Handbook of Experimental Pharmacology 276

Vijay Kumar *Editor*

Toll-like Receptors in Health and Disease

Springer

Handbook of Experimental Pharmacology

Volume 276

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HEP is indexed in PubMed and Scopus.

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Toll-like Receptors in Health and Disease

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-031-06511-8 ISBN 978-3-031-06512-5 (eBook)
https://doi.org/10.1007/978-3-031-06512-5

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The story of Toll-like receptors started almost 35 years ago with the discovery that the *Toll* protein plays a crucial role in embryonic development, including the dorsoventral body patterning in the *Drosophila melanogaster* (the common fruit fly). In 1991, Gay and Keith showed that the cytoplasmic domain of the Drosophila Toll protein is homologous to the human interleukin-1 receptor (IL-1R). Later studies established that, both Toll and IL-1R share common amino-acids (AAs) that are crucial for nuclear factor-kappa B (NF-κB) signaling that plays a significant role in embryonic development, immunity, and in the inflammatory immune response. For example, Toll protein in the *D. melanogaster* plays a crucial role in antifungal and antibacterial immunity through regulating the production of drosomycin, an antifungal peptide and Dif (a member of NF-κB) that induces the production of antimicrobial peptide cecropin. In 1997, the first report of the expression of human homolog of Toll protein called hToll (human Toll) came from the laboratory of Charles A. Janeway Jr. that is now called Toll-like receptor 4 (TLR4). This discovery filled the long existing gap in the recognition of pathogens and pathogen-derived molecules called microbe or pathogen-associated molecular patterns (MAMPs or PAMPs) by immune cells. The discovery of TLRs was recognized for the 2011 Nobel Prize in Physiology or Medicine to Jules Hoffmann and Bruce Beutler along with Ralph A. Steinman, who first discovered dendritic cells (DCs), very crucial innate immune cells. Hence, TLRs are very important pattern recognition receptors (PRRs) with diverse role in the biological process, including immunity and inflammation.

Fourteen years ago, the *Handbook of Experimental Pharmacology* published its first volume on TLRs that described different aspects of TLRs in immunity and inflammation. This second volume of the TLRs series is the continuation of that emphasis and has been developed to provide the updates made in the field of TLRs in different aspects of biology and disease varying from pain, stem cell biology, innate immune memory, mast cell biology and activation, brain immunity to drug targeting.

Overall recent advancements in the field of TLRs biology has increased our understanding in the field of immunology (target organ immunity), infection biology, neurodevelopment, neurodegeneration, reproduction, and stem cell biology. This understanding has opened different avenues for TLR-based therapeutics, adjuvants, and immunotherapeutic molecules to target different diseases varying from infections to cancers to autoimmune or autoinflammatory diseases.

Durham, North Carolina, USA Vijay Kumar

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Toll-Like Receptors (TLRs) in Health and Disease: An Overview

Vijay Kumar and James E. Barrett

Contents

Abstract

Toll-like receptors were discovered as proteins playing a crucial role in the dorsoventral patterning during embryonic development in the *Drosophila melanogaster* (*D. melanogaster*) almost 40 years ago. Subsequently, further research also showed a role of the Toll protein or Toll receptor in the recognition of Gram-positive bacterial and fungal pathogens infecting *D. melanogaster*. In 1997, the human homolog was reported and the receptor was named the Toll-like receptor 4 (TLR4) that recognizes lipopolysaccharide (LPS) of the Gram-negative bacteria as a pathogen-associated molecular pattern (PAMP).

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_568

Identification of TLR4 in humans filled the long existing gap in the field of infection and immunity, addressing the mystery surrounding the recognition of foreign pathogens/microbes by the immune system. It is now known that mammals (mice and humans) express 13 different TLRs that are expressed on the outer cell membrane or intracellularly, and which recognize different PAMPs or microbe-associated molecular patterns (MAMPs) and death/damage-associated molecular patterns (DAMPs) to initiate the protective immune response. However, their dysregulation generates profound and prolonged pro-inflammatory immune responses responsible for different inflammatory and immune-mediated diseases. This chapter provides an overview of TLRs in the control of the immune response, their association with different diseases, including TLR single nucleotide polymorphisms (SNPs), interactions with microRNAs (miRs), use in drug development and vaccine design, and expansion in neurosciences to include pain, addiction, metabolism, reproduction, and wound healing.

Keywords

COVID-19 · inflammation · MAMPs · PAMPs · PRRs · TLRs

1 Introduction

Toll-like receptors (TLRs) are one of the several pattern recognition receptors (PRRs) that recognize different pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) and death/damage-associated molecular patterns (DAMPs). Initially they were discovered in 1985 by *Christiane Nüsslein-Volhard* (Max Planck Institute in Tübingen, Germany) and *E.F. Wieschaus* (Lewis Thomas Laboratory Princeton University, Princeton, NJ, USA) as the genes controlling the dorsoventral embryonic patterning during development in the *Drosophila melanogaster* (*D. melanogaster*) or fruit fly (Nüsslein-Volhard and Wieschaus 1980). They observed the strange mutant phenotype in fly larvae and exclaimed, "Das war ja toll!" which, in German, means "That was strange or weird!", hence, the name "Toll" was given to the protein (Nüsslein-Volhard and Wieschaus 1980; Hansson and Edfeldt 2005). *Christiane Nüsslein-Volhard* and *E.F. Wieschaus* shared the Nobel Prize in Physiology and Medicine for their discovery with E.B. Lewis in 1995 (Valanne et al. 2011). The fruit fly genome encodes eight additional (total nine) Toll-related receptors (including Toll, tube, pelle, cactus, and dorsal, which also control immune function) controlling embryonic development more specifically than immune function (Bilak et al. 2003; Belvin and Anderson 1996). However, only Toll-9 in Drosophila shares structural and functional similarities with mammalian TLRs (Bilak et al. 2003). Of note, Drosophila Toll-related receptors do not serve as PRRs, as is the case with mammals but instead serve as cytokine (cleaved Spaetzle) receptors (Ferrandon et al. 2004; Ligoxygakis et al. 2002).

Later studies showed the role of Spaetzle/Toll/cactus gene cassette in the defense against Gram-positive bacterial (the Toll protein recognizes lysine-type peptidoglycan or PGN) and fungal infections in the adult *D. melanogaster (*Ferrandon et al. 2004*;* Ligoxygakis et al. 2002*;* Michel et al. 2001*;* Leulier et al. 2003*;* Rutschmann et al. 2002*)*. For example, peptidoglycan recognition protein SA (PGRP-SA) mediates the Toll-dependent defense against Gram-positive bacterial infections in the fruit fly (Gobert et al. 2003). Fruit flies with a mutation called Osiris that disrupts Gram-negative binding protein 1 (GNBP1) gene activating Toll-dependent defense against Gram-positive bacterial infections show a decreased survival after the Gram-positive bacterial infection without any defect during the Gram-positive bacterial and fungal infections (Gobert et al. 2003). Hence, PGRP-SA and GNBP1 can jointly activate the Drosophila Toll pathway. A revolution in the pathogen recognition by immune cells occurred when the group led by Charles A. Janeway Jr. in 1997 showed the expression of TLR4 in humans (Medzhitov et al. 1997). This discovery filled the long-lasting gap pertaining to the pathogen recognition by immune cells and the generation of the immune response required to clear the infection. Thus, TLRs are present in both achordates and chordates. We will discuss here only mammalian (human and mice) TLRs and provide an overview of how TLRs have revolutionized biology and medicine.

Humans have ten TLRs (TLR1-TLR10) and laboratory mice have 13 TLRs (Table 1), which regulate immunity under diverse conditions through regulating other PRRs [Nucleotide-binding and oligomerization domain (NOD)-like receptors or NLRs forming inflammasomes, complement receptors and components (CR3, CRIg, gC1qR, C5aR)] (Fitzgerald and Kagan 2020; Kumar 2019a). The activation of TLRs (TLR2) plays a crucial role in the functioning of absent in melanoma-like receptors (ALRs) that also form inflammasomes upon the recognition of self-DNA and the pathogen (bacteria and viruses)-derived DNA by the absent in melanoma-2 (AIM-2) protein during sterile inflammatory conditions or infections (*Francisella tularenesis* or *F. tularenesis* and *F. novicida*) (Fernandes-Alnemri et al. 2010; Jones and Weiss 2011; Rathinam et al. 2010). However, AIM-2 inflammasome activation (through impeding the STING (stimulator of interferon (IFN) genes) and TBK1 (TANK-binding kinase 1, a serine//threonine-protein kinase) interaction and depleting the intracellular K^+ level through the formation of gasdermin-D or GSDMD pore formation) serves as a negative regulator of another intracellular PRR signaling called cGAS (cyclic GMP-AMP synthase)-STING signaling pathway crucial for type 1 interferon (IFN) generation as an antiviral immune response (Kumar 2020a; McNab et al. 2015; Wu and Metcalf 2020). The decreased production of type 1 IFNs and the presence of autoantibodies (AutoAbs) against type 1 IFNs induce a severe COVID-19 phenotype in the severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) infected people (Bastard et al. 2020; Kumar 2020b). Hence, TLRs via regulating different PRRs (function) directly or indirectly regulate the immune response in diverse inflammatory conditions to maintain immune homeostasis.

Mice have a non-functional TLR10, whereas TLR10 (located in endosomes) in humans is functional and exerts an anti-inflammatory action. TLR1, TLR2, TLR4,

Table 1 Different TLRs (Mouse and Humans), their localization, and ligands with their origin

TLRs	TLR Localization	Ligands (PAMPs and DAMPs)	Origin of Ligands
TLR1	Plasma membrane	Triacyl lipopeptide Soluble factors	Bacteria and mycobacteria
TLR2	Plasma membrane	Peptidoglycan (PGN), lipoteichoic acid (LTA), lipoproteins or lipopeptides, lipoarabinomannan, A phenol-soluble modulin, Glycoinositolphospholipids, glycolipids, porins, zymosan, atypical lipopolysaccharide (LPS), heat shock protein 70 (Hsp70), eosinophil-derived neurotoxin (EDN) acts an alarmin	Gram +ve bacteria, mycobacteria, *S. epidermidis, Trypanosoma cruzi, Treponema maltophilum, Neisseria, Fungi, Leptospira interrogans, Porphyromonas gingivalis*, host
TLR3	Endolysosome	dsRNA	Viruses
TLR4	Plasma membrane	LPS, Taxol, fusion protein, envelope proteins, high mobility group box 1 protein (HMG-B1), Hsp60, Hsp70, Hsp22, Hsp96, type III repeat extra domain A of fibronectin, oligosaccharides of hyaluronic acids, polysaccharide fragments of heparin sulfate, fibrinogen, saturated fatty-acids, fetuin-A, N-myc and STAT interactor (NMI) and interferon-induced protein 35 (IFP35)	Gram negative bacteria, Plant, RSV, MMTV, *Chlamydia pneumoniae*, *Chlamydia trachomatis,* host
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Di-acyl lipopeptides, Zymosan	Mycoplasma
TLR7	Endolysosome	ssRNA, loxoribine, Bropirimine	Viruses, synthetic compounds
TLR8	Endolysosome	ssRNA	Viruses
TLR9	Endolysosome	CpG oligodeoxyneucleotide (ODN), hemozoin pigment	Bacteria and viruses (HSV), malaria
TLR10	Endolysosome	dsRNA (Lee et al. 2018)	Viruses
TLR11	Endolysosome	Profilin-like protein	*Toxoplasma gondii*
TLR12	Endolysosome	Profilin-like protein	*Toxoplasma gondii*
TLR13	Endolysosome	23 s ribosomal RNA	Bacteria

TLR5, and TLR6 are expressed on the cell membrane, and TLR3, TLR7, TLR8, TLR9, and TLR10 in the cytosolic organelles, including endosomes, lysosomes, phagosomes, and phagolysosomes (Kumar 2018). TLR9 is emerging as a novel TLR in the pathogenesis of cardiometabolic diseases and obesity-induced benign prostatic hyperplasia (Nishimoto et al. 2020; Calmasini et al. 2020). Also, the activation of

TLR9 in macrophages of corpora cavernosa (CC) of the penis in obese mice (fed high fat diet for 12 weeks) alters its relaxation and predisposes them to develop obesity-associated erectile dysfunction (ED) (Priviero et al. 2021). Additionally, TLR1/TLR2 activation in the CC of the rat penis in vitro increases its contraction that inhibits penis erection through altering vascular function that plays role in vasculogenic ED (Stallmann-Jorgensen et al. 2015). The overexpression of TLR4 in the CC of diabetic rats has been observed and its activation causes diabetes-associated ED (Nunes et al. 2018). The blockade of TLR4 in diabetic mice improved the diabetes-induced ED through suppressing oxidative stress and increasing the cyclic guanosine monophosphate (cGMP) levels that enhances CC relaxation (Nunes et al. 2018). Hence, the TLR4, TLR9, and TLR1/TLR2 activation in the CC of the penis may cause ED in humans.

The expression of different TLRs varies with immune cell types (macrophages, dendritic cells or DCs, neutrophils, mast cells, B cells, and T cells) and non-immune cells, including epithelial cells. Neurons also express different TLRs as discussed elsewhere (Kumar 2018). The binding of the specific TLR ligand to its corresponding TLR may initiate two different signaling pathways: (1) Myeloid differentiation primary response protein MyD88 (MyD88)-dependent and (2) TIR domain-containing adaptor protein inducing IFN-β (TRIF)-dependent. The activation of these two signaling pathways may lead to the NF-κB activation-dependent pro-inflammatory molecules (cytokines (TNF-α, IL-1, IL-6, IL-8, etc.), chemokines (CXCL8 and CXCL10), reactive oxygen species or ROS generation, and antimicrobial peptides or AMPs) and interferon regulatory factor 3 (IRF3) or IRF7-dependent type 1 IFN generation (Fig. 1). The TLR signaling overactivation during systemic infections, including the current COVID-19 pandemic, may lead to the development of a cytokine storm and sepsis (Fig. 1). We are not describing here the TLR signaling pathway in detail as these have been described elsewhere (Kumar 2019b, 2020c, 2021). The overactivation of pro-inflammatory TLRs may prove harmful to the host in terms of inflammatory organ damage during acute infections and inflammation (Fig. 1).

