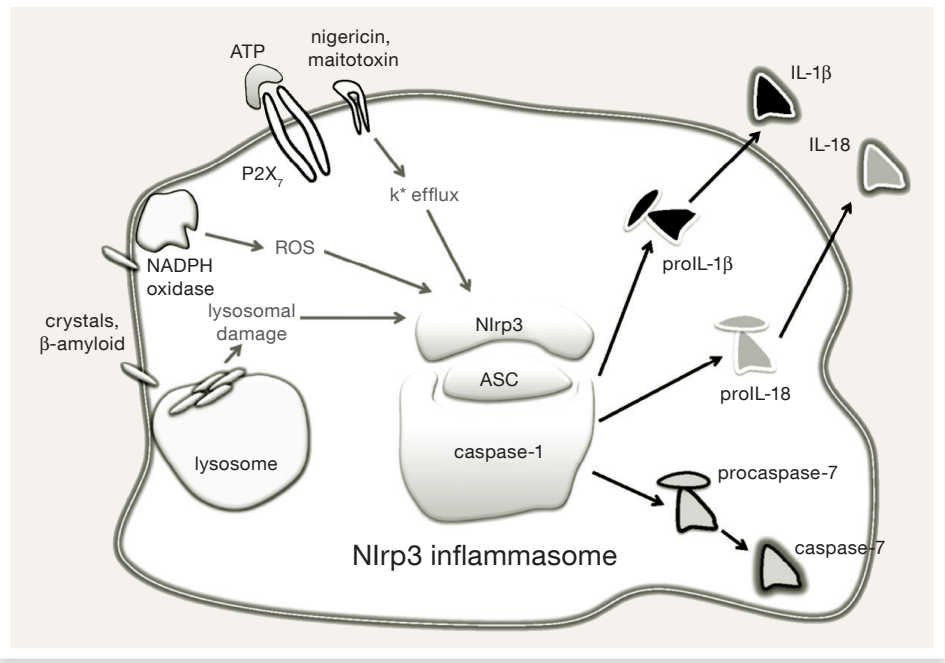


Figure 5. NLRP3 signaling pathway.
Taken from: *Int. J. Biochem. Cell Biol.* 42, 792–795 (2010).

1.1.3.4.2. AIM2 inflammasome

AIM2 belongs to the HIN-200 family that includes four members in humans (IFI16, MNDA, IFIX and AIM2) and six members in mice (IFI202, IFI203, IFI204, IFI205, PYHIN1 and AIM2) (77). HIN-200 family contains an amino-terminal pyrin domain (N-terminal PYD) and a carboxy-terminal oligonucleotide/oligosaccharide-binding domain (HIN domain). AIM2 is a cytoplasmic DNA sensor that directly binds DNA inducing proteolytic activation of caspase-1 and production and maturation of IL-1β. The HIN domain of AIM2 binds the DNA while the PYD domain associates with the PYD domain of the adaptor

molecule ASC, allowing procaspase-1 recruitment, via CARD-CARD interactions, and caspase-1 activation with subsequent maturation of IL-1β (24, 78, 79, 80) (**Figure 6**).

Several studies suggest an important role of AIM2 as a sensor for dangerous cytoplasmic DNA and they document the first example of a direct interaction between an inflammasome sensor and its ligand.

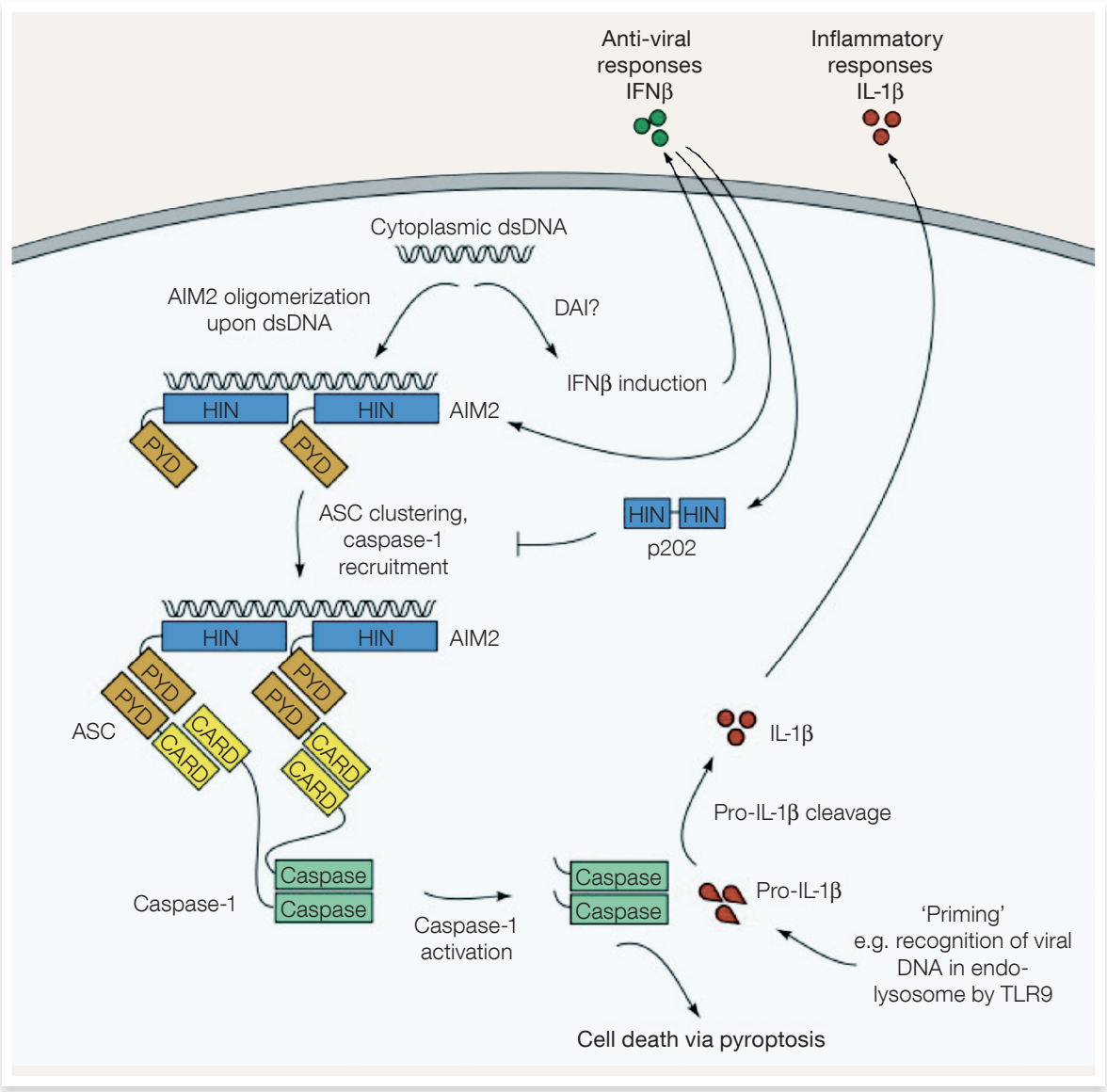


Figure 6. AIM2 signaling pathway.
Taken from: *Curr. Biol.* **19**, R262–R265 (2009).

1.1.4. Type I IFNs and other proinflammatory cytokines signaling viral invasion

All living organisms have developed mechanisms to protect themselves from invasion by exogenous pathogens, such as virus. Upon viral infection, the host innate immune system has the ability of acting as a first line defense in the prevention of viral invasion or replication before the more specific response by the adaptive immune system is generated.

After viral recognition, PRRs bind to PAMPs, leading to the activation of transcription factors, such as NF-κB, and IFN regulatory factors (IRFs). This event leads to the production and secretion of proinflammatory cytokines and type I IFNs (15, 16).

Cytokines are small cell-signaling molecules produced by cells at the site of infection and they are responsible for the first local inflammatory response and some systemic effects. There are evidences that during viral replication, the so-called early cytokines produced at the site of infection mediate many of the clinical and pathological manifestations. Among these cytokines, interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) α and β, interleukin-6 (IL-6), interleukin-8 (IL-8/CXCL8) and monocyte-attracting chemokines such as MCPs, seem to play an important role in the proinflammatory response against the virus (81). Besides being importante for the immediate defense, cytokine production also facilitates acquired immunity in several ways: first, promotes phagocytosis and antigen presentation by dendritic cells; secondly, proinflammatory cytokines, such as IL-1, IL-6 and TNF are co-mitogenic for lymphocytes.

Some cytokines are similar in their inflammatory effects. IL-1 and TNF are both involved in the upregulation of adhesion molecules (E-selectin, ICAM-1, VCAM-1) on vascular endothelial cells. They are also important to stimulate production of chemotactic

cytokines (chemokines) by connective tissue and endothelial cells (IL-8/CXCL8, MCP-1, etc.) and for their stimulating effects on neutrophil and macrophage function. IL-6 is a major product of IL-1- or TNF-stimulated cells and has some undeniable proinflammatory features, for example, promoting the fever induced by TNF and IL-1. However, it also shows some anti-inflammatory features, such as the capacity to partly downregulate TNF production. IL-6 also has major effects on the immune system, activating B cells to produce immunoglobulin, and T cells to upregulate some essential receptors (82).

Although, as it was described above, several cytokines are produced by host cell in response to a viral infection, type I IFNs are the principal cytokines involved in the viral response. Type I IFNs are represented by multiple isoforms of IFN-α and by IFN-β and other members such as IFN-ε, -ω and -κ (83). They can be produced by all nucleated cells in response to a viral infection. Type I IFNs exert their function after binding the receptor IFNAR 1/2 and after activation of JAK/STAT signaling pathway (84). IFN induction requires the conjugated action of the three transcription factors: ATF2/c-jun and NF-κB, which are activated in response to various stimuli such as proinflammatory molecules, and IRF3, which is activated by viral infection (85).

In a viral infection progress, the production of IFNs increases up to the levels required to control virus replication. Since virus replication is controlled, IFN production returns to basal levels. For an effective host antiviral response, in addition to a stimulation of type I IFNs production that prevents the replication of the virus, it is also necessary an attraction of inflammatory cells to the site of infection, which prevents the virus from spreading.

However, viruses have developed strategies to counterattack, finding ways to proliferate within host cells. In this sense, viruses have to identify the host targets that are key players in the antiviral response and find mechanisms to block host molecules that connect

virus recognition to downstream effectors of type I IFNs production and inflammatory cytokines expression. One example of this is the cleavage of MAVS at its C-terminal end by the NS3-4A protease of hepatitis C virus (HCV), which interrupts MAVS interaction with the outer mitochondrial membrane, disrupting signal transmission to downstream antiviral molecules (i.e. IRF3, IFN-β) and activation of NF-κB (86, 87, 88). The influenza virus NS1 protein is also able to antagonize RIG-I/MAVS pathway through the interaction of NS1 with the complex, blocking IRF3 activation and IFN-β expression (89). Viruses without the ability to suppress the host type I IFN responses are generally low pathogenic and available for vaccine strains.

1.1.5. Innate immune recognition and control of adaptive immune responses to virus

The immune system of mammals effectively fights infection through the cooperation of two connected systems that act together to provide both immediate and long-term immunity to pathogens: the innate immune system, which was described before, provides critical mechanisms for the rapid sensing and elimination of pathogens, and the adaptive immune system which is highly specific and is involved in elimination of pathogens in the late phase of infection as well as in the generation of immunological memory. The interaction between these two arms of immunity enables the immune system to efficiently recognize and eliminate invading pathogens with minimal damage to self, and provides protection from re-infection with the same pathogen (90).

The innate immune system, by the expression of germ-line encoded receptors (PRRs), recognizes viral PAMPs and limits viral replication via type I IFNs production and cytokines and chemokines expression, being in most cases enough for the elimination

of the pathogen. However, some viral infections outrun the innate immune response and when this occurs, the innate immune response works to slow the spread of infection while it calls for a stronger and more specific response. In addition to direct activation of innate-host defense mechanisms, signals induced by PRRs are also responsible for the activation of adaptive immune responses (5). These adaptive immune responses demonstrate specificity, discrimination and acquired “memory” for distinct viral molecules and respond more vigorously after repeated exposure to the same viral antigens.

The adaptive immune responses are mediated by antigen receptors that are clonally distributed on the two types of lymphocytes: T cells and B cells (91). The lymphocytes and their products coordinate the effector phases of adaptive immune responses and may eliminate the virus, primarily by generating neutralizing antibody that is specific for the virus and CD8+ cytotoxic T lymphocytes (CTL) that directly kill virally infected cells (92). The activation of these cells depends on the induction of co-stimulatory molecules and secretion of cytokines and chemokines by the cells of the innate immune system. The key cellular players in translating information from the innate response into adaptive immunity are the members of dendritic cell (DC) family (93, 94). Upon infection, DCs express a repertoire of PRR on their surface and in response to signals from these receptors they become antigen-presenting cells (APCs) competent to sustain the expansion and differentiation of antigen-specific cells into appropriate effector cells (11). When a DC captures a viral antigen, it migrates from the periphery into a secondary lymphoid organ, such as the spleen or a lymph node, and presents the viral antigen for recognition to naïve CD4+ lymphocytes to promote a T helper type 1 (Th1) immune response to the virus. The proliferation and differentiation of naïve CD4+ lymphocytes into CD4+ Th1 lymphocytes enables the stimulation of viral antigen-specific B lymphocytes and CD8+ lymphocytes to differentiate into plasma cells and cytotoxic T cells (CTL), respectively. DCs activated by pathogens normally express high levels of major histocompatibility complex (MHC) molecules bearing pathogen-derived peptides, which

can engage T cell receptors on naïve pathogen-specific T cells. While CD4+ Th1 lymphocytes (that recognize viral antigen presented by MHC class II molecules) are activated to perform localized effector functions that are designed to eliminate virus at the site of antigen encounter, CTL (that recognize viral antigen presented in the context of MHC class I molecules) directly kill the cell that presents the viral antigen (92).

In addition to DCs, certain types of nonimmune cells, apart from playing an important role in the defense of the host against infection at the front line, can also influence the development and direction of the adaptive immune response (9). It is reported that when epithelial cells at mucosal sites encounter a microorganism, they produce essential chemokines, such as CCL20, that chemoatract immature DCs and inflammatory monocytes to the site of antigen exposure. In addition, ECs also produce critical maturation cytokines such as IL-1, GM-CSF, and TSLP that can activate the recruited monocytes to differentiate in DCs and induce their maturation into fully competent antigen-presenting cells (APCs) that are capable of interacting with naïve T cells, thereby instructing the outcome of antigen-specific immunity (95).

Lamentably, the role of ECs in influencing the development of antigen specific immunity against a viral infection is still poorly understood. However, considering the available data, it is licit to speculate that ECs, being at the interface between the internal and external environment, may be involved in the development of a primary immune response against the virus and, probably, be important for the instruction of antigen-specific responses. Nevertheless, future studies are crucial to elucidate this topic.

1.1.6. Innate immune response in virus-induced kidney diseases

Viral infections are important causes of various kidney diseases often responsible for significant morbidity and mortality. In this sense, it is very important to understand how infections trigger or aggravate renal pathology. Epstein-Barr virus (EBV), cytomegalovirus (CMV), adenovirus, BK virus (BKV), hepatitis-C virus (HCV) and human immunodeficiency virus (HIV) infections are commonly associated with specific kidney diseases (44, 96).

Pathogen recognition is not restricted to immune cells and renal cells can also contribute to this phenomenon. However, the mechanisms involved remain unclear.

The role of innate immune receptors in kidney infections is, so far, largely unknown and has been the target of many studies. RNA viruses are highly associated with kidney pathologies. During the course of infection induced by RNA viruses, viral RNA alone or as part of immune complexes could reach the mesangium and trigger glomerular inflammation. One good example is HCV-associated glomerulonephritis (97). Although mesangial cells don't express TLR7/8, receptors that should be essential for the activation of these cells by viral ssRNA, previous studies from Wörnle *et al.* (44) showed a robust increase of TLR3 mRNA levels and proinflammatory cytokines and chemokines in microdissected glomeruli from biopsies of hepatitis C-associated but not idiopathic membranoproliferative glomerulonephritis. In addition, the treatment of cultured mesangial cells with poly(I:C) also resulted in a robust expression of TLR3 and production of cytokines and chemokines proposing a role of this receptor in some forms of glomerulonephritis. The activation of mesangial TLR3 during the course of HCV infection can also affect the expression of factors, which are relevant for adhesion, mesangial matrix metabolisation and fibrosis (98, 99, 100).

HIV-associated nephropathy (HIVAN) is also one of the most common causes of chronic kidney disease. The primary targets of classical HIVAN are glomerular cells, which typically show a focal and segmental glomerulosclerosis (FSGS) of the “collapsing type” (101). HIV replicates in glomerular cells, however these cells lack TLR7/8, the receptors described as being involved in the recognition of viral ssRNA (102). This observation suggests an involvement of receptors other than TLR7/8 in the recognition of HIV in renal cells but which receptors and mechanisms are activated during HIVAN are unknown so far.

DNA viruses, such as human herpes virus (HHV), adenovirus or polyomavirus, are also commonly associated with renal pathology. CMV or polyomavirus can be an important cause of renal allograft nephropathy with the virus replication in tubular epithelial cells. During infection by CMV, expression of TLR9 showed to be involved in the activation of dendritic cells. In addition, CMV is known to generate RNA transcripts activating dendritic cells via TLR3, but the involvement of TLR-independent viral recognition receptors to DNA virus remains to be elucidated (103). Also BKV is known to produce dsRNA during its replication (42) and recently a study showed the presence of BKV RNA in all BKV DNA positive biopsies, indicating an active viral replication, and a possible involvement of the dsRNA recognition receptors sensing this virus (104). In this sense, the role of viral receptors in the recognition of viral RNA and in the activation of innate immune signals during renal infection must be clarified and is the subject of the present study.

1.2. BK virus-associated nephropathy

Polyomavirus-associated nephropathy (PVAN) is an emerging cause of significant kidney transplant dysfunction affecting up to 10% of patients, often leading to graft loss. Most cases of PVAN are elicited by BK virus (BKV) in the context of intense

immunosuppression. Primary BKV infection occurs during childhood, and after infection the virus persists in the kidney and, upon immunosuppression of the human host, causes significant morbidity, particularly in bone marrow and renal transplant patients (105, 106, 107, 108).

BKV belongs to the polyomavirus family along with other five polyomaviruses detected in humans: JC virus (JCV), KI virus, WU virus, Merkel cell carcinoma virus, and the primate pathogen simian virus 40 (SV40) (109, 110, 111, 112). JCV is associated with progressive multifocal leukoencephalopathy, KI virus and WU virus are associated with respiratory illness and Merkel cell polyomavirus is associated with Merkel cell carcinoma. Although most cases of PVAN are due to BKV, JCV has also been implicated in a very small percentage (113).

BK virus-associated nephropathy (BKVAN) is by far the most important infectious complication affecting kidney transplants.

