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Sensing of MPyV infection by innate immunity sensors

Studium rozpoznáni polyomavirové infekce sensory vrozené imunity

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Prohlášení

Prohlašuji, že jsem disertační práci vypracoval samostatně a uvedl všechny použité informační zdroje a literaturu. Tato práce nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze……. Boris Rjabčenko…….

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Abstract

Host sensors that recognize pathogen associated molecular patterns and the mechanisms of innate immune response to mouse polyomavirus (MPyV) infection were the main topics of current work. We found that MPyV did not induce interferon (IFN) production during early events of infection, but induced interleukin-6 (IL-6) and other cytokine production without inhibiting virus multiplication. Cytokine microenvironment changed the phenotype of adjacent non infected fibroblasts toward the cancer-associated fibroblast (CAF)-like phenotype. We identified Toll-like receptor 4, a sensor of the innate immunity system, to be responsible for infection dependent IL-6 production. In an effort to determine whether and where virions are released from endosomal compartments into the cytosol, we found that the hydrophobic domains of minor capsid proteins, exposed on the surface of virions after their partial disassembly in the ER, play an important role in effective escape of virions from the lumen part of endoplasmic reticulum into the cytosol, Although naked, partially disassembled virions appear before translocation to the nucleus in the cytosol, viral DNA is not recognized by cytosolic sensors at this phase of infection Sensing of MPyV resulting in IFN production occurs first during viral replication. Mutant virus, defective in nuclear entry, was not able to induce interferon production. Both, p204 and cGAS DNA sensors, but not the endosomal sensor of methylated DNA -Toll like receptor 9, were involved in recognition of replicating phase of MPyV infection. Although p204 and cGAS colocalized in the nucleus with MPyV genomes, only p204 sensed DNA in the nucleus. Unexpectedly, cytosolic viral DNA leaked from the nucleus and micronucleus-like bodies (induced by genotoxic stress during MPyV infection) were the targets for cGAS and induced its activation. The absence of cGAS in cells did affect IFN production but not interaction of p204 with viral DNA. The outcome of results highlights the complex interactions between the virus and the host innate immunity sensors.

Key words: polyomaviruses; innate immunity; DNA sensing; TLR4; cGAS; p204; IFI16; interferon-β; interleukin-6

Abstrakt

Senzory hostitelských buněk rozpoznávající molekulární motivy vlastní patogenům a mechanismy vrozené imunitní odpovědi na infekci myším polyomavirem (MPyV), byly hlavními tématy této práce. Zjistili jsme, že během ranné faze infekce neindukuje MPyV produkci interferonu (IFN) ale indukuje produkci interleukinu-6 (IL-6) a dalších cytokinů aniž by byla inhibována replikace virů. Cytokinové mikroprostředí změnilo fenotyp sousedních neinfikovaných fibroblastů směrem k fenotypu podobnému fibroblastům spojeným s buněčnou transformací (cancer-associated fibroblast; CAF). Identifikovali jsme Toll-like receptor 4, senzor vrozené imunity, který je zodpovědný za produkci IL-6 během infekce. Ve snaze určit, zda a kde se viriony uvolňují z endozomálních kompartmentů do cytosolu, jsme zjistili, že hydrofobní domény minoritních kapsidových proteinů vystavené na povrchu virionů po jejich částečném rozložení v ER, hrají důležitou roli v účinné translokaci virionů z lumen endoplazmatického retikula do cytosolu. Ačkoliv se částečně rozložené viriony objevují před transportem do jádra "nahé" v cytosolu, virová DNA není v této fázi infekce rozpoznána cytosolickými sensory. Rozpoznání MPyV vedoucí k produkci IFN nastává až během replikace virových genomů v buněčném jádře. Mutantní virus, defektní ve vstupu do jádra, nebyl schopen vyvolat produkci interferonu. Senzory DNA p204 i cGAS, ale nikoliv endosomální senzor methylované DNA - Toll like receptor 9, byly zapojeny do rozpoznávání replikační fáze infekce MPyV. Přesto, že sensory p204 a cGAS kolokalizovaly v jádře s virovými genomy, pouze p204 patrně rozpoznal DNA v jádře. Cílem pro cGAS rozpoznání a aktivaci se stala virová a patrně I hostitelská DNA uniklá z jádra do cytosolu a DNA z mikrojader (process vyvolaný genotoxickým stresem během infekce MPyV). Absence cGAS v buňkách ovlivnila negativně produkci interferonu, ale neovlivnila interakci p204 sensoru s virovou DNA. Získané výsledky zdůrazňují složité interakce mezi virem a senzory vrozené imunity hostitele.

Klíčová slova: polyomaviry; vrozená imunita; DNA sensory; TLR4; cGA;, p204; IFI16; interferon-β; interleukin-6

CONTENT

List of abbreviations

ADAR	adenosine deaminases acting on RNA
Agno	agnoprotein
AIM2	absent in melanoma 2
APC	antigen-presenting cell
APOBEC3	apolipoprotein B editing complex 3
AP1	activator protein 1
αSMA	alpha smooth muscle actin
ATP	adenosine triphosphate
BKPyV	BK polyomavirus
B cell	B Lymphocyte
CAC	Colitis-associated cancer
CAF	cancer-associated fibroblast
cGAS	cyclic GMP-AMP synthase
CLR	C-type lectin receptor
CRP	C-reactive protein
DAI	DNA-dependent activator of interferon-regulatory factor
DDX41	DEAD-Box Helicase 41
DAMP	damage-associated molecular pattern
DDR	DNA damage response
dsRNA	double-stranded viral RNA
ER	endoplasmic reticulum
GAF	gamma interferon factor
GAS	gamma-activated site
GTP	guanosine triphosphate
JAK1	Janus kinase1
JCPyV	JC polyomavirus
HCMV	human cytomegalovirus
HD	hydrophobic domain
HIN-200	IFN-inducible nuclear protein 200
HPyV	human polyomavirus
HR	homologous recombination
IFI16	interferon-inducible protein 16
IFIX	interferon–inducible protein X

IFN	interferon
IFNAR	interferon α/ β receptor
IFNLR1	interferon lambda receptor 1
IKK	IκB kinase
IL	interleukin
IL10RB	IL10 receptor B
IRAK	IL-1 receptor-associated kinase
IRF	interferon-regulating factor
ISG	interferon stimulated gene
ISGF3	interferon-stimulated gene factor 3
JNK	c-Jun N-terminal kinase
KIPyV	KI polyomavirus
KSHV	Kaposi sarcoma-associated herpesvirus
LIPyV	Lyon IARC polyomavirus
LPS	lipopolysaccharide
LT	large T-antigen
LTA	lipoteichoic acid
MAP	mitogen-activated protein
MCPyV	Merkel cell polyomavirus
MDA5	melanoma differentiation-associated protein 5
MEF	mouse embryo fibroblasts
MOI	multiplicity of infection
MPyV	Murine polyomavirus/ mouse polyomavirus
MT	middle T-antigen
Mx	myxovirus resistance
MWPyV	Malawi polyomavirus
NCCR	non-coding control region
NEMO	NF-kB essential modulator
NF-kB	nuclear factor kB
NJPyV	New Jersey polyomavirus
NK	natural killer cell
NKT	natural killer T cell
NLR	NOD-like receptor
NLS	nuclear localization signal

OAS	2′–5′-oligoadenylate synthetase
ORF	open reading frame
ORI	origin of replication site
PAMP	pathogen-associated molecular pattern
PKR	protein kinase R
PML	promyelocytic leukemia protein
PRR	pattern recognition receptor
PSC	pancreatic stellate cell
PYD	pyrin domain
PyV	polyomavirus
pRB	retinoblastoma protein
RIG-I	RNA helicase retinoic acid inducible gene I
RLR	RIG-I-like receptor
RSV	respiratory syncytial virus
SAA	serum amyloid A
ssRNA	single-stranded RNA
ST	small T-antigens
STING	stimulator of interferon genes
STLPyV	Saint Louis polyomavirus
SV40	simian virus 40
TAK1	transforming growth factor-β-activated kinase 1
TBK1	TANK-biding kinase 1
TGF-β	transforming growth factor β
TIR	Toll–interleukin-1 receptor
TIRAP	TIR-domain-containing adaptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor receptor associated factor 6
TRAM	TRIF-related adaptor molecule
Treg	regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSPyV	Trichodysplasia spinulosa-associated polyomavirus
TYK2	tyrosine kinase 2
WUPyV	WU polyomavirus

1. Introduction

The first virus proven to be able to infect human was discovered in 1901. Since that time more than 200 other virus species harmful for humans have been described and every year additional 2 or 3 species are found (Woolhouse et al., 2012). Limited number of specific antiviral drugs (Razonable, 2011) makes the therapy of viral infection challenging. A current example is SARS-CoV-2 pandemic. Multiple casualties of SARS-CoV-2 infection were not connected with direct destruction of the cells and tissues by the virus, but by severe inflammation response run out of control (Hojyo et al., 2020). The adequate immediate immune response maintained by the components of innate immune system is the key factor of successful protection against viral infection determining the difference between moderate, severe and critic disease. The study of the mechanisms of virus sensing by innate immunity and viral strategy to contradict it, is utmost importance.

Here we use Murine polyomavirus (MPyV) as a model for study of innate immunity mechanisms contradicting the infection of small non enveloped dsDNA viruses. The inspiration for me and my colleagues to start the research came with observation that naked viral DNA being introduced into the cells caused robust interferon response, while viral genomes were hidden from the innate immune recognition when packed inside of capsid. We followed the infection and found that virus protective mechanisms are not perfect and, viral infection, in the case of polyomaviruses, becomes “visible” for protective immune system during late phases. Our research reminds me of an endless detective story in which criminals (virus components and products) try to hide or outwit detectives (innate immunity sensors) who aim to track them down and restrain them. In the following chapters, the results of our latest work are presented and discussed.

1.1. Polyomaviruses, discovery and importance

Polyomaviridae is the family of small, non-enveloped, DNA tumor viruses widely spread among animals. The honor of discovery of the first polyomavirus (PyV) - Murine polyomavirus (MPyV) - belongs to Ludwig Gross. In 1953, he isolated and characterized infectious agent capable of causing cancer in laboratory mice (Gross, 1953). Shortly after that, simian virus 40 (SV40) was accidentally found as a contaminant of monkey cell line used in production of vaccine against poliovirus (Sweet and Hilleman, 1960). Since that time, polyomaviruses were intensively studied and were used as unique tool for understanding of different biological processes.

The existence of human polyomaviruses was first confirmed in 1971 by discovery of BK and JC polyomaviruses (BKPyV and *JCPyV*) in the samples taken from immunocompromised patients (Gardner et al., 1971) (Padgett et al., 1971). Up to date, another 12 human polyomaviruses have been found and characterized: KI polyomavirus (KIPyV) (Allander et al., 2007), WU polyomavirus (WUPyV) (Gaynor et al., 2007), Merkel cell polyomavirus (MCPyV) (Feng et al., 2008), Trichodysplasia spinulosa-associated polyomavirus (TSPyV) (van der Meijden et al., 2010), human polyomaviruses 6 and 7 (HPyV6 and HPyV7) (Schowalter et al., 2010), human polyomavirus 9 (HPyV9) (Scuda et al., 2011), Malawi polyomavirus (MWPyV) (Siebrasse et al., 2012), Saint Louis polyomavirus (STLPyV) (Lim et al., 2013), human polyomavirus 12 (HPyV12) (Korup et al., 2013), New Jersey polyomavirus (NJPyV) (Mishra et al., 2014) and Lyon IARC polyomavirus (LIPyV) (Gheit et al., 2017).