The most current scenario is the cytokine storm generation in the patients with severe corona virus disease 2019 (COVID-19), who die eventually with respiratory failure due to acute lung injury (ALI) occurring due to the profound inflammatory damage as well with multiorgan failure (MOF) originating due to sepsis (Fig. 1). For example, both TLR2 and MyD88 expression are associated with the severity of the COVID-19 due to their role in the β-Coronavirus-induced inflammatory immune response (Zheng et al. 2021; Sariol and Perlman 2021). The pro-inflammatory action of TLR2 (induction of pro-inflammatory cytokine (TNF-α, IL-6, IL-1β, and IFN-γ) and chemokine generation) during severe COVID-19 takes place independent of the severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) (Zheng et al. 2021). This is because TLR2 recognizes the SARS-CoV-2 envelope protein as its ligand to initiate the pro-inflammatory gene transcription responsible for cytokine storm generation. As mentioned previously, TLR2 activation also initiates AIM2 inflammasome that further aggravates the inflammation through dampening the protective type 1 IFN-mediated antiviral immune response generated due to the

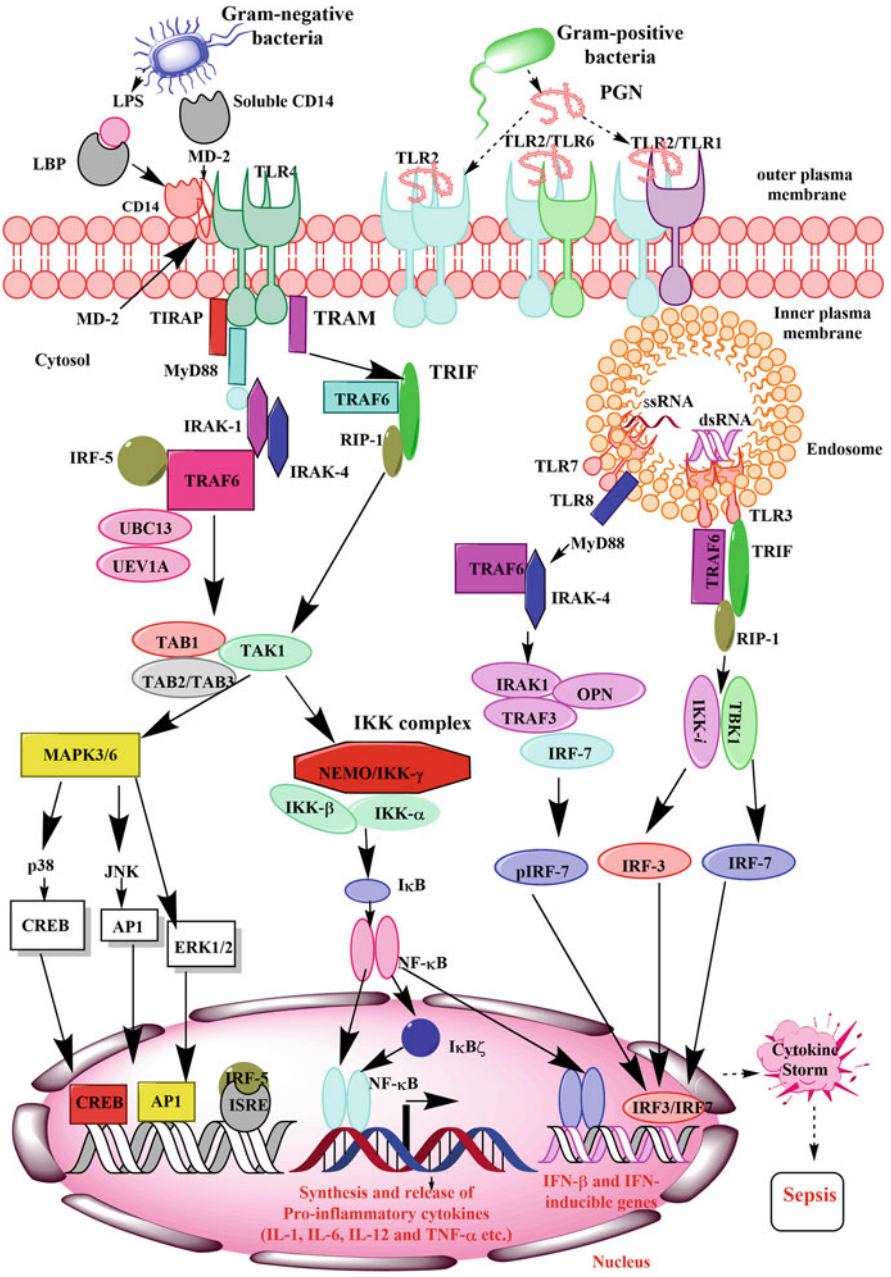

Fig. 1 Schematic representation of TLR (MyD88-dependent and MyD88-independent or TRIF-dependent) signaling pathways. The activation of different TLRs (shown in the figure) upon ligation to their corresponding ligands (PAMPs/MAMPs and DAMPs) initiates the cascades of events through activating the downstream signaling molecules to generate different pro-inflammatory molecules (cytokines, chemokines, IFNs, and ROS) required to remove the invading pathogen or DAMPs. However, their overactivation induces cytokine storm leading to the development of

activation of cGAS-STING signaling pathway. A recent study has indicated that AIM2 inflammasome activation in the blood monocytes of COVID-19 patients that proves TLR2 and AIM2 inflammasome activation in COVID-19 patients increases the disease severity through enhanced pro-inflammatory molecules generation and the suppression of type 1 IFN generation through cGAS-STING signaling pathway (Junqueira et al. 2021).

The severity of COVID-19 increases with the GSDMD levels, which form a GSDMD pore that decreases the cytosolic K^+ level that further inhibits cGAS-STING signaling pathway activation to synthesize type 1 IFNs (Junqueira et al. 2021). On the other hand, TLR7 plays a crucial role in the Middle-East respiratory syndrome-Coronavirus (MERS-CoV)-induced type 1 IFN production that protects host via clearing the infection (Channappanavar et al. 2019). Also, a previous study has shown the rapid production of type 1 IFNs by plasmacytoid dendritic cells (pDCs) through the activation of TLR7 against the highly cytopathic human SARS-CoV to control the potentially lethal CoV infections (Cervantes-Barragan et al. 2007). Hence, timing, intensity, and the type of TLR activation during microbial infection determine its protective and harmful action. TLR signaling is a very controlled process as its over activation may prove detrimental to the host due to the exaggerated inflammatory immune response that may also induce autoimmune diseases along with other inflammatory conditions, including neurodegeneration (Kumar 2019b; Farrugia and Baron 2017; Gao et al. 2017). To overcome this, the host has several endogenous negative regulators of TLR signaling to prevent their exaggerated activation and inflammatory damage. These different endogenous negative regulators of TLRs have discussed extensively somewhere else (Kumar 2020c). Hence, the host has endogenous negative regulators of TLRs to prevent their exaggerated activation to maintain the immune homeostasis.

2 Single Nucleotide Polymorphisms (SNPs) As Well As Primary Genetic Defects in TLR Signaling, Predisposition to Different Infectious and Inflammatory Diseases

SNPs are common in different human population for different genes. This phenomenon has also been reported for TLRs. We will not discuss this in detail as it is discussed at length elsewhere (Lin et al. 2012). The first case of TLR polymorphism was observed in a laboratory mouse strain C3H//HeJ as a consequence of its hyporesponsiveness to LPS in response to the substitution of proline amino acid (AA) with the histidine at AA 712 in the TLR4 (Poltorak et al. 1998; Hoshino et al. 1999). We have discussed different TLR polymorphisms associated with different infectious and inflammatory diseases somewhere else (Kumar 2018). Table 2

Fig. 1 (continued) sepsis that is also seen in patients with severe COVID-19 (current SARS-CoV-2 infection-based pandemic)

Table 2 TLR polymorphism in humans and their impact of infection, immunity, and inflammation

TLRs	TLR polymorphism (SNP and nucleic acid variant)	TLR polymorphism and disease association
TLR1	T1805G (I602S) R80T	602S SNP in TLR1 is protective against Leprosy caused by *Mycobacterium leprae* (Johnson et al. 2007) R80T is associated with increased risk of Aspergillosis (Kesh et al. 2005; Mezger et al. 2010)
	TLR1-7202G/A (rs5743551)	Increased susceptibility to sepsis and associated mortality (Wurfel et al. 2008; Thompson et al. 2014)
TLR2	T597C R753Q, SNP – 15607A/G of haplotype 2 of TLR2	T597C SNP protects against leprosy but increases susceptibility to tuberculosis (Bochud et al. 2008; Thuong et al. 2007) R753Q increases susceptibility to *S. aureus* infection (Moore et al. 2004), 15607A/G of haplotype 2 increases viral shedding and lesional rate in patients with genital herpes simplex virus (HSV) Type 2 infection (Bochud et al. 2007a)
TLR3	rs1879026 (G/T), rs13126816, rs3775291, L412F TLR3 polymorphism, 299,698 T/G, 293248A/A, 299698 T/T	rs1879026 (G/T) – increased prevalence of hepatitis B virus (HBV) infection (Al-Qahtani et al. 2012), rs13126816 and rs3775291 – protect from human herpes simplex virus type 2 (HSV-2) infections (Svensson et al. 2012), L412F TLR3 polymorphism – resistance against HIV infection/AIDS in the Italian population (Sironi et al. 2012), and same SNP in Japanese population protects against acute graft rejection (Citores et al. 2011), L412F increases severity of COVID-19 in male European patients of COVID-19 (Croci et al. 2021), 299,698T/G, 293248A/A, 299698T/T SNPs in the exon of TLR3 are associated with Stevens-Johnson syndrome (SJS), mucocutaneous disease in Japanese patients (Ueta et al. 2007), the TLR3 mutant (rs3775291) is associated with susceptibility, severe COVID-19 phenotype, and increased mortality due to the poor recognition of the SARS-CoV-2 dsRNA (Dhangadamajhi and Rout 2021)
TLR4	A896G or D299G C1196T or T399I are non-synonymous SNPs and are linkage disequilibrium (Misch and Hawn 2008)	D299G TLR4 polymorphism – increased Gram negative bacteremia and sepsis prevalence (Agnese et al. 2002) and is also associated with Mediterranean spotted fever due to *Rickettsia conorii* infection (Balistreri et al. 2005)

(continued)

Table 2 (continued)

TLRs	TLR polymorphism (SNP and nucleic acid variant)	TLR polymorphism and disease association
		T399I TLR4 polymorphism – hyporesponsive to LPS (Arbour et al. 2000) Co-segregation of both D299G and T399I TLR4 SNPs more severe hyporesponsiveness to LPS and higher incidence of sepsis and severe form of RSV infection (Tal et al. 2004; Awomoyi et al. 2007; Lorenz et al. 2002)
	rs4986790 (*TLR4*) polymorphism (AG and GG genotypes)	Less neutrophil extracellular traps (NETs) formation or NETosis in patients with community acquired pneumonia (Karnaushkina et al. 2021)
TLR5	C1174T (R392) (replacement of sequence encoding Arg392 with a stop codon) Present in 10% of European population (Netea et al. 2012; Wlasiuk et al. 2009)	More susceptible to develop *Legionella pneumophila* infections or Legionnaires' disease (Legionellosis) (Hawn et al. 2003) and recurrent cystitis (Hawn et al. 2009)
TLR6	A1401G (a TLR6 promoter SNP) C744T	A1401G – High risk of prostate cancer (Sun et al. 2006) TLR6 C744T – protects against asthma (Tantisira et al. 2004)
TLR7	Intron I, c.IT-120G	c.1-120G TLR7 allele – protects inflammation and fibrosis development in males with chronic HCV-infection (Schott et al. 2007)
TLR8	129G	Less inflammation in male patients of chronic HCV infection due to less IFN-α production (Wang et al. 2011)
TLR9	G-1174A and A1635G	People with these two SNPs in TLR9 exhibit rapid progressor phenotype for HIV-1 infection (Bochud et al. 2007b; Medvedev 2013)
TLR10	Two SNPs (rs11466617 and rs4129009)	Decreased risk of tuberculosis (TB) in Tibetan Population, but did not in Chinese Han Population (Wang et al. 2018b)
	Three SNPs in TLR10, I775L, I399L, and N241H	Reduced susceptibility to complicated skin and skin structure infections (cSSIs) in White East European people (Stappers et al. 2015)
	rs2101521, rs10004195, rs11725309 and rs6841698 in TLR10 gene	Chinese Han people are susceptible to pediatric idiopathic uveitis (PIU) (Lv et al. 2020)

indicates diseases linked to SNPs of different TLRs in humans. More recently, the polymorphism L412F in TLR3 in European (Italy) males has been shown to be associated with the increased severity of COVID-19 due to impaired autophagy, increased TNF-α production, and an increased frequency of autoimmune disorders as co-morbidity in males with specific class II HLA haplotypes prone to autoantigen presentation (Croci et al. 2021). Another TLR3 mutation with rs3775291 increases the susceptibility and severity of COVID-19 and also increases the mortality in COVID-19 patients due to the poor recognition of the SARS-CoV-2 dsRNA (Dhangadamajhi and Rout 2021). The loss of a function mutation in TLR7 (Ser301Pro and Ala1032Thr variants) in the White European patients with COVID-19 in Italy has increased the disease severity and mortality in young male patients (Fallerini et al. 2021). The details of TLR7 variants due to loss of function mutation are described further by Fallerini et al. (2021). Another recent study has shown the association of the TLR-4 (Asp299Gly and Thr399Ile) minor alleles 299Gly (G) and 399Ile (T) with the severity of the COVID-19 in the Egyptian patients due to the higher IL-6 production (Taha et al. 2021). Hence, polymorphism in different TLRs plays a significant role in the pathogenesis and severity of the COVID-19.

In addition to SNPs, patients with primary immunodeficiency diseases (PIDs), which affect TLRs signaling also become susceptible to different microbial infections (Maglione et al. 2015). For example, the defect in interleukin-1 receptor-associated kinase (IRAK)-4 and MyD88 increases the susceptibility to bacterial infections, whereas mutations in NF-κB essential modulator (NEMO) and other downstream mediators increase susceptibility to a broad range of infections caused by bacteria, viruses, and fungi (Maglione et al. 2015). Chronic granulomatous disease (CGD) and X-linked agammaglobulinemia (XLA) patients show a defective TLR2 and TLR4 signaling. Whereas, the CGD patients show increased TLR5 and TLR9 signaling and patients with XLA show an increased TLR4, 7, 8, and 9 signaling (Maglione et al. 2015). Also, the neutrophils of CGD patients show lower TLR5 and TLR9 expression due to the fact that these innate immune cells show a defective immune response against flagellated bacteria (TLR5 recognizes flagellin as a PAMP) (Hartl et al. 2008). The details of PIDs resulting due to the genetic defects of NF-κB and TLR (MyD88-dependent and independent) signaling have been described somewhere else (Maglione et al. 2015; Aluri et al. 2021). Even the dysregulation of TLR signaling pathways (due to genetic defects) through different TLRs has been associated with different myelodysplastic syndromes (cluster of hematopoietic stem cell disorders involving abnormal hematopoiesis with high risk of transformation to acute myeloid leukemia or AML) due to their regulatory role in hematopoietic stem and progenitor cell (HSPCs) functions (Paracatu and Schuettpelz 2020). Hence, the genetic defects in TLR signaling predispose the host to different infectious diseases, PIDs, and autoimmunity. For example, Y-chromosome-linked autoimmune accelerator (*Yaa*) mutation in mice doubles TLR7 expression and accelerates the incidence of autoimmune disease in mice and TLR9 deficiency also aggravates the autoimmune disease systemic lupus erythematosus or SLE, and cancers, including the myeloid leukemia (Marshak-Rothstein 2006; Zhang et al. 2021).