1.2.1. Virological and epidemiological aspects of BKV

BKV is a small nonenveloped virus, with a 40- to 44-nm sized icosahedral capsid that contains a circular double-stranded DNA genome of 5000-base pair. It encodes for two early regulatory proteins — small T antigen and large T antigen — which are involved in transformation, viral replication and gene regulation and expression, and three late structural capside proteins — viral proteins 1, 2 and 3 (VP-1, VP-2 and VP-3). It also encodes for a late protein called agnoprotein, which is probably involved in processing of late mRNAs and assembly of viral particles, and the non-coding control region (NCCR), which contains the viral promoters and origin of replication (114, 115, 116).

The molecular events involved in BKV invasion of host cells and subsequent intracellular trafficking are an important area of study. In various cell culture models, BKV uses an N-linked glycoprotein containing an alpha(2,3)-linked sialic acid as a receptor and enters cells through caveolae-mediated endocytosis (117, 118, 119). After this relatively slow and cholesterol-dependent internalization, BKV migrates through the cytoplasm and via cellular cytoskeletal components, i.e. the endoplasmic reticulum and microtubules to the nucleus, where viral transcription, replication and assembly take place (120, 121) (Figure 7).

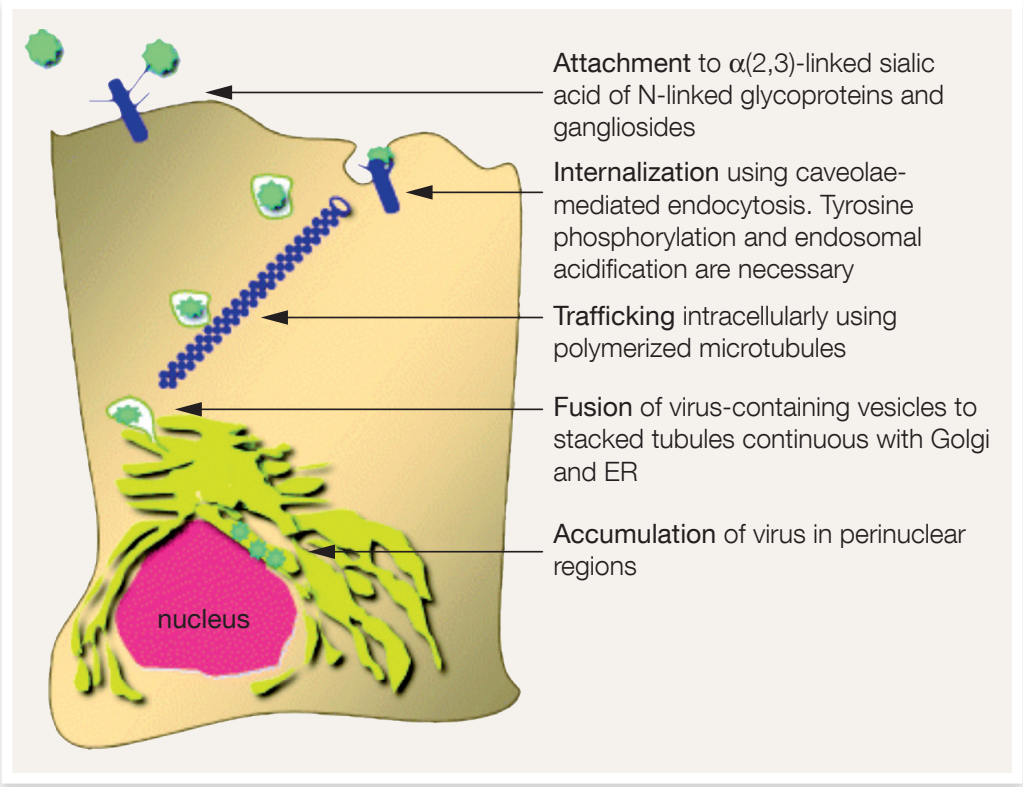


Figure 7. Infectious entry pathway of BKV.
Taken from: *Transpl. Infect. Dis.* 8, 62–67 (2006).

BKV is typically acquired during childhood and transmission occurs via oral and respiratory routes, but some data suggests transmission via cells and tissues, particularly by kidney transplantation (116, 122). After primary replication, BKV remains in the

renourinary tract with intermittent reactivation and low-level DNA load (viruria) in immunocompetent adults (123, 124). The levels of viruria increase in immunocompromised individuals often with the presence of decoy cells in urine (125). After kidney transplantation the virus normally replicates in urothelial cells, which remains asymptomatic in two-thirds of infected patients (126), but also exhibits tropism for the renal tubular epithelium, where it can establish latent infection (127). Approximately 30%–50% of kidney transplant patients with high level viruria progress to BKV viremia (BKV DNA in plasma) and the prevalence of BKVAN oscillates between 1% and 10% (128, 129) mostly due to immunosuppression treatment and diagnostic methods rather than true differences in BKV epidemiology.

1.2.2. Clinical features and risk factors of BKVAN

The most frequent symptom associated with primary infection by BKV is an upper respiratory infection. After primary infection has resolved, the virus remains latent in the renourinary epithelium, where viral replication is controlled by mechanisms of immune surveillance. BKV tends to persist indefinitely in different organs such as kidney, ureter, brain, and lymphoid cells, but changes in immune functions may cause viral reactivation (130). BKV reactivation is associated to pregnancy, human immunodeficiency virus, cancer, diabetes, and transplantation (131, 132).

BKVAN has been recognized as an early event and occurs within the first year after transplantation (128, 133). Reactivation of the virus may start as early as 4 months post-transplantation and run a course until graft failure with a medium diagnosis time of 9.5 months (134). The clinical features of BKVAN most closely resemble those of acute rejection. Most kidney transplant recipients with BKVAN manifests with renal dysfunction

resulting in either an asymptomatic acute or slowly progressive rise in serum creatinine concentration (135, 136). The presence of decoy cells in the urine is strongly suggestive of polyomavirus infection but they are not entirely sensitive and specific for BKV infection (129). BKV DNA is detected in the urine and plasma in nearly all cases of BKVAN (137). Occasionally, subjects may show ureteric obstruction and hydronephrosis and cases of cystitis have also been reported. 30%–60% of the cases presented with progressive renal failure (138). BKVAN is also associated with characteristic histologic findings on kidney biopsies (cytopathic changes described posteriorly) (139).

BKVAN was rare until late 1990s and an emergence of the disease became evident with the introduction of high doses of new immunosuppressive protocols often including mycophenolate mofetil (antiproliferative agent) and tacrolimus (calcineurin inhibitor agent) (128, 140, 141, 142). However, some studies suggest that the intensity of immunosuppressors rather than a specific agent is the key risk factor for the emergence of BKVAN (143, 144). There are other risk factors that appear to be associated with BKVAN. Seronegative subjects who received kidneys from seropositive donors (BKV D+/R-) appear to have a higher risk of nephropathy in children (145). Deficient humoral and/or cellular immunity may also contribute to structural organ damage and allograft dysfunction (146, 147). Recipients with greater donor and recipient HLA mismatching appear to have an increase vulnerability to the BKV infection and BKVAN (148). In the context of immunosuppression, other risk factors including ongoing graft injury as a result of allograft rejection, ischemia, and drug toxicity also have been implicated in the development of the disease (149).

1.2.3. Histological diagnosis of BKVAN

The renal involvement in PVAN is multifocal and distal nephron segments, i.e. medullary collecting ducts and distal cortical tubules, are more severely affected than proximal segments (150). To achieve a productive infection, the BKV genome has to be delivered to the nucleus, where early genes are expressed, followed by DNA replication, late protein expression, and virion assembly. Histologically, viral replication results in tubular epithelial cell enlargement, karyomegaly and basophilic nuclear inclusion bodies. These cytopathic changes are often associated with lysis of tubular epithelial cells, denudation of the basement membrane and a strong interstitial inflammatory response, similar to that seen in acute rejection (AR). Since this disease is very often misdiagnosed as AR, the diagnosis is very difficult but essential. The diagnosis includes detection of BKV DNA on urine (viruria) and plasma (viremia) by polymerase chain reaction (PCR) or quantitative PCR, presence of “decoy cells” in the urine sediment by urine cytology, histopathology of renal allograft biopsies, viral culture, and serology (146, 151, 152, 153, 154). However, decoy cells, viruria and viremia only indicate viral replication and not nephropathy. In this sense the histological diagnosis of BKVAN requires evaluation of a renal biopsy with demonstration of cytopathic changes and confirmation with immunohistochemical stains for the simian virus 40 (SV40) large T antigen (LT-ag), which identifies all PV infections due to cross-reactivity between SV40 and both the BKV and JCV (139) (Figure 8). Distinction between the different types of polyomavirus requires the use of species-specific antibodies, in situ hybridization (ISH) or in situ PCR.

Because of the detrimental impact of BKV infection and its association with premature graft loss, an early diagnosis is essential to avoid the progression of the disease.

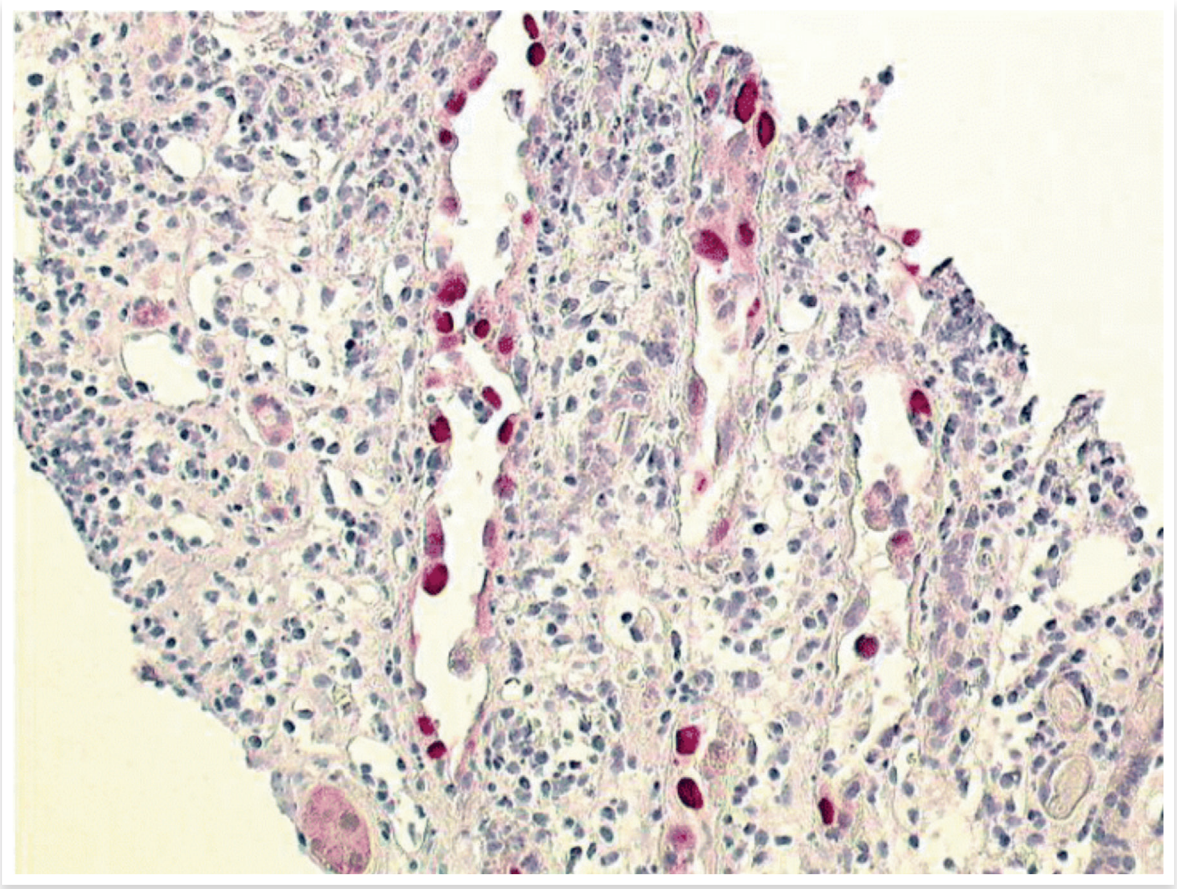


Figure 8. : Immunohistochemical staining for SV40 (large T) in human renal biopsies with PVAN shows strong nuclear positivity in distal tubular and collecting duct epithelial cells.
Taken from: *Am. J. Trans.* 5, 1562–1568 (2005).

1.2.4. Histological progression of BKVAN

BKVAN can present with different histological patterns and progress through various stages (141, 155, 156, 157, 158). Three histological patterns of BKVAN (A, B and C) can be identified in renal biopsies. Pattern A (early changes) corresponds to the early disease and is characterized by viral cytopathic changes only, in near-normal renal parenchyma. The lack of virally induced epithelial cells lysis is typical for this pattern. Negligible or absent tubular atrophy, interstitial fibrosis and inflammation are also features

of this stage of the disease. Pattern B (florid changes) identifies intermediate disease and is characterized by combination of viral cytopathic changes and focal/multifocal areas of tubular atrophy, interstitial fibrosis and inflammation. Pattern C (late sclerosing changes) corresponds to very advanced histological changes and is characterized by very scarce cytopathic changes in diffusely scarred renal tissue, and extensive tubular atrophy, interstitial fibrosis and inflammation (158, 159).

A progression of pattern A to pattern B or C can be observed if productive viral replication spreads and virally induced tubular injury persists over weeks to months. A regression of pattern B to pattern A may be observed during the resolution of BKVAN but fibrosis and tubular atrophy in pattern C are irreversible and are associated with severe allograft dysfunction or loss (160).

1.2.5. Treatment and prevention of BKVAN

Despite the critical role PVAN may play in long-term graft loss, there is little known about the immunology of this infection or its acute or chronic effects on the allograft.

After a renal transplant with an immunologically non-identical kidney almost all recipients are submitted to an immunosuppressive therapy in order to prevent acute rejection and the loss of the renal allograft. In this situation, an adequate level of immunosuppression is required to dampen the immune responses to the allograft. However, to lower the risk of infection and malignancy, the level of immunosuppression is slowly decreased over time, as the risk of acute rejection decreases. The risk of infection directly correlates with the degree of immunosuppression therapy and the intensity of the therapy must also take in consideration the development of chronic allograft nephropathy, which is

the most common underlying long-term cause of allograft loss. Currently, the major immunosuppressive agents being used in various combination regimens are: corticosteroids (prednisone), antiproliferative agents (azathioprine, mycophenolate mofetil), calcineurin inhibitors (tacrolimus, cyclosporine), and mammalian target of rapamycin inhibitors (mTOR [sirolimus, everolimus]) (Table 2) (161, 162, 163).

Treatment of BKVAN is still evolving and, as the optimal maintenance immunosuppressive therapy is still not well established, the best treatment so far is an early diagnosis of the disease in order to act before renal damage is caused. The first-line treatment of BKVAN is the reduction of the immunosuppression (106, 116, 164) but current evidences suggest that prevention based upon a screening and preemptive strategy is superior to an approach that relies upon therapy of established disease (165).

Given the frequency of polyomavirus infection and the potential for graft loss, randomized trials of treatment and prevention strategies are absolutely needed to better guide therapy and improve outcomes for kidney transplant patients with BKVAN.

1.2.5.1. Treatment

Many studies are being developed in order to find effective treatment for BKVAN and at the same time to reduce the risk of rejection and to preserve renal function. The mainstay of therapy for BKVAN in kidney transplant recipients is to improve BKV-specific immunity by reducing or discontinuing immunosuppressive drugs (166). Several strategies and their combinations have been reported in the context of immunosuppressive therapy in patients with definitive diagnosis of nephropathy. One of the strategies involves first the reduction of the calcineurin inhibitor (tacrolimus or cyclosporin) by 25%–50%, followed by the reduction of the antiproliferative agent (MMF or azathioprine) by 50% or

discontinuation of the last if viremia persists (135, 167, 168) (**Figure 9**). Another strategy is based in the reduction of the antiproliferative agent by 50% followed by the reduction of calcineurin inhibitor by 25%–50% followed by discontinuation of the antiproliferative agent if necessary (168, 169) (**Figure 9**). A substitution of the calcineurin inhibitor tacrolimus by a lower dose of cyclosporine or switching from calcineurin inhibitor to low-dose of sirolimus (mTOR agent) can also be an effective additional strategy. Steroid (prednisone) avoidance has also been suggested to decrease the prevalence of BKVAN (170). The reduction of immunosuppression strategy can also be considered for patients with presumptive BKVAN defined by sustained viremia (10^4 copies/mL) for more than three weeks. The protocol appears to be safe and effective for preventing BKVAN and clearing BKV viremia with low rates of acute rejection (144). Few reports suggest the use of adjuvant drugs such as cidofovir, leflunomide, intravenous immunoglobulin (IVIG) or fluoroquinolones in combination with immunosuppression reduction in patients with sustained high-level of plasma BKV load (171, 172). However, larger randomized control trials proving that adjunctive use of these agents with antiviral activity is superior over timely reduced immunosuppression are still missing.

1.2.5.2. Prevention strategies

A screening and preemptive strategy is based in the idea that periodic monitoring of viruria (Vr) and viremia (Vm) allows the detection of early systemic infection, which permits a prompt treatment including a decrease of immunosuppression and/or administration of antiviral agents, and may limit morbidity and mortality (129, 158). The appearance of BK Vr is a characteristic that precedes the development of BKVAN, reflecting viral activation and replication in the renourinary tract. However, BK Vr is not by itself diagnostic of renal parenchymal involvement. In contrast the identification of BK Vm is pathognomonic of renal parenchymal disease and allows a diagnosis of presumptive

BKVAN (164). Higher prevalence of Vr, as opposed to Vm, and lack of good correlation with Vr induced investigators to use Vm as a better marker for preemptive reduction in immunosuppressive therapy (136).

Current guidelines for the screening and diagnosis have been published (128, 168). Screening for BKV replication must be performed at least every three months, for up to two years post-transplant and then annually until the fifth year post-transplant (128). BKV replication in the urine (Vr) can be studied either by cytology for decoy cells or by quantitative PCR for BKV DNA or VP1 mRNA (128, 137, 156, 168, 173, 174). Patients with a positive screening test (decoy cells or urine DNA load greater than 10^7 copies/mL) should be tested for plasma BKV DNA load (Vm). In patients with plasma DNA load greater than 10^4 copies/mL and persistent for more than three weeks, an allograft biopsy should be considered to definitively diagnose BKVAN (128, 168) (**Figure 9**). Vigorous post-transplantation screening and preemptive strategies allows the identification of patients at risk for BKVAN before significant parenchymal damage occurs by the detection of the disease at an early stage, and improves graft survival of patients with BKVAN.