Polyomaviruses are strongly associated with development of severe pathological conditions. BKPyV infection may lead to the occurrence of different nephropathies, especially in recipients of donor kidneys (Coleman et al., 1978; Vigil et al., 2016) . JCPyV infection causes virus associated brain demyelinating disease – progressive multifocal leukoencephalopathy (PML) in immunocompromised patients (Tan and Koralnik, 2010). MCPyV infection is a key factor in development of one of the most fatal types of skin cancers – Merkel cell carcinoma (Feng et al., 2008). The genomes of TSPyV were identified in the skin samples of patients with trichodysplasia spinulosa (van der Meijden et al., 2010). HPyV7 was found to be associated with thymic epithelial tumors (Rennspiess et al., 2015).

Seroprevalence of polyomaviruses in healthy human population varies from 40% for MCPyV up to 80% for BKPyV (Kean et al., 2009). Nevertheless, no specific treatment for polyomavirus infections is yet available.

1.2. Structure and life cycle of polyomaviruses

. The capsid of polyomaviruses, approximately 45 nm in diameter, has icosahedral symmetry (with triangulation number T equal 7) and consists of 72 pentamers. Each pentamer contains five molecules of VP1 protein. One molecule of minor capsid proteins, either VP2 or VP3, is bound to the central cavity of each pentamer in the interspace of virion (Figure 1) (Stehle and Harrison, 1996; Chen, 1998).

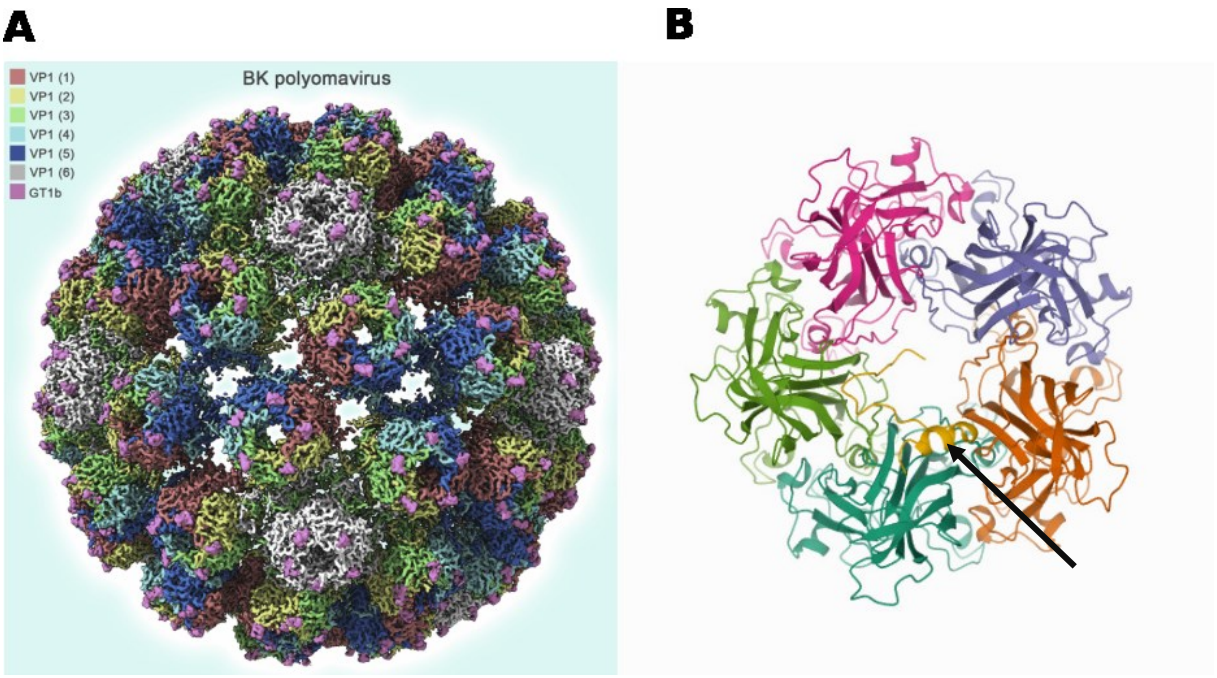


Figure 1. **A**. The structure of polyomavirus capsid shown on example 3.4-Å cryoelectron microscopy structure in complex with the fragment of receptor (GT1b ganglioside). Taken from Daniel L. Hurdiss (Hurdiss et al., 2018). **B**. Visualization of Interaction of MPyV internal protein VP2 (shown in yellow color, pointed by arrow) with the major capsid protein VP1 (shown as differently colored molecules of VP1 pentamer. Taken from protein data bank PDB, the model was made based on the structure resolved by Chen et.al (Chen, 1998).

The genome of polyomaviruses is presented by circular, double stranded DNA, approximately 5kbp long. It is composed of non-coding control region (NCCR) and two transcription regions encoding early and late proteins. The NCCR includes the origin of replication site (ORI), early and late promoters and enhancer (Figure 2). The region of early genes encodes early regulatory proteins large T-antigen (LT), small T-antigens (ST) and in case of mouse and hamster polyomaviruses, middle T-antigen (MT). The region of the late, structural, genes encodes viral major capsid protein, VP1, and the

minor capsid proteins VP2 and VP3. Late region of polyomaviruses infecting primates BKPyV, JCPyV or SV40 also encodes agnoprotein from small ORF placed upstream of VP1 coding region. Multiple transcript variants are generated from primary transcripts by alternative splicing. Molecule of DNA is packed in virion in form of condensed minichromosome in complex with histone proteins: H2A, H2B, H3 and H4 (Cole; Dalianis and Hirsch, 2013).

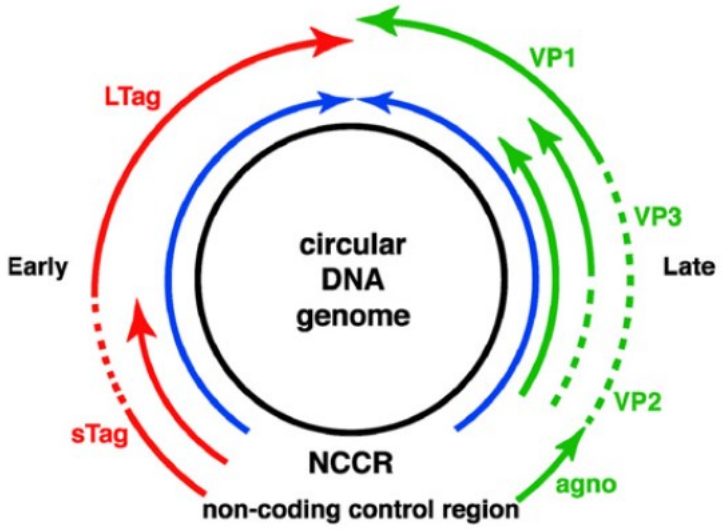


Figure 2. Scheme of PyV genome. Including the NCCR and the transcripts of the viral early and late genes (blue arrows). The spliced variants of mRNA for the early proteins (large and small T antigen) are shown by red arrows and for capsid proteins (VP1, VP2 and VP3) by green arrows. Solid green and red lines represent the open reading frame (ORF) and the introns are shown by dotted line. Scheme includes the small ORF encoding the agnoprotein (agno). According to Dalianis et al. (Dalianis and Hirsch, 2013)

PyVs differ in the length of their replication cycle in the range of 2 -5 days. Their life cycle starts by internalization l by receptor mediated endocytosis (Figure 3). The known receptors utilized by PyVs are, mainly, different types of gangliosides, glycosphingolipids with residues of sialic acids, (GM1, GD1b, GT1b) reviewed in (Giannecchini, 2020). After internalization, virions are transported inside of initially early and then late endosomal compartments using microtubule network to the ER where they become partially disassembled (Mannová and Forstová, 2003). From ER, virions translocate to the cytosol and their transport to the cell nucleus occurs via nuclear pore complex (Soldatova et al., 2018)

The nuclear events start by the transcription from the early coding part of PyV genome and follow by the induction of PyV DNA replication. The early regulatory protein, LT antigen is responsible for initiation of viral replication from ORI and, together with other early antigen(s), it regulates viral transcription and reprograms cell cycle. LT antigen immortalizes cells by negatively regulation of retinoblastoma protein (pRb) and virus takes advantage of cell interphase to S-phase switch in favor of its replication (Dyson et al., 1990; Freund et al., 1992). Early regulatory proteins also contradict the function of tumor suppressor protein, p53, resulting in cell transformation ant tumorigenesis (Asselin et al., 1983; Treisman et al., 1981). With the onset of genome replication, transcription of the late region is markedly enhanced Late structural proteins are delivered into the nucleus and assembled in to the virions together with viral genomes in complexes with histones. The viral exit is associated with lytic phase of viral infection. Nevertheless, a part of viral progeny is transported actively from infected cells without lysis of cellular membranes (Evans et al., 2015).

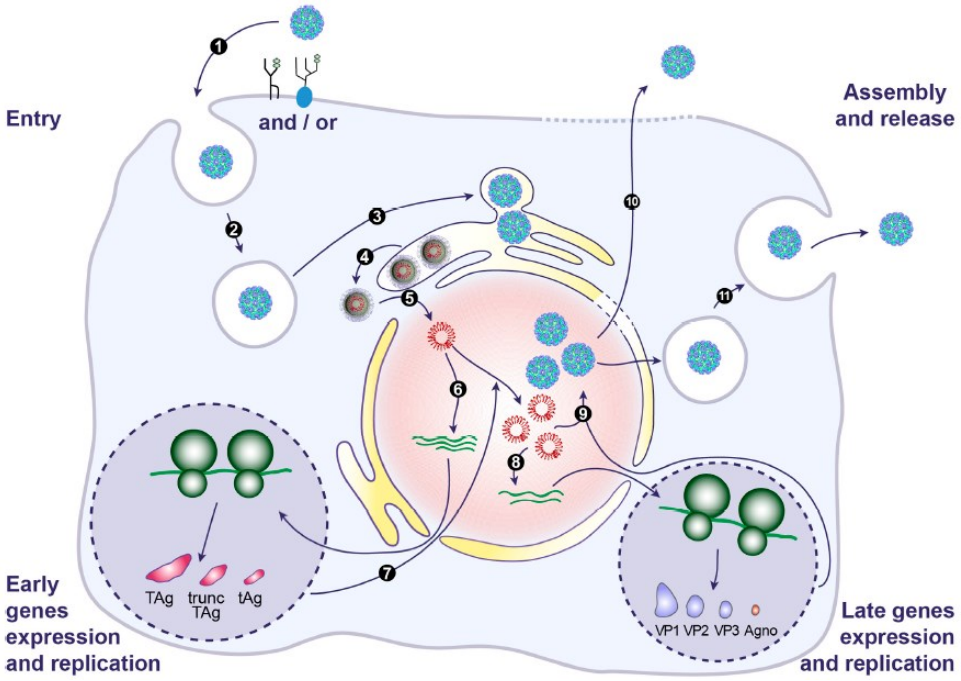


Figure 3. PyV life cycle on the example of BKPyV. Virus binds to receptor (particularly GT1b and GD1b) and/or an N-linked glycoprotein) and becomes internalized by cells (1,2). It is transported into ER in endosomes (3). Partially disassembled virions leave ER and are delivered I into the nucleus via nuclear pores (4,5). Early transcription starts and early antigens come into the nucleus to start viral genome replication (7,8). Production of the late proteins, their delivery in to the nucleus, assembly and release of virions occurs during the final steps of infection (9,10,11). According to Francois Helle (Helle et al., 2017).