3 The Expanding World of TLRs in the Field of microRNA (miR) Biology

MicroRNAs (miRNAs or miRs) are non-coding small RNAs, which are involved in almost all known processes (apoptosis, cell-cycle, autophagy, tumorigenesis, and immune response) taking place in a living body through the regulation of gene expression (Bayraktar et al. 2019; Xu et al. 2012; Gozuacik et al. 2017). They serve as critical regulators of posttranscriptional processes through targeting the defective antisense complementary portions of coding and non-coding transcripts (Bayraktar et al. 2019). They are secreted by different cells, including cancerous and immune cells (macrophages, DCs, and T cells, etc.) through the release of extracellular vesicles (EVs), including exosomes, microvesicles (MVs), which are released to communicate with adjacent as well as distant cells to maintain homeostasis, including immune homeostasis and gut–brain–microbiota axis (Kumar et al. 2021; Tesovnik et al. 2020; Zhao et al. 2021). Some miRs can also bind to different TLRs and initiate pro- or anti-inflammatory immune responses (Tables 3 and 4) (Bayraktar et al. 2019). For example, recently different miRs derived from EVs in humans have been shown to bind and activate TLR7/TLR8 expressing blood immune cells in patients with type 1 diabetes mellitus (T1DM) (Tesovnik et al. 2020).

The miR-146a is involved in endotoxin or lipopolysaccharide (LPS) tolerance through targeting the TLR4 downstream signaling pathway mediated by MyD88, IRAK1, IRAK2, and TRAF6 (Nahid et al. 2011). Also, miR-21, miR-146, miR-155, and the let-7 family target TLRs or their signaling components crucial in TLR signaling pathways playing a significant role in the regulation of complex immune process involved in the maintenance of immune homeostasis under diverse conditions (infection, inflammation, cancer, and autoimmunity) (O'Neill et al. 2011; O'Connell et al. 2010). In addition to the regulation of TLR signaling-mediated immune response by miRs, the miRs (their expression and function) are also regulated by TLRs. For example, lack of TLR4 significantly alters the quantity and diversity of the miRs expressed in the cerebral cortex as compared to wild type (WT) mice (Ureña-Peralta et al. 2020). For example, the TLR4 KO mice have decreased levels of the miR200 family of miRs and the cluster of miR-99b/let-7e/miR-125a gets upregulated (Ureña-Peralta et al. 2020). Thus, TLR4 KO mice may exert a dysregulated inflammatory immune response during neuroinflammation due to the lack of TLR4 as well as the dysregulation associated miRs and other immunoregulatory molecules (NF-κB, MyD88, TLR7/8, and ion channels and MAPK signaling pathways) (Ureña-Peralta et al. 2020). Hence, the interaction between miRs and TLRs is a two-step process that depends on their types and physiological or pathophysiological status of the host. The details of this complex process are discussed in detail somewhere else (Bayraktar et al. 2019; O'Neill et al. 2011; Li and Shi 2013; Chen et al. 2013; Liang et al. 2019; Virtue et al. 2012). Tables 3 and 4 mention different miRs and TLRs interacting and affecting each other at different levels.

Table 3 TLRs and miRs regulating each other

TLRs	TLR Localization	miRs regulating/inhibiting TLRs at transcription or translation level	miRs as TLR ligand	TLRs regulating miRs	
TLR1	Plasma membrane	ND	ND		
TLR2	Plasma membrane	miR105 binds to TLR2 mRNA and decreases its translation to TLR2 protein. Hence, miR105 serves as anti-inflammatory miR.	ND	↑	miR155, miR146, miR147, miR9
TLR3	Endolysosome	miR155, miR21, miR223	ND	↑	miR155, miR146, miR147
TLR4	Plasma membrane	Let-7i and let-7e miRs bind to the TLR4 mRNA and inhibit its translation to TLR4 protein. Thus, let-7i and let-7e miRs are also an anti-inflammatory miR. miR146, miR223	ND	↑	miR155, miR146, miR132, miR21, miR223, miR147, miR9, miR27b
				↓	miR125b Let-7i, miR98
TLR5	Plasma membrane	ND	ND	↑	miR146
TLR6	Plasma membrane	ND	ND		
TLR7	Endolysosome	Let-7	miR29a, miR21, let-7b	↑	miR9
TLR8	Endolysosome	ND	miR21, miR29a	↑	miR9
TLR9	Endolysosome	ND	ND	↑	miR155, miR132

ND, Not detected/Not Known; ↑ Upregulated; ↓ Downregulated

Table 4 miRs inhibiting downstream signaling or adaptor molecules of TLR signaling pathways

miRs inhibiting TLR downstream signaling or adaptor molecules	
miRs	Adaptor or signaling molecule
miR155	MyD88, TAB2, IKKε
miR145	MAL
miR146a and miR146b	IRAK1 and TRAF6
miR10a	TAK1
miR223, miR15a, and miR16	IKKα
miR9	NF-κB1
miR199	IKKβ

4 Development and Implications of Different TLR-Based Therapeutics (Drugs and Vaccines)

The TLR4-based therapeutic called Eritoran or E5564 (a TLR4 antagonist, developed by the Japanese pharmaceutical company Eisai Co. Ltd.) was developed to target Gram-negative bacterial sepsis that worked well in animal models but failed in a 28-day mortality study in clinical trials of sepsis with effects comparable to those of placebo (Kumar and Sharma 2008; Opal et al. 2013). However, Eritoran has shown promising results in preventing acute lung injury (ALI) associated with severe influenza infection and secondary bacterial infection after primary influenza infection (Shirey et al. 2013; Shirey et al. 2019). The rate of development of secondary infection in severe COVID-19 is higher than influenza patients along with ALI induction that complicates the disease and increases the severity and associated mortality against influenza infection (Shafran et al. 2021; De Santis et al. 2021; Sopirala 2021). Based on these findings, Eisai is planning to use Eritoran in clinical studies of patients with severe COVID-19 alone or along with other therapeutics that will help to decrease ALI, incidence of secondary bacterial infections, and the worst outcome of COVID-19 (mortality). Other TLR4 antagonists have shown a protective action different viral infections causing ALI along with influenza one (Shirey et al. 2021).

Additionally, TLR agonists, including a TLR4 agonists called AS04 (a monophosphoryl lipid A (MPLA)-based molecule) have been used as an adjuvant in hepatitis B vaccine (FENDrix®), in human papillomavirus (HPV) vaccine called Cervarix® and different cancer (metastatic melanoma and non-small cell lung carcinoma) vaccines (Kumar 2018). Imiquimod (Aldara, 3M Health Care Limited), a TLR7/TLR8 agonist is used to treat warts caused by human papilloma virus (HPV), basal cell carcinoma, and actinic keratitis (Kumar 2018; Beutner et al. 1998; Schulze et al. 2005). The use of different TLR agonists and antagonists in different conditions as immunomodulator and immunotherapeutics, including vaccines at pre-clinical evaluation stage and clinical trials level has been discussed elsewhere (Kumar 2018; Farooq et al. 2021; Sartorius et al. 2021; Anwar et al. 2019). Additionally, exploration of the interaction between different miRs at different levels that have potential to antagonize or activate the TLR signaling has opened new avenues to regulate the TLR signaling through targeting specific miR associated specific TLR depending on the disease conditions. Also, the establishment of crucial role of different TLRs (TLR2, TLR4, TLR7, TLR8, and TLR9) in the pathogenesis and severity of the COVID-19 has indicated their importance in targeting the pandemic through drugs/molecules targeting specific TLR.

5 Conclusion

TLRs were first recognized in *D. melanogaster* (fruit fly) as genes controlling dorsoventral patterning of the body during embryonic development. However, later studies have shown their potential role in pathogen recognition in mice and humans.

To date 13 TLRs have been reported in humans, which recognize different PAMPs and DAMPs. This recognition induces pro-inflammatory signaling pathways crucial to generate inflammation and clearing the potential threat to the host. Thus, the journey of TLRs has started from the embryonic development in the fruit fly and reached to the regulation of immune response under diverse conditions, including infections, cancer, neurodegeneration, autoimmunity, and immunodeficiency. The advancement in TLR biology has established their role in neural plasticity and in the regulation of behaviors, including learning and memory, and anxiety, chronic persistent pain, addiction to drugs, obesity and metabolism, including cancer metabolism, reproduction, acute wounds, and wound healing (Chen and DiPietro 2017; Huebener and Schwabe 2013; Dasu and Rivkah Isseroff 2012; Crews et al. 2017; Peirs and Seal 2015; Lacagnina et al. 2018; Könner and Brüning 2011; Lauterbach et al. 2019; Lancaster et al. 2018; Huang et al. 2018; Kannaki et al. 2011; Okun et al. 2011; Lathia et al. 2008; Li and Hidalgo 2021). TLRs and miR also interact with each other at different stages and regulate their functions. Hence, miRs can be used to target different TLRs during different inflammatory diseases or other conditions. For example, miR146a-5p has been shown to alleviate chronic neuropathic pain associated with injury through inhibiting TLR signaling pathway (Interleukin 1 receptor-associated kinase 1 or IRAK1 and tumor necrosis factor receptor (TNFR)-associated factor 6 or TRAF6) (Wang et al. 2018a). On the other hand, the let-7e miR interacts with TLR7 and induces neurodegeneration, including Alzheimer's disease (AD) in humans (Lehmann et al. 2012). Hence, preventing this interaction may prove helpful to prevent the incidence of neurodegeneration in people with higher levels of let-7e miR in their brain or cerebrospinal fluid (CSF). The continuous research in the field of different areas of biology and medicine is further increasing the evidence for the role of TLRs in diverse conditions and their regulation, including ED. Hence, discovering different ways to target TLRs, including through miRs are opening many avenues use as therapeutic targets as well as vaccine candidates for both sterile and infection-induced inflammatory conditions. This indicates that almost 40 years following their discovery TLRs are sitting over the top as PRRs and controlling diverse biological processes along with immune regulation.

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Signal-Strength and History-Dependent Innate Immune Memory Dynamics in Health and Disease

Shuo Geng, Kisha Pradhan, and Liwu Li

Contents

Abstract

Innate immunity exhibits memory characteristics, reflected not only in selective recognition of external microbial or internal damage signals, but more importantly in history and signal-strength dependent reprogramming of innate leukocytes characterized by priming, tolerance, and exhaustion. Key innate immune cells such as monocytes and neutrophils can finely discern and attune to the duration and intensity of external signals through rewiring of internal signaling circuitries, giving rise to a vast array of discreet memory phenotypes critically relevant to managing tissue homeostasis as well as diverse repertoires of inflammatory conditions. This review will highlight recent advances in this rapidly expanding field of innate immune programming and memory, as well as its translational implication in the pathophysiology of selected inflammatory diseases.

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_485

Keywords

Exhaustion · Inflammatory diseases · Innate immunity · Innate memory · Memory dynamics · Priming · Signal strength · Tolerance

Abbreviations

ABCA1	ATP-binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ACE	Angiotensin-converting enzyme
APC	Antigen presenting cells
ApOE	Apolipoprotein E
ATG	Autophagy-related gene
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
CCL	C-C motif chemokine ligand
CCR	C-C Chemokine receptor
CLP	Cecal ligation and puncture
CMP	Common myeloid progenitor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptors
DAMP	Damage-associated molecular pattern
ERK1/2	Extracellular signal-regulated kinase 1/2
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte-monocyte progenitors
GPNMB	Glycoprotein-Nmb
GRK2	G protein-coupled receptor kinases
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cell
ICAM1	Intercellular adhesion molecule 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK-M	Interleukin-1R-associated-kinase- M
JMJD3	Jumonji domain containing 3
KDM6B	Lysine demethylase 6B
LAG-3	Lymphocyte-activating gene
Ldlr	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAL	MyD88-adapter-like
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage-colony-stimulating factor
MEP	Megakaryocyte-erythrocyte progenitor

MMP	Matrix metalloproteinases
MYD88	Myeloid differentiation factor 88
NET	Neutrophil extracellular trap
NOX2	NADPH oxidase 2
oxLDL	Oxidized low-density lipoprotein
PAMP	Pathogen associated molecular pattern
PD-1	Programmed cell death protein-1
PD-L1	Programmed death-ligand 1
PI3K/AKT	Phosphatidylinositol-3-kinase and protein kinase B
SFK	Src family kinases
SR-A	Scavenger receptor class A
SR-B1	Scavenger receptor class B type 1
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophages
TAN	Tumor-associated neutrophils
TCR	T cell receptor
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAM	Toll/IL-1R domain-containing adaptor-inducing IFN-β-related adaptor molecule
TRIF	Toll/IL-1R domain-containing adaptor-inducing IFN-β
VEGF	Vascular endothelial growth factor

1 Introduction

The establishment of "memory" is the cardinal and classical features of adaptive immunity and has served as the guiding principle of empirical vaccine generation for millennium. Adaptive immunity develops lasting memory responses toward highly specific antigens through somatic recombination-mediated generation of T cell receptors (TCR) and/or B cell receptors (BCR), followed by clonal expansion via interaction with selective antigen presenting cells. In contrast, innate immune cells can only respond to general molecular patterns associated with pathogens through innate receptors (Kawai and Akira 2007). Given limited repertoire of innate receptors, innate immune cells were not historically considered to be memory generating entities. However, emerging data from the last decade reveal fascinating, complex, and dynamic "memory"-like behaviors of innate immune cells that transcend beyond the classical adaptive immune memory phenotypes. The distinct features of innate memory are reflected in signal-strength and history-dependent behaviors such as priming, tolerance, and exhaustion (Li et al. 2020). The generation of innate memory may have profound consequence related to pathophysiology of both acute and chronic inflammatory diseases (Morris et al. 2014).

2 Mechanisms for the Generation of Innate Immune Memory

In the classical sense of immune memory, adaptive immune cells such as T cells and B cells gain the capability to uniquely recognize and memorize highly distinct antigens through somatic VDJ recombination. In sharp contrast, innate immune cells do not have the machinery for VDJ recombination and thus rely upon limited innately encoded receptors to recognize general molecular patterns (e.g., PAMPs – pathogen-associated molecular patterns; DAMPs – damage associated molecular patterns). Despite its limited specificity, innate immune cells can differentiate the signal strength and history of challenges, exhibiting "memory-like" behavior of priming, tolerance, and exhaustion (Geng et al. 2016; Yuan et al. 2016a; Xiong and Medvedev 2011; Foster et al. 2007; Lin et al. 2020). The establishment of such memory-like behavior is clearly distinct from the acquisition of adaptive memory and does not require genetic recombination. Instead, closely intertwined intracellular circuitries involving redox signaling, sub-cellular trafficking, metabolic and epigenetic processes are likely involved to establish transient memory states with limited stability and plasticity (Yuan et al. 2016b; Baker et al. 2014, 2015; Maitra et al. 2012; Chan et al. 2005; Netea et al. 2016) (Table 1).