Table 2. Immunosuppressive drugs used in kidney transplantation (162)

Group	Drug	Mechanisms
Calcineurin inhibitors	Cyclosporine	Binds to cyclophilin; complex inhibits calcineurin phosphatase and T cell activation
	Tacrolimus	Binds to FKBP12; complex inhibits calcineurin phosphatase and T cell activationn
Antiproliferative agents	Mycophenolate mophetil	Inhibits synthesis of guanosine monophosphate nucleotides; blocks purine synthesis, preventing proliferation of T and B cells
	Azathioprine	Converts 6-mercaptopurine to tissue inhibitor of metalloproteinase, which is converted to thioguanine nucleotides that interfere with DNA synthesis; thioguanine derivates may inhibit purine synthesis

Corticosteroids	Prednisone	Inhibits T cell activation with subsequent prevention of T cells from attacking the transplanted organ
Mammalian target of rapamycin inhibitors (mTOR)	Sirolimus	Binds to FKBP12; complex inhibits target of rapamycin and interleukin-2-driven T cell proliferation
	Everolimus	Derivate of sirolimus

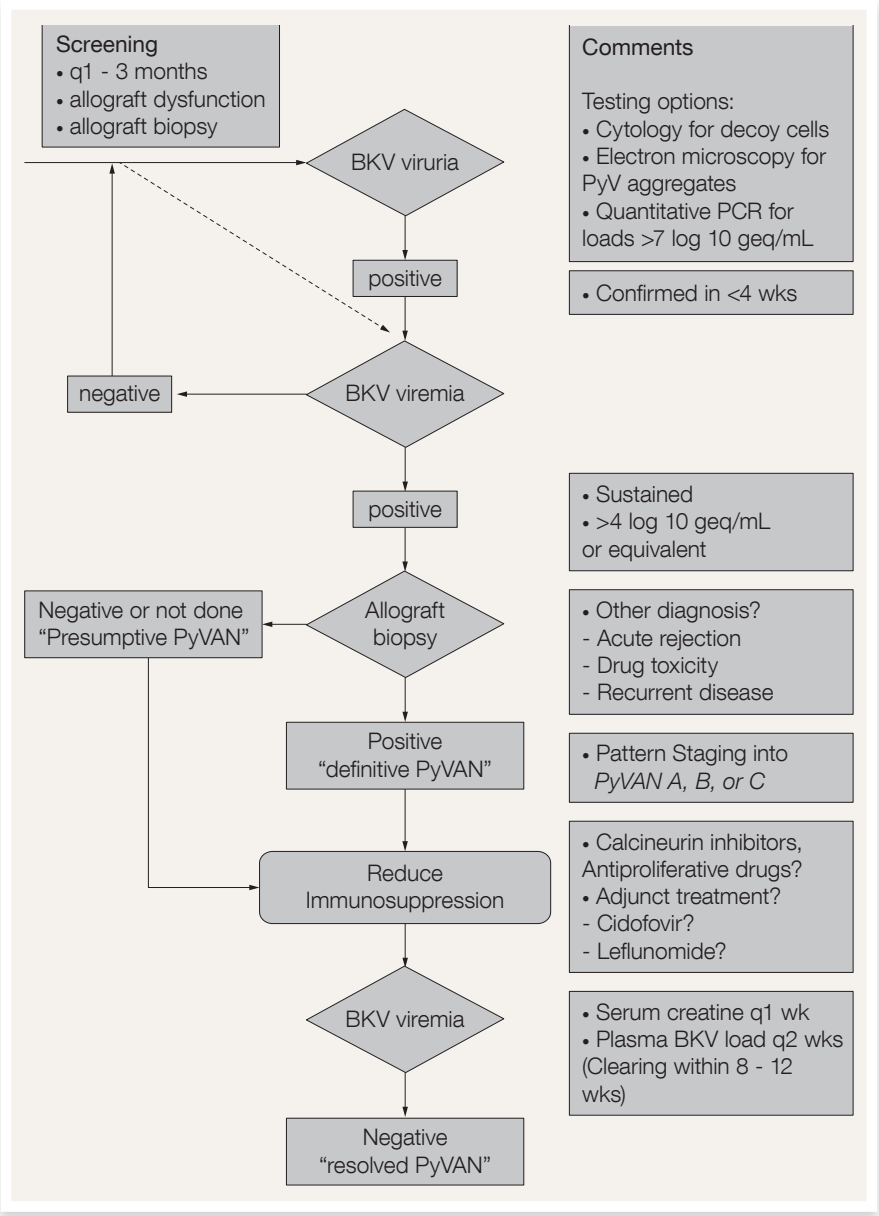


Figure 9. Screening and treating BKV replication and disease in kidney transplant patients.
Taken from: *Am. J. Transplant.* 9, S136–S146 (2009).

1.2.6. Polyomavirus BK and the activation of innate immune defense mechanisms leading to BKVAN

For polyomavirus BK, dsDNA and dsRNA are critical targets. The dsRNA is a molecular pattern associated with viral infection because most viruses at some point produce it during their replication cycle (42, 175). In this study, we restricted our search for receptors that could be involved in PVAN to those that sense nucleic acids in the cytoplasm and endosomal compartments. Among the several intracellular dsRNA sensors described above, TLR3 and RIG-I seem to be good candidates to play an essential role in PVAN. TLR3 recognizes viral dsRNA, which is generated during life cycle of many virus, and its synthetic analogue poly(I:C) through its unique adaptor protein TRIF (28, 176, 177). Recognition of viral RNA by TLR3 triggers activation of the transcription factors NF-κB and IRF3 and induction of type I IFNs, which are critical for cellular antiviral responses. RIG-I is a highly inducible cytoplasmic RNA helicase that signals antiviral responses after binding dsRNA and ssRNA containing a 5`triphosphate (5`-ppp). This pathway has been implicated in antiviral responses to Sendai virus, vesicular stomatitis virus, Newcastle disease virus, as well as different flaviviruses and Kaposi's sarcoma-associated herpesvirus (54, 55, 56) and the cytosolic B form of dsDNA, poly(dA-dT) · poly(dA-dT) via the cytosolic DNA-dependent RNA polymerase III (PolIII) (57).

Because of the characteristics involved in BKV invasion of host cells and subsequent intracellular trafficking we hypothesized that particularly TLR3 could be a receptor candidate to mediate activation of innate immunity in PVAN. First, TLR3 resides in the endosomal membrane and the endoplasmic reticulum and moves to dsRNA-containing endosomes in response to dsRNA (178). As BKV has previously been shown to enter cells through endocytosis, viral dsRNAs could activate TLR3 during BKV infection of tubular epithelial cells upon viral entry and uncoating in the endosome. Second, upregulation of the TLR3 pathway was reported in response to various viruses

containing a dsDNA genome: KSHV upregulates TLR3 expression in human monocytes during primary infection (179); TLR3 can control herpesvirus infection, since children with a TLR3 deficiency were very susceptible to HSV-1-induced encephalitis (180); Infection of a neuron cell line with HSV-1 triggers IL-6 and IRF1 mRNA production (181). In addition, in a mouse model of murine CMV infection, the TLR3-mediated TRIF signaling pathway is activated on viral inoculation, contributing to innate defense against systemic viral infection (103).

In this study, we examined the activation of TLR3 and RIG-I in allograft biopsy samples of PVAN and in human collecting duct epithelial cells after poly(I:C) and cytokine stimulation, as well as after infection with BKV. Our findings indicate that activation of innate immune defense mechanisms, that is, TLR3, contributes to the inflammation in PVAN.

In summary, the detailed immunopathogenesis as well as specific pathways activated during the viral infection of the kidney leading to significant inflammatory glomerular and tubulointerstitial injury are poorly understood. In particular, the role of the innate immune system, the function of Toll-like receptors, in particular TLR3, and the involvement of the recently identified IFN-induced RNA helicases (RIG-I and MDA5) and oligomerization domain (NOD)-like receptors, sensing the presence of viral dsRNA remain to be fully elucidated. To study the molecular and cellular pathways initiated in the PVAN was, therefore, the goal of this current research project. The identification of specific signaling cascades activated during the viral infection of renal cells should allow us to identify new target molecules for a specific antiviral and anti-inflammatory therapy in this disorder.

CHAPTER 2

OBJECTIVES

2. Objectives

As PVAN is a significant complication after kidney transplantation, which can often lead to graft dysfunction and may cause morbidity and even mortality, and innate immune system has a critical role in recognizing viral infections and evoking initial antiviral responses, the aim of this study was to identify antiviral responses of the renal tubular epithelium during the course of this pathology. For this, we studied:

- The activation of TLR3 and RIG-I in allograft biopsy samples of PVAN and in human collecting duct epithelial cells after poly(I:C) and cytokine stimulation as well as after infection with BKV;

- The activation of proinflammatory cytokines and type I IFNs produced downstream TLR3 and RIG-I;

- The BK virus–host cell interaction and the activation of signaling cascades leading to renal inflammation.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

3.1. Materials

3.1.1. Equipments and instruments

Balances:

- Analytical Balance, BP 110 S (Sartorius, Göttingen, Germany)

Cell Incubators:

- Type B5060 EC-CO2 (Heraeus Sepatech, München, Germany)

Centrifuges:

- Heraeus, Megafuge 1.0R (Thermo Scientific, Langenselbold, Germany)
- Hettich Zentrifugen, Rotante 460 R (Hettich Zentrifugen, Adelsried, Germany)
- Hettich Zentrifugen, Universal 16 (Hettich Zentrifugen, Adelsried, Germany)
- Eppendorf, Centrifuge 5415 D (Eppendorf, Hamburg, Germany)

ELISA-Reader:

- Tecan, GENios Plus (Tecan, Crailsheim, Germany)

Spectrophotometer:

- Beckman DU® 530 (Beckman Coulter, Fullerton, CA, USA)

TaqMan Sequence Detection System:

- ABI prism ™7700 sequence detector (PE Biosystems, Darmstadt, Germany)

Microscopes:

- Leica SP5 (Leica Microsystems, Solms, Germany)

3.1.2. Other equipments

- Thermomixer 5436 (Eppendorf, Hamburg, Germany)
- Vortex genie 2™ (Bender&Hobein AG, Zurich, Switzerland)
- Water bath HI 1210 (Leica Microsystems, Bensheim, Germany)

3.1.3. Chemicals and reagents

Cell culture:

- Dulbeccos's Modified Eagle's medium (GIBCO/Life technologies, Paisley, Scotland, UK)
- Ham's F12 medium (GIBCO/Life technologies, Paisley, Scotland, UK)
- Insulin (Sigma-Aldrich, Steinheim, Germany)
- Dexamethasone (Sigma-Aldrich, Steinheim, Germany)
- Sodium Selenite (Sigma-Aldrich, Steinheim, Germany)
- Transferrine (Sigma-Aldrich, Steinheim, Germany)
- L-Glutamine (GIBCO/Life technologies, Paisley, Scotland, UK)
- Hepes Buffer (GIBCO/Life technologies, Paisley, Scotland, UK)

- Heat-inactivated Fetal Bovine Serum (Biochrom KG, Berlim, Germany)
- Dulbecco's PBS (1x) (PAA laboratories, Cölbe, Germany)
- Trypsine/EDTA (1x) (PAA laboratories, Cölbe, Germany)
- Penicillin/Streptomycin (PAA laboratories, Cölbe, Germany)

Stimulation experiments:

- Poly(I:C) (Invivogen, San Diego, CA, USA)
- Poly(dA:dT) (Sigma-Aldrich, Steinheim, Germany)
- TNF-α (PeproTech, Hamburg, Germany)
- IL-1β (PeproTech, Hamburg, Germany)
- IFN-α (PeproTech, Hamburg, Germany)
- Opti-MEM I medium (GIBCO/Life technologies, Paisley, Scotland, UK)
- Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany)

Molecular biology techniques:

- PureLink RNA Mini Kit (Invitrogen, Karlsruhe, Germany)
- RNase free DNase (Qiagen, Hilden, Germany)
- Superscript II (Invitrogen, Karlsruhe, Germany)
- Hexanucleotide Mix (Roche, Mannheim, Germany)
- Recombinant RNasin Ribonuclease inhibitor (Promega, Mannheim, Germany)
- dNTP Set (Fermentas, St. Leon-Rot, Germany)
- Acrylamide (Ambion, Tokyo, Japan)
- 5 × buffer (Invitrogen, Karlsruhe, Germany)
- DTT (Invitrogen, Karlsruhe, Germany)
- Qubit RNA assay Kit (Invitrogen, Karlsruhe, Germany)
- RT-PCR primers (Applied Biosystems, Darmstadt, Germany)
- DC protein assay kit (Bio-Rad Laboratories, Munich, Germany)

- Western Lightning Chemiluminescence reagent plus Kit (PerkinElmer, Massachusetts, USA)

ELISA Kits:

- Human IL-6 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA)
- Human CXCL8/IL-8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA)
- Human IL-1β/IL-1F2 Quantikine HS ELISA kit (R&D Systems, Minneapolis, MN, USA)
- Human IFN-β ELISA Kit (PBL Interferon Source, New Jersey, USA)

Antibodies:

- Human TLR3 antibody (Acris Antibodies, Herford, Germany)
- Human RIG-I antibody (provided by Dr. Simon Rothenfusser (182))
- Human IL-1β/IL-1F2 antibody (R&D Systems, Minneapolis, MN, USA)
- Rabbit anti-mouse IgG [H&L]-HRP (Acris Antibodies; Hiddenhausen, Germany)
- Goat anti-rat IgG+IgM [H+L] (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA, USA)
- Rabbit anti-goat IgG Peroxidase conjugate (Calbiochem, San Diego, CA, USA)
- Rabbit polyclonal to beta-Actin (Abcam, Cambridge, UK)
- Goat anti-rabbit IgG Peroxidase Conjugate (Calbiochem, San Diego, CA, USA)
- Mouse anti-T-Ag (Calbiochem, San Diego, CA, USA)

Knockdown experiments:

- siPort NeoFX Transfection Agent (Ambion, Tokyo, Japan)
- Pre-designed siRNA for TLR3 or RIG-I (Ambion, Tokyo, Japan)
- Silencer negative control siRNA (Ambion, Tokyo, Japan)

3.1.4. Other Chemicals

- Bovine Serum Albumin (Roche Diagnostics, Mannheim, Germany)
- DMSO (Merck, Darmstadt, Germany)
- EDTA (Calbiochem, San Diego, USA)
- Ethanol (Merk, Darmstadt, Germany)
- Isopropanol (Merk, Darmstadt, Germany)
- Mercaptoethanol (Roth, Karlsruhe, Germany)
- Trypan blue (Sigma, deisenhofen, Germany)

3.1.5. Miscellaneous

- Accu-jet Pro Pipette controller (Brand, Wertheim, Germany)
- Pipette's tip 1-1000 µl (Eppendorf, Hamburg, Germany)
- PVDF membrane (Millipore Immobion, Schwalbach, Germany)
- Tissue culture flasks 15 and 75 cm^2 (TPP, Trasadingen, Switzerland)

3.2. Methods

3.2.1. Preparation of human tissue

For mRNA expression analysis, parts of human renal biopsies were obtained according to the local ethical committees directives and samples were processed according to

the protocol of an European multicenter study for gene expression analysis (the European Renal cDNA Bank — Kroener-Fresenius Biopsy Bank, ERCB-KFB) (183). Microdissected tubulointerstitial specimens from 21 patients were analyzed, including 10 allograft biopsies with acute cellular rejection and 5 allograft biopsies with PVAN. In both groups the Banff histologic scores and the immuno-histochemical analysis of inflammatory infiltrates were similar. For control biopsies, renal tissue was derived from pretransplantation kidney biopsies during cold ischemia time from three cadaveric (CON CDx, n=3) and three living donors (CON LDx, n=3).

For double immunofluorescence staining, paraffin-embedded tissue sections from a total of 23 renal allografts including 19 cases with a histological secured diagnosis of PVAN and 4 randomly chosen transplant biopsies with AR were obtained from the Department of Cellular and Molecular Pathology at the German Cancer Research Center following guidelines of the respective Ethics Committees.

3.2.2. Immunofluorescence technique for TLR3 and SV40 double staining

For immunofluorescence double staining, kidney needle biopsies from patients with PVAN were fixed and embedded in paraffin. 5 µm sections were prepared and mounted on glass slides. After microwave antigen retrieval with citrate buffer, the specimens were treated with blocking buffer and subsequently with primary antibodies against SV40 ((1:20) mouse T Antigen) or TLR3 ((1:100) anti-human guinea pig), followed by species-specific secondary antibodies as described (184). Specimens were analyzed on a Leica SP5 confocal microscope and confocal stacks were modeled using Volocity software (Waltham, MA).

3.2.3. Cell culture and stimulation experiments

3.2.3.1. Cell culture of immortalized human collecting duct epithelial cells (HCDCs)

Immortalized human cortical collecting duct epithelial cells (HCDCs) were provided by H. Debiec (185). Cell line was maintained under standard cultural conditions (in an incubator set at 37°C supplied with 5.0% CO2) in culture medium containing a 1:1 mixture of Dulbecco's Modified Eagle's medium and Ham's F12 medium supplemented with 5 µg/ml Insulin, 5×10^{-8} M Dexamethasone, 3×10^{-8} M Sodium Selenite, 5 µg/ml Transferrine, 2 mM Glutamine, 10 M HEPES Buffer and 2% heat-inactivated fetal bovine serum.

3.2.3.2. Stimulation experiments

Extracellular poly(I:C) or cytokine stimulation of HCDCs

For stimulation experiments, HCDCs were plated in culture six-well plates one day before the experiment. For ELISA and RNA extraction, cells were incubated with medium control or a combination of TNF-α (25 ng/ml), IL-1β (10 ng/ml) and IFN-γ (20 ng/ml) for 24 h, washed with PBS, incubated in culture medium for 6 hours and washed again with PBS. Subsequently, HCDCs were incubated with medium control or medium containing poly(I:C) (10 µg/ml) for 12 h. After the incubation period, aliquots of the supernatant medium were removed for ELISA analysis at the times indicated and cells were washed with PBS and prepared for RNA isolation.

Intracellular poly(I:C) or poly(dA:dT) transfection in HCDCs

24 h before transfection, cells were plated in a culture 6-well plate in 2 ml growth medium. To examine the effect of poly(I:C) or poly(dA:dT) intracellularly, HCDCs were treated with medium control or transfected with poly(I:C) (5 µg/ml) or poly(dA:dT) (5 µg/ml) in different time intervals (3, 6, 9, 12 and 24 h) using Lipofectamine 2000. After stimulation, cell supernatants were collected for ELISA and cells were washed with PBS and prepared for RNA isolation

BKV infection

For infection of the cells, virus prepared previously was dissolved in culture medium and 1 ml was added to HCDCs that were plated 24 h before. Cells were incubated with medium alone (control) or medium containing the virus for different time intervals (3, 6, 9, 12 and 24 h). After each time point, corresponding supernatants were collected for ELISA analysis and cells were prepared for RNA isolation.