1.3. Molecular components of innate immunity

1.3.1. Pattern recognition receptors

Pattern recognition receptors (PRRs) are components of innate immunity representing the first line of defense against invasion of pathogens. They are responsible for either recognition of specific pathogen-associated molecular patterns (PAMPs) or for recognition of damage-associated molecular patterns (DAMPs). The activation of PRRs occurs in case if pathogens (bacteria, fungi, viruses) overcome the anatomical barriers including, for example, skin, bacterial microflora, pH, mucosal wash, digestive enzymes or mucus. Concerning viral infection, there is another barrier – the presence of virus aggregating small peptides, defensines, in mucosa. They are produced by immune competent cells and able to neutralize both enveloped and non-enveloped viruses, even prior their receptor binding and internalization into by the cells (Daher et al., 1986; Wilson et al., 2013).

The history of PRRs started in the 1995 when the group of Pamela Ronald discovered receptor kinase-like protein, the product of the rice gene **Xa2**, inducing resistance to the infection by Gram-negative bacterium (Xanthomonas oryzae pv. Oryzae) (Song et al., 1995). Since then, a number of PRRs in both plants and animals have been discovered and characterized. Currently they include membrane binding Toll-like receptors (TLRs), C-type lectin receptors (CLRs) or NOD-like receptors (NLRs) and nucleic acids sensors e.g. RIG-I-like receptors (RLRs) or DNA sensors, some of which occurs both in cytoplasm and in the nucleus. Different PRRs were shown to recognize different types of molecules including carbohydrates, lipids, proteins and nucleic acids (Kumar et al., 2011).

The first evidence that PRRs might also respond to viruses came with the finding that the fusion protein of the Respiratory syncytial virus (RSV) stimulated the secretion of interleukin (IL)-6 from mouse macrophages depending on **TLR4** signaling (Kurt-Jones et al., 2000). Shortly after that, Vaccinia virus (VACV) proteins, A46R and A52R, were shown to antagonize TLR signaling in cultured cells (Bowie et al., 2000). Since that time, various PRRs were identified in connection with virus infection. For example, **TLR2** was shown to recognize viral proteins (Oliveira-Nascimento et al., 2012), and, recently, was found to sense viral SARS-CoV-2 infection (Zheng et al., 2021); **TLR7** was proved to detect single-stranded RNA (ssRNA) of RNA viruses (Lund et al., 2004); **TLR3,** RNA helicase retinoic acid inducible gene I (**RIG-I**) and melanoma

differentiation-associated protein 5 (**MDA5**) were shown to be responsible for double-stranded viral RNA (dsRNA) detection (Yoneyama et al., 2004; Barral et al., 2009; Goubau et al., 2014); **TLR9** was demonstrated as sensor of unmethylated CpG DNA in the genomes of DNA viruses (Lund et al., 2003; Krug et al., 2004).

TLR9 remained the only known PRR for DNA sensing till the year 2007, when Takaoka *et al.* found the existence of putative cytoplasmic DNA sensor, DNA-dependent activator of *interferon*-regulatory factors (**DAI**) (Takaoka et al., 2007). Then, numerous of other putative DNA sensors absent in melanoma 2 (**AIM2**), cyclic GMP-AMP synthase (**cGAS**), interferon-inducible protein 16 (**IFI16**), interferon–inducible protein X **IFIX)**, DEAD-Box Helicase 41 (**DDX41**), **RNA polymerase III**, **Ku70/Ku80**, leucine repeat containing protein, DEAH box containing proteins **DHX36** and **DHX9** were identified (Bürckstümmer et al., 2009; Unterholzner et al., 2010; Zhang et al., 2011b; Sun et al., 2013; Diner et al., 2015; Kim et al., 2010; Chiu et al., 2009; Zhang et al., 2011a).

PRRs recognition of PAMPs triggers the production of cytokines - small peptides responsible for activation and control of inflammatory response and for the induction of expression of multiple genes involved in restriction of pathogen's invasion. The cytokines manage their functions by binding to the specific receptors, placed on cell membrane and activation on downstream cascade. There are five main types of cytokines: interferons (IFNs), ILs, chemokines, lymphokines and tumor necrosis factors (TNFs). While lymphokines are restricted by production in lymphocytes and TNFs mainly produced by macrophages, the other types of cytokines may be produced by variety of cell types (Zhang and An, 2007).

The mechanisms of sensing by cGAS, IFI16 and TLR4, the mechanisms of interferon stimulated gene (ISG) induction by IFNs as well as brief overview of IL-6 cytokine are described below.

1.3.2. DNA sensing by cGAS and IFI16 proteins

cGAS is a DNA sensor containing in its structure two positively charged DNA binding domains and single catalytic domain, placed in between. Interaction with DNA causes allosteric structural change of protein conformation leading to dimerization and activation of catalytic center of the protein. The active catalytic center then catalyzes synthesis of a phosphodiester-linked cyclic dinucleotide 2',3'cGAMP from guanosine

triphosphate (GTP) and adenosine triphosphate (ATP) (Ablasser et al., 2013; Sun et al., 2013).Dinucleotide 2',3'cGAMP plays the role of second messenger and activates the stimulator of interferon genes (STING) protein by ligand-binding dependent oligomerization and subsequent translocation of STING from membrane of ER to the Golgi apparatus (Mukai et al., 2016; Shang et al., 2019). During translocation, STING interacts with the TANK-biding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF-3) stimulating phosphorylation and oligomerization of IRF3 (Tanaka and Chen, 2012). Then, by phosphorylation activated IRF3 translocates to the nucleus and initiates transcription of IFN genes in association with CBP/p300 coactivator (Hiscott et al., 1999) (Figure 4). Triggered by STING, activation of TBK1 causes, besides phosphorylation of IRF3, also activation of nuclear factor kB (NF-kB), resulting in induction of inflammatory cytokine production (Yum et al., 2021).

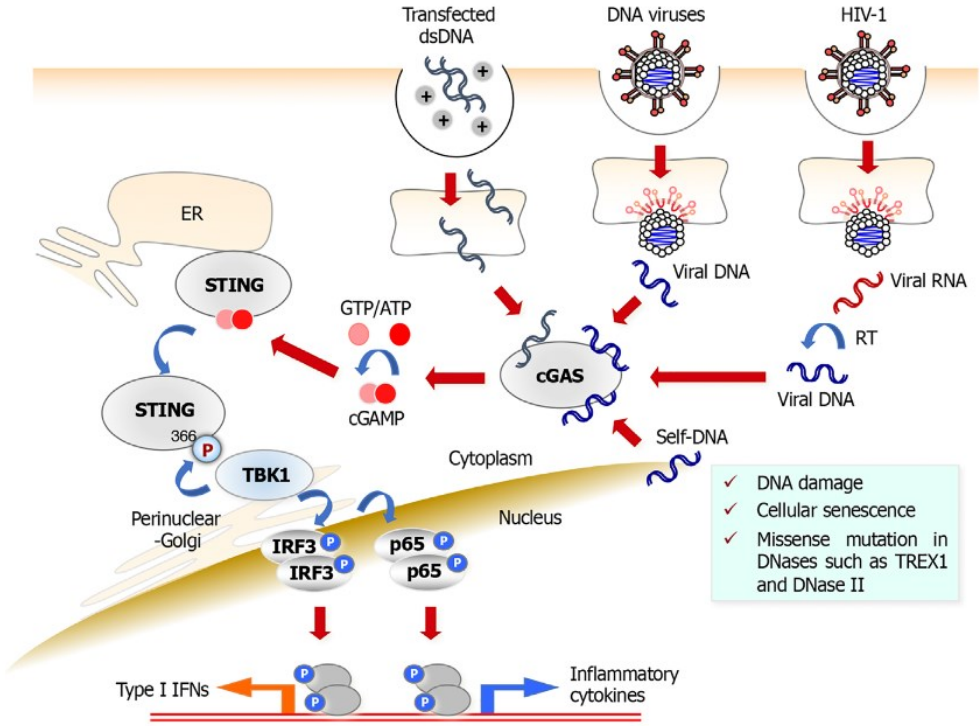
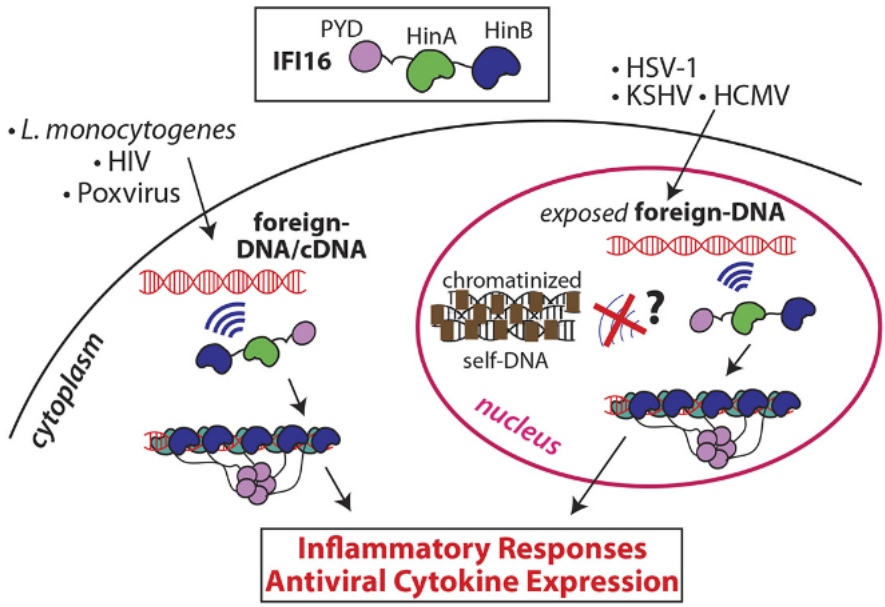


Figure 4. dsDNA of different origin (transfected, viral, self-DNA or reverse-transcribed viral RNA) is sensed by cGAS in the cytoplasm, resulting in cGAMP production and STING activation. Activated STING translocates into perinuclear Golgi and stimulates TBK1 dependent phosphorylation of either IRF3 or subunits of NF-kB (p65). The activated complexes of IRF3/IRF3 and p65/p65 then translocate to the nucleus and induce transcription of genes for IFN Type I and Inflammatory cytokine, respectively. Picture is taken and adapted from Takayuki Abe (Abe et al., 2019)

IFI16 belongs to the family of hemopoietic IFN-inducible nuclear proteins sharing similar 200 long amino acid motifs (HIN-200). These motifs contain DNA binding and PYRIN domains (Dawson and Trapani, 1996). The involvement of other members of the HIN-200 family, AIM2 and IFIX, in DNA sensing was mentioned above.

IFI16 senses foreign dsDNA both in the nucleus and in the cytoplasm initiating inflammatory response and antiviral cytokine expression (Figure 5). In the nucleus, it interacts with both naked, nucleosome free viral DNA and viral DNA present in the form of nucleosomes. In the first case, IFI16 is consequently acetylated by histone acetyltransferase, p300. Acetylation occurs within nuclear localization signal (NLS) of the protein and drives its translocation to cytosol (Li et al., 2012). Acetylated IFI16 either forms inflamasome complex in the nucleus, which is then translocated to the cytoplasm and induces activation of caspase-1, cleavage of pro-IL-β and secretion of IL-β (Kerur et al., 2011; Singh et al., 2013) or is directly translocated to the cytoplasm and mediates , STING-TBK1 dependent activation of IRF3 and induction of IFN Type 1 gene expression (Ansari et al., 2015; Diner et al., 2015; Orzalli et al., 2012). The recognition of viral chromatinized genomes by IFI16 is associated with epigenetic silencing of viral chromatin (Lo Cigno et al., 2015).