A cardinal example of innate memory can be seen with monocyte/macrophage responses to rising dosages of bacterial endotoxin (Yuan et al. 2016b; Lu et al. 2015). While a prolonged challenge with higher dosages of lipopolysaccharide (LPS) can lead to reduced expression of pro-inflammatory cytokines, commonly known as endotoxin tolerance (Morris et al. 2014), prolonged stimulation with a subclinical super-low dose LPS can polarize monocyte/macrophage into a "primed" low-grade inflammatory state with sustained expression of inflammatory mediators (Yuan et al. 2016a, b). The mechanisms of endotoxin tolerance likely involve the activation and induction of molecular suppressors at multiple levels such as cytoplasmic signaling suppressors interleukin-1R-associated-kinase (IRAK)-M, and phosphatidylinositol-3-kinase and protein kinase B (PI3K/AKT) (Xiong and Medvedev 2011; Piao et al. 2009), as well as nuclear transcriptional suppressor RelB (Maitra et al. 2012; Chan et al. 2005). On the other hand, the generation of primed low-grade inflammatory monocyte/macrophage requires the clearance of suppressors such as IRAK-M and PI3K/AKT (Geng et al. 2016; Maitra et al. 2012). At the sub-cellular level, subclinical super-low dose LPS preferentially disrupts the homeostatic processes of autophagic flux as well as pexophagy, leading to the accumulation of reactive oxygen species involved in the establishment of low-grade inflammation (Yuan et al. 2016a; Geng et al. 2019). Innate leukocytes

Table 1 Key features of innate and adaptive immune memories

	Innate immune memory	Adaptive immune memory
Generation mechanism	Competitive signaling circuitries	Genetic recombination
Propagation mechanism	Intercellular communications	Clonal expansion
Duration	Relatively short-lived	Long-lasting
Stability	Prone to adaptation	Stable

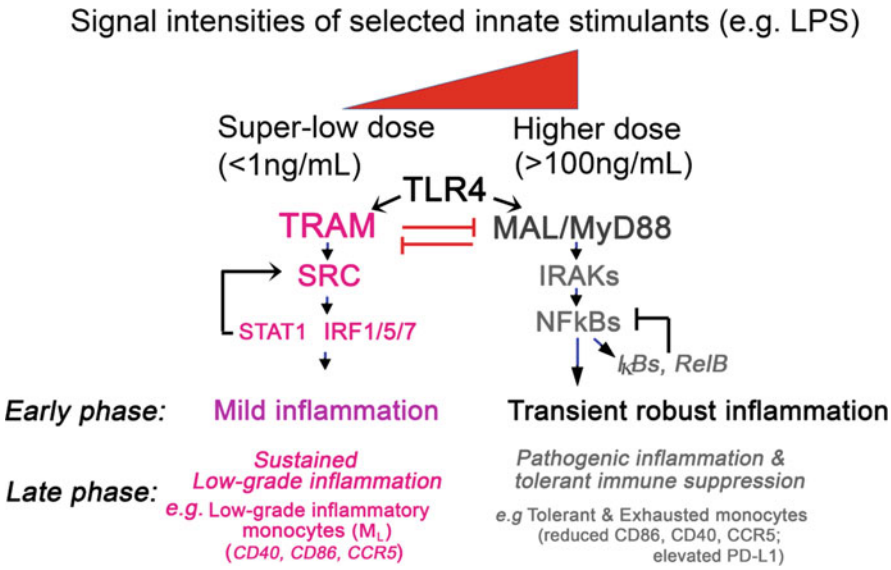

Fig. 1 Illustration of innate memory dynamics based on signal strength and duration. Innate immune leukocytes such as monocytes and macrophages can finely sense the strength and duration of external danger signals (e.g., lipopolysaccharide, LPS) and undergo distinct adaptations to generate dynamic memory states. In the case of LPS, a prolonged challenge with subclinical super-low dose LPS (<1 ng/mL) will induce a sustained low-grade inflammatory states due to the positive-feedback signals involving mutually activating TRAM adaptor, SRC kinases, IRF1/5/7, and STAT1. In contrast, while higher dose LPS acutely induces a transient and robust inflammatory response through the activation of NFkB, prolonged stimulation with higher LPS signals will trigger the expression of inhibitory kBs (IkBs) and RelB, leading to a tolerant state with reduced expression of inflammatory mediators such as CD86, CD40, and CCR5. Tolerant leukocytes still maintain a skewed expression of profile of selected immune suppression genes such as PD-L1, and eventually adopt an exhausted state characterized by pathogenic inflammation and immune suppression

may sense the signal strength and duration of LPS via distinct usage and assembly of intra-cellular adaptor molecules such as myeloid differentiating factor 88 (MyD88) and TRIF-related adaptor molecule (TRAM), with TRAM preferentially directing the cellular response to sustained stimulation of super-low dose LPS (Yuan et al. 2016b; Rahtes and Li 2020). On the other hand, MyD88 is preferentially involved in response to higher dose LPS during both the acute response phase and the compensatory phase of tolerance (Cheng et al. 2015; Laird et al. 2009). The intra-cellular processes responsible for priming and tolerance may likely compete with each other forming multi-tiered competitive circuitries, assisting the decision-making processes of innate leukocytes in adopting dynamic activation behaviors (Morris et al. 2014; Fu et al. 2012; Kadelka et al. 2019) (Fig. 1). The generation of mutually competitive circuitries is also a fundamental principle for the clear differentiation and activation of other immune cells such as T helper cells (Hong et al. 2011, 2012).

Sustained challenges with higher dose endotoxin lead to not only endotoxin tolerance, but also an exhausted state characterized by pathogenic inflammation and immunosuppression often seen during the progression of sepsis (Efron et al. 2018; Horiguchi et al. 2018). "Endotoxin tolerant" cells are not inert and can still robustly respond to endotoxin stimulation, with a significantly altered landscape of gene expression potentially contributing to pathogenic inflammation and immune exhaustion (Foster et al. 2007; Lu et al. 2015). For example, monocyte/macrophage with prolonged LPS stimulation exhibits robust induction of iNOS and PD-L1 (Foster et al. 2007; Lu et al. 2015). Persistent iNOS expression may contribute to pathogenic inflammation, and PD-L1 is a major contributor mediating immune suppression. Recently, we demonstrate that endotoxin exhaustion is not limited to monocyte/macrophage and can also be seen in neutrophils with prolonged challenge of higher dose LPS (Lin et al. 2020). Exhausted neutrophils with prolonged LPS treatment manifest enhanced expression of pathogenic inflammatory mediators such as LTB4 and ICAM1, contributing to altered migratory and swarming behaviors reminiscing septic neutrophils (Lin et al. 2020). Exhausted neutrophils similarly express elevated PD-L1, potentially contributing to immune suppression (Lin et al. 2020).

3 Innate Immune Memory During the Pathogenesis of Acute and Chronic Diseases

3.1 Low-Grade Inflammatory Memory Monocyte in Atherosclerosis

Atherosclerosis and related cardiovascular complications are among the leading causes of morbidity and mortality in the world (Libby et al. 2019). Previously considered as a lipid storage disease, atherosclerosis is nowadays well recognized as a chronic low-grade inflammatory disease that occurs within the arterial wall (Back et al. 2019). The programming of low-grade inflammatory monocytes is crucially involved in the pathogenesis of atherosclerosis. Non-resolving low-grade inflammatory monocytes and monocyte-derived macrophages are the key mediators for the formation and progression of atherosclerotic plaques (Jongstra-Bilen et al. 2006; Libby and Hansson 2015). Monocytes can be primed by risk factors present in the circulation and in the vessel wall, such as pathogen-associated molecular patterns, oxidized lipoproteins, shear stress, and oxidative stress. Excessive inflammatory signals tend to trigger compensatory anti-inflammatory tolerance and therefore the expression of pro-inflammatory mediators in monocytes is transient and subsequently suppressed due to the induction of homeostatic negative regulators (Nathan and Ding 2010; Biswas and Lopez-Collazo 2009; Adib-Conquy and Cavaillon 2009). In contrast, under non-resolving low-grade inflammatory conditions, monocytes may fail to develop tolerance and are programmed into a sustained inflammatory state that favors the development of atherosclerosis (Baker et al. 2014; Maitra et al. 2012; Deng et al. 2013).

LPS, also known as endotoxin, is the major stimulant to prime monocytes, which are the primary immune cells responding to LPS given their relatively high expression of TLR4. Trace amount of gut microbiota-derived LPS may leak into circulation via increased gut permeability, leading to subclinical endotoxemia (Frazier et al. 2011; Lassenius et al. 2011). According to epidemiological studies endotoxemia levels as low as 50 pg/mL may serve as a strong risk factor for the development of atherosclerosis (Stoll et al. 2004). Indeed, atherosclerosis patients have low but significantly elevated serum LPS level as compared with healthy individuals (79.0 ± 10.7 vs. 43.5 ± 11.9 pg/mL, $p < 0.001$). This concentration of LPS is sufficient to up-regulate Nox2 expression and elevate oxidative stress in human monocytes (Carnevale et al. 2018). In the murine model of atherosclerosis, $ApoE^{-/-}$ mice fed with high-fat diet exhibit significantly higher level of serum LPS as compared to the counterparts fed with regular diet. Oral administration of *Akkermansia muciniphila* decreases the circulating LPS level, alleviates atherosclerosis progression, as well as reduces monocyte/macrophage accumulation in the plaques (Li et al. 2016). These findings indicate that low-grade inflammatory monocytes primed by low-dose LPS are critically involved in the pathogenesis of atherosclerosis.

Chronic injection of subclinical dose LPS to high-fat diet-fed $ApoE^{-/-}$ mice (a murine model of atherosclerosis) significantly exacerbates the pathogenesis of atherosclerosis accompanied by higher levels of circulating $Ly6C^{Positive}$ low-grade inflammatory monocytes as well as increased number of macrophages within the plaque areas. The surface level of inflammatory chemotaxis receptor CCR5 is significantly elevated while the surface expression of SR-B1, a modulator for anti-inflammation and lipid metabolism, is reduced on circulating monocytes from the high-fat diet-fed $ApoE^{-/-}$ mice conditioned with super-low dose LPS. The monocytes that are primed with subclinical dose LPS for a long-term exhibit similar phenotype, as characterized by enhanced levels of CCR5 and reduced levels of SR-B1. Adoptive transfer of these LPS primed monocytes to high-fat diet-fed $ApoE^{-/-}$ mice results in significant elevation of plaque size and lipid deposition, suggesting that these low-grade inflammatory monocytes programmed by subclinical dose LPS can directly contribute to atherosclerosis progression. Mechanistically, super-low dose LPS treatment induces increased level of miR-24, which mediates the suppression of SR-B1, and reduction of IRAK-M, which is a critical negative-feedback regulator. IRAK-M deficiency in turn leads to elevated miR-24 levels, forming a positive-feedback loop sustaining the low-grade inflammatory state conducive to atherosclerosis (Geng et al. 2016). There are two competitive pathways transducing signals following LPS stimulation, namely the MyD88-dependent pathway and the MyD88-independent pathway mediated by TRIF and TRAM (Palsson-McDermott and O'Neill 2004). Intriguingly, the low-grade inflammatory monocyte primed by super-low dose LPS is dependent upon TRAM/TRIF but not MyD88 (Yuan et al. 2016b). By employing a bone marrow transplantation strategy, Lundberg et al. have shown that hematopoietic deficiency of TRAM and TRIF but not MyD88 adaptor-like (MAL) significantly reduces atherosclerosis in $Ldlr^{-/-}$ mice (another murine model of atherosclerosis). TRAM deficiency also leads to

down-regulated level of pro-inflammatory mediators, such as TNF-α, IL-6, IL-12, CCL2, CCL5, and CXCL10, in the aorta of atherosclerotic mice (Lundberg et al. 2013). These data suggest that the priming of low-grade inflammatory monocytes by subclinical dose LPS during atherosclerosis is mainly mediated by TRAM, and targeting TRAM may promote effective generation of resolving monocytes for the prevention and treatment of atherosclerosis.

In addition to low-dose LPS, low concentrations of oxidized low-density lipoprotein (oxLDL) can also induce epigenetic reprogramming of monocytes into a pro-inflammatory state. Primary human monocytes trained with low doses of oxLDL (below 10 μg/mL) for 24 h exhibit an enhanced response to secondary stimulation 6 days later by expressing a series of pro-inflammatory mediators, including IL-6, TNFα, IL-8, MCP-1, MMP-2, and MMP-9. These trained monocytes have enhanced capacity to generate foam cells, elevated expressions of scavenger receptors (CD36 and SR-A), and reduced expression of cholesterol efflux transporters (ABCA1 and ABCG1). Therefore, these pro-inflammatory monocytes may contribute to the pathogenesis of atherosclerosis. The oxLDL-induced long-lasting proatherogenic profile can be significantly attenuated if the monocytes are pre-treated with histone methyltransferase inhibitor, suggesting that epigenetic histone modification is crucial for this innate immune memory of monocytes (Bekkering et al. 2014). It has been found that oxLDL treatment can cooperatively boost the activation of macrophages induced by low-dose LPS. Costimulation with oxLDL and low-dose LPS significantly up-regulates the genes transcribed by promoters containing an AP-1 binding site as well as induces the activation of ERK1/2. The combined effects of subclinical endotoxemia and oxLDL result in the establishment of pro-inflammatory state of macrophages and production of a series of inflammatory cytokines within atherosclerotic lesions (Wiesner et al. 2010).

3.2 Exhausted Memory Innate Leukocytes During the Pathogenesis of Sepsis

Sepsis is a systemic inflammatory response to severe infection and injury leading to multi-organ failure and remains one of the primary causes of death in hospitalized patients (Rhee et al. 2019; Perner et al. 2016). In 2017, global incidence of sepsis was around 48.9 million cases and sepsis-related deaths were estimated at 11.0 million cases (Rudd et al. 2020). The new coronavirus (SARS-CoV-2) in the ongoing outbreak and its associated disease COVID-19 pose tremendous threats to public health and drastically affect worldwide economies and societies (Kumar 2021a, b). Particularly, sepsis is the leading cause of death by COVID-19, which has been observed in nearly all deceased patients in numerous cohorts (Lopez-Collazo et al. 2020; Kumar 2020). The immune response of sepsis patients consists of a hyperinflammatory phase featured by "cytokine storm" and an immunosuppressive phase exemplified by immune cell exhaustion and dysfunction (Hotchkiss et al. 2016). Many clinical trials have been conducted to attenuate the hyperinflammatory effects by using anti-cytokine or anti-inflammatory agents, such as anti-IL-1β,

anti-TNF-α, anti-LPS, and TLR inhibitors. Unfortunately, none of these approaches produces robust curative outcomes, and in some cases, the survival rate was even reduced (Brady et al. 2020; Abraham et al. 1997; Opal et al. 2013). A hallmark of sepsis is diminished clearance of primary pathogens and increased risk of secondary infection due to pathogenic inflammation and immune suppression (Efron et al. 2018). Over 70% of deaths occur after the first 3 days of sepsis, many of which occur weeks after sepsis onset (Otto et al. 2011). Thus, immunosuppression caused by leukocyte exhaustion has been increasingly recognized as a major factor for sepsis-induced mortality. A recent single cell study revealed that moribund COVID patients tend to have higher numbers of exhausted classical monocytes (Schulte-Schrepping et al. 2020).