3.2.4. RNA isolation, cDNA synthesis and real-time RT-PCR

3.2.4.1. RNA isolation from cultured cells

After stimulation with the combination of cytokines and/or poly(I:C) or poly(dA:dT), RNA isolation was performed using a RNeasy Mini Kit, according to the manufacture protocol. Cells were washed with PBS to remove residual medium and 350 µl of RLT buffer (lysis

buffer) containing 1% β-mercaptoethanol were added to the cells. Cells were scraped and immediately transferred to microcentrifuge tubes and frozen at -80°C until RNA isolation. For RNA isolation, cell lysates were thawed and 350 µl of 70% ethanol were added. The mixture was mixed well by pipetting to blend thoroughly and to disperse any visible precipitate that may form after adding ethanol. This mixture was transferred to RNeasy mini-columns held in 2 ml collection tubes and centrifuged for 15 s at 10000 rpm. The flow-through was discarded and 350 µl of RW1 buffer (washing membrane-bound RNA buffer) were added to the column and centrifuged again for 15 s at 10000 rpm. After discarding the flow-through RNase-free DNase was added to the samples for an efficient on-column digestion of DNA during RNA purification. After 15 min incubation at room temperature, 350 µl of RW1 buffer were added again to the column and centrifuged 15 s at 10000 rpm. The flow-through was discarded together with the collection tubes and the column was transferred in new 2 ml collecting tubes. 500 µl of RPE buffer (washing membrane-bound RNA buffer) were added to the column and centrifuged for 15 s at 10000 rpm and the flow-through was discarded. This step was once again repeated. The column was placed in a new 1.5 ml collection tube, 35 µl of RNase free water were added to the column and centrifuged for 1 min at 10000 rpm to elute the RNA.

3.2.4.2. Complementary DNA synthesis

The RNA samples isolated according to the procedure detailed above were diluted in DEPC water (1 µg/30 µl) and a mastermix containing 9 µl of 5 × buffer, 1 µl of 25 mM dNTP mixture, 2 µl of 1.1 M DTT, 1 µl of 40U/µl RNasin, 0.5 µl of 15 µg/ml acrylamide, 0.5 µl of hexanucleotides and 1 µl of superscript (or ddH_2O in case of the negative controls) was prepared. 15 µl of mastermix were added to each RNA sample and incubated at 42°C on a thermal shaker incubator for 90 min. After incubation, cDNA samples were collected and stored at -20°C. For real-time RT-PCR samples were diluted for 10 times in ddH_2O.

3.2.4.3. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Real-time RT-PCR analysis was performed on a TaqMan ABI 7700 sequence detection system using heat-activated *Taq*DNA polymerase. After an initial hold of two minutes at 50°C and ten minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. For all quantitative analyses the cDNA content of each sample was compared with another sample following the ΔΔCt technique. This procedure uses the formula $A_0/B_0 = (1 + E)^{(Ct,B-Ct,A)}$, where A_0 is the initial copy number of sample A; B_0 is the initial copy number of sample B; E is the efficiency of amplification; Ct,A is the threshold cycle of sample A; and Ct,B is the threshold cycle of sample B. The amplification efficiency was defined as 1, as all analyses were performed during the same run, including control dilution series. Following the formula above, the cDNA content of sample A compared to sample B was calculated by subtracting the mean Ct (triplicates) of sample A from the mean Ct of sample B (= ΔCt). Copies of the individual transcripts in sample A were defined as $2^{\Delta Ct}$ copies of transcripts in sample B.

Commercially available predeveloped TaqMan reagents were used for the human target genes TLR1-8, TLR10, RIG-I, IL-6, RANTES/CCL5, MCP-1/CCL2, IL-8/CXCL8, IFN-β, IP-10/CXCL10, IL-1β, NLRP3, AIM2, caspase-1, TRIF and two endogenous control genes (18S rRNA, GAPDH) (**Table 3**). For human TLR9 detection a primer pair was used: forward primer: 5`-CGGAGGCTGGATGTCAGC-3`; reverse primer: 5`-GGAGTGGTCCACT GTCTTGAGG-3`; specific probe: 5`-AGCTGCGAGAGCTCAACCTTAGCGC-3`; FAM and TAMRA labeled. For detection of BKV Large T-antigen sequence, the following primer pair was used: forward primer: 5`-GGAAAGTCTTTAGGGTCTTCTACCTTT-3`; reverse primer: 5`-GGTGCCAACCTATGGAACAGA-3`; specific probe: 5`-AATCTGCTGTTGCT TCTTCATCACTGGCA-3`, VIC and TAMRA labelled. The normalization to both reference

(housekeeping) genes gave comparable results. All measurements were performed in duplicates. Controls consisting of bidistilled H_2O were negative in all runs.

Table 3. Taqman gene expression assays used in the study

Gene	Accession number	Assay ID	Context Seq AoD
IL-6	NM_000600	Hs00174131_m1	TGGATTCAATGAGGAGACTTGCCTGGTGAA
RANTES/ CCL5	NM_002985.2	Hs00174575_m1	TCCAACCCAGCAGTCGTCTTTGTCACCCG
MCP1/ CCL2	NM_002982.3	Hs00234140_m1	TCAGCCAGATGCAATCAATGCCCCA
IL-8/CXCL8	NM_000584.2	Hs00174103_m1	GCTCTGTGTGAAGGTGCAGTTTTGCCAAG
IP-10/ CXCL10	NM_001565.2	Hs00171042_m1	GTGGCATTCAAGGAGTACCTCTCTCTAGAAC
IFN-β	NM_002176.2	Hs00277188_s1	TACCTCCGAAACTGAAGATCTCCTAGCCT
TLR3	NM_003265.2	Hs00152933_m1	TAGCAGTCATCCAACAGAATCATGAGACAG
RIG-I	NM_014314.3	Hs00204833_m1	CCCTAGACCATGCAGGTTATTCTGGACTTT
IL-1β	NM_000576.2	Hs00174097_m1	TGGAGCAACAAGTGGTGTTCTCCATG
NLRP3	NM_183395.2 NM_001079821.2 NM_001127462.2 NM_001127461.2 NM_001243133.1 NM_004895.4	Hs00918082_m1	GAGCCGAAGTGGGGTTCAGATAATG
AIM2	NM_004833.1	Hs00175457_m1	GATCAGGAGGCTGATCCCAAAGTTGTCA
Caspase-1	NM_001223.4 NM_001257118.1 NM_00125719.1 NM_033292.2 NM_033293.2 NM_033294.2 NM_033295.2	Hs00354836_m1	CCGCAAGGTTCGATTTTCATTTGAG
TRIF	NM_182919.2	Hs01090712_m1	GCAGCCCCGGATCCCTGATCTGCTT
TLR1	NM_030956.3	Hs00374069_g1	TTGAAACTTCCATCTGTAAGGCTAT
TLR2	NM_003264.3	Hs00152932_m1	TGGTAGTTGTGGGTTGAAGCACTGG

TLR4	NM_003266.3	Hs00370853_m1	CTGCGTGGAGACTTGGCCCTAAACC
TLR5	NM_003268.5	Hs00152825_m1	ACAGTCACCAAACCAGGGATGCTAT
TLR6	NM_006068.4	Hs00271977_s1	CCAGCTACTTGATTTAAGTGTTTTC
TLR7	NM_016562.3	Hs00152971_m1	GACTAAAAATGGTGTTTCCAATGTG
TLR8	NM_016610.2	Hs00152972_m1	GGAAAGGAGACTAAAAAGGAAAACA
TLR10	NM_030956.3	Hs00374069_g1	TTGAAACTTCCATCTGTAAGGCTAT
GAPDH	NM_002046.3	4310884E	
18S rRNA	X03205.1	4310893E	

3.2.5. Fluorescence-activated cell sorting (FACS) analysis for TLR3

HCDCs were cultured under basal conditions for 24 h. For FACS analysis cultured cells were detached with PBS/10 mM EDTA (pH 8) and incubated for 30 min on ice with a monoclonal antibody against TLR3 or the appropriate isotype controls. After two washing steps with PBS, cells were incubated with a fluorescein isothiocyanate (FITC) rabbit secondary antibody against mouse $F(ab)_2$ fragment and incubated on ice for 30 min. After washing the cells twice with PBS, the tubes were centrifuged for 2 min at 1200 rpm and finally the pellet was suspended in 200 µl of PBS and FACS analysis was carried out. For intracellular staining, HCDCs were treated with Cytofix/Cytoperm Reagent followed by monoclonal antibody against TLR3 and secondary antibody incubations in saponin buffer. The TLR3 signal was analyzed using a FACSCalibur with CellQuest analysis software. Appropriate IgG isotype preparations (mouse IgG1 clone MOPC 21) were used to control for unspecific staining.

3.2.6. Western immunoblot analysis for TLR3, RIG-I and IL-1β

After incubation with poly(I:C) or poly(dA:dT) with or without prestimulation with proinflammatory cytokines, HCDCs were lysed with RadioImmunoPrecipitation assay (RIPA) buffer (50 mM Tris-HCL, pH 8; 150 mM NaCl; 1% NP-40; 0,5% Sodium Deoxycholate; 0,1% SDS) containing protease inhibitors. The lysates were cleared by centrifugation at 13.000 X g for 15 min at 4°C and protein concentration was determined with DC protein assay kit. Proteins (40 µg/ml) were separated by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) on an 8% polyacrylamide gel and transferred to a PVDF membrane for western blotting. The membranes were blocked in 3% milk solution in Tris-buffered saline-Tween 20 (TLR3) or 5% Bovine Serum Albumin (RIG-I, IL-1β, beta-Actin loading control) for 1 h and probed overnight with a monoclonal antibody against human TLR3 or human RIG-I or human IL-1β.

After washing four times in TBST (Tris-buffered saline-Tween 20), the membranes were incubated with secondary purified rabbit anti-mouse IgG [H&L]-HRP (TLR3) or secondary peroxidase-conjugated AffiniPure goat anti-rat IgG+IgM [H+L] (RIG-I) or anti-goat IgG peroxidase conjugate (IL-1β) for 1 h and washed again four times with TBST. Signals were visualized with a Western Lightning Chemiluminescence reagent plus Kit.

Beta-Actin was used as a loading control and the secondary antibody was an anti-rabbit IgG Peroxidase conjugate.

3.2.7. ELISA

ELISA for IL-6, IL-8/CXCL8, IL-1β and IFN-β were performed on cell culture supernatants using commercial assay kits and according to the supplied instructions.

For IL-6 and IL-8/CXCL8, 100 µl of Assay Diluent were added to each well. Next, 100 µl (IL-6) or 50 µl (IL-8/CXCL8) of standard, sample or control were added per well and the plate was incubated for 2 h at room temperature. After the incubation time, each well was washed four times with 400 µl of Wash Buffer followed by the complete removal of the liquid. 200 µl (IL-6) or 100 µl (IL-8/CXCL8) of Conjugate solution was added to each well and the plate was incubated again for 2 h (IL-6) or 1 h (IL-8/CXCL8) at room temperature. After the incubation time, the wells were washed again four times with 400 µl of Wash Buffer and this was followed by the complete removal of the liquid. 200 µl of Substrate solution were added to each well and the plate was incubated for 20 min (IL-6) or 30 min (IL-8/CXCL8) at room temperature and protected from light. 50 µl of the Stop solution were then added to each well and the optical density of each well was determined using a microplate reader set to 450 nm with a wavelength correction of 570 nm.

The results were determined by calculating the average of the duplicate readings for each standard, control and sample. A standard curve was created by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best curve through the points on the graph.

For IL-1β, 100 µl of Assay Diluent were added to each well followed by 150 µl of standard, sample or control. The plate was incubated for 3 h at room temperature on a horizontal orbital microplate shaker set at 500 rpm. After the incubation time, the wells were washed six times with 400 µl of Wash Buffer followed by the complete removal of the liquid. Next, 200 µl of Conjugate solution were added to the wells and the plate was

incubated for 2 h at room temperature on the shaker. After the incubation time, 50 µl of Substrate solution were added to each well and the plate was incubated again for 30 min at room temperature and on the shaker. 50 µl of Amplifier solution were added to each well followed by a new incubation period of 30 min at room temperature on the shaker. Finally, 50 µl of Stop solution were added to the wells and the optical density of each well was determined at 490 nm with a wavelength correction of 690 nm. The results were determined as described above.

For IFN-β, 100 µl of interferon standards, blanks and samples were added to the each well and the plate was incubated for 1 h at room temperature. After 1 h the wells were washed three times with 300 µl of diluted Wash Buffer. Next, 100 µl of diluted antibody solution were added to all wells and the plate was incubated for 1 h at room temperature. After the incubation time, the wells were washed again three times with 300 µl of diluted Wash Buffer and 100 µl of diluted HRP solution were added to all wells. The plate was incubated for 1 h. After the incubation time the wells were washed three times with diluted Wash Buffer solution and 100 µl of TMB Substrate solution were added to each well. The plate was incubated in the dark for 15 min at room temperature. After the 15 min incubation, 100 µl of Stop solution were added to each well and the absorbance was determined at 450 nm. The results were determined as described above.

3.2.8. Knockdown of gene expression with short interfering RNA (siRNA)

Cells were trypsinized one hour or less before transfection and set aside at 37°C. siPort NeoFX was diluted in Opti-MEM I medium using the suggested amounts from the company and incubated for 10 min at room temperature. Next, small RNA was diluted in

Opti-MEM I medium for a final concentration of 10 nM. Diluted siNeoFX was combined with the diluted RNA, mixed by pipetting up and down and incubated 10 min at room temperature. The newly formed transfection complexes were dispensed into the empty wells of a culture 6-well plate. Cells prepared previously were seeded into the culture plate wells (at a density of 2.3×10^5 cells/well) containing transfection complexes. Without swirling, the plate was gently rocked back and forth to evenly distribute the complexes.

Transfection of siRNA into the cells was performed with pre-designed siRNA for TLR3 or RIG-I over 24 h. The following day, cells were stimulated with poly(I:C) (10 µg/ml) for 12 h. The culture supernatants and cells were separated and then processed for real-time RT-PCR analysis. Scrambled siRNA was used as the nonspecific negative control of siRNA.

3.2.9. Polyomavirus BK preparation and infection of HCDCs with polyomavirus (type BK) derived from patients with BKV reactivation

For BK virus isolation, a urine sample with a viral load of $>10^8$ copies/ml from a patient with a known BKV reactivation was used. The sample was inoculated for 1 h on Vero cells before minimal essential medium (MEM)/2% FCS was added. Viral propagation was monitored in the supernatant by quantitative real-time PCR with an in house-test on a 7500 Fast Real-Time PCR-System using primers from the Large T antigen region and a VIC-labeled probe (see above). At a load of 10^9 copies/ml, virus was harvested by centrifugation of the supernatant and subsequently passaged on HEK 293 cells with MEM/2% FCS for 60 days. Infected cells were split at 10-day intervals. Viral growth

was monitored by quantitative PCR and by immunofluorescence against T antigen. To isolate BKV, HEK 293 cells were lysed by repeated freeze-thaw cycles (3 times) and subsequent centrifugation at 800g for 30 min. The supernatant was adjusted to 5000 FFUs/ml with PBS and stored at -80°C. For experiments virus was dissolved in culture medium and 1 ml was added to HCDCs. HCDCs were stimulated as described before.

3.2.10. Statistical analysis

Values are provided as mean ± SEM. Statistical analysis was performed by unpaired *t*-test if applicable or by analyses of variance. Significant differences are indicated for *P*-values <0.05 (*) or 0.01 (**), respectively.

CHAPTER 4

RESULTS

4. Results

4.1. In biopsies with PVAN, staining for polyomavirus (Anti-SV40) and TLR3 colocalized in epithelial cells of cortical tubules and the collecting duct

Polyomavirus BK exhibits a tropism for the renal tubular epithelium, particularly in distal nephron segments, where it establishes latent infection. Immunofluorescence double staining for the SV40 large T antigen (T-Ag) of polyomavirus and TLR3 in allograft biopsies with histological diagnosis of PVAN showed colocalization of nuclear signals for polyomavirus and intracytoplasmatic signals for TLR3 in epithelial cells of distal cortical tubules and the collecting duct (**Figure 10**). Intensity of immunofluorescence signals and SV40/TLR3 colocalization was semiquantitatively and separately assessed in medullary collecting ducts and cortical tubules. The results are summarized in table 4. In general, we found a great variation in SV40 and TLR3 signal intensity in the different biopsies, with an overall most intense TLR3 staining in medullary collecting ducts of PVAN. Highest semiquantitative SV40/TLR3 colocalization rates (partially >75%) were found in collecting ducts of the medulla in biopsies with few interstitial infiltrates. TLR staining in renal transplant biopsies without BKV infection was significantly less intense than in PVAN, and revealed a more cortical accentuated pattern with a most prominent TLR3 expression in cortical atrophic and dilated tubules.

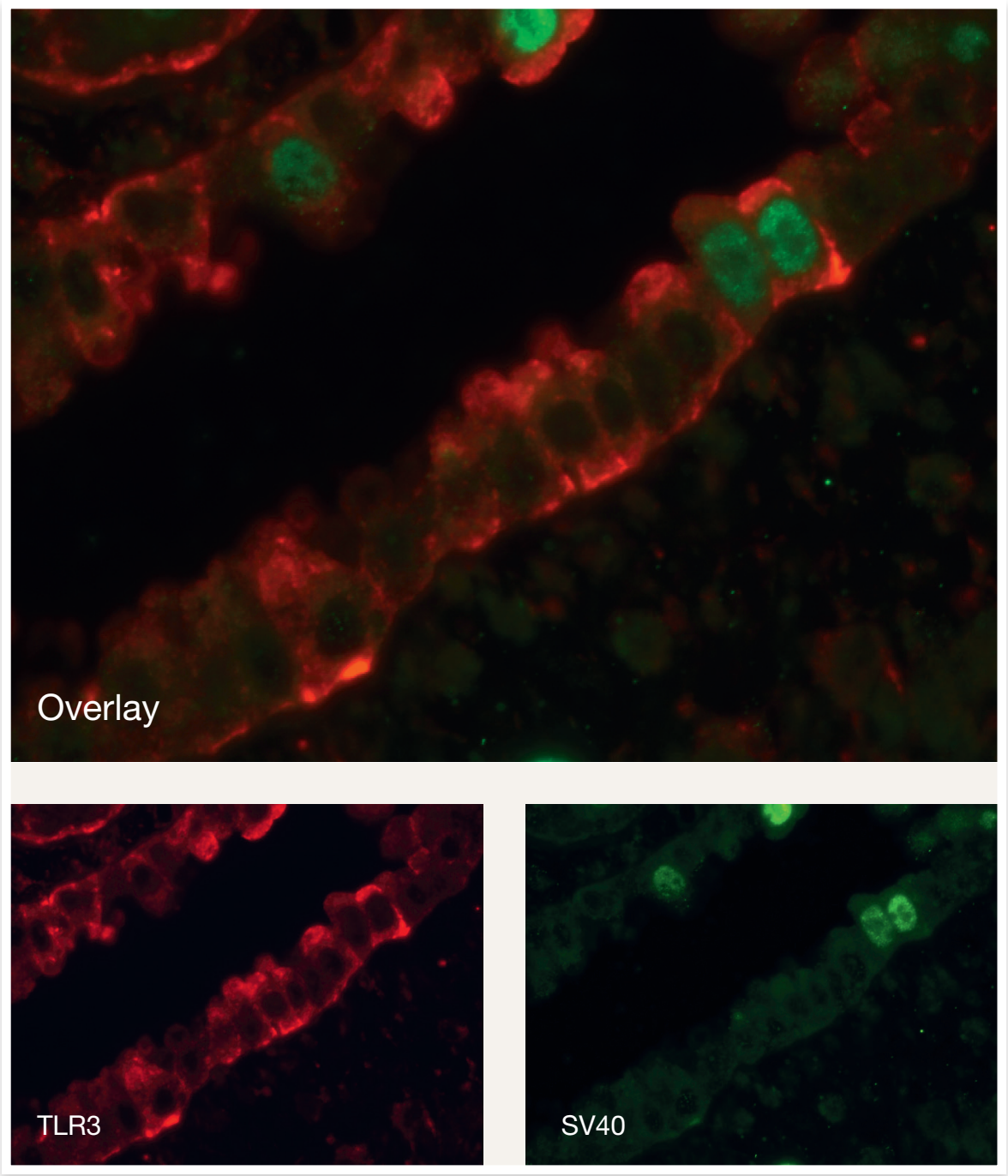


Figure 10. Immunofluorescence double staining for Toll-like receptor 3 (TLR3) and SV40 in human kidney biopsies with clinical and histological diagnosis of polyomavirus-associated nephropathy (PVAN): colocalization of polyomavirus (anti-SV40) and TLR3 protein expression in epithelial cells of distal cortical tubules and collecting duct. Double immunofluorescence staining of paraffin-embebed tissue sections of kidney needle biopsies from individuals with PVAN. Colocalization of nuclear signals for polyomavirus (SV40, green) and intracytoplasmic signals for TLR3 (red) in tubular epithelial cells of distal cortical tubules and collecting duct.