Figire 5. IFI16 assembles faster on longer dsDNA. Top: IFI16 is composed of three functional domains flanked by unstructured linkers, namely one pyrin domain (PYD) and two dsDNA-binding Hin domains (HinA and HinB). Bottom: IFI16 detects foreign dsDNA from invading pathogens in both the host cell nucleus and cytoplasm. According to Sarah A Stratmann (Stratmann et al., 2015)

IFI16 has broad spectrum of affinity to DNA, but preferentially binds to a quadruplex structure of DNA present in telomeric regions (Wang and Patel, 1993), promoters of oncogenes (Siddiqui-Jain et al., 2002) and found in genome of DNA viruses (Murat et al., 2014; Tlučková et al., 2013). The one-directional scanning movement and oligomerisation of IFI16 along targeted foreign DNA is required for formation of effective sensing platform. The optimal length of DNA molecule is 200bp and longer (Stratmann et al., 2015).

1.3.3. Sensing by TLR4

TLR4 was the first member of the large group of Toll-like receptors (only in human 13 members described) discovered in 1990 by Shumann et al. (Schumann et al., 1990). It is featured by presence of trimodular structure, composed of the extracellular leucine-rich repeats, a single transmembrane region, and the intracellular Toll-IL-1 receptor (TIR) domain required for downstream signal transduction domain (Kawai and Akira, 2010; Pålsson-McDermott and O'Neill, 2004).

TLR4 is a receptor mainly for lipopolysaccharides (LPS) and, thus, it plays an important role in defense against gram-negative bacteria, but it is also able to recognize lipoteichoic acid (LTA), fibronectin and taxol (Pålsson-McDermott and O'Neill, 2004) The fusion protein RSV was shown to be sensed by TLR4 as well (Kurt-Jones et al., 2000).

Stimulation with the ligands causes dimerization or oligomerization of two receptor chains followed by recruitment of myeloid differentiation primary response protein 88 (MyD88) and TIR-domain-containing adaptors (TIRAP) to the intracellular part of the receptor (Saitoh et al., 2004). MyD88 forms complexes with IL-1 receptor-associated kinases (IRAKs), tumor necrosis factor receptor associated factor 6 (TRAF6), and interferon-regulating factor 5 (IRF-5). TRAF6 acts as an E3 ubiquitin ligase and catalyzes the polyubiquitination of itself and NF-kB essential modulator (NEMO) protein also known as inhibitor of NF-kB kinase subunit gamma (IKK-γ). Followed activation of transforming growth factor-β-activated kinase 1 (TAK1) results in the phosphorylation of NEMO and activation of the IκB kinase (IKK) complex. Phosphorylated IkB is then sent to ubiquitin dependent degradation and free nuclear NF-kB is translocated into the nucleus and triggers the expression of pro-inflammatory cytokine genes (Israël, 2010; Akira et al., 2006). TAK 1 also activates MAP kinase depending cascade resulting in activation of the activator protein 1 (AP-1) and

enhanced expression of pro-inflammatory cytokine genes (Lee et al., 2000, 2002). There is MyD88-independent TLR4 mediated induction of pro-inflammatory cytokines and type I IFNs. In this case, the activation of NF-kB and IRF-3 proceed through TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). TRIF interacts with TRAF6 and receptor interacting protein 1 (RIP1) (Cusson-Hermance et al., 2005) and mediates NF-kB activation. In parallel, TRIF initiates TBK1/IKK-I dependent phosphorylation of IRF-3 throught TRAF3. Phosphorilated IRF3 then translocates to the nucleus to regulate transcription (Kawai and Akira, 2007) (Figure 6).

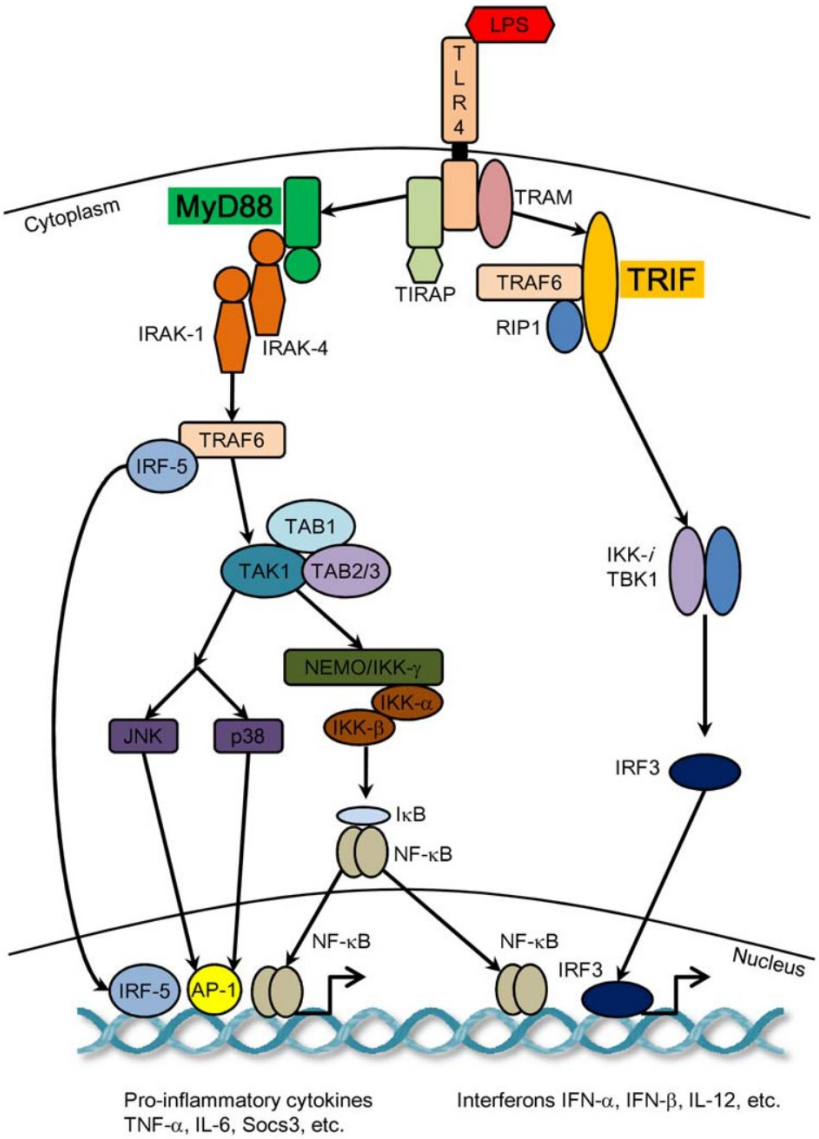


Figure 6. Simplified overview of LPS-induced signaling through TLR4. LPS binds with TLR4 and activates transcription factors AP-1, NF-kB and IRF3 through MyD88 - and TRIF- dependent pathways. This leads to the induction of proinflammatory cytokines and interferons. According to (Tsuchiya et al., 2009).

1.3.4. IL-6

IL-6 is small (26kDA) protein, produced in different immune competent and somatic cell lines. The production of IL-6 is stimulated by activation of TLRs and RIG-1 like receptors but can be also induced by tumor necrosis factor (TNF)-α, and IL-1β. After binding to the specific receptor, IL-6 initiates signaling mainly associated with the JAK/STAT3 pathway (Wang et al., 2013), resulting in induction of transcription of multiple downstream genes and production of cytokines, receptor proteins, protein kinases and adaptor proteins (Brocke-Heidrich et al., 2004; Mauer et al., 2015). IL-6 signalization is associated with multiple functions in different processes, reviewed by Tanaka and Kishimoto (Tanaka and Kishimoto, 2014) and shown in Figure 7. Therefore, the consequences of IL-6 induction varies depending of virus, cell type, tissue and organism; reviewed by Lauro Velazquez-Salinas here (Velazquez-Salinas et al., 2019).

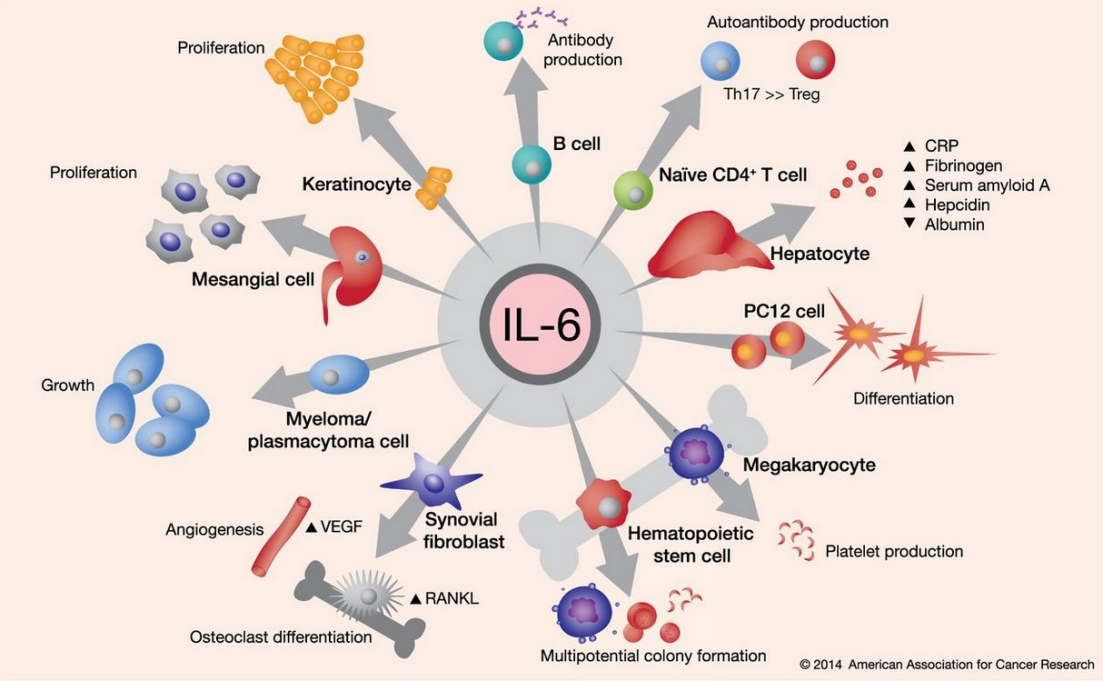


Figure 7. Pleiotropic activity of IL-6. IL-6 induces activated B cells to antibody production. IL-6, combined with transforming growth factor (TGF-β), preferentially promotes the differentiation of naïve CD4⁺ T cells into Th17 cells, but inhibits TGF-β–induced Regulatory T cell (Treg) development. As a consequence, Th17/Treg imbalance may cause the onset and progression of immune-mediated diseases. IL-6 also induces production of acute-phase proteins, such as C-reactive protein (CRP), serum amyloid A (SAA) protein, fibrinogen, and hepcidin, but reduces synthesis of albumin in hepatocytes. In bone marrow, IL-6 induces maturation of megakaryocytes into platelets and activation of hematopoietic stem cells. Moreover, IL-6 promotes the differentiation of osteoclasts and angiogenesis, stimulates collagen production by dermal fibroblasts, and stimulates the growth of myeloma cells and mesangial cells. According to Tanaka and Kishimoto (Tanaka and Kishimoto, 2014).