T cell exhaustion driven by persistent exposure to infections during sepsis has been well documented in the literature. The exhausted T cells are defined by a progressive loss of T cell effector function, a state of vigilant transcription distinct from functional effector or memory T cells. A typical alteration of exhausted T cells is the overexpression of a series of inhibitory molecules, such as PD-1, CTLA-4, LAG-3, and TIM-3 (Wherry 2011). PD-1 is a critical negative regulator involved in suppressing lymphocyte responses. PD-1/PD-L1 pathway plays an important role in the initiation and promotion of immunosuppression (Liu and Li 2017). Multiple studies using mouse model of cecal ligation and puncture (CLP) have unveiled elevated PD-1 expression on splenic $CD4^+$ and $CD8^+$ T cells. There is a continuously increased PD-1 expression on $CD4^+$ and $CD8^+$ T cells with the progression of sepsis, associated with a drastic reduction of total T cell population. Similarly, sepsis patients also have significantly increased PD-1 expression on T cells in the peripheral blood, spleen as well as injured organs (Patil et al. 2017). These exhausted T cells from sepsis patients fail to efficiently produce inflammatory cytokines and their secretory profiles are potently compromised (O'Sullivan et al. 1995; Wick et al. 2000; Heidecke et al. 1999). The ploy-functionality of $CD8^+$ cells is also significantly impaired in severe sepsis patients, and PD1 expression is inversely correlated with the number of poly-functional $CD8^+$ T cells (Choi et al. 2017). PD-1 is considered as one of the most promising targets for immunomodulatory therapy to resume T cell function. However, anti-PD-1 treatment alone does not yield expected outcomes because multiple negative costimulatory molecules are expressed on the surface of exhausted T cells. For example, a recent study demonstrates that T cells co-expressing LAG3 and PD-1 are more significantly exhausted as compared to LAG3 or PD-1 single positive T cells in patients with acute sepsis. Furthermore, the frequency of co-expressing T cells is positively associated with the mortality and the length of hospital stay (Niu et al. 2019). Thus, therapies targeting these suppressor molecules may maximize the recovery of T cells.

Correspondingly, monocytes in sepsis patients tend to express higher levels of immune receptors including CD63, CD163, CD206, TLR2, and TLR4, presumably rending them with elevated responses to infections (Armstrong et al. 2004; Hirsh et al. 2001). However, studies reveal that monocytes from sepsis patients are less responsive than those from healthy individuals. They cannot efficiently produce TNF-α, IL-1β, and IL-8 when challenged with LPS ex vivo, and the reduced TNF-α

production by monocytes is employed as an index to evaluate the immune suppression of patients with sepsis (Ryan et al. 2017). The diminished capacity to produce pro-inflammatory cytokines may be due to the elevated expression of IRAK-M, an inhibitory Toll receptor signaling molecule, in the monocytes from sepsis patients. The patients with higher IRAK-M levels on admission have a higher mortality rate (Wiersinga et al. 2009). Monocytes are specialized antigen presenting cells (APCs) that present surface MHC molecule-bound antigens to activate T cells. The exhaustion of these APCs potentially facilitates the immunosuppression during sepsis. Sepsis induces altered monocyte–T cell interactions because of reduced expressions of co-stimulatory molecules on monocytes. Indeed, monocytes in sepsis patients are found to express much lower levels of CD40, CD80, and CD86 (Sugimoto et al. 2003; Sinistro et al. 2008; Lissauer et al. 2009). On the contrary, PD-L1 surface expression is up-regulated on monocytes from septic mice models as well as sepsis patients, correlated with T cell exhaustion and immunosuppression via PD-1/PD-L1 signaling pathway (Patil et al. 2017). Monocyte PD-L1 expression can be used as an independent predictor of 28-day mortality in patients with septic shock (Shao et al. 2016).

Neutrophils are the most abundant leukocytes in the circulation and play a crucial role in sepsis as the first line of defense in protecting the body from microbial invasion. The interaction between neutrophils and other immune cells is necessary for the resolution of excessive inflammation as well as effective host defense (Serhan and Savill 2005). Exhausted neutrophils with aberrant immune responses to infection have been observed in septic animal models and patients. Excessive bacterial products and pro-inflammation cytokines in sepsis induce the loss of CD62L expression but elevated integrin expression (e.g., CD11b) on the surface of neutrophils (Rosenbloom et al. 1999; Kovach and Standiford 2012). In addition, CXCR2 expression is reduced in the neutrophils of mice and patients with severe sepsis. Prolonged neutrophil stimulation results in up-regulation of iNOS and GRK2, which further promotes CXCR2 internalization (Paula-Neto et al. 2011). Therefore, neutrophils exhibit reduced rolling and migratory capacity and fail to be recruited to the primary infection foci. Instead, these exhausted neutrophils tend to form pathogenic aggregations in the vital organs, which can be mimicked through in vitro examination (Lin et al. 2020). The antimicrobial activities of neutrophils are also compromised during sepsis. Excessive bacterial load activates complement system, and high levels of C5a suppress the phagocytic function and ROS production of neutrophils. Various studies with septic mouse models and sepsis patients have revealed impaired phagocytosis, oxidant production as well as oxidative burst capacity of septic neutrophils (Shen et al. 2017; Bhan et al. 2016). Furthermore, neutrophils from septic mice and patients can induce immunosuppression and T cell apoptosis in a cell-contact dependent manner via the surface expression of PD-L1. Thus, the PD-L1 level on neutrophils, which is positively associated with sepsis severity, may serve as a biomarker for the prognosis of septic patients (Wang et al. 2015). Despite its clinical significance, the mechanisms of neutrophil exhaustion during sepsis are still poorly understood. We have recently reported that neutrophils treated with LPS in vitro for a prolonged period develop a phenotype of elevated

ICAM1, CD11b, and PD-L1 expression as well as enhanced swarming and aggregation, which resembles the exhausted neutrophil phenotypes seen in sepsis. Importantly, the exhaustive profiles are significantly alleviated in TRAM deficient neutrophils after prolonged LPS challenge as compared with wild-type neutrophils. TRAM mediated neutrophil exhaustion may be dependent upon Src family kinases (SFK) and STAT1 activation, since SFK inhibitor can effectively block neutrophil exhaustion caused by prolonged LPS treatment. Furthermore, TRAM deficiency is protective against the development of severe systemic inflammation and multi-organ damage in mice (Lin et al. 2020). These data unveil a critical function of TRAM in promoting neutrophil exhaustion.

3.3 Innate Immune Memory During the Pathogenesis of Cancer

The phenotype and functionalities of myeloid cells (e.g., monocytes, macrophages, and neutrophils) are substantially changed by tumor-induced systemic environment and microenvironment, so that these cells usually acquire pro-tumor functions to promote cancer progression. On the other hand, the tumor-associated innate immune cells may provide ideal targets for fighting against cancer.

The number of circulating monocytes significantly increases in both humans and mice bearing tumors. Among several cancer types, patients with high blood monocyte counts have a poorer disease prognosis, and the ratio of lymphocytes to monocytes has become a prognostic factor for lung cancer, colorectal cancer, and ovarian cancer (Kiss et al. 2020; Olingy et al. 2019). The increased monocyte levels may be caused by two reasons: enhanced migration from bone marrow to circulation, and increased myelopoiesis. Patients with pancreatic cancer exhibit elevated circulating monocyte levels associated with decreased monocyte abundance in the bone marrow. CCL2, a critical chemokine for monocyte recruitment, is commonly present at higher levels in serum of both mice and humans with cancer, facilitating the egress of monocytes from the bone marrow (Kishimoto et al. 2019). Cancer is usually accompanied by elevated serum levels of cytokines that are involved in the myeloid cell differentiation and survival, such as macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage-colony stimulating factor (GM-CSF) (Scholl et al. 1996; Ribechini et al. 2017; Katsumata et al. 1996). Excessive production of these cytokines and tumor-associated low-grade inflammation promote reprogramming of myelopoiesis. As a result, hematopoietic stem cell (HSC), common myeloid progenitor (CMP), and granulocyte-monocyte progenitor (GMP) populations are expanded, while common lymphoid progenitor (CLP) and megakaryocyte-erythrocyte progenitor (MEP) are not significantly altered. For example, increased frequency of HSC and GMP populations is observed in peripheral blood of patients with various types of solid tumors, indicating that tumor-associated environment favors myeloid hematopoiesis and expansion of circulating monocytes (Wu et al. 2014; Manz and Boettcher 2014; Casbon et al. 2015; Strauss et al. 2020). In addition to increased numbers, the phenotype of monocytes is also profoundly influenced by tumors. One of the

well-documented features of cancer-educated monocytes is the acquisition of immunosuppressive properties. The monocytes from healthy individuals express high level of HLA-DR (the protein of MHC II) on the surface, while HLA-DR level is significantly down-regulated in the monocytes of cancer patients (Luczynski et al. 2004; Ugurel et al. 2004). The high level of CD14 HLA-DR^{low} monocytes is correlated with the lower levels of tumor-specific T-cells in the circulation of cancer patients. The patients with lower levels of CD14^{+} HLA-DR^{low} monocytes are more responsive to immune checkpoint blockade therapy (Weide et al. 2014; Weber et al. 2016). The surface expression of CD86, a co-stimulatory molecule for T cell activation, is also reduced on cancer-educated monocytes, inhibiting T cell function (Luczynski et al. 2004; Ugurel et al. 2004). Furthermore, monocytes in cancer patients have enhanced expression and activity of arginase-1, which limits the availability of L-arginine to T cells (Trovato et al. 2019; Hoechst et al. 2008). Up-regulation of PD-L1 and GPNMB may also contribute to the immunosuppressive activity of monocytes in cancer (Kobayashi et al. 2019). Intriguingly, monocytes from cancer patients exhibited increased level of phosphorylated STAT3, and STAT3 is potentially activated in the healthy monocytes after co-culture with cancer cells. Inhibition of STAT3 attenuates the immunosuppressive activity of cancer-educated monocytes (Trovato et al. 2019; Poschke et al. 2010). Therefore, STAT3 may be a crucial transcription factor for the reprogramming of monocytes by tumor-specific environment.

Based on the pro-tumor characteristics of monocytes, a series of regimens have been developed to reverse monocyte reprogramming. Specific antibody against CCR2 or small molecules that block CCR2 signaling remarkably restrain the growth and metastasis of tumor cells in mouse models of lung, breast, prostate, pancreatic, and liver cancers (Olingy et al. 2019). A recent clinical study has revealed that oral administration of PF-04136309, a small molecule CCR2 antagonist, effectively reduces circulating monocytes and tumor-associated macrophages (TAMs) in pancreatic cancer (Nywening et al. 2016). Angiotensin II serves as a key regulator of cancer-induced myelopoiesis, and angiotensin-converting enzyme (ACE) inhibitors may suppress the excessive generation of pro-tumor monocytes. Enalapril, an ACE inhibitor, has been shown to reduce monopoiesis and TAMs as well as prolong the survival of mice with lung tumors (Cortez-Retamozo et al. 2013). Neutralization of tumor-derived GM-CSF reduces the emergence of CD11b^{+} Gr-1^{+} myeloid cells, leading to elevated anti-tumor activity of T cells and restrained tumor growth (Bayne et al. 2012). Arginase inhibition may also be an alternative approach to mitigate monocyte-mediated arginine depletion and consequent immunosuppression. Accordingly, a small molecule arginase inhibitor has been developed, which can increase plasma and tumor arginine levels, enhance anti-tumor T cell responses, prime immunity toward a pro-inflammatory state, and reduce tumor growth in mouse cancer models (Steggerda et al. 2017). Application of STAT3 inhibitors has also yielded promising anti-tumor outcomes by boosting anti-tumor immunity in mice. TAM receptor tyrosine kinases promote STAT3 phosphorylation, and administration of UNC4241, an inhibitor against TAM receptor tyrosine kinases, significantly

alleviates the immunosuppressive activity of monocytes in a mouse model of melanoma (Holtzhausen et al. 2019).

Neutrophil is one of the major components of tumor-infiltrating innate immune cells. Cancer patients with poor prognosis often have an expanded pool of tumor-associated neutrophils (TANs), which exhibit complex and contradictory functions, promoting or limiting tumor growth (Powell and Huttenlocher 2016). The pro-tumor neutrophils produce high levels of VEGF, MMP9, and HGF, which facilitate tumor angiogenesis. MMP9 production and NET formation from neutrophils may provide ideal environment and niche for tumor cell intravasation and metastasis. Similar as cancer-educated monocytes, some TANs also possess immunosuppressive properties and suppress T cell proliferation via deprivation of L-arginine (Granot 2019). Compared with circulating and splenic neutrophils, TANs secrete higher amount of CCL7 (a Tregs chemoattractant) that promotes the recruitment of Tregs to tumor, thereby forming an immunosuppressive microenvironment (Fridlender and Albelda 2012). On the other hand, some neutrophils also exert anti-tumor activities by secreting cytotoxic mediators (ROS) to induce tumor cell apoptosis (Granot 2019). Intriguingly, accumulating data have suggested that the interaction between neutrophils and T cells is indispensable for the proper anti-tumor response of adaptive immunity (Eruslanov et al. 2014; Stoppacciaro et al. 1993). A subset of TANs has been found to exhibit both neutrophil and APC characteristics in the patients with lung cancer, and these cells can boost anti-tumor T cell responses (Singhal et al. 2016). The molecular mechanisms underlying the differential function of neutrophils are not well understood. A recent study has indicated that Tollip, an innate immunity signaling adaptor molecule, may contribute to the neutrophil reprogramming in a mouse model of colorectal cancer. Tollip-deficient neutrophils have STAT5-dependent elevation of CD80 and reduction of PD-L1 as compared to wild-type counterparts. Therefore, Tollip-deficient neutrophils may potently activate T cells to exert anti-tumor activity. Adoptive transfer of Tollip-deficient neutrophils but not Tollip-deficient monocytes promotes tumor immune surveillance and reduces colorectal cancer burden in vivo. Thus, Tollip may serve as a target to modulate the decision-making process of neutrophils for future cancer therapy (Zhang et al. 2019).

In recent years, the concept of trained immunity has drawn increasing attention due to its potential for the treatment of diseases such as cancer. Trained immunity represents an epigenetic and metabolic reprogramming process of innate immune cells to acquire long-term function and memory property (Netea et al. 2017). Instillation of Bacillus Calmette-Guérin (BCG) can prime monocytes from the patients with bladder cancer into a pro-inflammatory and anti-cancer profile, mediated by the autophagy genes ATG2B and ATG5 (Buffen et al. 2014). The patients who exhibit low responsiveness to trained immunity have a higher chance of recurrence and tumor progression (Netea et al. 2016). In LPS primed macrophages, 70% of the inducible genes are the targets of Jumonji domain containing 3 (JMJD3), also known as lysine demethylase 6B (KDM6B) (De Santa et al. 2009). KDM6B can stabilize tumor suppressor gene p53 (Ene et al. 2012), and higher KDM6B expression is a prognostic indicator for better survival in neuroblastoma patients (Yang

et al. 2019). Thus, LPS-trained macrophages via KDM6B may have clinical potential for cancer therapy. Fungal-derived polysaccharide β-glucans have been used to treat various cancers for a long time, and they are also potential inducers to promote trained immunity. A series of studies have revealed that β-glucan treatment leads to marked phenotypical and functional alterations of monocytes/macrophages, such as elevated cytokine production, enhanced phagocytic capacity, and increased ROS generation (Netea et al. 2017; Lerias et al. 2019). A recent study has shown that training with β-glucan leads to a transcriptomic and epigenetic rewiring of granulopoiesis in mice with melanoma, which subsequently induces the anti-tumor phenotype of TANs. Importantly, the mice receiving a single injection of β-glucan still exhibit a significant inhibition of tumor growth after 28 days, indicating long-term anti-tumor effects of neutrophil-mediated trained Immunity (Kalafati et al. 2020).