Table 4. TLR3/SV40 double immunofluorescence staining in PVAN biopsies.

	Immunofluorescence		TLR3/SV40 colocalization		
PVAN case no.	SV40 positivity	TLR3 positivity	Medullary collecting duct	Cortex	Interstitial infiltrates
1	+++ Strong	+ Few, focal	++	++	Moderate
2	+ Few, focal	+ Few	++++	+++	Few
3	(+) Focal	++ Moderate	+++	++++	Few, focal
4	+++ Strong	+ Few	++++	++	Few
5	+ Few	++ Moderate	++	+	Moderate
6	+++ Strong	++ Moderate	++	++	Moderate
7	(+) Focal	+ Few	++++	+	Few, focal
8	(+) Focal	+ Few	+	++	Moderate
9	(+) Focal	++ Moderate	+	+	Moderate
10	+++ Strong	+++ Strong	++++	++++	Absent
11	+ Few	+++ Strong	++	+	Moderate
12	+++ Strong	+++ Strong	+++	++	Severe, diffuse
13	+++ Strong	+ Few	++	++	Moderate
14	+++ Strong	+ Few	+++	+	Few, focal
15	+++ Strong	+ Few	+++	+++	Absent
16	+ Few	+ Few	++++	+++	Few, focal
17	++ Moderate	+++ Strong	++++	++++	Few
18	++ Moderate	+++ Strong	+++	++	Severe, diffuse
19	(+) Focal	++ Moderate	++++	+++	Few, focal

Abbreviations: PVAN, polyomavirus-associated nephropathy; TLR3, Toll-like receptor 3.
TLR3/SV40 colocalization was evaluated semiquantitatively as: +, <25%; ++, ≥25 to <50%; +++, ≥50 to <75%; ++++, ≥75%.
Highest medullary TLR3/SV40 colocalization rates were found in PVAN biopsies with absent or few and focal interstitial infiltrates. In randomly chosen renal transplant biopsies with negative immunohistochemical SV40 staining, TLR3 expression was nearly absent in proximal tubules, and was strong in dilated atrophic tubules.

4.2. TLR3 mRNA expression, but not RIG-I mRNA expression is significantly induced in biopsies with PVAN compared to allograft biopsies with ongoing acute rejection (AR)

To compare the activation of innate immune defense mechanisms, we analysed the mRNA expression of TLR3 and RIG-I in the tubulointerstitial compartment of manually microdissected kidney biopsies. We observed a significant induction of TLR3 mRNA expression in the tubulointerstitial compartment of PVAN compared to renal allograft biopsies with AR and pretransplant donor biopsies. Despite similar inflammatory infiltrates by immunohistochemical analysis (**Table 5**), the increased expression of TLR3 mRNA in PVAN clearly differentiated them from those with AR (**Figure 11a**). In contrast, RIG-I mRNA expression was significantly induced in renal allograft biopsies with AR when compared to PVAN and controls (**Figure 11b**). As expected, according to the equally intense inflammatory infiltrate, the mRNA expression of CCL5/RANTES was significantly upregulated in both AR and PVAN as compared to controls (**Figure 11c**).

Table 5. Histological findings and diagnoses in renal transplant biopsies included in the mRNA expression analysis.

PVAN Case Nr.	Interstitial Infiltrates	Histological diagnosis
I	(+) few Mononuclear lymphocytes, minimal edema, no tubulitis	PVAN, no viral inclusions
II	+ lymphocytes (>25%), focal, no tubulitis	PVAN, viral inclusions, No Rejection
III	++ lymphocytes, plasma cells, few granulocytes (30%)	PVAN, no viral inclusions
IV	++ mononuclear lymphocytes (30%), minimal edema	PVAN, no viral inclusions
V	++ focal moderate (20%)	PVAN, no viral inclusions moderate interstitial Rejection

AR Case Nr.	Interstitial Infiltrates	Histological diagnosis
I	+++ mononuclear lymphocytes, histiocytes (>50%), cortical and medulla, intense tubulitis	Acute cellular Rejection BANFF Ib-II
II	++ (30%) focal, mononuclear lymphocytes, massive edema	Acute tubulointerstitial Rejection BANFF I-II
III	++ focal moderate lymphocytes, monocytes, plasma cells, focal tubulitis	BANFF Borderline
IV	+++ (>50%) lympho-plasmacellular infiltrates	Acute tubulointerstitial Rejection BANFF IIa
V	++ plasma cells, histiocytes	Acute cellular Rejection BANFF Ib
VI	++ (>25%) lymphocytes, histiocytes, few plasma cells, focal tubulitis, diffuse edema	Acute cellular Rejection BANFF Ib
VII	+ focal lymphocytes, leucocytes, tubulitis	Acute vascular cellular Rejection BANFF IIa
VIII	++ moderate lymphocytes, few granulocytes	Acute vascular cellular Rejection BANFF IIb
IX	+++ (50%) lymphocytes, histiocytes, few granulocytes, minimal edema	Acute cellular Rejection BANFF Ia
X	+++ (50%) lymphocytes, histiocytes, few granulocytes, diffuse edema	Acute vascular cellular Rejection BANFF IIa

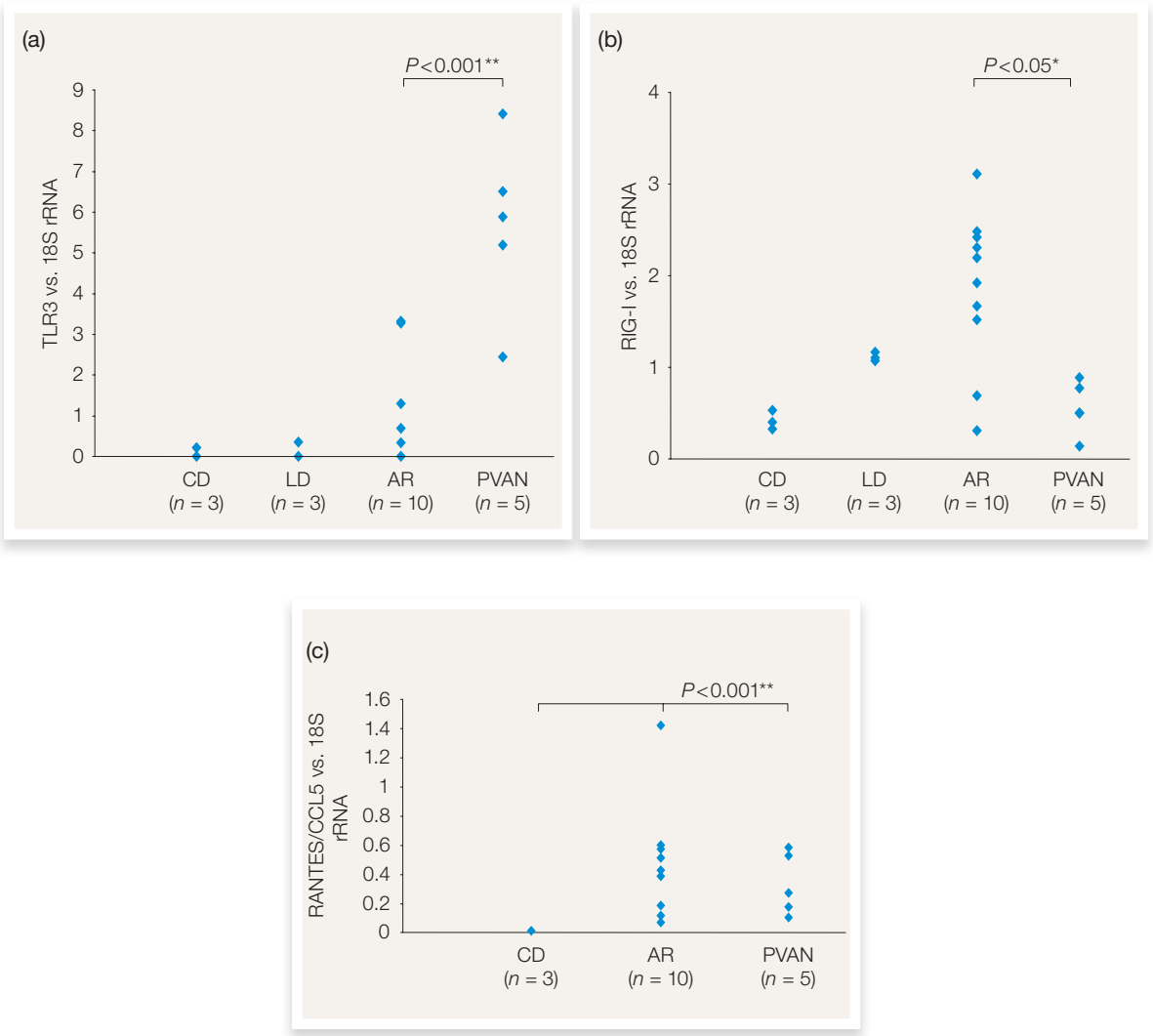


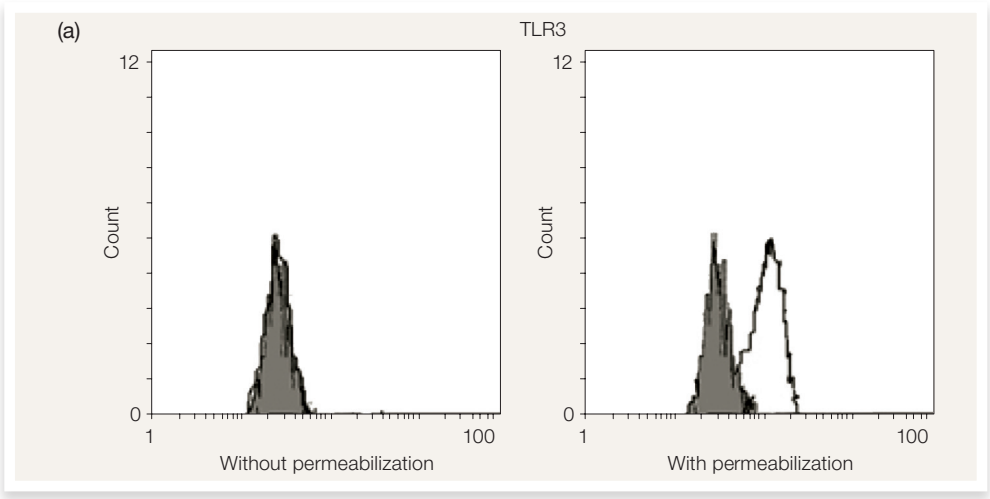
Figure 11. Toll-like receptor 3 (TLR3), retinoic acid inducible gene-I (RIG-I) and RANTES/CCL5 mRNA expression in the tubulointerstitial compartment of manually microdissected renal transplant biopsies. **(a)** Expression of mRNA for TLR3 shows a significant induction in polyomavirus-associated nephropathy (PVAN) compared with renal allograft biopsies with acute cellular rejection (AR) and pretransplant donor biopsies (cadaveric donor (CD) biopsies and living donor (LV) biopsies). **(b)** In renal allograft biopsies with AR, RIG-I mRNA is significantly induced when compared with both controls and biopsies with PVAN. **(c)** RANTES/CCL5 (regulated upon activation, normal T cell expressed and secreted)/(chemokine (C-C motif) ligand 5) was significantly upregulated in AR and PVAN compared with pretransplant donor biopsies from living donors. *P<0.05; **P<0.001.

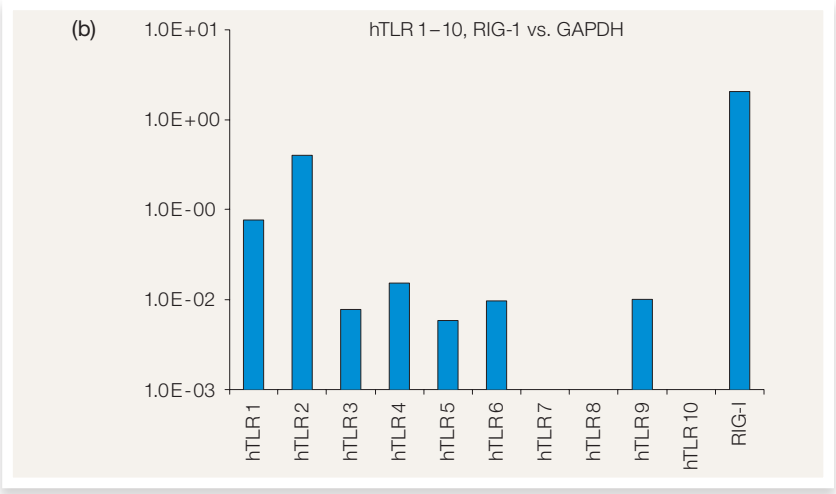
4.3. Expression of TLR3 and RIG-I protein on HCDCs in culture is enhanced after stimulation with poly(I:C) and proinflammatory cytokines

To confirm the expression of TLR3 protein on HCDCs, we performed FACS analysis with a TLR3 specific monoclonal antibody. No detectable surface staining for TLR3 was found on HCDCs under basal conditions. As TLR3 has been described as an intracellular, vacuolar receptor (43), HCDCs were permeabilized prior to TLR3 staining and FACS analysis. This resulted in a robust TLR3 staining of HCDCs (**Figure 12a**).

In order to test whether HCDCs might express other TLRs under basal conditions, we screened for mRNA of TLR1 through TLR10 by real-time RT-PCR. In addition to TLR3, HCDCs also robustly expressed TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 (**Figure 12b**). TLR7 and TLR10 expression was too low to allow evaluation and no expression was found for TLR8 (**Figure 12b**).

To confirm TLR3 expression and to test the expression of RIG-I protein on HCDCs, we conducted western blot analysis with specific monoclonal antibodies for TLR3 and RIG-I (**Figure 12c**). Non-stimulated HCDCs showed slight TLR3 and moderate RIG-I basal protein expression. Stimulation by poly(I:C) (10 µg/ml) as a mimetic of viral dsRNA, induces a significant increase in the TLR3 protein expression, whereas RIG-I protein expression showed only a marginal increase. Stimulation of HCDCs with a combination of proinflammatory cytokines (TNF-α, IL-1β and IFN-γ) combined with poly(I:C) (10 µg/ml) enhanced both TLR3 and RIG-I expression. HEK 293 cells were used as controls and showed a robust TLR3 and RIG-I protein expression.





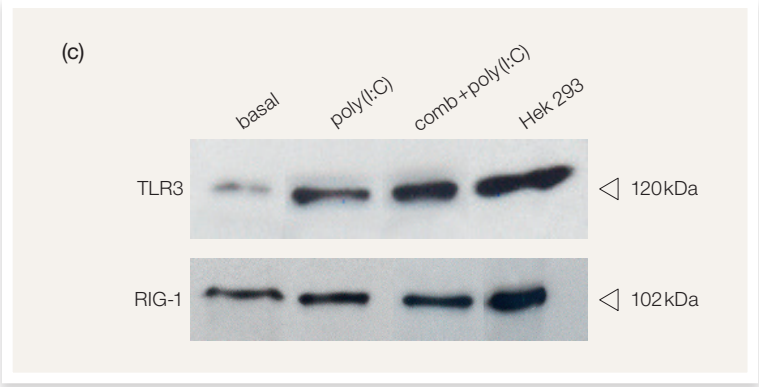


Figure 12. Localization of Toll-like receptor 3 (TLR3) and expression of TLR1 to 10 and retinoic acid inducible gene-1 (RIG-I) in cultured human collecting duct epithelial cells (HCDCs). **(a)** The localization of TLR3 protein on HCDCs was analysed by flow cytometry without and with prior permeabilization. No TLR3 signal was found on HCDCs without permeabilization, whereas a clear signal for TLR3 was detected after permeabilization. **Open histograms** represent the fluorescence activity after

incubation with a monoclonal anti-TLR3 antibody (Ab). **Filled histograms** denominate the signal of the appropriate isotype control. Results shown are from one of two independent experiments, which showed reproducible staining patterns. **(b)** HCDCs were cultured under basal conditions for 24 h before RNA extraction. Real-time reverse transcriptase (RT)-PCR was performed using primers specific for the human TLR1–10 and RIG-I as indicated in material and methods. HCDCs express TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9 and RIG-I mRNA under basal conditions. No expression of TLR7, TLR8 and TLR10 mRNA was observed under the same conditions. **(c)** Western blot analysis for TLR3 and RIG-I protein expression was performed in HCDCs incubated with 10 µg/ml polyriboinosinic: polyribocytidylic acid (poly(I:C)) with or without prestimulation with a combination of proinflammatory cytokines (tumor necrosis factor-α (TNF-α), interleukine-1β (IL-1β) and interferon-γ (IFN-γ)), or medium alone. HEK 293 cells were used as positive control. Poly(I:C), mimicking viral double-stranded RNA (dsRNA), induced TLR3 and RIG-I protein expression. This effect was potentiated after prestimulation with the combination of cytokines (comb + poly(I:C)).