1.3.5. Interferons

IFNs were discovered by Alick Isaacs and Jean Lindenmann, in 1957, when they found that incubation of 10-day-old chick chorioallontoic membrane with inactivated influenza virus caused release of a substance that isable to protect another chorioallontoic membrane from infection by active influenza virus. They termed the interfering substance “interferon” (Isaacs and Lindenmann, 1957). Nevertheless, it is worth to note, that 3 years earlier, the group of scientists from Japan working with VACV showed similar effect, isolated the substance and named it “inhibitory factor” (Nagano and Kojima, 1954).

At present, there are 3 known types of IFN, divided on the base of differences in receptor used, structure and functions. Type I IFNs are represented in mammals by at least 13 different subtypes of IFN-α, single type of IFN-β and by less studied IFN-ε, IFN-ω, IFN-κ, IFN-δ, IFN-τ and IFN-ζ (also known as limitin). IFN-α is mainly produced by plasmacytoid dendritic cells (pDCs), IFN-β is mainly produced by non-immune cells, such as fibroblasts and epithelial cells in response to viral infection but can be also produced by the innate immune cells e.g. macrophages and pDCs.
The cellular sources of other members of IFN Type I are summarized in Table 1.

IFN-ε	IFN-ω	IFN-τ	IFN-κ	IFN-δ	limitin
Cells of CNS, female reproductive organs	PDCs, Hematopoetic Cells, mainly leukocytes	Trophoblasts and Endometrial Cells	Epidermal Cells, Keratinocytes	Blastocyst Cells	Mature T Lymphocytes, Bronchial Epithelial and Salivary Duct Cells

Table 1. Main cellular source of IFN-ε, IFN-ω, IFN-κ, IFN-δ, IFN-τ and IFN-ζ.
Adapted accordingly to Shi-fang Li (Li et al., 2018).

Type II IFNs consist of single member - IFN-γ, produced by natural killer (NK) and T (NKT) cells. Type III IFNs are preferentially produced by epithelial cells, hepatocytes and myeloid dendritic cells and are represented by 4 molecules: IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4 (Pestka et al., 2004; de Weerd and Nguyen, 2012).

Type I IFNs initiate downstream pathway through interaction with interferon α/ β receptors (IFNAR) 1 and 2. It is followed by Janus kinase1 (JAK1) and tyrosine kinase 2 (TYK2) dependent activation of signal transducer and activator of transcription (STAT) 1 or 2. Activated STAT1, STAT2, or heterodimers, form STAT1-STAT2-IRF9 (interferon-regulatory factor 9) complexes, known as interferon-stimulated gene factor 3

(ISGF3) complexes. Then, ISGF3 are translocated to the nucleus and act as transcription factor for initiation of transcription of multiple interferon stimulated genes (ISGs) (Novick et al., 1994; Aaronson and Horvath, 2002).

Type II IFNs utilize different type of receptors – interferon gamma receptors (IFNGR) 1 and 2 (Bach et al., 1997; Platanias, 2005). The receptor activation is followed by JAK1-JAK2 dependent formation of gamma interferon factor (GAF), composed of STAT1 homodimers and translocation of phosphorylated GAF to the nucleus, where it binds interferon gamma-activated site (GAS) located in the promoter region of some ISGs (Bach et al., 1997; Platanias, 2005). Type II IFNs are pro-inflammatory cytokines produced by activated NK, antigen-presenting cells (APC) and B Lymphocytes (B cells). (Chan et al., 1991; Scharton and Scott, 1993). They participate in differentiation and stimulation of immune competent cells, especially macrophages (Heise and Virgin, 1995) and T and B lymphocytes (Abed et al., 1994; Sercan et al., 2010).

Type III IFNs activate signaling pathway similar to that activated by IFNs Type I. This results in ISGF3 translocation and corresponding induction of ISG expression (Kotenko et al., 2003; Syedbasha and Egli, 2017). The signaling of IFN Type III occurs through unique heterodimeric receptor composed from interferon lambda receptor 1 (IFNLR1) and IL10 receptor B (IL10RB) (Syedbasha and Egli, 2017) (Figure 8).

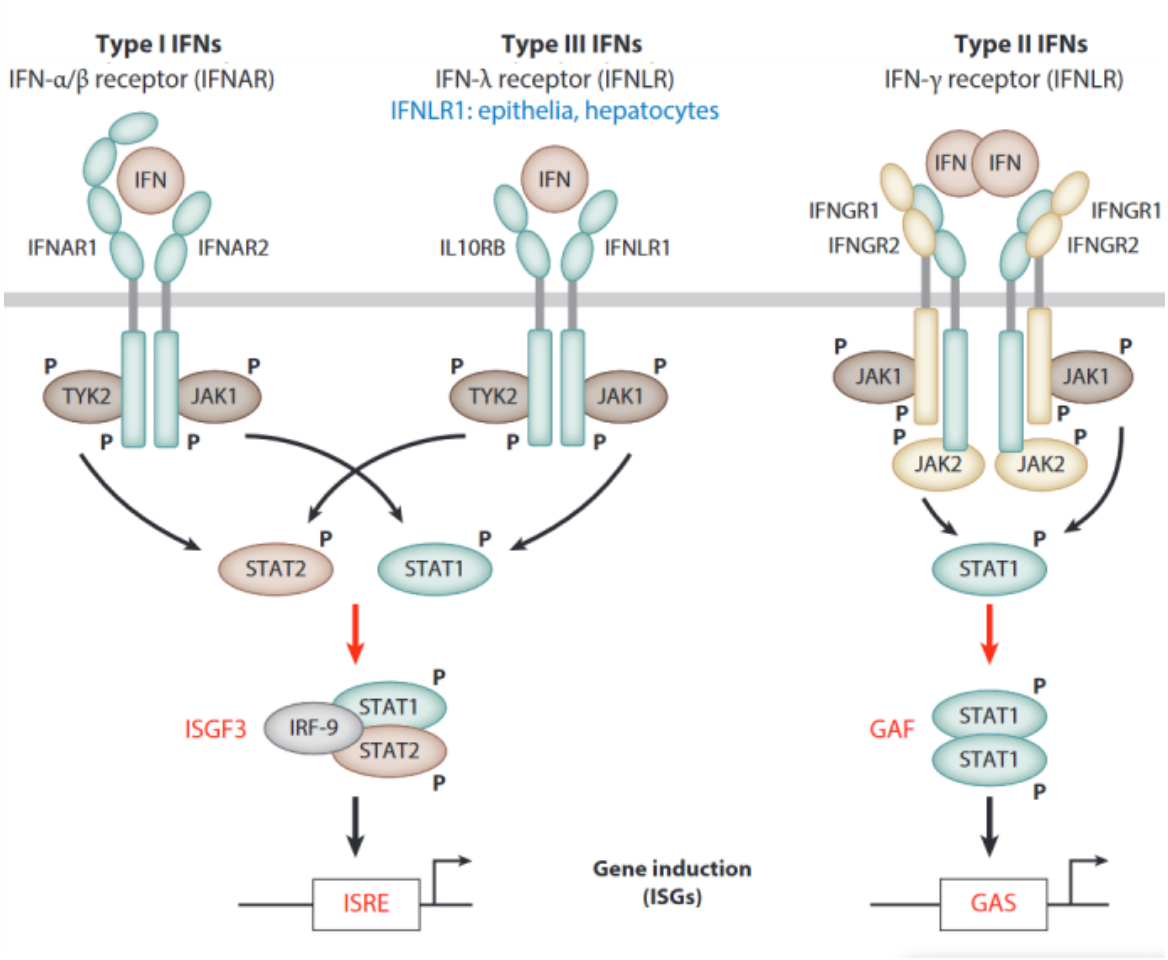


Figure 8. The schematic representation of signalings mediated by receptors of IFNs Type I, II and III. According to Volker Fensterl (Fensterl et al., 2015).

1.3.6. Restriction of virus infection by IFN-β induced ISGs

IFNs Type I trigger expression of multiple ISGs. They stimulate expression of, both, autocrine and paracrine restriction factors including expression of MHC molecules and the presentation of foreign antigens to cytotoxic and helper T cells. They contradict viral infection at different phases (Fensterl et al., 2015; Ivashkiv and Donlin, 2014; McNab et al., 2015).

Induced expression of **RIG-I**, **MDA5** and **DAI** contributes to enhanced ability of cytosolic recognition of short dsRNA, long dsRNA and dsDNA, respectively (Saito and Gale, 2008; Takaoka et al., 2007). **TRIM proteins** either directly contradict virus infection or are involved in regulation of innate immune signaling pathways. For example, TRIM19 (also known as promyelocytic leukemia protein (PML)) is a nuclear scaffoldprotein maintaining IRF3 mediated IFN gene transcription and epigenetic regulation of viral replication (Chen et al., 2015; Tsai and Cullen, 2020). TRIM5α

directly binds viral capsids and TRIM21 binds viruses opsonized by antibodies. Both cases are followed by induction of proteosomal degradation (Sastri and Campbell, 2011; Foss et al., 2019). TRIM32 and TRIM 25 contradict function of viral polymerases (Fu et al., 2015; Meyerson et al., 2017). **MicroRNAs** are involved in the regulation of innate immune signaling and/or in degradation of viral RNAs (Fani et al., 2018). The enzyme, 2′–5′-oligoadenylate synthetase (**OAS**) was the first characterized IFN-induced antiviral protein, discovered by Walden K. Roberts in 1976. It activates the latent RNase L which then results in degradation of viral and cellular RNAs (Roberts et al., 1976; Coccia et al., 1990; Eskildsen et al., 2003). Induced expression of dsRNA-dependent protein kinase R (PKR) results in inhibition of proteosynthesis (Dauber and Wolff, 2009). Dynamin-like GTPases – myxovirus resistance (**MX**) family of proteins are able to detect the nucleoproteins and/or nucleocapsid proteins of different viruses and prevent viral replication (Nigg and Pavlovic, 2015). Adenosine deaminases acting on RNA (**ADAR**) modify mRNA transcripts by changing the nucleotide content of the RNA molecules, resulting in RNA structure unwinding and destabilization (Samuel, 2012). Apolipoprotein B Editing Complex 3 (**APOBEC3**) is cytidine deaminase catalyzing the deamination of cytidine to uridine in the single stranded DNA. This results in degradation of reversed transcribed DNA (Sheehy et al., 2002; Stavrou and Ross, 2015). Ubiquitin-like interferon stimulated gene 15 (**ISG15**) protein modifies certain viral proteins, including capsid proteins, by covalent binding (ISGylation). It results in dominant-negative interfering with assembly of higher order structures (Skaug and Chen, 2010; Perng and Lenschow, 2018)

The summarized scheme of IFN induced ISG expression and restriction of virus infection is shown in Figure 9.