4 Concluding Remarks

Despite these exciting advances, the field of innate immune memory has only been revealed as the very tip of an iceberg closely intertwined with almost all aspects of human pathophysiology. Innate leukocyte may adopt highly diverse disease and context-dependent memory states, requiring single cell approaches to further clarify their unique contribution to distinct pathogenesis of inflammatory diseases. Memory leukocytes may further propagate their phenotypes to neighboring cells, establishing unique memory niche within local environments (Ballesteros et al. 2020). Future efforts are needed to finely map the establishment as well as propagation of innate memory through genetic and chemical approaches in tracking the ontogeny and propagation of memory innate leukocytes, in response to varying degrees of signal strengths and intensities within complex host immune environments. Harnessing the potential of reprograming innate memory would hold enormous promise in the treatment of both acute and chronic human diseases.

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Innate Neutrophil Memory Dynamics in Disease Pathogenesis

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Contents

Abstract

Neutrophils, the most abundant leukocytes in circulation and the first responders to infection and inflammation, closely modulate both acute and chronic inflammatory processes. Resting neutrophils constantly patrol vasculature and migrate to tissues when challenges occur. When infection and/or inflammation recede, tissue neutrophils will be subsequently cleaned up by macrophages which collectively contribute to the resolution of inflammation. While most studies focus on the anti-microbial function of neutrophils including phagocytosis, degranulation,

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_538

and neutrophil extracellular traps (NETs) formation, recent research highlighted additional contributions of neutrophils beyond simply controlling infectious agents. Neutrophils with resolving characteristics may alter the activities of neighboring cells and facilitate inflammation resolution, modulate long-term macrophage and adaptive immune responses, therefore having important impacts on host pathophysiology. The focus of this chapter is to provide an updated assessment of recent progress in the emerging field of neutrophil programming and memory in the context of both acute and chronic diseases.

Keywords

Chronic diseases · Dynamics · Neutrophil memory

Abbreviations

AASV	Anti-neutrophil cytoplasmic antibody-associated systemic vasculitis
AAU	Acute anterior uveitis
ADCC	Antibody-dependent cell-mediated cytotoxicity
AMPK	AMP-activated protein kinase
ANCAs	Anti-neutrophil cytoplasmic antibodies
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
CLP	Cecal ligation and puncture
COVID-19	Coronavirus Disease 2019
DAMP	Damage-associated molecular patterns
ECM	Extracellular matrix
EIU	Endotoxin-induced uveitis
ERK1/2	Extracellular signal-regulated protein kinase 1/2
FcγRII	Fc gamma receptor II
fMLP	N-Formylmethionyl-leucyl-phenylalanine
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HNSCC	Head and neck squamous cell carcinoma
HO-1	Heme oxygenase-1
IDO	Indoleamine 2,3-dioxygenase
IL-1β	Interleukin 1 beta
LAMP-2	Lysosome-associated membrane protein-2
LPS	Lipopolysaccharides
Mcl-1	Myeloid cell leukemia 1
MMP-9	Matrix metallopeptidase 9
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-κB

NSCLC	Non-small-cell lung carcinoma
P2RX1	P2X purinoceptor 1
PBS	Phosphate buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide 3-kinase
PICS	Persistent inflammation, immunosuppression, and catabolism syndrome
PMN	Polymorphonuclear leukocytes
PR3	Proteinase-3
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SLE	Systemic lupus erythematosus
TGF-β1	Transforming growth factor beta 1
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

1 Introduction

Through Toll-like receptor (TLR) signaling circuitries, host neutrophils can sense the nature, strength, and duration of TLR agonists and adopt diverse activation phenotypes such as primed, immune-enhancing, resolving, tolerant or immune-exhausted states (Table 1). This may explain the diverse functional implications of neutrophils within complex pathophysiological settings that range from acute and chronic infectious/inflammatory diseases, including sepsis, COVID-19 (Coronavirus disease 2019), cardiovascular diseases, and cancer, to effective tissue repair and resolution of inflammation (Albrecht and Petty 1998; Bratton and Henson 2011; Greenlee-Wacker 2016; Netea et al. 2016; Van Spriel et al. 2001; Yang et al. 2017). Neutrophils likely adapt complex activation status through dynamic interactions with distinct environmental cues as well as neighboring cells (Chen et al. 2014; Kumar and Sharma 2010; Pillay et al. 2012).

Emerging basic and translational studies reveal that differentially polarized neutrophil subsets may be closely correlated with the pathogenesis of various diseases. For instance, both pro- and anti-tumor activities of neutrophils have been reported in a variety of cancers (Brandau et al. 2013; Mackey et al. 2019). Most notably, MMP9 and VEGF secreted by neutrophils enhance angiogenesis in pancreatic adenocarcinoma (Nozawa et al. 2006), fibrosarcoma (Bekes et al. 2011; Jablonska et al. 2010), prostate carcinoma (Bekes et al. 2011), and hepatocellular carcinoma (Kuang et al. 2011). Tumoral invasion can be promoted by extracellular matrix (ECM) degradation contributed by neutrophil enzymes and cytokines in fibrosarcoma and breast carcinoma (Queen et al. 2005; Shamamian et al. 2001). Reactive oxygen species

Table 1 Neutrophil activation dynamics related to pathophysiological states

	Molecular and cellular features	Physiological roles	Pathological roles
Primed neutrophils	↑ Anti-microbial activity (Park et al. 2017; Shah et al. 2017) ↑ Respiratory burst (Shah et al. 2017) ↑ Pro-inflammatory markers (Yao et al. 2015)	Beneficial during the infection but detrimental in infection-free inflammation (Jennings et al. 2014; Perl et al. 2007)	Blocking neutrophil priming alleviates sepsis-induced acute lung injury in human (Bai et al. 2015) Lead to multi-organ failure but may facilitate the recovery in post-trauma patients (Bhatia et al. 2006; Hietbrink et al. 2009; Peyssonnaux et al. 2005) Elevated degranulation exacerbates various autoimmune responses (Kessenbrock et al. 2009; Lande et al. 2011; Mohr and Wessinghage 1978) Potentially enhances tumor progression and regression depending on the accessibility of primed neutrophils (Bubenik et al. 1970; Demers et al. 2016; Kuwabara et al. 2019)
Tolerance neutrophils	↑ Immunosuppressive phenotype (Lewkowicz et al. 2013; McCall et al. 1993) ↓ TLR4 expression (Parker et al. 2005) ↓ ROS production (Parker et al. 2005) ↓ Bacterial clearance (Haraoka et al. 1999) ↓ Respiratory burst (McCall et al. 1993)	Actively suppress typical immune reactions (Lewkowicz et al. 2013; Mashimo et al. 2008; Parker et al. 2005; Wang et al. 2021) Might be advantageous in terms of constraining excessive inflammatory reactions (Ariga et al. 2014)	Induced in a hypoxia environment to protect the body from excessive autoimmune reactions (Sureda et al. 2004) Increases the risk of late-onset septic shock or secondary infection in post-injury and post-sepsis patients (Hietbrink et al. 2013; Salkowski et al. 1998) The elevated frequency of tolerant neutrophils indicates the poor prognosis for different cancers (Choi et al. 2012; Dumitru et al. 2012)
Exhausted neutrophils	↑ Pro-inflammatory markers (Navarini et al. 2009) ↑ Immunosuppressive markers (Lin et al. 2020; Patera et al.	Excessive pathogenic inflammation and immunosuppression	Associated with the severity of sepsis (Patera et al. 2016) Increases the susceptibility to infections post-trauma

(continued)

Table 1 (continued)

	Molecular and cellular features	Physiological roles	Pathological roles
	2016) ↑ Atypical swarming ability (Hopke et al. 2020; Lin et al. 2020) ↓ Chemotaxis (Lin et al. 2020) ↓ Micro-organism killing function (Hopke et al. 2020; Knooihuizen et al. 2021)		(Kleinveld et al. 2019; Liao et al. 2013) Contributes to derailed immune reactions and aggravates pneumonia and tissue damages in COVID-19 patients (Aghbash et al. 2021; Aschenbrenner et al. 2021; Wang et al. 2020)

(ROS) produced by neutrophils can exacerbate tumor-promoting inflammation by damaging proliferating epithelial cells (Antonio et al. 2015). Furthermore, neutrophils can "prepare" the premetastatic niches to facilitate the metastatic spread of fibrosarcoma and mammary adenocarcinomas (Psaila and Lyden 2009; Sceneay et al. 2012). Moreover, both immature and mature neutrophils can suppress T-cell activation to dampen anti-tumor immunity (Kusmartsev et al. 2005; Pillay et al. 2012), with the elevated arginase I generated by the immunosuppressive neutrophils potentially responsible (Rotondo et al. 2009). On the other hand, neutrophils can also exert substantial cytotoxic activity against cancer cells via antibody-dependent cell-mediated cytotoxicity (ADCC) with their Fc receptors (Hubert et al. 2011), tumoricidal mediator secretion (Mantovani et al. 2011), and recruitment of other effector cells from both arms of immunity (Brandau et al. 2013; Pickaver et al. 1972; Souto et al. 2011).

Emerging studies established the paradigm of neutrophil functional diversity based on their polarization status ranging from naïve, primed, immune-enhancing, resolving, tolerant/immune-suppressive, to exhausted states. Recent studies further suggested that additional context-dependent neutrophil heterogeneities may exist which are closely related to human pathophysiology, through complex interactions with other immune cells (Kumar and Sharma 2010). This chapter will address some emerging concepts related to the adaptation of heterogeneous neutrophils upon challenges related to health and disease pathogenesis, with a particular focus on the emerging properties of neutrophil priming, immune tolerance, and exhaustion.

2 Primed/Immune-Enhancing Neutrophils

Resting neutrophils in circulation are non-adherent and with limited activities of bacterial clearance. Neutrophils primed with microbial materials via TLRs or inflammatory agents via intracellular signaling processes will undergo complex phenotypic changes including chemotaxis, adhesion, transmigration, degranulation, and cell

survival, collectively undergoing a transformational priming process in order to mount a robust anti-microbial defense (Shah et al. 2017). Neutrophil priming comes in two distinct phases with an early acute phase and a late delayed phase (Cerasoli Jr et al. 1990; Ichinose et al. 1990; Yao et al. 2015). The early priming phase usually does not require de novo gene transcription or new protein synthesis and primarily relies upon the translocation of pre-existing intracellular proteins to the cell surface, thus enabling their rapid activation. On the other hand, during the late phase of delayed priming, epigenetic modification may be involved to allow alterations of gene expression profiles and prolonged maintenance of neutrophil priming. Common priming agents include cytokines (e.g., TNF-α); chemoattractants (e.g., fMLP); microbial substances (e.g., lipopolysaccharides (LPS)), and others (e.g., small molecules, ATP, and other metabolites) (Miralda et al. 2017). Subsequently, distinct signaling processes involving stress kinases such as p38 and inflammatory transcription factors such as NF-κB can be differentially activated upon the stimulation of diverse priming agents (Dang 2006; Vogt et al. 2018). Neutrophil priming also alters the course of cell apoptosis, through the upregulation of the anti-apoptotic protein Mcl-1 in primed neutrophils, leading to delayed neutrophil apoptosis (Fotouhi-Ardakani et al. 2010; Lu et al. 2008).

Primed neutrophils can be both beneficial and harmful, depending upon the nature and magnitude of priming signals as well as subsequent pathological contexts. For example, Perl et al. demonstrated that the presence of primed neutrophils was advantageous during subsequent bacterial infection but detrimental during sterile lung inflammation without infection (Perl et al. 2007). A similar paradigm was also demonstrated in the study where the entry of primed neutrophils to lungs provided a protective effect during Influenza virus infection but not during infection-free inflammation (Jennings et al. 2014). Detailed underlying molecular mechanisms remain to be further elucidated and may beget distinct subsets of primed and/or immune-enhancing neutrophils with unique and diverging phenotypes in the future. This section of the chapter aims to provide an overview regarding the critical and selective involvement of neutrophil priming in acute and chronic diseases.

2.1 Neutrophil Priming in Trauma and Sepsis

Sepsis is a systemic inflammatory syndrome caused by injury coupled with polymicrobial infection, which may ultimately lead to multi-organ failure and acute mortality. Cytokine storms produced by the host through the acute phase of sepsis may be responsible for the initial priming of neutrophils. Primed neutrophils may hasten the pace and aggravate the magnitude of degranulation and modulate the intensity of extracellular trap formation, collectively altering the course and severity of sepsis (Fortunati et al. 2009). For instance, neutrophils from septic patients were shown to exhibit an elevated degranulation response to subsequent chemotactic stimuli fMLP (Drost et al. 1999). Primed neutrophils from septic patients also exhibited elevated levels of pro-inflammatory markers, such as CD54, Dectin-2, and IL-1β, and accelerated motility in a sequential manner (Yao et al. 2015). With

potential therapeutic implications, it was reported that the blockage of neutrophil priming may reduce tissue injury in experimental animals with sepsis-induced acute lung injury (Bai et al. 2015). On the other hand, neutrophils collected from sepsis survivors demonstrated enhanced autophagy, increased potential of generating NET, and effective microbial-killing function (Park et al. 2017), suggesting compound effects of neutrophil priming in distinct phases of sepsis progression and recovery.

Systemic injury sustained from trauma may similarly prime neutrophils that in turn impact the survival outcome of the patients (Irak et al. 2003). The extent of neutrophil priming induced by major trauma can vary depending upon the magnitude of the traumatic insult, as reviewed by Mortaz et al. (Mortaz et al. 2019). Phenotypic changes of neutrophils upon priming, such as elevation of CD11b and IL-6, have been regarded as a prognostic marker for trauma injuries (Mortaz et al. 2019; Nast-Kolb et al. 1997). Patients with circulating primed neutrophils are susceptible to multi-organ failure induced by the secondary challenge due to the infiltration of hyper-activated neutrophils, also known as a "double-hit model." Studies have shown a significant elevation of elastase and adhesion molecules from patients suffering from major trauma, contributing to increased neutrophil degranulation, extended neutrophil lifespan, and accumulation of hyperactive neutrophils within tissues which collectively lead to multi-organ failure (Bhatia et al. 2006; Peyssonnaux et al. 2005). However, insufficient neutrophil priming may dampen the late-phase recovery and increase the risk of developing secondary complications in post-trauma patients (Hietbrink et al. 2009). The complex dynamics of neutrophil adaptation during the course of sepsis and trauma still require extensive future studies.