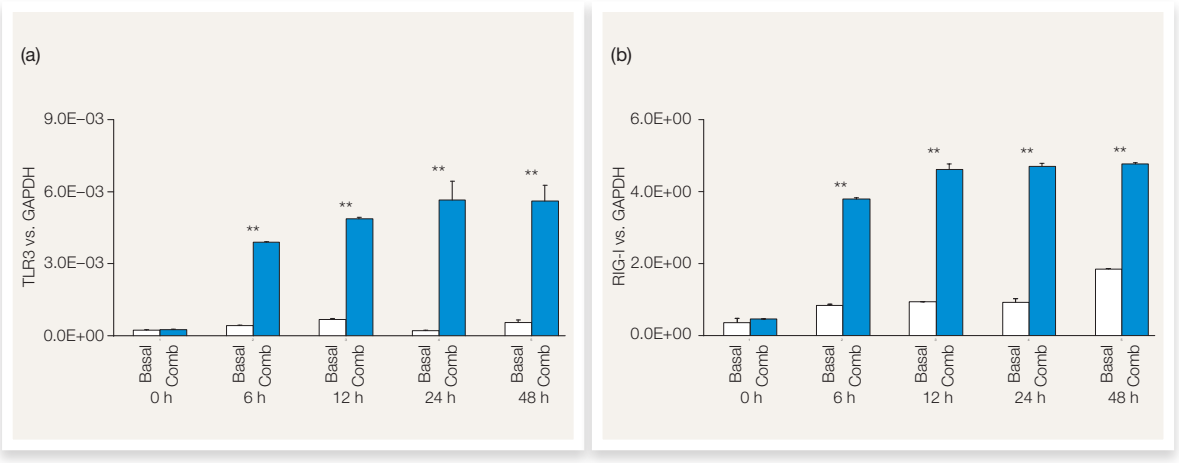
4.4. Expression of TLR3 and RIG-I mRNA increases after stimulation with poly(I:C) and proinflammatory cytokines

To verify our results on a transcriptional level, HCDCs were also examined for TLR3 and RIG-I mRNA expression.

RNA was prepared from HCDCs growing under standard conditions as well as from HCDCs that had been stimulated with a combination of the cytokines TNF-α, IL-1β and IFN-γ for different time intervals (0, 6, 12, 24, 48 h) to simulate a proinflammatory milieu as would occur during ascending polyomavirus infection. By real-time RT-PCR, TLR3 mRNA was amplified from both unstimulated (basal) and stimulated (comb) cells. The low basal expression for TLR3 was significantly increased with the cytokine combination in a time-dependent manner up to 48 h (**Figure 13a**). Similar results were found for RIG-I mRNA expression (**Figure 13b**).

To test the effect of poly(I:C) on the expression of TLR3 and RIG-I mRNA, HCDCs were preincubated with (comb) or without (basal) the combination of proinflammatory cytokines (TNF-α, IL-1β and IFN-γ) for 24 hours and posteriorly incubated with or without poly(I:C) (10 µg/ml) for 12 hours prior to RNA extraction and real-time RT-PCR. The low basal expression of TLR3 (**Figure 13c**) and RIG-I mRNA (**Figure 13d**) was significantly enhanced by stimulation with proinflammatory cytokines (comb). Incubation with poly(I:C) significantly increased basal TLR3 but not RIG-I mRNA expression (**Figures 13c, 13d**). When HCDCs had been pretreated with proinflammatory cytokines, incubation with poly(I:C) (comb + poly(I:C)) markedly enhanced TLR3 and RIG-I mRNA expression. This effect was highly significant compared to basal conditions and to cytokine stimulated conditions (comb), respectively.

Stimulation of HCDCs with increasing concentrations of poly(I:C) (1, 5, 10 100 µg/ml) for 12 h (white columns) slightly enhanced the basal mRNA expression of TLR3 and RIG-I with a maximal increase upon stimulation with 100 µg/ml of poly(I:C). Prestimulation with the cytokine combination (blue columns) strongly increased the poly(I:C)-induced TLR3 and RIG-I mRNA expression (**Figures 13e, 13f**). Taken together, prestimulation with a combination of proinflammatory cytokines was a potent inducer of significant TLR3/RIG-I upregulation.



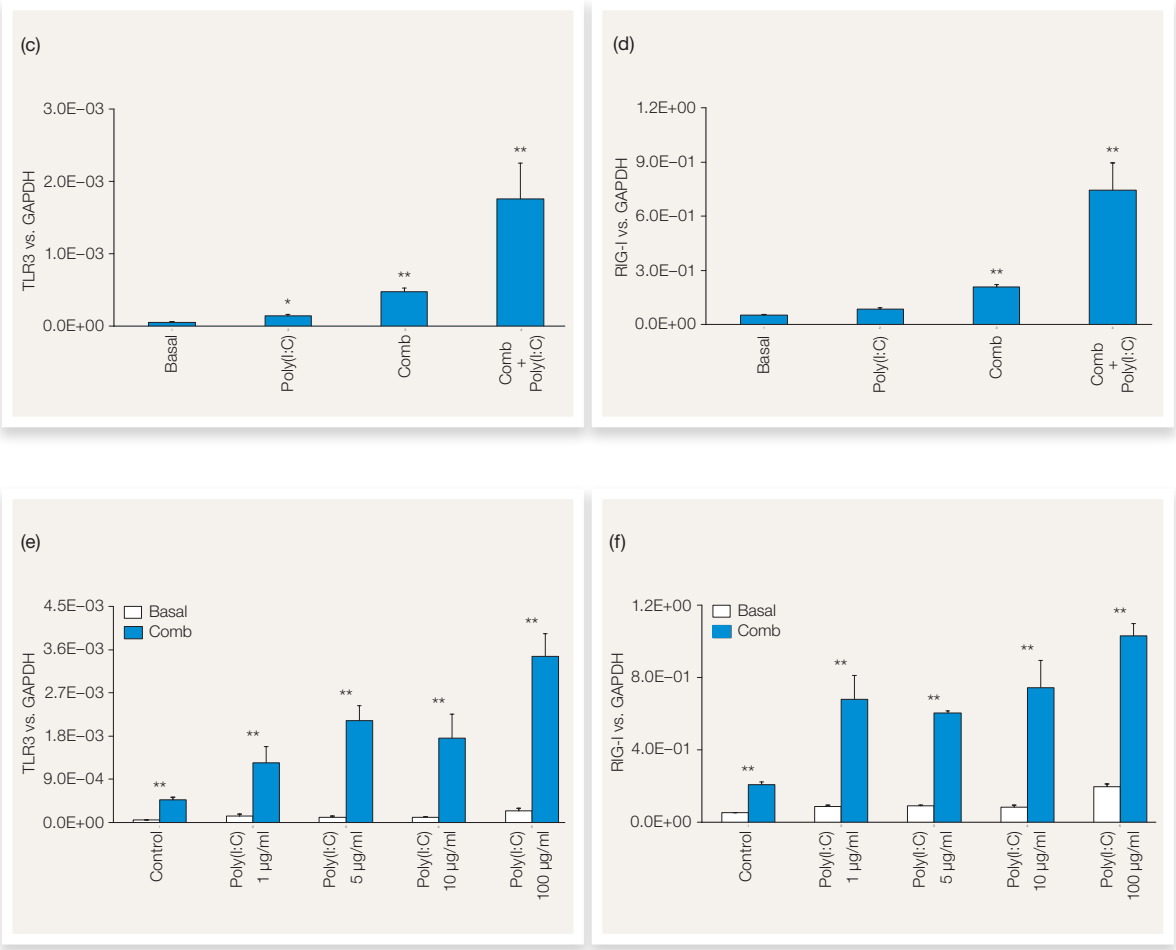


Figure 13. Expression of Toll-like receptor 3 (TLR3) and retinoic acid inducible gene-I (RIG-I) mRNA on cultured human collecting duct epithelial cells (HCDCs). HCDCs were stimulated with (Comb) or without (Basal) a combination of cytokines (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-g (IFN-γ)) for the times indicated and levels of mRNA expression for TLR3 and RIG-I were analyzed by Real-time reverse transcriptase (RT)-PCR. **(a)** The low basal expression for TLR3 was increased with the cytokine combination in a time-dependent manner up to 48 h. **(b)** Similar results were found for RIG-I mRNA expression. To test the effect of TLR3 and RIG-I activation by polyriboinosinic:polyribocytidylic acid (poly(I:C)), mimicking viral double-stranded RNA (dsRNA), HCDCs were preincubated with (Comb) or without (Basal) the cytokine combination (TNF-α, IL-1β, and IFN-γ) and subsequently stimulated with and without poly(I:C) for 12 h. The mRNA expression for TLR3 and for RIG-I was analyzed by real-time RT-PCR. HCDCs showed low basal expression for **(c)** TLR3 and **(d)** RIG-I mRNA, which was induced by stimulation with proinflammatory cytokines (Comb). Incubation with poly(I:C) revealed a significant increase of basal TLR3, but not RIG-I, expression. **(e, f)** After pretreatment with proinflammatory cytokines, incubation with different concentrations of poly(I:C) (Comb + poly(I:C)) markedly enhanced TLR3 and RIG-I mRNA expression. Data are representative of three independent experiments. *P<0.05; **P<0.01. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

4.5. Activation of TLR3 and RIG-I by poly(I:C) significantly enhanced the mRNA expression of the proinflammatory cytokine IL-6, the chemokines RANTES/CCL5, MCP-1/CCL2, IL-8/CXCL8, and IP-10/CXCL10 and the antiviral IFN-β

To test the "downstream" effects of TLR3 and RIG-I activation by poly(I:C) mimicking viral dsRNA in HCDCs, we studied mRNA expression of selected cytokines and chemokines (IL-6, RANTES/CCL5, MCP-1/CCL2, IL-8/CXCL8 and IP-10/CXCL10). HCDCs showed low basal expression for IL-6 mRNA. IL-6 expression was significantly induced by stimulation with proinflammatory cytokines (comb). Stimulation with poly(I:C) increased IL-6 expression without preconditioning, an effect that was markedly potentiated when HCDCs had been preconditioned with proinflammatory cytokines (comb + poly(I:C)) (**Figure 14a**) in order to enhance their TLR3 expression. Similar results were obtained for MCP-1/CCL2 (**Figure 14c**) and IL-8/CXCL8 (**Figure 14d**). Expression of RANTES/CCL5 (**Figure 14b**) could not be increased by poly(I:C) stimulation alone, but was increased when cells were preconditioned with cytokines alone (comb) and even more after cytokine preconditioning followed by additional poly(I:C) stimulation (comb + poly(I:C)). Basal expression for IP-10/CXCL10 mRNA (**Figure 14e**) was not detectable and after poly(I:C) stimulation only a slight mRNA expression for IP-10/CXCL10 was found. Pretreatment with the cytokine combination (comb) for 24 h resulted in a significantly increased IP-10/CXCL10 mRNA expression, but remained similar after additional poly(I:C) stimulation (comb + poly(I:C)). For the antiviral IFN-β, basal expression was moderately increased after poly(I:C) stimulation, and in parallel to the chemokines studied afore, preconditioning with the cytokine combination revealed a significant increase in IFN-β mRNA expression (**Figure 14f**).

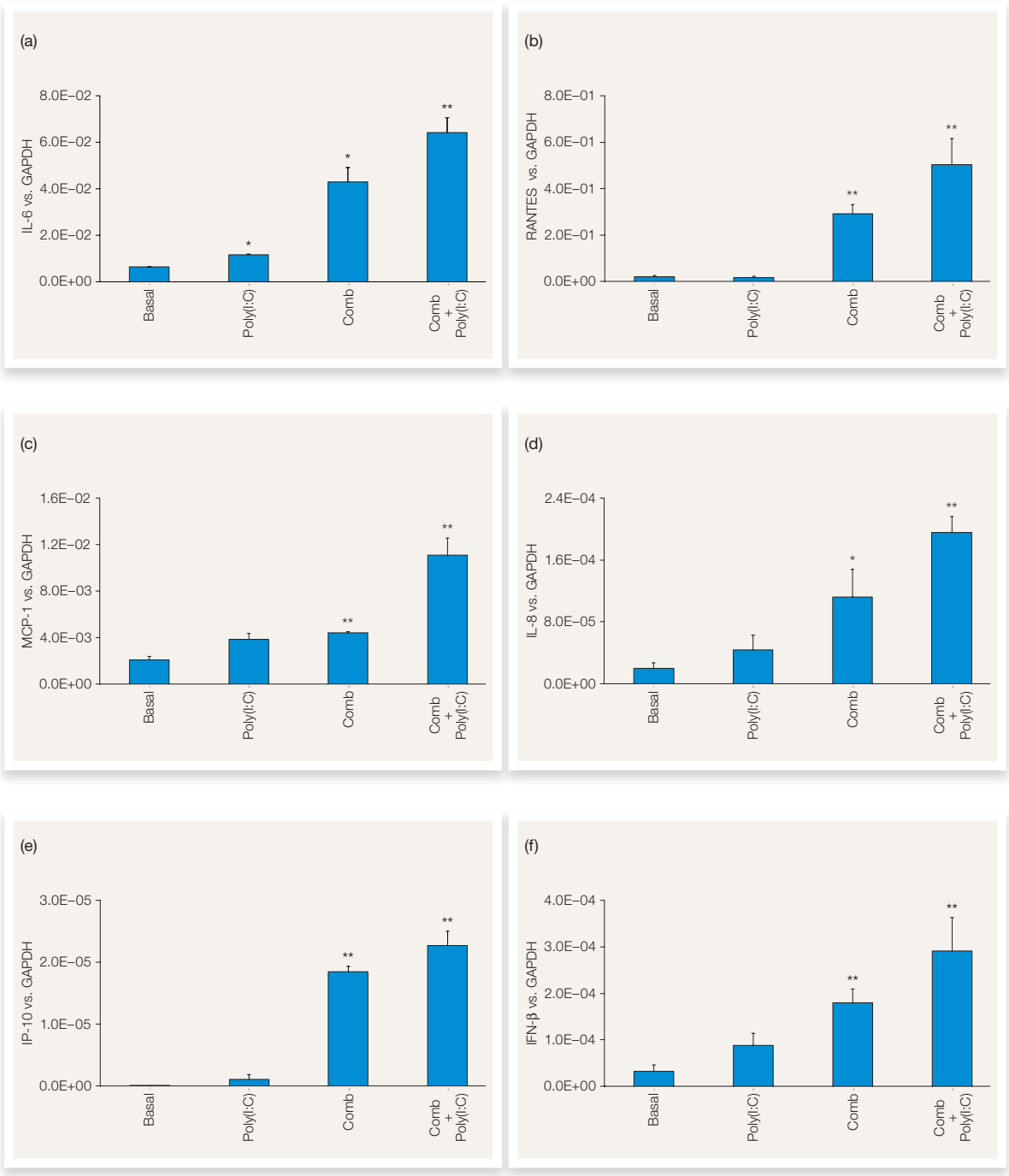
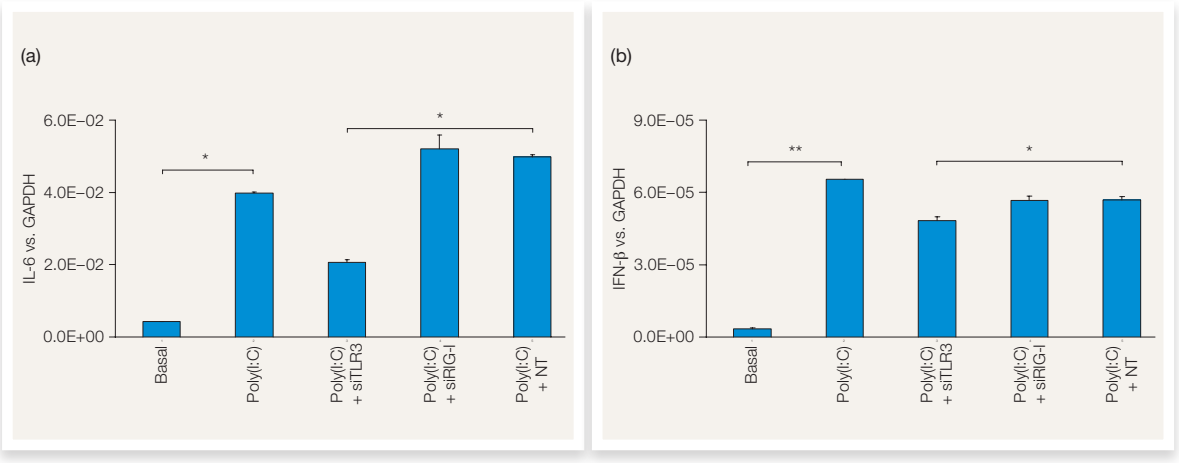


Figure 14. Effect of polyriboinosinic:polyribocytidylic acid (poly(I:C)) and cytokine prestimulation on the mRNA expression of selected cytokines and chemokines. Human collecting duct epithelial cells (HCDCs) were pretreated with (Comb) or without (Basal) a cytokine combination (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ)) for 24 h and incubated with or without poly(I:C) for 12 h. mRNA levels for IL-6 **(a)**, MCP-1/CCL2 **(c)**, Il-8/CXCL8 **(d)**, IP-10/CXCL10 **(e)** and IFN-β **(f)** slightly increased after stimulation with poly(I:C), an effect potentiated after pretreatment with the cytokine combination. **(b)** Expression of RANTES/CCL5 was not increased by poly(I:C) without preconditioning, but was increased by preconditioning with cytokines alone (Comb) and even more after cytokine preconditioning followed by additional poly(I:C) stimulation (Comb + p(I:C)). Data are representative of three independent experiments. *P<0.05; **P<0.01.

4.6. Knockdown of TLR3 with siRNA inhibits the poly(I:C) induced expression of IL-6 and IFN-β in cultured HCDCs, whereas knockdown of RIG-I had no effect on the expression of both cytokines

Knockdown experiments using the respective short interfering RNAs (siRNAs) were performed to evaluate which of the two receptors (TLR3 or RIG-I) might be involved in the IL-6 and IFN-β response to poly(I:C) stimulation. HCDCs were transfected with siRNA for TLR3 (siTLR3) or RIG-I (siRIG-I) and were stimulated with poly(I:C) (10 µg/ml), mimicking viral dsRNA, for 12 h. IL-6 and IFN-β expression was analysed by real-time RT-PCR (**Figure 15a**, **15b**) and ELISA (**Figure 15c**).

The poly(I:C) induced IL-6 and IFN-β mRNA expression was significantly reduced in cells pretreated with siRNA for TLR3. In contrast, siRNA for RIG-I did not significantly decrease the poly(I:C) induced IL-6 or IFN-β mRNA expression (**Figure 15a**, **15b**). ELISA for IL-6 confirmed the poly(I:C)-induced upregulation of IL-6 protein, which was also only attenuated by siRNA for TLR3, but not by siRNA for RIG-I (**Figure 15c**). ELISA for IFN-β was also performed but no protein expression was observed maybe due to the low sensitivity of the used ELISA kit.