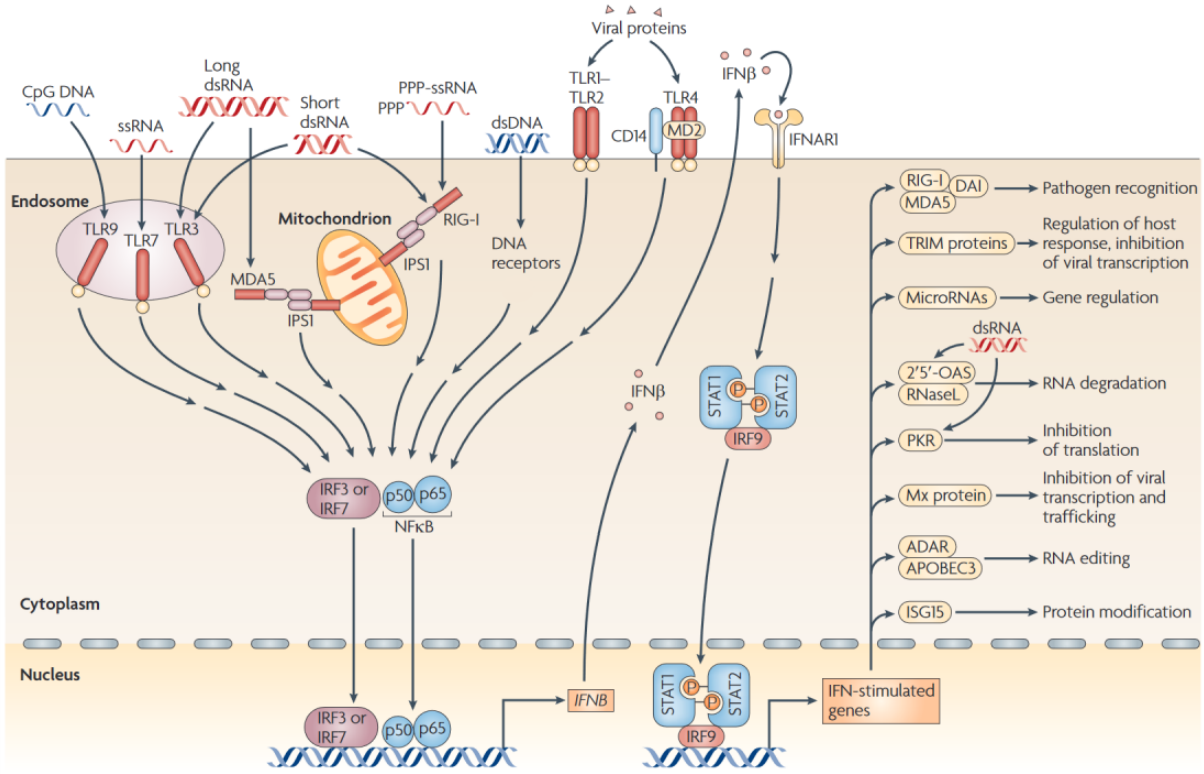


Figure 9. Activation of the interferon response (triggered by PRRs detection of viral PAMPs) initiates ISG expression and restriction of viral infection.
IPS1, IFNB-promoter stimulator1; MD2, myeloid differentiation protein 2; PPP, 5′ triphosphate. According to Andrew G.Bowie (Bowie and Unterholzner, 2008).

1.3.7. Recognition of DNA viruses by cGAS or IFI16

Herpesviruses were the first viruses shown to be sensed by cGAS with following STING-IRF3 dependent induction of IFN production. It was shown, that knock out (KO) of cGAS prevents activation of IRF3 and production of IFN-β in response to Herpes simplex virus 1 (HSV-1) and KSHV infection (Ma et al., 2015; Sun et al., 2013). cGAS was also found as a dominant cytosolic DNA sensor recognizing internalized adenovirus and initiating STING-TBK1-IRF3 dependent IFN production (Lam et al., 2014). Hepatitis B virus (HBV) DNA was found to be sensed by cGAS but IFN induction in hepatocytes was limited due to limited STING expression in this cells (Lauterbach-Rivière et al., 2020).

The VACV infection, characterized by massive production of viral DNA in cytoplasm, was shown to be sensed mainly by cGAS (Meade et al., 2019).

While cGAS mediating sensing of DNA viruses have been shown to occur in the cytoplasm of infected cells, IFI16 recognizes viral DNA both, in the cytoplasm and the

nucleus. VACV DNA fragments transfected into the cells, were shown to be recognized by IFI16 or by its mouse analogue, p204, and cause STING, TBK1 and IRF3 dependent IFN-β production. The same authors found that HSV-1 DNA was sensed by IFI16/p204 during infection and suggested that both cytoplasmic and nuclear sensing take place (Unterholzner et al., 2010). IFI16 was found to sense Human cytomegalovirus (HCMV) infection. Its silencing resulted in increased virus replication (Gariano et al., 2012). IFI16 was shown to be associated with inflamasome induction during HSV-1 and Kaposi sarcoma-associated herpesvirus (KSHV) infection (Kerur et al., 2011).

1.4. Sensing of polyomaviruses

Polyomaviruses have been known and studied since 1950, but the importance of innate immunity in maintenance and/or restriction of polyomavirus infection, has just began to be properly evaluated.

Recently, it was shown that transient, virus free expression of LT antigen of SV40, BKPyV or JCPyV is sufficient for STAT1 dependent stimulation of expression of ISGs and establishment of antiviral state (Giacobbi et al., 2015). Both, IFNα and IFNβ restrict replication of JCPyV in Primary Human Fetal Glial Cells (Co et al., 2007). By the same group, it was demonstrated previously that JCPyV infection can induce transcription of multiple ISGs I (Verma et al., 2006). BKPyV infection of endothelial cells results in cell type dependent activation of IFNβ, together with IRF3 and STAT1 (An et al., 2019). Treatment by IFNγ inhibits both BKPyV and JCPyV infection already on the level of early antigen expression (Abend et al., 2007; De-Simone et al., 2015).

MCPyV ST antigen contradicts NF-kB signaling by binding NEMO adaptor protein, resulting in decreased transcription of NF-kB dependent genes and collapse of inflammatory signaling (Griffiths et al., 2013). Another mechanism protective against innate immunity was recently found for BKPyV. It was shown that agnoprotein of BKPyV diminishes the nuclear translocation of IRF3, resulting in decreased expression of IFN genes (Manzetti et al., 2020).

A number of evidence of induction of innate immunity by polyomavirus infection and even the mechanisms by which PyVs overcome innate immunity processes have been described. However, there is a gap in the understanding of at what stage of PyV infection, the virus is recognized by PRRs and what viral components are sensed. In our study, we addressed these questions.

2. Aims of the study

The main aim of our study was to find the way(s) of MPyV recognition by the components of cell innate immunity.

To achieve it, we aimed:

- to determine the phase of MPyV infection sensitive to recognition of the virus by innate immunity sensors;

- to find components of cell innate immunity involved in polyomavirus sensing and following signaling;

- to evaluate the possible consequences of MPyV sensing for infection and cell transformation.

3. Results

The results are presented in form of incorporated in this work articles (**Appendix I**), published by me and my colleagues in peer reviewed journals.

3.1. The list of publications

Huerfano, S., **Ryabchenko, B**., and Forstová, J. (2013). Nucleofection of Expression Vectors Induces a Robust Interferon Response and Inhibition of Cell Proliferation. DNA Cell Biol *32*, 467–479.

Huérfano, S., **Ryabchenko, B**., Španielová, H., and Forstová, J. (2017). Hydrophobic domains of mouse polyomavirus minor capsid proteins promote membrane association and virus exit from the ER. The FEBS Journal *284*, 883–902.

Ryabchenko, B., Soldatova, I., Šroller, V., Forstová, J., and Huérfano, S. (2021) Immune sensing of mouse polyomavirus DNA by p204 and cGAS DNA sensors. The FEBS Journal.

Janovec, V., **Ryabchenko, B**., Škarková, A., Pokorná, K., Rösel, D., Brábek, J., Weber, J., Forstová, J., Hirsch, I., and Huérfano, S. (2021). TLR4-Mediated Recognition of Mouse Polyomavirus Promotes Cancer-Associated Fibroblast-Like Phenotype and Cell Invasiveness. Cancers *13*, 2076.

Journal Impact Factor 2020 (5 years) (Clarivate Analytics)

DNA and Cell Biology	3.3
The FEBS Journal	*5.5*
Cancers	*6.9*

In virtue of difference in transliteration in Czech and English languages the author's surname is spelling as Rjabčenko or Ryabchenko.

3.2. The highlights of results

- Naked, MPyV DNA, introduced in to the cell by nucleofection induces robust interferon response

- MPyV genomes are invisible for innate immunity DNA sensors during their endosomal trafficking from the plasma to the endoplasmic reticulum

- Prior translocation of virions to the cell nucleus, the minor capsid proteins, VP2 and VP3 mediate escape of the partially disassembled virions from ER to the cytosol, utilizing properties of their hydrophobic domains

- MPyV genomes are recognized by cGAS and IFI16 sensors during replication of MPyV genomes

- The sensing is followed by STING dependent IFN type 1 production

- IFI16 co-localizes with MPyV DNA in the nucleus

- cGAS co-localizes with MPyV genomes both, in nucleus (within replication foci) and in the cytoplasm (sensing of DNA leaked from the nucleus and micronucleus-like bodies)

- Detectable level of cGAMP, the product of activated cGAS, was found exclusively in the cytoplasm.

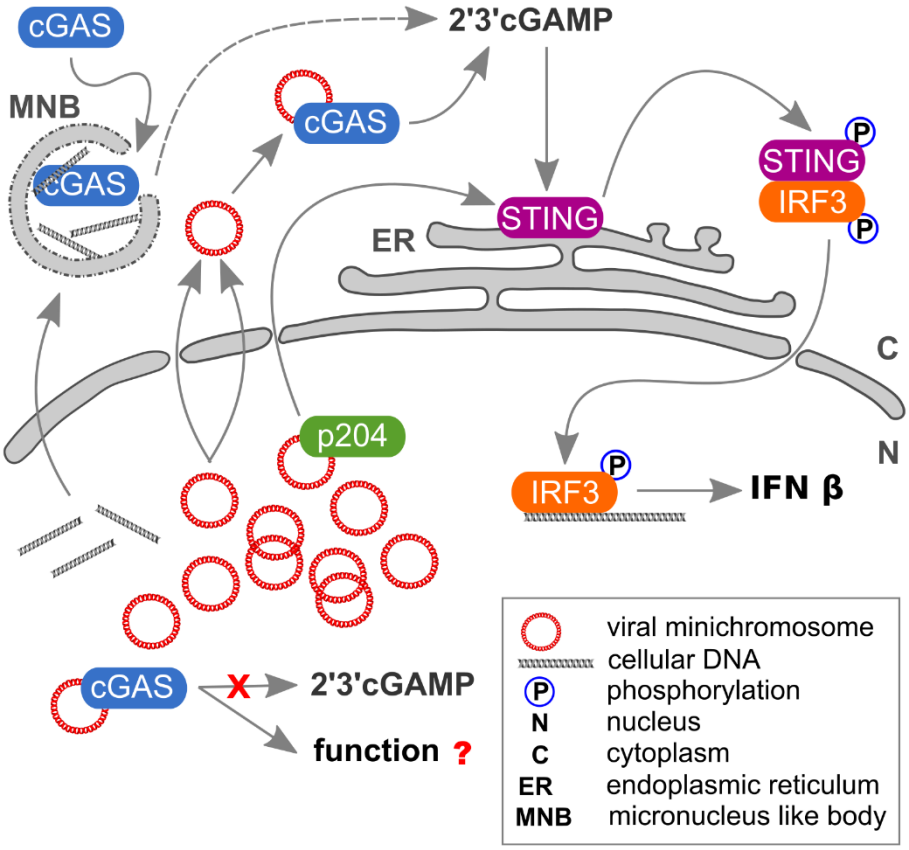
- TLR4 senses MPyV virion during immediate early stages of infection and causes massive IL6 and other cytokine production

- Cytokine microenvironment changes the phenotype of neighboring fibroblasts toward the cancer-associated fibroblast (CAFs).