2.2 Neutrophil Priming in Autoimmune Disorders

Autoimmune diseases can result from a dysregulation of the immune system leading to an immune attack on the self-tissues and organs (Antonelli et al. 2015). Neutrophils are involved in the pathophysiology of the dysregulated autoimmunity in diverse systems (Vogt et al. 2018). Circulating neutrophils are primed in patients with anti-neutrophil cytoplasmic antibody-associated systemic vasculitis (AASV) (Charles et al. 1991). Primed neutrophils have increased PR3, MPO, and LAMP-2 translocation to the cell membrane. LAMP-2 translocation further facilitates the binding of anti-neutrophil cytoplasmic antibodies (ANCAs) (Gabillet et al. 2012; Kain et al. 1995). Endothelial injury caused by hyper-activated neutrophils, the dysregulation of neutrophil cell death, and NET formation can collectively exacerbate autoimmune responses and vascular inflammation (Kessenbrock et al. 2009).

Primed neutrophils have also been observed in patients with systemic lupus erythematosus (SLE). In contrast to the "classical" priming, lupus neutrophils displayed accelerated apoptosis and impaired phagocytic capacity (Armstrong et al. 2006; Brandt and Hedberg 1969). A subset of neutrophils from patients with SLE exhibited elevated NET formation (Lande et al. 2011). However, the aberrant clearance of NET material due to the accumulation of immune complexes (i.e.,

autoantibodies) in SLE could further exacerbate cytokine storm and promote pro-inflammatory responses leading to tissue damage related to lupus pathogenesis (Villanueva et al. 2011).

Furthermore, elevated neutrophil priming was observed in rheumatoid arthritis (RA) synovial fluid and synovial tissue (Kaplan 2013). Cytokines, autoimmune antibodies, and GM-CSF are critical factors for priming neutrophils in the pathogenesis of joint destruction (Chatham et al. 1990; Cook et al. 2011; Sadik et al. 2012). Degranulation by primed neutrophils is involved in cartilage destruction and bone resorption (Chakravarti et al. 2009; Mohr and Wessinghage 1978). In addition, the progression of RA is associated with the upregulated expression of membrane-bound activating receptors and adhesion molecules on neutrophils collected from synovial fluid (Chakravarti et al. 2009; Dominical et al. 2011). The accelerated NETosis and ROS production from primed neutrophils also contribute to aggravated immune responses in the joint and exacerbate RA (Dang et al. 2006; Khandpur et al. 2013).

2.3 Neutrophil Priming in Cancer

Differentially polarized neutrophils have conflicting roles during the pathogenesis of cancer. Neutrophils can enhance tumor progression by stimulating angiogenesis and invasion. On the other hand, neutrophils may also display cytotoxicity effects by producing inflammatory mediators and ROS or activating other immune cells, which lead to tumor regression. One potent priming agent in the cancer setting is G-CSF which can prime neutrophils for the enhanced NETosis (Shen et al. 2014). Neutrophils collected from head and neck squamous cell carcinoma (HNSCC) patients had increased chemokines and chemotaxis (Trellakis et al. 2011). Studies in animal cancer models also reported the local expression of G-CSF in the tumor micro-environment (Berger-Achituv et al. 2013; Boone et al. 2015). Demers et al. illustrated that both locally produced and systemic G-CSF can facilitate neutrophil priming. Collectively, these studies suggested that neutrophils primed by G-CSF can enhance NET formation which leads to thrombosis and promotes tumor growth resulting in an adverse outcome for cancer patients (Demers et al. 2016). On the other hand, cytotoxicity of primed neutrophils against tumor cells has been described in the 1970s, and it was widely attributed to the production of ROS and cytotoxic enzymes, including MPO (Bubenik et al. 1970; Demers and Wagner 2013). A recent study further supports this intriguing paradigm of opposing neutrophil functions during tumor pathogenesis with the differential accessibility of neutrophils to the tumor micro-environment as a crucial determinant for tumor development (Kuwabara et al. 2019).

2.4 Neutrophil Priming in Metabolic Diseases

The metabolic changes during diabetes pathogenesis induce oxidative stress in endothelial cells, macrophages, and neutrophils, which collectively contribute to diabetic complications (Rendra et al. 2019). A potent neutrophil priming agent, TNF-α, was increased in diabetic individuals and could prime neutrophils for enhanced generation of ROS and cytokines (Alexandraki et al. 2008; Hanses et al. 2011; Karima et al. 2005). Diabetes predisposes neutrophils to NETosis and ROS production resulting in impaired wound healing and diabetic complications (Omori et al. 2008; Wong et al. 2015). Glucose-mediated low-grade systemic inflammation was shown to activate neutrophils in high-fat-diet-fed-mice (Yano et al. 2012). Primed neutrophils with elevated expression of inflammatory lipid mediators such as leukotriene B4 (LTB4) were shown to exacerbate the pathogenesis of atherosclerosis (Geng et al. 2019).

3 Immune Suppressive/Tolerant Neutrophils

The concept of immune tolerance was largely appreciated in the field of adaptive immunity, as reflected in central and peripheral tolerance. Central tolerance involves the negative selection process within the thymus through the presentation of self-antigens, while peripheral tolerance is mediated by inducible T regulatory cells (Treg) which can suppress self-reactive T or B cells. Both scenarios of immunological tolerance prevent excessive inflammatory reaction and avoid autoimmune disorders. Emerging studies revealed that innate immune cells can adopt an analogous "tolerance" phenotype to prevent excessive inflammation. Tolerant monocytes/macrophages have reduced ability to express selected inflammatory cytokines such as TNF-α and IL-1β as well as compromised ability for phagocytosis (Freudenberg and Galanos 1988; Pena et al. 2011). The development of macrophage tolerance perhaps serves as a compensatory mechanism to avoid excessive inflammation. For example, Svensson-Arvelund et al. demonstrated that macrophage tolerance developed in the human fetal placenta can create a homeostatic environment that may avoid the rejection of the semi-allogeneic fetus during pregnancy (Svensson-Arvelund et al. 2015). Regarding neutrophils, limited studies suggest the development of neutrophil tolerance in diverse circumstances, including patients with acute anterior uveitis (AAU) and lung infection (Chang et al. 2007; Sahoo et al. 2014). The following section addresses some salient features of neutrophil tolerance from selected experimental and clinical studies in vitro and in vivo.

3.1 Experimental Analyses of Neutrophil Tolerance

In the in vitro experimental setting, prolonged LPS stimulation can generate tolerant neutrophils with reduced expression of TLR4 and reactive oxygen species (ROS) in response to neutrophil-priming cytokine GM-CSF (Parker et al. 2005). TLR4

deficiency in neutrophils is associated with compromised bacterial clearance (Haraoka et al. 1999), suggesting that tolerant neutrophils could have an impaired bacterial-killing function. This is supported by the study showing tolerant neutrophils being ineffective in clearing *Shigella sonnei* and prone to necrosis (Torraca et al. 2019). In addition to TLR agonists, immunosuppressive properties of tolerant neutrophils, including elevated production of IL-10, TGF-β1, IDO, and HO-1, can also be induced by intercellular communication with Treg cells (Lewkowicz et al. 2013). This cross-talk between innate and adaptive immunity suggests that innate immune tolerance can be induced by the adaptive immune cells other than prolonged stimulation by microbial/chemical substances. The effects of neutrophil tolerance are not limited to compromised anti-microbial defense. Rather, under a certain scenario, an enhanced microbial-killing capability of tolerant neutrophils was reported by Landoni et al. *(*Landoni et al. 2012*)*. Such discrepancy might result from the difference of stimulating agents, and/or varying dosages and durations used in separate studies during their induction of tolerant neutrophils. Likewise, the discrepant effects of tolerant neutrophils were also noticed from in vivo studies. Tolerant neutrophils from septic mice induced by the cecal ligation and puncture (CLP) had a deficiency in multiple bactericidal processes (Chiswick et al. 2015). Tolerance might also be beneficial despite impairing neutrophil's bacterial-killing function, as reflected in reduced mortality of LPS tolerant mice following CLP. Perhaps reduced inflammation due to neutrophils tolerance might blunt tissue damage, leading to better survival outcomes (Ariga et al. 2014; McCall et al. 1993).

Tolerant neutrophils, while being immunosuppressive, are not completely inert. This is analogous to macrophage tolerance in which instead of being immunoparalyzed, tolerant macrophages are alternatively activated and able to recover back to a hybrid pro- and anti-inflammatory state (Lopez-Collazo and del Fresno 2013). The "immunosuppressive but responsive" characteristic of tolerant neutrophils was also present in endotoxin-induced uveitis (EIU) rat models caused by consecutive LPS stimulations (Mashimo et al. 2008) and in mice with pancreatic ductal adenocarcinoma (PDAC) (Wang et al. 2021) in which neutrophils secreted more IL-10 and exhibited an elevated level of PD-L1 respectively.

3.2 Clinical Implication of Neutrophil Tolerance

Tolerant neutrophils with a reduced TLR4 expression can be found in AAU patients (Chang et al. 2007). Sahoo et al. also reported that reducing elastase production may similarly induce neutrophil tolerance upon lung infection and minimize host tissue damage (Sahoo et al. 2014). Neutrophil tolerance can also be achieved under chronic hypoxia where neutrophils have delayed oxidative burst and less MPO activity, which renders protection from autoimmune disorders (Sureda et al. 2004).

On the other hand, growing evidence revealed that tolerant neutrophils may also increase host susceptibility to secondary infections. For example, post-trauma patients with tolerant neutrophils were more likely to develop late-onset septic

shock. Tolerant neutrophils from these patients expressed less FcγRII as well as CXCR-1, with impaired responsiveness potentially responsible for elevated post-trauma sepsis severity (Groeneveld et al. 2017; Hietbrink et al. 2013). Mitochondria-derived damage-associated molecular patterns (mtDAMPs) were identified to be potential tolerizing agents that suppress NET formation in post-trauma patients, through modulating the activity of AMPK (Hazeldine et al. 2019). The deficiency in neutrophil effector functions may increase the risk of secondary infections in certain cohorts of sepsis patients (Skelton and Purcell 2021; Wafaisade et al. 2011).

In the context of tumor progression, mediators secreted by the tumor micro-environment may be critically involved in the generation of either immune-enhancing or immune-suppressing tolerant neutrophils. This led to the earlier concept of N1 (anti-tumor) and N2 (pro-tumor) neutrophils. However, recent studies suggested that neutrophil diversity is far more complex and exceeds the simplistic description of N1 vs N2 neutrophils. Our understanding of neutrophil diversity is still limited, and the field lacks clear molecular markers that can differentiate distinct subsets of functionally unique neutrophils. Although some studies suggested that neutrophils may be separated by differences in their density, functional analyses reveal that density difference is not the relevant marker to differentiate unique neutrophil subsets (Scapini et al. 2016). Future integrated analyses that utilize single-cell sequencing and functional characterization are needed to define relevant molecular markers that can represent various neutrophil subsets. The cardinal features of tolerant neutrophils in the context of tumor micro-environment include reduced oxidative burst, decreased chemotaxis, and impaired bactericidal activity; lower pro-inflammatory cytokine secretion but greater immunomodulatory molecule such as TGF-β generation have been reported in neutrophils from cancer patients with acute lymphoblastic leukemia (Tanaka et al. 2009), hepatocellular carcinoma (Uehara and Sato 1994), lung cancer (Shirai et al. 1998), bladder carcinoma (Kastelan et al. 2003), or squamous cell carcinoma of the oral cavity (Jablonska et al. 2001, 2009). Furthermore, the frequency of tolerant neutrophils expressing $CD11b^{+}CD15^{+}CD16^{low}$ could be an indicator for the poor prognosis of the terminal lung, breast, and gastrointestinal cancer patients (Choi et al. 2012; Dumitru et al. 2012). Considering that the levels of CC ligands reversely correlate with the survival rates for cancer patients, tolerant neutrophils may also propagate the immunosuppressive state to other immune cells (Tsuda et al. 2012), including macrophages and T cells, through the secretion of CC ligands to further promote tumor growth (Akgul et al. 2001; Wu et al. 2019).

Being cognizant of primed neutrophils presenting in septic patients, a growing amount of literature reported that tolerant neutrophils can also be found in septic patients entering the protracted immunosuppressive phase. Experimental studies in patients, likewise in animal models, revealed the neutrophil functional impairment as in decreased bacterial clearance, defective ROS generation, paralyzed chemotactic ability, and augmented production of IL-10 (Alves-Filho et al. 2010; Hotchkiss et al. 2013; Stephan et al. 2002). The reduction of neutrophil function has been associated with the susceptibility to secondary infections. Stephan et al. demonstrated that patients with compromised neutrophil functions had a higher risk of acquiring

Pseudomonas aeruginosa-induced nosocomial infection (Salkowski et al. 1998). While most of the pre-clinical studies showed that septic mice with the pretreatment of endotoxin as a prophylactic approach had the reduced severity of sepsis due to reduced production of cytokines and chemokines by tolerant neutrophils (Chen et al. 2015; Kopanakis et al. 2013; Landoni et al. 2012), a recent publication unveiled that the effects of pre-programming neutrophils are in an LPS-dosage-dependent manner in which the pretreatment of "tolerant" dose LPS can induce neutrophil tolerant phenotype to provide protective effects in septic mice; on the other hand, neutrophils from mice pre-conditioned with super-low dose LPS, instead of exhibiting tolerant properties, polarize to a "primed" state to generate greater pro-inflammatory cytokines and exacerbate sepsis outcome (Chen et al. 2015). This in vitro study provides novel insight focusing on LPS-dosage-dependent neutrophil plasticity mediated by distinct intracellular signaling cascades and could clarify the diverging observations between animal models and human clinical scenarios.

4 Exhausted Neutrophils

In contrast to neutrophil priming and tolerance, studies regarding neutrophil exhaustion and its underlying mechanisms are scarce. Existing data suggested that the nature of exhausted neutrophils, to a certain degree, overlaps with the primed and/or tolerant neutrophils (Craciun et al. 2010; Parker et al. 2005). With the recent development of single-cell RNA sequencing techniques, exhausted neutrophils in diverse circumstances are getting better defined. Clinically, exhausted neutrophils are getting better appreciated during the chronic phases of diseases. For instance, in the scenario of sepsis, advanced ICU care and supportive treatments may rescue the initial hyper-activated inflammatory response-induced multi-organ failure. However, persistent immunosuppression-related complications may linger long past the initial septic injury. The exhausted neutrophil phenotype may persist during the prolonged phase of immune suppression. The phenotype regarding the exhaustion state of pathogenic inflammation coupled with refractory immune suppression in chronically ill patients has been recently coined as PICS (persistent inflammation, immunosuppression, and catabolism syndrome) (Rosenthal and Moore 2015, 2016), with exhausted neutrophils critically impacting the process of PICS. A clear definition for neutrophil exhaustion is warranted in order to provide basic and translational perspectives to related pathophysiological processes. Here, we briefly evaluate current literature regarding exhausted neutrophils, regarding their phenotypic and functional alterations in vitro and in vivo (Table 1).