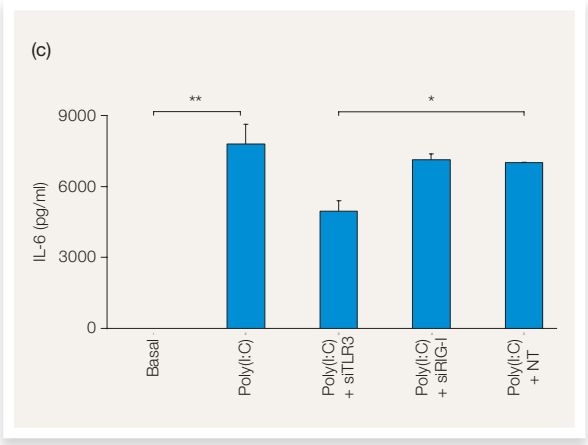
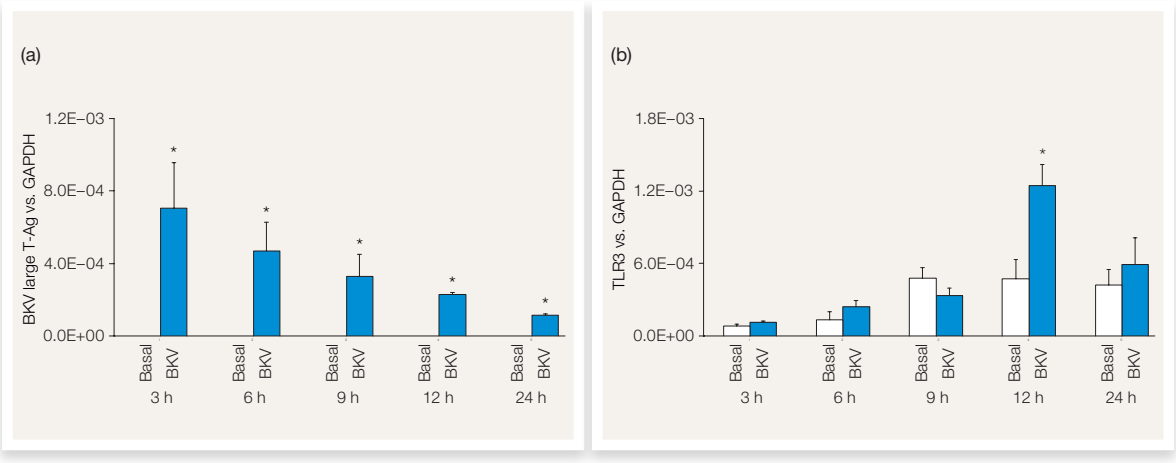


Figure 15. Effect of transfection with short interfering RNA (siRNA) for Toll-like receptor 3 (TLR3) or retinoic acid inducible gene-I (RIG-I) on the expression of IL-6 and IFN-β. Human collecting duct epithelial cells (HCDCs) were transfected with siRNA specific for TLR3 or RIG-I and stimulated with polyriboinosinic:polyribocytidylic acid (poly(I:C)) for 12 h. **(a)** Poly(I:C) significantly increased IL-6 mRNA expression, an effect that was significantly attenuated by siRNA specific for TLR3. In contrast siRNA for RIG-I did not significantly reduced IL-6 mRNA expression. **(b)** IFN-β mRNA expression was significantly increased after poly(I:C) stimulation, an effect that was significantly attenuated in cells pretreated with siRNA for TLR3 but not in cells pretreated with siRNA for RIG-I. **(c)** IL-6 mRNA expression was confirmed by enzyme-linked immunosorbent assay (ELISA) but IFN-β mRNA expression could not be confirmed on protein level. Data are representative of three independent experiments. *P<0.05; **P<0.01; NT, non-targeting Negative Control siRNA.

4.7. Infection of cultured HCDCs with BKV resulted in a significant increase of IL-6, IL-8/CXCL8 and TLR-3 and RIG I expression

In order to simulate an '*in vivo*' model of BKV infection of the distal nephron segment, HCDCs were infected with BKV as described above. To evaluate the expression of TLR3, RIG-I and cytokines in response to a viral infection, the cells were incubated with BKV for different time intervals (3, 6, 9, 12, 24 h). As a read-out system for effective viral infection of HCDCs, we analyzed the expression of BKV Large T-Antigen (large T-Ag), representing a marker protein of the early phase of BKV infection. Highest expression of Large T-Antigen was detected at 3 h post infection by real-time RT-PCR and decreased in a time-dependent manner (**Figure 16a**).

Basal TLR3 and RIG-I mRNA expression showed a slight increase during HCDC culture over 24 h and after infection with BKV the mRNA expression of both receptors was markedly upregulated at 12 h, and then immediately decreased (**Figures 16b, 16c**). The TLR3 adaptor molecule TRIF showed a robust constitutive basal expression in HCDCs but BKV exposure was not able to enhance TRIF expression in our model, as determined by real-time RT-PCR (**Figure 16d**). To test potential effects of BKV infection on selected cytokine and chemokine generation by HCDCs, we determined IL-6 and IL-8/CXCL8 expression by real-time RT-PCR. Under basal conditions, HCDCs in culture showed a slight constitutive expression of both IL-6 and IL-8/CXCL8, an effect that was potentiated after BKV exposure. IL-6 mRNA showed a marked increase at 3 h and a moderate, but still highly significant increase 6 h after infection (**Figure 16e**). Similar results were obtained for the chemokine IL-8/CXCL8 with a prominent mRNA expression at 3 and 6 h and a return towards baseline at 9 h post infection (**Figure 16f**). IL-6 and IL-8/CXCL8 protein expression was determined by ELISA. Under basal conditions, HCDCs showed a constitutive expression of both IL-6 and IL-8/CXCL8 (**Figures 16g, 16h**). After BKV exposure, IL-6 showed a significant and robust increase at all time points, with highest absolute levels at 9 h post infection (**Figure 16g**). In contrast, IL-8/CXCL8 was not elevated at 3 h post infection, but showed a constant significant upregulation at 9, 12 and 24 h after BKV infection (**Figure 16 h**).



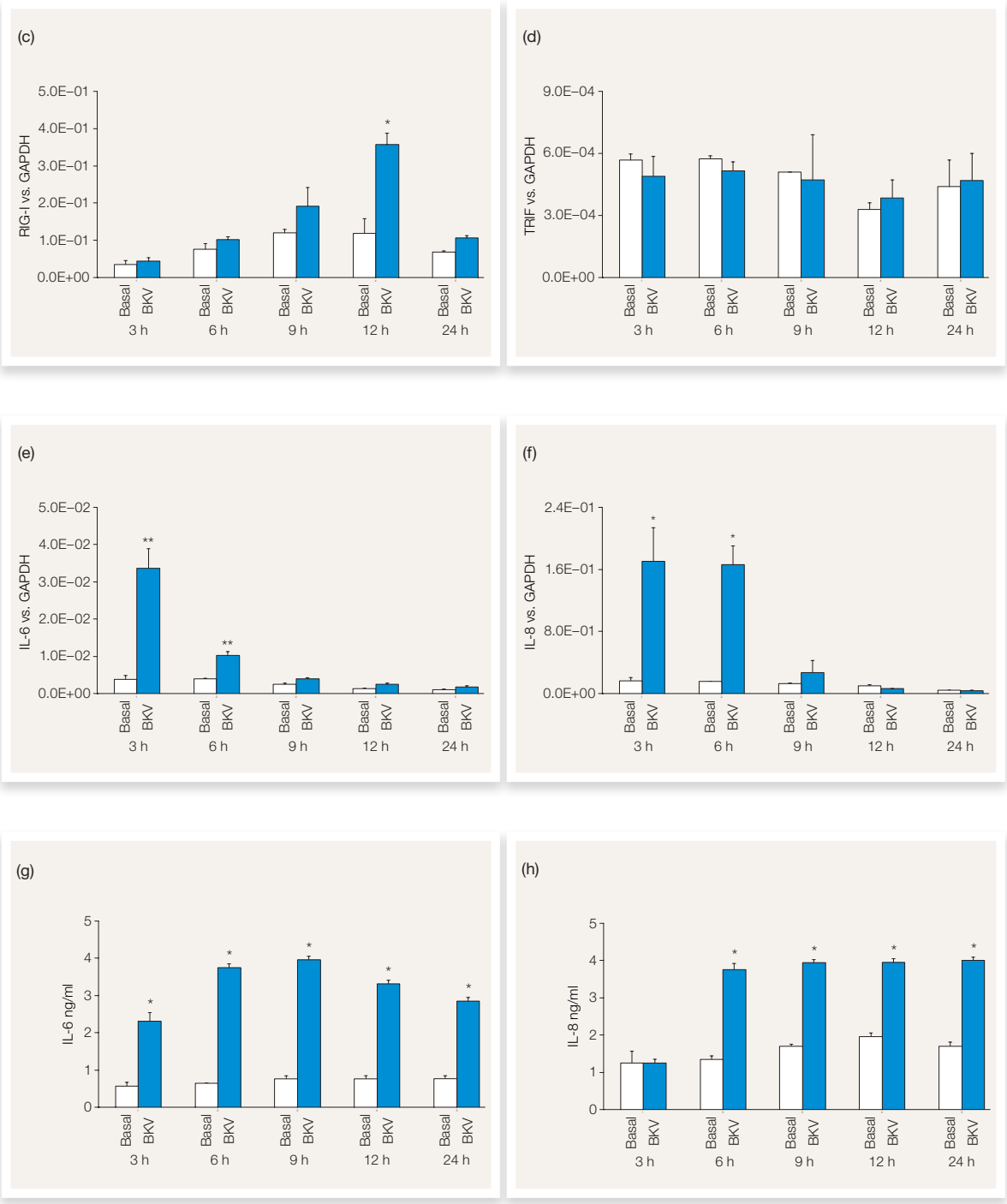


Figure 16. Infection of cultured human collecting duct epithelial cells (HCDCs) with BK virus (BKV) results in a significant increase of cytokine and chemokine expression and upregulation of double-stranded RNA (dsRNA) receptors Toll-like receptor 3 (TLR3) and retinoic acid inducible gene-I (RIG-I). HCDCs were infected with BKV and were then incubated for different time intervals (3, 6, 9, 12, and 24 h). **(a)** Highest levels of large T antigen (T-Ag), a marker protein of the early phase of BKV infection, were detected at 3 h post infection by real-time reverse transcriptase (RT)-PCR and decreased in a time-dependent manner. **(b, c)** Basal TLR3 and RIG-I mRNA expression showed a slight increase during incubation over 24 h. BKV infection upregulated both receptors at 12 h post infection with a sub-

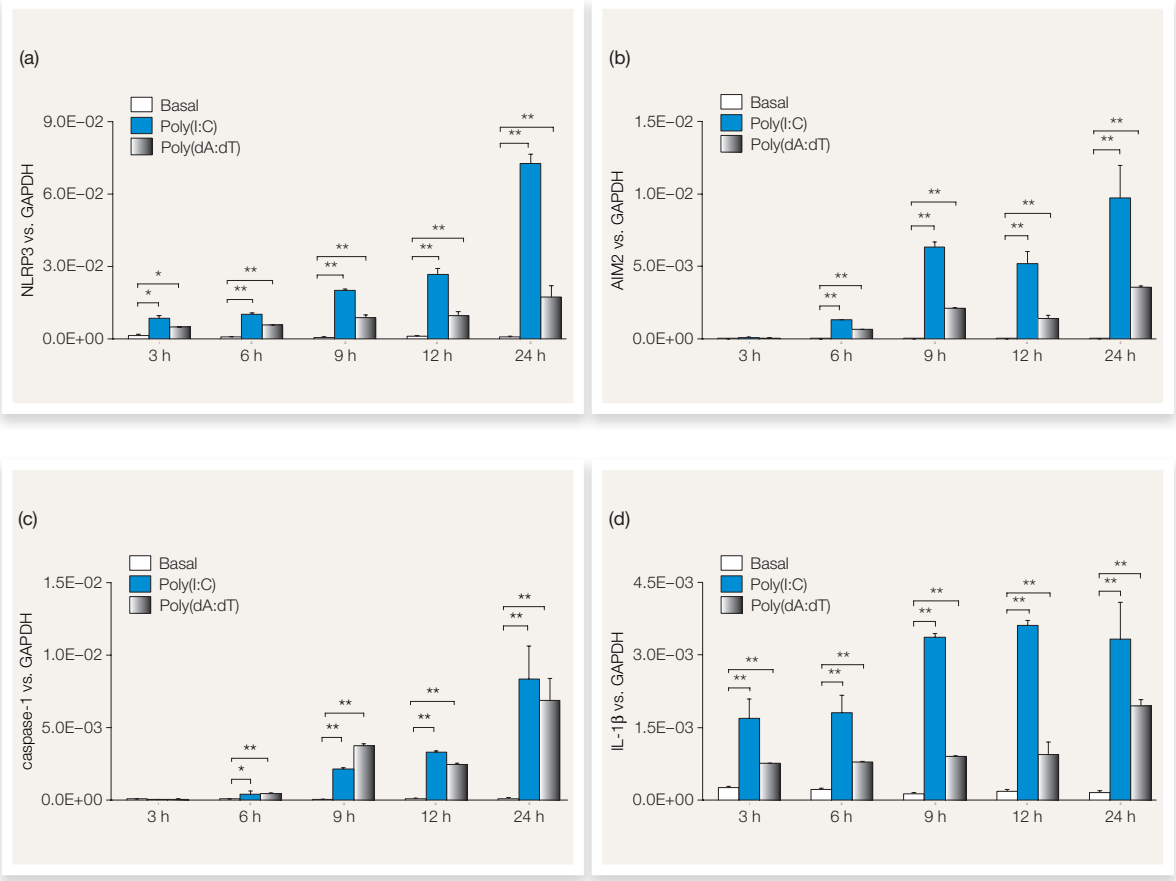
sequent decrease. **(d)** The basal expression of TIR-domain-containing adaptor-inducing interferon-β (TRIF) was not significantly affected over the time, after BKV infection. **(e)** Under basal conditions, cultured HCDCs showed a low expression of interleukin-6 (IL-6), which increased significantly after BKV exposure. **(f)** Similar results were obtained for the chemokine IL-8/CXCL8. **(g, h)** Protein secretion of IL-6 and IL-8/CXCL8 was confirmed by enzyme-linked immunosorbent assay (ELISA) technique. Data are representative of three independent experiments. *P<0.05; **P<0.01.

4.8. Poly(I:C) or poly(dA:dT) stimulation, but not BKV infection, induced the transcription of NLRP-3 and AIM2 in HCDCs

Activation of TLR3/RIG-I and robust IL-6 and IL-8/CXCL8 upregulation after BKV exposure was a consistent feature in our '*in vivo*' model of BKV infection. During initial BKV infection of HCDCs, it is likely that different DNA and RNA sensing pathways are triggered simultaneously in order to potentiate antiviral responses to infection. Recognition by these sensing pathways could finally lead to the assembly of inflammasome complexes that activate caspase-1, leading to production of IL-1β and IL-18. To test this hypothesis, we studied the expression of (i) IL-1β, an important proinflammatory cytokine that is secreted after assembly of inflammasome complexes, (ii) the inflammasome components NLRP-3 and caspase-1 and (iii) AIM2, a newly discovered pathogen recognition receptor involved in the sensing of cytosolic DNA produced by infection with DNA viruses (22, 78, 186). Since NLRP3 and AIM2 inflammasomes are located in the cytoplasm, we first examined whether transfected poly(I:C) or poly(dA:dT) can induce NLRP3 and AIM2 expression in HCDCs as well as caspase-1 and IL-1β, which are known to be activated downstream to these inflammasome signaling pathways. We found a clear increase of NLRP3 mRNA expression after treatment with poly(I:C), as well as after treatment with poly(dA:dT) (**Figure 17a**). NLRP3 expression induced by poly(I:C) or poly(dA:dT) is time dependent with the maximum expression at 24 h after

stimulation. AIM2 mRNA was also significantly upregulated by poly(I:C) or poly(dA:dT) in these cells being the maximum expression also after 24 h (**Figure 17b**). Concordant with these results, poly(I:C), as well as poly(dA:dT) were able to induce caspase-1 and IL-1β mRNA expression in HCDCs (**Figures 17c, 17d**). ELISA and western blot analysis for IL-1β were performed under exactly the same conditions in order to confirm these results on protein level but no protein expression was observed.

After BKV infection we found a very low basal IL-1β mRNA expression with a slight, but not significant increase at 3 and 12 h post infection and a significant increase at 6 and 9 h post infection, decreasing the expression almost until the basal levels after 24 h (**Figure 17e**). This marginal upregulation could not be confirmed by ELISA technique. Caspase-1, NLRP3 and AIM2 expression was not induced by BKV infection.



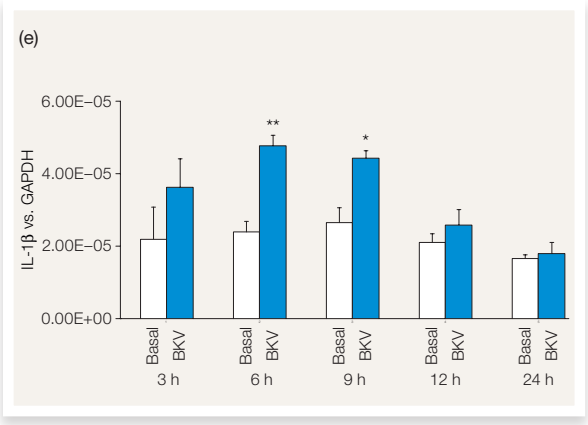


Figure 17. Polyriboinosinic:polyribocytidylic acid (poly(I:C)) and poly(deoxyadenylic-deoxythymidylic) acid (poly(dA:dT)) but not BK Virus (BKV) infection promoted the expression of NLRP3, AIM2, caspase-1 and IL-1β mRNA. Human collecting duct epithelial cells (HCDCs) were transfected with poly(I:C) or poly(dA:dT) or infected with BKV for different time intervals (3, 6, 9, 12 and 24 h). **(a)** NLRP3 mRNA expression induced by poly(I:C) or poly(dA:dT) increased in a time dependent manner up to 24 h after transfection. **(b, c, d)** Similar results were found for AIM2, caspase-1 and IL-1β mRNA expression. (f) BKV infection significantly upregulated the expression of IL-1β mRNA at 6 h and 9 h post infection, with a subsequent decrease up to 24 h. Data are representative of three independent experiments. *P<0.05; **P<0.01.

CHAPTER 5

DISCUSSION

5. Discussion

PVAN due to BKV reactivation is an increasingly recognized complication in renal transplant recipients leading to graft dysfunction and premature graft loss in up to 10% of the patients.

Stronger immunosuppressive regimens have decreased the rates of acute rejection in kidney transplantation, but have also led to the emergence of PVAN, by supporting viral replication (164). Unfortunately there are no drugs with proven efficacy for BKV replication. Although a number of centers have adopted the use of cidofovir or leuflunomide, some in vitro studies suggested a limited therapeutic efficacy of these agents associated to potential clinical toxicities (187, 188). Therefore, an early diagnosis of PVAN by histology in combination with timely reduction in immunosuppression are the only proven measures that can circumvent irreversible parenchymal damage (172).