- Transformation of normal fibroblast to CAFs is characterized by increased invasiveness and chemokine production

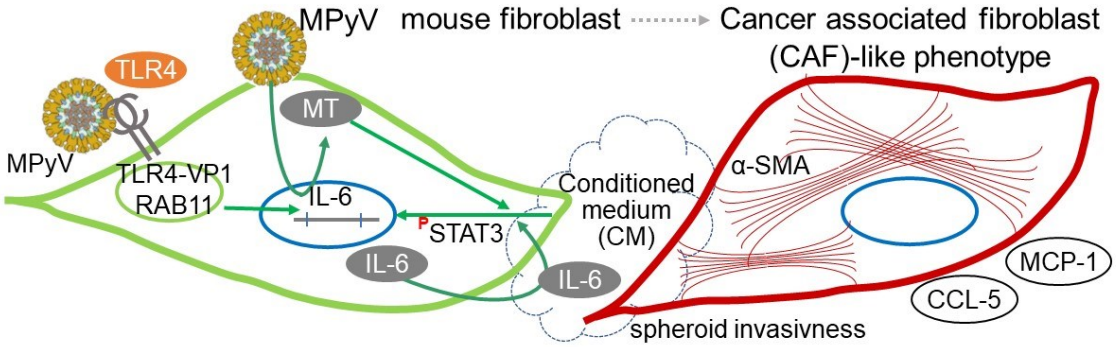
3.3. The graphical interpretation of results

P204 and cGAS mediated sensing.



Viral minichromosomes can be sensed in the nucleus by p204 and in the cytoplasm (in form of leaked DNA) by cGAS proteins. Both, p204 and cGAS recognition leads to the STING-IRF3 dependent IFNβ expression. cGAS can be also found associated with viral minichromosomes in the nucleus, but the functional consequence of it remains unclear, since 2'3'cGAMP, the mediator molecule, transducing signal from activated cGAS to the STING,, is found exclusively in the cytoplasm. cGAS protein also senses cellular DNA, presented in micronucleus-like bodies that are formed as a result of genomic stress caused by infection.

TLR4 induced MEFs-CAFs transformation



MPyV virions are sensed by TLR4 during their uptake and transport in early endosomes. TLR4 activation induces massive secretion of IL6 and other cytokines (without inhibition of replication of MPyV) and activation of STAT3. Cytokine microenvironment changes the phenotype of uninfected cells surrounding infected fibroblasts into cancer associated fibroblasts CAFs, characterized by increased invasiveness and upregulated expressions of MCP-1, CCL-5, and α-SMA.
Simultaneously, STAT3 is activated also by viral MT protein independently of IL6.

4. General discussion and conclusions

4.1. The early stages of infection are invisible for DNA sensors

There are no detectable levels of IFN type I production in MPyV infected cells up to 24hpi, when massive replication of viral genomes occurs. One of the most obvious explanation is that viral DNA is not accessible for PRRs during MPyV trafficking from the plasma membrane to the nucleus.

What happen if viral DNA stays in cytoplasm in naked form, becames clear from our study of nucleofection of plasmids carrying MPyV DNA. We showed that DNA delivered to the cells by nucleofection caused robust IFN type 1 response. We detected both, the induction of IFN production and ISG (STAT1, MX1, IRF1 and IRF7) expression. At the time these experiments were performed, very little was known about cytosolic DNA sensors. Based on the evidence that together with activation of IFN production, we detected the translocation of NF-kB transcription factor, we hypothesized the sensing via already known DNA sensor, TLR9, recognizing methylated motifs on the sequences of DNA.

However, we did not observe any significant differences in the levels of IFNβ, in the cells transfected by methylated and non-methylated plasmids. We finally concluded that observed IFN induction was TLR9 independent. Our following unpublished data, strongly supported the involvement of at least one of other discovered DNA sensor, p204 (IFI16 analogue) in recognition of naked forms of DNA, since we found multiple sites of co-localization between p204 protein and DNA in the cytoplasm of nucleofected cells (Figure 10).

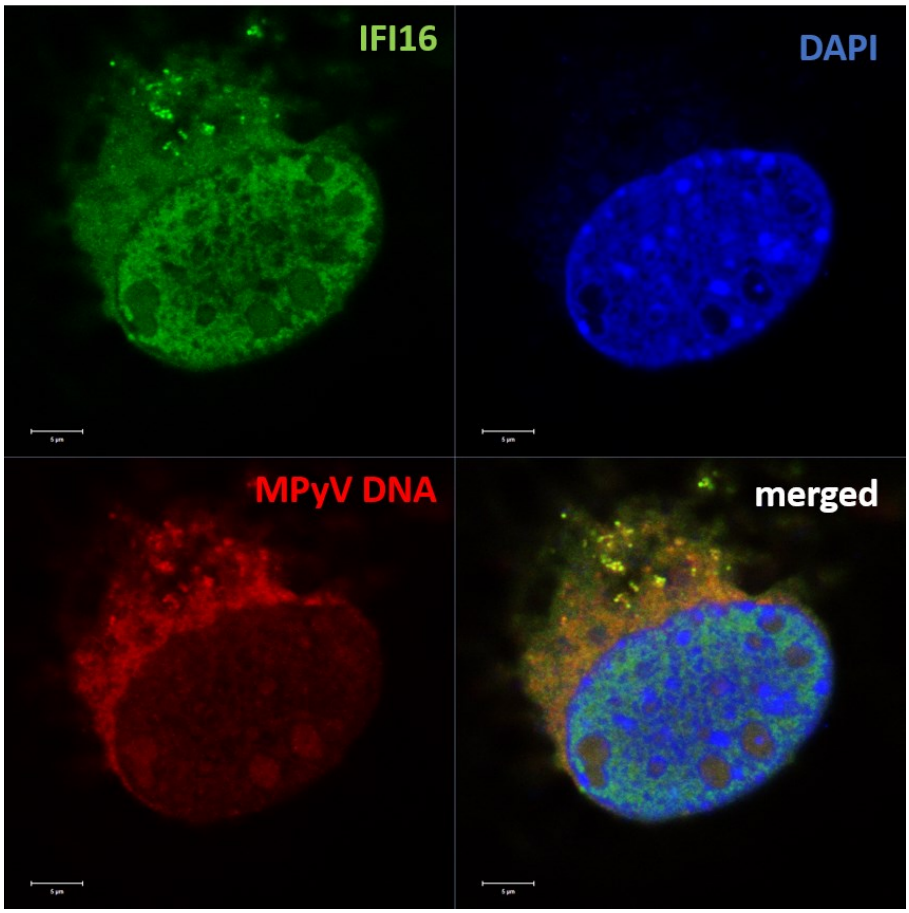


Figure 10. MEF cells were nucleofected by plasmid carrying MPyV DNA sequences and simultaneous detection of p204 (green) by *in situ* immunofluorescence and DNA (red) by FISH was performed 6 hours post transfection. Selected confocal section is presented. Bar 5um.

During infection, viral DNA stays apparently packed in virions during PyV trafficking inside the endocytic vesicles. Therefore, viral DNA is not sensed by TLR9 present in endosomes.

Partial disassembly of virions occurs only after the virions enter the ER. The manner in which partially disassemble virions are transported from the ER into the cell nucleus was unclear. There were two hypotheses, either that virions enter the nucleus by direct translocation across the inner nuclear membrane from the ER to the nucleus, or that partially disassembled virions enter the ER from the cytosol and then travel via importins through the nuclear pore. The next effort of our research group was to find out which hypothesis is valid and to understand the way virions cross the ER membrane. We found that the important role in efficient interaction with membranes of

ER and in consequent fast escape from ER belongs to the minor capsid proteins VP2 and VP3 in fact, more precisely, to the hydrophobic domains. (HDs) in their structure. In our study, we analyzed the variants of MPyV with mutated HDs of the minor capsid proteins. We found that in comparison with the wild type MPyV, the infection with the virus mutated in HD1 (unique for VP2 protein was delayed The virus, mutated in HD2 (common for VP2 and VP3 proteins) was not infectious. By proximity ligation assay (PLA), we found out that all these viruses were present in lumen of the ER at similar amounts and at the same time. However, for both mutants and especially the one with abolished HD2, the association of virions with the ER membrane was markedly reduced and, conversely, the presence of virions in ER lumen was increased

This observation was further confirmed by the use of transiently expressed vectors coding the EGFP-fused minor capsid proteins. The EGFP-fused VP2 mutated in HD2 was incapable of association with ER membrane. This mutant variant of VP2 protein also lost cytotoxic and viroporin-like properties of the minor capsid proteins.

Parallel study of our group proved that partially disassembled virions appear in the cytosol binding importins and that translocation across nuclear pores is the major way of transport of PyV genomes into the nucleus (Soldatova et al., 2018).

Incidentally, BKV agnoprotein, which has similar to minor capsid proteins of MPyV cytotoxic potential by its viroporin properties, was recently shown to impair nuclear IRF3 and IFNβ production, disrupt mitochondrial membranes and promote targeting the mitochondria for autophagy (Manzetti et al., 2020). Whether the minor capsid proteins have similar abilities remains to be revealed.

We expected that after translocation of PyV virions from the ER to the cytosol, prior the transport of their genomes to the cell nucleus, viral DNA could be sensed by cytosolic DNA sensors. However, even at this stage of infection, we did not find any convincing signal of IFN induction. A possible factor helping to keep the early cytoplasmic phase of infection hidden from recognition by DNA sensors is the fast and efficient process of virus translocation from ER to the nucleus.

In our previous (unpublished) experiments, MPyV nucleocores were isolated (by gradient ultracentrifugation) after disassembly of capsids of purified virions with dithiothreitol and EGTA. The nucleocores, composed of viral DNA condensed with cell histones and VP1, were then treated with benzonase and different DNases. No signs of viral genome cleavage were detected unless nucleocores had previously been treated with proteases (Forstová, Palková, unpublished data). Apparently, condensed

nucleocores, in which viral DNA is protected even after loss of capsid shells become relaxed in the nucleus.

4.2. MPyV genomes are sensed by cGAS and p204 at late stages of infection

We observed that detectable levels of IFN appeared at late phases of infection (24hpi and later). Absence of IRF3 phosphorylation and of IFN response in STIKG KO cell line confirmed that IFN induction is STING dependent and triggered by ISGF3 transcription complex. The indicated time of infection is associated with replication of viral genomes. Thus, we hypothesized that replication of MPyV is sensed by PRRs. The remarkable reduction of IFN response in case that we used infection by the virus mutated in nuclear localization signals (NLSs) of its capsid proteins (these mutations notably decreased translocation of virions from the cytosol to the nucleus) was another fact supporting our hypothesis. By that time, the function of HIN-200 family of proteins in nuclear sensing of foreign DNA was described (Roberts et al., 2009) and narrowed the area of searching for possible PRRs for MPyV infection. We found that mouse analogues of human IFI16 protein, p204, interacts with MPyV genomes in the nucleus. We also detected decreased transcription of genes for IFNβ and MX1 together with decreased levels of phosphorylated IRF3 in p204 silenced cells. All this suggests that MPyV genomes may be sensed in the cell nucleus. Nevertheless, the molecular mechanism of the recognition remains unclear. IFI16 protein requires long, nucleosome-free parts of DNA for effective sensing (Stratmann et al., 2015). MPyV genomes are delivered into the nucleus in form of chromatinised DNA and viral replication is associated with histone incorporation and nucleosome formation simultaneously with replication. The only part of viral DNA free of nucleosomes is NCCR (Kube and Milavetz, 1989). But this, approximately 300bp long sequence contains, different regulatory elements (transcription promoters and enhancer, replication origin) and has to be occupied by multiple complexes of proteins involved in transcription initiation and maintaining as well as in genome replication. n. Thus, further experiments, aimed to uncover the exact mechanism of p204 role(s) on IFN induction during MPyV infection have to be done.

The decision to check whether another DNA sensor, cGAS, plays a role in sensing of MPyV came with appeared publications that, besides cytoplasmic location, cGAS also occurs in the nucleus. It was found that nuclear localization of cGAS is associated

with the centromere regions and long interspersed nuclear element (LINE) DNA repeats, and that nuclear cGAS is able to induce immune response through cGAMP (Gentili et al., 2019).

By confocal microscopy, we found co-localization of viral DNA clusters and clusters of cGAS protein in the nucleus. We also found very strong decrease of IFNβ gene transcription in cGAS KO cell line. The presence of cGAMP in infected cells indicated that cGAS induced IFN response by the canonical way. But, despite of multiple attempts, we were not able to detect the presence of cGAMP in the nuclei of infected cells neither by mass spectrometry nor by ELISA assays. So, we hypothesized that cGAS sensed viral or cell structures appeared in cytoplasm following massive replication of viral genome and/or genotoxic stress caused by infection. We found, that infection is characterized by significant increase in amount of micronucleus-like bodies presented in cytoplasm. Also, we detected the small islets of viral DNA in the nuclei of infected cells, and distinguished them from viral DNA possibly remained in the cytoplasm after entry. By confocal microscopy, we observed that, both, micronucleus-like bodies and islets of viral DNA leaked from the nucleus to the cytoplasm co-localized with cytoplasmic clusters of cGAS. Thus, our findings strongly support the hypothesis that cGAS is the sensor of MPyV DNA and that it senses viral genomes that are leaked from the nucleus of infected cells and DNA present in micronucleus-like bodies appeared in cytoplasm as a result of genotoxic stress caused by infection. Our findings are in accordance with previously observed participation of cGAS in recognition of micronuclei followed by immune response induction (Mackenzie et al., 2017).

Nevertheless, the presence of cGAS on the clusters of replicating viral DNA in the nucleus remains unexplained. Nuclear function of cGAS is largely unknown but it becomes clear, that self-DNA sensing by cGAS is absent because cGAS is sequestered by chromatin (Michalski et al., 2020). Recently, it was shown that cGAS inhibits homologous recombination (HR) by contradicting Rad51-mediated strand invasion. PyVs induce DNA damage response (DDR) by multiple mechanisms and may even utilize DDR in their profit (Jiang et al., 2012; Sowd et al., 2014; Heiser et al., 2016). Thus, the involvement of cGAS in the DD) induced by MPyV infection is possible scenario explaining nuclear cGAS-PyV DNA association.

Recently, it was described that DNA damage induces immune response activation through non-canonical, p53 dependent, activation of STING, involving participation of

IFI16 (Dunphy et al., 2018). In contrast to canonical pathway, where activation of STING is associated with phosphorylation, non-canonical pathway is characterized by ubiquitination of STING. Our experiments of infection of p53 KO cells revealed the decreased level of IFN production in response to MPyV infection (unpublished data). The involvement of non-canonical signalization in activation of immune response to PyV infection is a task for our future research. Another recent finding, declares the essential role of IFI16 in cGAMP dependent activation of adaptor STING molecule (Almine et al., 2017). On the other hand, it was shown that cGAS mediates stability of IFI16 protein and promotes IFI16 dependent recognition of HSV genomes in the nucleus (Orzalli et al., 2015). However, we did observe the changes neither in p204 protein level in cGAS KO cells, nor in positioning of p204 protein into the nuclear MPyV genome foci. These results indicate either possible differences between IFI16 and its mouse analogue, p204, and/or possible cell type dependent mechanism of stabilization. Uncovering the exact role of IFI16 / p204 in IFN induction during PyV infection will require further experiments. These will, among other things, be the subject of our further research.

4.3. TLR4 recognition of MPyV and its consequences

TLR4 is involved in activation of different cytokine production in response to MPyV infection (Velupillai et al., 2006). The consequences of that activation has different effects, depending on the type of cytokine produced. For example, infection of the mouse strain, BR, resistant to MPyV induced tumorigenesis, activates production of IL12 in contrast to activation of IL10production in mouse PEA, strain which is polymorphic in TLR4 gene and susceptible to the formation of tumors (Velupillai et al., 2012). The MPyV structure recognized by TLR4 is probably a part of VP1, since TLR4 mediated cytokine production can be activated by virus-like particles, composed of VP1 protein only (Velupillai et al., 2006). The results of above studies led us to verify the hypothesis that TLR4 may be part of protective immunity against MPyV infection. First, we looked by confocal microscopy on to the interaction between TLR4 and MPyV, virions. We found sites of their co-localization as fast as 1,5 hours post infection with increased tendency with infection progression. The sites were identified by co-localisation of Rab 11 GTPase as recycling endosomes. Then, we found that the recognition of MPyV by TLR4 led to the induction of pro-inflammatory cytokine IL6 production and following activation of STAT3 protein. Fibroblasts transformed by

expression of polyomavirus MT antigen demonstrate constitutively activated STAT3 via c-Src protein kinase (Garcia et al., 1997; Schaffhausen and Roberts, 2009). However, MT activation of STAT3 is independent of IL6 production.

We did not find any protective role of TLR4 mediated IL6 production against MPyV infection when we used the treatment of infected cells with agonist of TLR4 or direct treatment by recombinant IL6. This is consistent with previous observation that BKPyV, infection is highly resistant to the treatment with cytokines, with the only exception of IFNγ (Abend et al., 2007). We further hypothesized that activation of IL6-STAT3 pathway by MPyV infection may be connected with cell transformation Involvement of IL6 secretion with tumorigenic cell transformation is well documented. For example, development of Colitis-associated cancer (CAC) is tightly connected with secretion of IL6. The transformation of Pancreatic stellate cells (PSC) into the cancer associated fibroblasts (CAFs) was shown to be characterized by the presence of IL6, together with another inflammatory cytokines. High level of IL6 expression was found in CAFs associated with HPV induced neoplasia (Erez et al., 2010; Grivennikov et al., 2009; Öhlund et al., 2017).

In our hands, the invasiveness of mouse embryo fibroblasts (MEFs) in a 3D collagen matrix treated with medium taken from infected cells and purified from the virus, was significantly increased. Moreover, the cells were characterized by higher production and altered distribution of alpha smooth muscle actin (αSMA) - one of the markers of cancer associated cell transformation. We also found the overexpression of other cancer associated chemokines, MCP1 and CCL5. Their involvement in cancer progression was previously documented (Soria et al., 2008). Thus, we confirmed that activation of TLR4 by MPyV infection resulted in formation of cytokine microenvironment boosting transformation of non-infected MEFs into the CAFs. The transformation is driven by secretion of IL6, since the inhibition of IL6 pathway by ruxolitinib prevents MEFs from transformation. Importantly, we also found the increased invasiveness of colon carcinoma CT26 cells, treated by the medium taken from infected cells. But, in contrast to MEFs, treatment by ruxolitinib did not drastically affect the invasiveness, suggesting existence of additional factors involved in developing of CT26 cell invasiveness. Massive pro-inflammatory response, including IL6 secretion, during BKPyV infection was previously found in renal transplant recipients (Sadeghi et al., 2009). Our findings point the existence of potential hazard connected with possible development of malignancies in those patients.

Another possible consequence of TLR4 mediated STAT3 activation is involvement of STAT3 in the silencing of IFNs type 1 response. It was shown, that STAT3 negatively regulates IFN type 1 genes expression by multiple mechanisms, including formation of heterodimers with transcription factor STAT1, induction of transcription of negative regulators of IFNs and even induction of miRNA transcription resulted in reduction of ISGs (Tsai et al., 2019). The antiviral effects of IL6 in response to acute viral infection is well characterized and explained by involvement of IL6 signalization in modulation of immune-competent cells (Velazquez-Salinas et al., 2019). Nevertheless, long term production of IL6 is associated with development of different pathologic conditions, including tumorigenesis. The absence of protective activity of IL6 against MPyV infection in our experiments is obviously the consequence of the limitation of in vitro cell culture method.

Final remarks

The important role of IFNs Type I in restriction of MPyV infection underlie our unpublished data, when we found that IFN-β treatment significantly (up to 5 times) decreases transcription of MPyV early antigens during infection of MEFs. Despite of high multiplicity of infection (MOI) used in our experiments, there is moderate to small activation of IFNs production in infected cells in comparison with IFN activation by treatment with known IFN stimulators (poly:IC, cGAMP or plasmid DNA), suggesting that the virus has an escape strategy to contradict innate immune response in favor of productive infection. The elements of that strategy include, among others, i) trafficking of MPyV protected from cytosolic DNA sensors in endosomes, ii) the viroporin like properties of viral minor capsid proteins facilitating late escape of the partially disassembled virus from ER to the cytosol just prior its translocation to the nucleus, iii) nucleocore where the genomic DNA is tightly condensed with histones and VP1, thus protected from recognition.

Based on our data, it is clear that innate immune recognition of MPyV infection is composed of non-protective and protective events. Non protective events are represented by TLR4 mediated recognition of incoming virions resulted in formation of cytokine microenvironment containing IL6 and capable in transformation of adjustment, not infected cells into the CAFs.

Protective events are represented by recognition of MPyV infection during replication of viral genomes by DNA sensors: cGAS and p204. Also, by LT antigen induced DDR, resulting in noncanonic induction of IFN, may be involved in protection against MPyV infection. However, it should be recalled that interferon induction is weak. This may be due to the intervention of early MPyV products. For example, the LT antigen is able to bind the phosphorylated p53 required in the STING complex. The moderate level of IFN response to MPyV infection in infected cells may be explained also by the fact that activation of TLR4-IL6-STAT3 and/or MT- c-Src-STAT3 pathways (in infected cells) negatively regulates IFNs production. Studying the ways in which viral products are involved in thwarting the mechanisms of innate immunity protection will be a major topic of our ongoing study.

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Appendixes

Appendix I: articles used by the author as the basis of PhD thesis
(in digital form of PhD thesis are attached as a separate file)

Appendix II: other publications of the author, not included in this work

1. *The major capsid protein, VP1, of the mouse polyomavirus stimulates the activity of tubulin acetyltransferase 1 by microtubule stabilization*
   Horníková, L., Bruštíková, K., **Ryabchenko, B.,** ...Lánský, Z., Forstová, J.
   Viruses, 2020, 12(2)

2. *Structural studies of PML nuclear bodies in polyomavirus infected cells*
   Ryabchenko, B., Forstova, J.
   Biopolymers and Cell, 2019, 35(3)

3. *Cytotoxic active constituents of essential oils of curcuma longa and curcuma zanthorrhiza*
   Schmidt, E., **Ryabchenko, B.,** Wanner, J., Jäger, W., Jirovetz, L.
   Natural Product Communications, 2015, 10(1)

4. *Replication timing of pseudo-NORs*
   Evgeny Smirnov, Dušan Cmarko, Lubomír Kováčik, Guy M Hagen, Alexey Popov, Otakar Raska, José-Luis Prieto, **Boris Ryabchenko,** Filipa Amim, Brian McStay, Ivan Raska
   Journal of Structural Biology, 2011, 173(2)

5. *Investigation of anticancer and antiviral properties of selected aroma samples*
   Ryabchenko, B., Tulupova, E., Schmidt, E., ...Buchbauer, G., Jirovetz, L.
   Natural Product Communications, 2008, 3(7)