Despite the critical role that PVAN may play in long-term graft loss, there is surprisingly little known about the immunology of BKV infection and particularly about the intra-graft cellular immune response after renal transplantation. In the current study, we present results suggesting that in PVAN critical viral dsRNA sensors are involved in the antiviral response in the infected renal epithelia.

The presence of viral dsRNA in the cell is a signature of virus infection, which activates cellular sensors of the innate immune system to induce host cellular responses that have a key role in controlling virus infection (16, 21). dsRNA can be generated as a replication intermediate for ssRNA virus or as a by-product of symmetrical transcription in DNA virus. Thus, for three reasons we evaluated the potential role of critical viral

sensors for dsRNA in PVAN: (i) dsRNA is produced by most viruses (including polyomavirus BK with a circular dsDNA genome) at some point during their replication (42); (ii) during promiscuous transcription, the intracellular concentration of dsRNA is further increased (about 20% of the polyomavirus RNA in infected cells consists of partially doublestranded molecules during transcription in the late stage) (189, 190); (iii) BKV RNA, indicating active viral replication, was found in all BKV DNA positive biopsies in a recent gene expression study of renal transplant biopsies (104).

During replication of DNA or RNA viruses and exposition with immune complexes, the host cells contact with viral (ss) or (ds) RNA triggering a specific immune response to the viral infection including NF-κB target genes, type I IFNs, and IFN response genes. Among the receptors involved in sensing viral particles, TLR3, which is expressed in the endosome of the cell, is known to be involved in the recognition of viral dsRNA and its synthetic analogue poly(I:C). TLR3 can be expressed by conventional DCs and macrophages (6) as well as by a variety of nonimmune cells such as glomerular mesangial cells (44), endothelial cells and epithelial cells (191). TLR3 deficiency is associated with susceptibility to several viral infections such as MCMV infection in mice and HSV-1 infection in humans (103, 180). However, evidence to date shows that TLR3 is not universally required for the generation of effective antiviral responses. Some studies have shown that TLR3-deficient mice were more resistant to infection by West Nile virus (WNV) and influenza virus suggesting that TLR3-mediated recognition contributes to the pathogenesis rather than protection in the case of these infections (192, 193). In this situation, RNA helicases represent an alternative major cellular sensor for several viral infections associated with dsRNA (47). When the virus enters in the cytoplasm, dsRNA generated during the course of viral replication is sensed by the host cell, an event that is independent of TLR3 (16). In this regard, RLRs seem to be critical to recognize viral RNA in the cytoplasm (6). We, therefore, looked at TLR3 and the RNA helicase RIG-I, which may also function as cytosolic alternative pattern recognition receptor of viral

motifs (13, 14, 194). However, a critical cooperation of the RIG-I/MDA5-type I IFN and the TLR3-type II IFN signaling axes may be required for efficient innate antiviral immune responses (195).

We previously reported that in human kidney, immunohistochemical staining shows TLR3 expression in glomeruli with a mesangial pattern, in vascular smooth muscle cells of preglomerular vessels and in collecting duct epithelial cells, suggesting a pivotal pathogenic role of this receptor (44). As BKV normally replicates in urothelial cells but it can also exhibit tropism for the renal tubular epithelium (126, 127), we first examined whether TLR3 and polyomavirus BK protein colocalize in the same cells of the collecting tubules. Immunofluorescent staining of renal biopsies with PVAN did, in fact, co-localize polyomavirus (Anti-SV40) and TLR3 protein expression in tubular epithelial cells of cortical collecting tubules. SV40 Large T-antigen showed strong nuclear positivity in the renal epithelium, whereas the staining pattern for TLR3 was most consistent with tubular membrane localization (Figure 10).

The features of PVAN are in many cases very similar to those associated to AR, and thus, a similar inflammatory response can be expected. Cellular inflammatory infiltrates and mRNA expression profiles of inflammatory cytokines and chemokines in PVAN and in biopsies with AR are similar in composition and quantity. This is particularly true for RANTES/CCL5 (146). During an episode of inflammation, several inflammatory markers are expressed by the cells at the site of infection. Among these markers, RANTES/CCL5 plays a primary role in the inflammatory immune response due to its ability to chemoattract leukocytes into inflammatory sites and to modulate their function (196). Here, we observed that RANTES was indeed significantly upregulated in both AR and PVAN biopsies compared to controls, confirming relevant inflammation (Figure 11c). On the other hand, TLR3 mRNA expression segregated PVAN from transplant biopsies with AR (Figure 11a). TLR3 mRNA expression, but not RIG-I mRNA expression, was significantly

induced in allograft biopsies with PVAN compared to those with ongoing AR (Figures 11a, 11b). In contrast, RIG-I mRNA was significantly induced in AR, an observation deserving further investigation (Figure 11b).

Based on these data, we hypothesized that particularly TLR3 could be a receptor candidate to mediate activation of innate immunity in PVAN. Our hypothesis was underscored by the following two recent findings: (i) TLR3 resides in the endosomal membrane and the endoplasmic reticulum and moves to dsRNA-containing endosomes in response to dsRNA (178). Since BKV has previously been shown to enter cells through endocytosis, viral dsRNAs could activate TLR3 during BKV infection of tubular epithelial cells upon viral entry and uncoating in the endosome; (ii) activation of the TLR3 pathway was described in various viruses containing a dsDNA genome, e.g. KSHV (179), HSV-1 (180, 181) and murine CMV (103).

To further elucidate a potential role of dsRNA receptors in PVAN, we used a cell culture model of collecting tubule origin (HCDCs), the predominant site of renal BK infection (150). First, we confirmed that the immortalized HCDC line expresses dsRNA receptors under basal conditions. A robust basal mRNA and protein expression for TLR3 as well as for RIG-I was demonstrated by real-time RT-PCR and western blot analysis (Figures 12b, 12c). TLR3 was localized by FACS analysis in HCDCs intracellularly (Figure 12a). Localization of TLR3 in the endosomal membranes of the collecting duct would expose the receptor to the endocytosed virus during BKV infection, as discussed above.

To characterize the activation of innate immune mechanisms in PVAN we prestimulated HCDCs with a cytokine combination to establish a proinflammatory milieu in cell culture, as might occur during viral infection. In addition we exposed the cells to poly(I:C), a synthetic mimetic of viral dsRNA, to simulate aspects of viral infection. The basal expression of both TLR3 and RIG-I mRNA in HCDCs was increased by prestimulation

with the combination of cytokines in a dose-dependent manner (Figures 13a, 13b). The expression of TLR3, and RIG-I mRNA was also enhanced after stimulation with poly(I:C) (Figures 13c, 13d, 13e, 13f). The concentration of poly(I:C) required for these effects are in the range of those reported by others for these receptors (197).

Human tubular epithelial cells are known to interact both with neighbouring cells and with the immune system through the production of cytokines and chemokines, such as IL-6, IL-8/CXCL8, IL-15, TNF-α, MCP-1, RANTES, and TGF-β (198, 199, 200), which are responsible for local inflammatory reactions, as well as some systemic effects. These responses are required to initiate a cellular and humoral immune response to control infection, but can also result in tissue injury and dysfunction, as evidenced in PVAN (139). We, therefore, investigated the role of selected cytokines and chemokines in HCDCs. Activation of viral receptors by poly(I:C) significantly enhanced the mRNA expression of various cytokines and chemokines (IL-6, IL-8/CXCL8, RANTES/CCL5, MCP-1/CCL2 and IP-10/CXCL10) in these cells after poly(I:C) stimulation (Figures 14a, 14b, 14c, 14d, 14e). These findings are in accordance with data from Mannon and coworkers, who reported elevated levels of RANTES, TNF-α, TNF-β and activation markers of macrophages in PVAN (146). In addition, increased urinary excretion of MCP-1 is associated with intra-graft tubulointerstitial inflammation in patients with PVAN (201).

In order to identify which viral receptors might contribute to the generation of cytokines and chemokines, we knocked down the receptor expression by transfection with the corresponding siRNA. The poly(I:C) induced expression of IL-6 in cultured HCDCs was significantly inhibited by transfection with siRNA for TLR3, whereas transfection with siRNA for RIG-I had no significant effect on IL-6, suggesting a pivotal role of TLR3 in this scenario (Figures 15a, 15b).

Finally, we established an infection model using cultured HCDCs and BKV isolated from urine of patients with known BKV reactivation. After BKV infection, the mRNA expression for IL-6 and IL-8/CXCL8 increased (Figures 16e, 16f). This early cytokine and chemokine activation was followed by a time-dependent significant increase of TLR3 and RIG-I expression with maxima at 12 h post infection, whereas BKV infection could not enhance the robust constitutive basal expression of TRIF (Figures 16b, 16c, 16d). Recently, Low and colleagues described primary human proximal tubule epithelial (HPTE) cells, another major cell type that can be infected by BKV '*in vivo*', as a model system for lytic BKV infection (202). Subsequent studies using this model found a primary activation of genes involved in cell cycle regulation and apoptosis (203). Of particular note, the HPTE model was not able to elicit an antiviral immune response in the given experimental context.

Our results differ in two major points from the HPTE model. First, HCDCs significantly expresse large T-antigen, a marker protein of the early phase of BKV infection, at a very early stage of infection (3 h post infection) (Figure 16a), whereas in the HPTE model early viral gene expression was first detectable at 24 h post infection with levels increasing out to 72 h post infection. These findings suggest that BKV infection of HCDCs involves particularly the early phase of BKV infection with a more rapid viral trafficking through the cytoplasm than in HPTE cells. Second, the distinctive cytokine and chemokine activation after BKV infection, demonstrated by significant IL-6 and IL-8/CXCL8 upregulation, was a prominent early signature in our model. In contrast, a cytokine-targeted PCR array analysis of the HPTE model performed at 4 h post infection revealed no proinflammatory response to BKV infection (203).

Even basal TLR3 and TRIF expression was sufficient for significant early cytokine/chemokine activation in our infection model. This initial early cytokine and chemokine activation seems to be independent of efficient virus replication, as demonstrated also,

in for example, rhinovirus-induced airway epithelial cells (204, 205) and apparently starts an efficient antiviral response in HCDCs, leading to a transient robust dsRNA receptor expression as demonstrate by significant TLR3 and RIG-I expression.

Activation of TLR3 and RIG-I after stimulation with synthetic dsRNA was a consistent feature in cultured HCDCs, and upregulation of both receptors was confirmed in our infection model mimicking the early infection phase of the collecting duct by BKV. In contrast, we found a robust TLR3, but unexpectedly no significant RIG-I expression in biopsies with PVAN. How can this obvious discrepancy be reconciled? Although infection models try to roughly copy the BKV infection of target cells, these are acute models and may not exactly reproduce the '*in vivo*' situation in clinically conspicuous PVAN captured by renal biopsy in a late phase of the viral infection. During initial BKV infection of HCDCs, it is likely that different DNA- and RNA-sensing pathways are triggered simultaneously, leading to a parallel upregulation of various receptors. This crosstalk between different innate immunity pathways could eventually potentiate antiviral responses to infection. Potential signaling pathways other than NF-κB or IFN that are activated upon viral recognition and mediate antiviral responses have been recently suggested (23, 24, 206). This includes the cytoplasmic multiprotein complexes called inflammasomes, which mediate activation of caspase-1 and promotes the secretion of the proinflammatory cytokine IL-1β. After secretion from the cell, IL-1β induces several biological effects that are associated with infection and inflammation (207). Recently, Thomas and coworkers and Allen and coworkers (208, 64) suggested an involvement of NLRP3 inflammasome in the host response against influenza virus and Kanneganti and colleagues (209) have also proposed a role for NLRP3 in response to viral infection and dsRNA. Poly(I:C) was also suggested as being able to activate NLRP3 (210). In addition Alnemri and coworkers (78) identified AIM2 inflammasome as a potential candidate to sense cytoplasmic DNA. To confirm if HCDCs would express NLRP3 or AIM2 in response to dsRNA or dsDNA, we stimulated the cells with poly(I:C) or poly(dA:dT). Consistent with

previous results we observed that poly(I:C) is able to induce NLRP3 mRNA expression in HCDCs (**Figure 17a**). Poly(dA:dT) also induces NLRP3 mRNA expression in this cell line model (**Figure 17a**). AIM2, known as a receptor for cytoplasmic DNA, was upregulated after exposure to poly(dA:dT) (**Figure 17b**), confirming previous data from other work groups. However, interestingly, AIM2 mRNA expression appears to be also induced by poly(I:C) on these cells (**Figure 17b**), but as no other data supporting this observation is available, further studies are absolutely necessary in order to elucidate the mechanisms of activation of AIM2 by dsRNA. As expected, caspase-1 and IL-1β mRNA expression was also induced by poly(I:C) and poly(dA:dT) in HCDCs (**Figures 17c**, **17d**) suggesting an inflammatory response initiated by the activation of the inflammasome pathway. However, no results were obtained on protein level impeding us of taking conclusions about the activation of inflammasome pathway by poly(I:C) or poly(dA:dT) on these cells.

As it was mentioned above, NLRP3 and AIM2 appear to be involved in the recognition of several viruses. In this sense, and after observation that HCDCs express NLRP3 and AIM2 mRNA after exposure to poly(I:C) or poly(dA:dT), we hypothesized an involvement of these receptors in PVAN. However, we were not able to demonstrate activation/upregulation of NLRP3 inflammasome pathway or AIM2 pathway in our '*in vivo*' model of BKV infection. Although we observed an increase of IL-1β mRNA expression after BKV infection (**Figure 17e**), this data could not be confirmed on protein level suggesting that inflammasome pathway is not activated by BKV on these cells.

A detailed analysis of the observed differences in dsRNA receptor activation between PVAN and AR should now be an important area of future study and could also have a significant impact on the demanding histological differentiation of these two inflammatory conditions. The striking upregulation of RIG-I mRNA expression in AR has now to be confirmed in larger-scale studies and with immunohistochemical or immunofluorescence techniques. On the basis of our findings, it is questionable whether TLR3 mRNA

expression analysis or TLR3 immunohistochemistry has the potential to serve as an additional diagnostic tool to discriminate PVAN biopsies from those with acute ongoing rejection, as TLR3 expression is not a unique feature of PVAN. Although TLR3 immunofluorescence signals were most intense in medullary collecting ducts of PVAN, and were independent of concomitant inflammation, we also observed distinct TLR3 staining in cortical atrophic and dilated tubules of AR.

Taken together, we were able to show significant activation of proinflammatory cytokines, chemokines and the dsRNA receptors TLR3 and RIG-I in cultured HCDCs after poly(I:C) and cytokine stimulation and in a BKV infection model, mimicking polyomavirus BK infection of collecting duct tubules. Immunofluorescence double staining of BKV and TLR3 in the collecting tubular epithelial cells and mRNA expression analysis of biopsies with PVAN suggest that activation of innate immune defense mechanisms, particularly via TLR3, is involved in the antiviral and inflammatory response.

In future studies, the interaction of TLR3 with additional viral sensors, for example cytosolic sensors of dsDNA, and the role of RIG-I for driving inflammation in PVAN will have to be elucidated. To better identify the cellular and molecular events of BK polyomavirus infection it would also be important to investigate how the activation of viral receptors of innate immunity regulates an adaptive response in PVAN. Finally, as the use of immunosuppressor agents in the treatment of AR increases the emergence of PVAN, it is also an attractive topic for future studies to elucidate how the immunosuppressor agents can influence the innate immune response to the virus.

Concluding, the goal of this study was to identify innate antiviral responses initiated in PVAN. The identification of specific signal cascades activated during the viral infection of renal cells should allow us to identify novel markers that could play an essential role in this disease and then identify therapeutic strategies and potentially unique targets of

intervention. In the future it will be important to determine which viral immune evasion genes are critical for persistence '*in vivo*', so that these genes can be specifically targeted for pharmacologic intervention.

CHAPTER 6

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APPENDICES

Abbreviations

Ab – Antibody
AIM2 – Absent in melanoma 2
AR – Acute rejection
APC – Antigen presenting cell
BIR – Baculovirus “inhibitor of apoptosis” repeat domain
BKV – BK virus
BSA – Bovine serum albumine
CARD – Caspase recruitment domain
Cardif – CARD adaptor inducing IFN-β
CCL – Chemokine (C-C motif) ligand
cDNA – Complementary DNA
CMV – Cytomegalovirus
CpG – Cytidine-phosphate guanosine
CTL – Cytotoxic T lymphocytes
DC – Dendritic cell
DED – Dead effector domain
DMEM – Dulbecco’s modified Eagle´s medium
DNA – Deoxyribonucleic acid
DNTPs – Deoxynucleotide triphosphates
dsDNA – Double stranded DNA
dsRNA – Double stranded RNA
EC – Epithelial cell
EBV – Epstein-Barr virus
ELISA – Enzyme-linked immunosorbent assay
EMCV – Encephalomyocarditis virus
FACS – Fluorescence-activated cell sorting
FADD – FAS-associated death domain-containing protein
FCS – Fetal calf serum
FITC – Fluorescein isothiocyanate
HCDCs – Human collecting duct epithelial cells
HIV – Human immunodeficiency virus
HSV-1 – Herpes simplex virus
IKK – IκB kinase
IL – Interleukin
IL-1R – Interleukin-1 receptor
IFN – Interferon
IPS-1 – IFN-β promoter stimulator 1

IRAK – Interleukin-1 receptor associated kinase
IRF – Interferon regulatory factor
ISRE – IFN-sensitive response element
KSHV – Kaposi´s sarcoma-associated herpesvirus
LGP2 – Laboratory of genetics and physiology-2
MAVS – Mitochondrial antiviral signaling protein
MCP-1 – Monocyte chemoattractant protein
MDA5 – Melanoma differentiation associated protein 5
MyD88 – Myeloid differentiation protein 88
NALP3 – NACHT-LRR-PYD-containing protein 3
NF-κB – Nuclear factor κB
NK – Natural killer
NLR – Nod-like receptor
NOD – Nucleotide-binding oligomerization domain
PAMP – Pathogen associated molecular pattern
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
Poly(dA:dT) – Poly(deoxyadenylic-deoxythymidylic) acid
Poly(I:C) – Polyriboinosinic:polyribocytidylic acid
PRR – Pathogen recognition receptor
PVAN – Polyomavirus-associated nephropathy
PYD – Pyrin domain
RANTES – Regulated upon activation, normal T cells expressed and secreted
RSV – Respiratory syncytial virus
RIG-I – Retinoic acid-inducible gene-I
RIP-1 – Receptor-interacting protein 1
RIPA – RadioImmunoPrecipitation assay
RLR – RIG-I-like receptor
RNA – Ribonucleic acid
RT – Reverse transcriptase
siRNA – Small interfering RNA
ssRNA – Single stranded RNA
ssDNA – Single stranded DNA
TBST – Tris-buffered salin-Tween
TIR – Toll/interleukin-1 receptor domain
TLR – Toll-like receptor
TNF – Tumor necrosis factor
TRAF – TNF receptor associated factor
TRAM – TRIF related adaptor molecule
TRIF – TIR-domain-containing adaptor-inducing interferon-β
VISA – Virus-induced signaling adapter
WNV – West Nile virus

Curriculum Vitae

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