Exhausted neutrophils may develop following persistent and overwhelming stimulations. Navarini and his colleagues showed that a high inoculum of *Listeria monocytogenes* led to neutrophil exhaustion in mice in which the expression of CD11b (a cell surface marker associated with pathogenic inflammation) was persistently elevated post-infection. Excessive neutrophil infiltration was also noticed in spleens and livers, resulting in tissue damage (Navarini et al. 2009). Another independent study demonstrated a similar exhaustion phenotype in vitro with

neutrophils, challenged with prolonged LPS stimulation, possessing elevated levels of CD11b and PD-L1 (an immunosuppressive marker), and a reduction in CXCR2 expression (correlated with neutrophil paralysis in chemotaxis) (Lin et al. 2020). Collective studies, therefore, suggested a complex signature of exhausted neutrophils with a pathogenic inflammation and immune-suppression phenotype. An ex vivo study with human neutrophils complements murine studies described above (Knooihuizen et al. 2021). Neutrophils from patients with cirrhosis, which is a late stage of liver fibrosis caused by chronic hepatitis and alcoholism, secreted greater inflammatory cytokines but possessed impaired fungicidal capacity. Exhausted neutrophils also exhibit abnormal migratory and swarming phenotypes (Knooihuizen et al. 2021), and these abnormalities were also noticed in chronic granulomatous disease (Hopke et al. 2020), suggesting a prevalent presence of exhausted human neutrophils in various disease settings.

4.1 Neutrophil Exhaustion During Sepsis and Trauma

Abnormal neutrophil migration is one of the hallmarks of neutrophil exhaustion. Neutrophils from severe sepsis patients were deficient in chemokine-mediated directional migration, potentially correlated with the severity of sepsis (Drost et al. 1999). The degree of neutrophil dysfunction correlates to the severity of the septic insult, which persists beyond physiological recovery from the initial injury. Patel et al. reported that defects in neutrophil NETosis, migration, and delayed apoptosis may all persistent throughout the course of sepsis, and the suppression of NETosis is associated with both early- and late-onset of sepsis mortality (Patel et al. 2018). Exhausted neutrophils are also immunosuppressive with progressively reduced phagocytic ability as well as increased PD-L1 levels. The frequency of exhausted neutrophils with high PD-L1 expression closely correlates with the reduction of $CD8^+$ T cells and NK cells as well as the severity of sepsis, suggesting that sepsis-induced innate immune exhaustion can be propagated to adaptive immune cells via ligand–receptor interaction (PD-1/PD-L1) (Patera et al. 2016). Hence, the susceptibility to infections increases due to the lack of functional patrolling neutrophils in the bloodstream, which results in an exhausted state with persistent pathogenic inflammation and immune suppression.

Exhausted neutrophils with features of pathogenic inflammation may also be rapidly recruited to tissues subjected to severe trauma and injury. In this regard, a study reported that excessive pro-inflammatory cytokines were secreted following traumatic brain injury (Liao et al. 2013). Exhausted neutrophils may further propagate systemic tissue damage beyond the original lesion site. Exhausted neutrophils contribute to pathogenic inflammation in which neutrophils with elevated expression of adhesion markers accumulate in vital tissues causing organ injury. The observation done by Kleinveld et al. also illustrated that both pro-inflammatory and anti-inflammatory cytokines were elevated in post-trauma patients with multi-organ failure, indicative of dysregulated host immune responses associated with severe complications (Kleinveld et al. 2019).

4.2 Neutrophil Exhaustion in COVID-19

COVID-19 was a global pandemic caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection, with over 168 million confirmed infected individuals and over 3.5 million deaths from COVID-19 worldwide as of May 2021. Increasing research in the epidemiology and pathophysiology of COVID-19 suggests that the severity of the disease is related not only to the alveolar epithelial cell damage caused by the viral infection but also to a dysregulated inflammatory reaction which drives multi-organ dysfunction and subsequent bacterial superinfections, resulting in complex disease manifestations, ranging from asymptomatic cases to severe morbidities and mortalities (Cavalcante-Silva et al. 2021). The derailed immune reaction is complex and characterized by altered numbers of effector cells and levels of inflammatory mediators (Cavalcante-Silva et al. 2021; Shambat et al. 2020).

Neutrophilia was reported to be present in the peripheral blood of severe and non-surviving COVID-19 patients and that the neutrophil-to-lymphocyte ratio (NLR) can predict poor outcomes in patients with COVID-19 (Wang et al. 2020). Excessive neutrophil infiltration in pulmonary capillaries and unrestrained neutrophil degranulation and cytokine production serve as contributors to pathological inflammation of pneumonia to exacerbate tissue injuries (Aghbash et al. 2021; Wang et al. 2020). In addition, using the protein association network software, a recent paper showed that seven putative SARS-CoV-2 receptors (ACE2, DPP4, ANPEP, CD209, CLEC4G, CLEC4M, and CEACAM1) are mainly involved in the ontology of "Neutrophil Degranulation" signaling network (Didangelos 2020), suggesting a close connection between SARS-CoV-2 and neutrophil-related inflammation. Meanwhile, RNA-seq analyses of granulocyte transcriptomes revealed that not only do the inflammatory features, including elastase, MPO, alarmin, and type I interferon (IFN)-induced genes, but also immunosuppressive signatures, such as expression of IL10, SOCS3, ARG1 (Arginase), and PD-L1, simultaneously increase in severe COVID-19 patients (Aschenbrenner et al. 2021). Jointly, the dysregulated neutrophil features in COVID-19 patients are consistent with hallmarks of exhaustion, which may compromise host defense and potentially lead to long-term complications.

5 Conclusion and Future Direction

Decades of studies on neutrophils provide complex adaptation of neutrophils in response to diverse challenges both in vitro and in vivo, which bear significant relevance to health and disease. Neutrophil memory dynamics ranging from priming, tolerance, resolution to exhaustion may alter the course of disease pathogenesis in sepsis, chronic inflammatory diseases, and cancer. Despite significant advancement, fine-tuning of neutrophil adaptation to challenges and underlying mechanisms are still not fully understood. Previous studies suggest that dynamically-integrated competitive intracellular signaling circuitries may enable

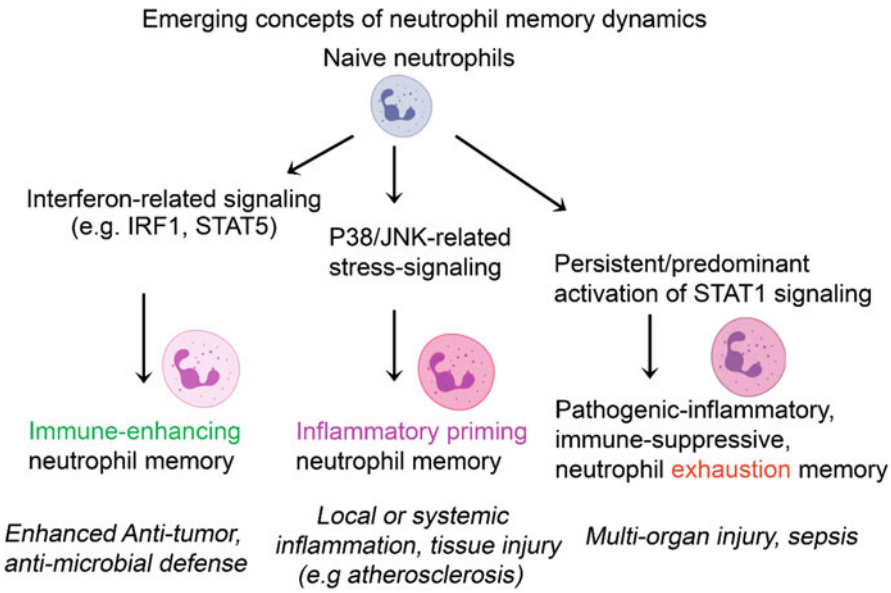

Fig. 1 Neutrophil signaling dynamics that potentially underlie the differential establishment of diverse neutrophil memory states. In addition to priming and tolerance, emerging studies suggest that neutrophils may adopt additional unique activation states such as an immune-enhancing state with robust anti-cancer and anti-microbial functions. The immune-enhancing state may be induced by interferon-related signaling processes involving IRF1 and STAT5. The inflammatory priming state, discussed extensively in this review, may be triggered by p38/JNK-related stress signaling processes. The persistent activation of STAT1 may skew activated neutrophils (including primed and tolerant) into an exhausted state characterized by pathogenic inflammation and immune suppression. (The illustration of neutrophils were adapted through a creation from the BioRender software)

complex neutrophil activation states. For example, enhanced interferon-related signaling may correlate with the establishment of immune-enhancing neutrophil phenotype conducive for anti-tumor and anti-microbial functions (Kalafati et al. 2020; Zhang et al. 2019; Zhang et al. 2020), while activation of stress-related kinases such as p38 and JNK may prime neutrophils into an inflammatory memory phenotype implicated in the pathogenesis of systemic inflammatory diseases such as sepsis and atherosclerosis (Chen et al. 2015; Guo et al. 2016). On the other hand, persistent activation of STAT1 may drive the exhausted neutrophil phenotype characterized by pathogenic inflammation and immune suppression (Lin et al. 2020) (Fig. 1). With the emergence of next-generation single-cell analyses and integrative bioinformatics approaches, future efforts are warranted to provide a clear understanding of context-dependent neutrophil programming dynamics closely related to the pathophysiology of diverse human diseases.

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Cell-Specific Expression Pattern of Toll-Like Receptors and Their Roles in Animal Reproduction

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2022_584

Abstract

Toll-like receptors (TLRs), a part of the innate immune system, have critical roles in protection against infections and involve in basic pathology and physiology. Secreted molecules from the body or pathogens could be a ligand for induction of the TLR system. There are many immune and non-immune types of cells that express at a least single TLR on their surface or cytoplasm. Those cells may be a player in a defense system or in the physiological regulation mechanisms. Reproductive tract and organs contain different types of cells that have essential functions such as hormone production, providing an environment for embryo/fetus, germ cell production, etc. Although lower parts of reproductive organs are in a relationship with outsider contaminants (bacteria, viruses, etc.), upper parts should be sterile to provide a healthy pregnancy and germ cell production. In those areas, TLRs bear controller or regulator roles. In this chapter, we will provide current information about physiological functions of TLR in the cells of the reproductive organs and tract, and especially about their roles in follicle selection, maturation, follicular atresia, ovulation, corpus luteum (CL) formation and regression, establishment and maintenance of pregnancy, sperm production, maturation, capacitation as well as the relationship between TLR polymorphism and reproduction in domestic animals. We will also discuss pathogen-associated molecular patterns (PAMPs)-induced TLRs that involve in reproductive inflammation/pathology.

Keywords

Domestic animals · Polymorphism · Reproductive cells · Toll-like receptors

1 Introduction

Following puberty, domestic animals show reproductive cyclicity continuously in males or during a certain period in females. Although the time and the duration could be different among species, the main goal of the reproductive cycle is to conceive/become pregnant. However, the current concept of manipulating reproduction in domestic animal species is established on either contraception or improvement of pregnancy chances. Like the other organs and tracts of the body, the reproductive system and organs in both males and females intertwined the immune system at

many points. Many components of the immune system have been described in reproductive organs and tissues. Apart from their roles protecting from inflammation caused by bacteria or viruses in reproductive systems, we have known that the immune system components regulate many physiological functions of reproduction systems such as the production of gamete cells, ovulation, transport of semen/spermatozoa and oocyte, the establishment and maintenance of pregnancy, and labor, etc., which are also considered as anti/pro-inflammatory process. Among immune system components, the innate immune system components are the first responsive part and later induce the acquired immune system. Toll-like receptors (TLRs), a critical part of the innate immune system, have roles in protection against infections and involve in basic pathology and physiology. Besides pathogen-associated molecular patterns (PAMPs), there are numerous endogenous ligands from intracellular content or extracellular matrix breakdown products that can be recognized by TLRs (for a detailed review, see Yu et al. (2010)). Induction of endogenous ligand-TLR complex can induce sterile inflammation by stimulating downstream cytokine production which changes the physiological status of cells and tissues in many organs and systems (Pineau and Lacroix 2009). Compared to roles of TLR in human reproduction that are well explained in all parts of reproductive tract components (please see reviews by Girling and Hedger (2007); Koga and Mor (2010), data on TLR patterns in reproductive physiology and pathology of domestic animals are very limited. Because sequencing of the TLR indicates a high homology (95% nucleotide sequence identity between cattle and human) between human and domestic animal species, it could be suggested that there are conserved and similar roles of TLRs among mammalians (Roach et al. 2005; Werling and Coffey 2007; Kannaki et al. 2011; Menzies and Ingham 2006). Therefore, in this chapter, we will provide current information about physiological functions of TLR in the cells of reproductive organs and tract (Fig. 1), especially their roles in follicle selection, maturation, follicular atresia, and ovulation, corpus luteum (CL) formation and regression, establishment and maintenance of pregnancy, sperm production, maturation, capacitation as well as the relationship between TLR polymorphism and reproduction in domestic animals. Additionally, we will also discuss PAMPs induced TLRs involving in reproductive inflammation/pathology.

2 Roles of TLRs in Female Reproductive Cells

The female reproductive system is composed of the primary sex organ (ovary) and secondary sex organs (known as genital tract components including the vulva, vagina, cervix, uterus, and oviduct). The female genital tract plays crucial roles such as establishment and maintenance of pregnancy and protection from invading microorganisms. The female genital tract has two main parts as the lower genital tract (ectocervix, vagina, and vulva) and the upper genital tract (endocervix, endometrium, and oviduct) which are lined with squamous and columnar epithelium, respectively (Pineda 2003). The upper genital tract, composed of a site-specific mucosal immune system, contributes to support/regulation of sperm and embryo/

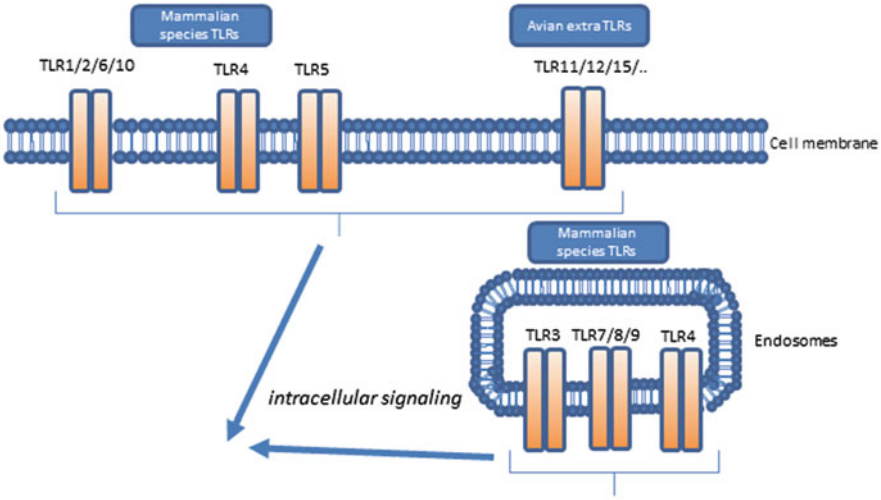

Fig. 1 Schematic model for reproductive physiological events regulated by TLRs in domestic animals

fetus life. Spreading of microorganisms from the lower to the upper parts of genital tract results in endometritis and salpingitis. Compared to the upper part, the lower genital tract that is always exposed to microorganisms represents a complex immune balance providing a barrier against the pathogens and participating in both innate and acquired immune defense systems (Nasu and Narahara 2010). In this part, we will describe cell/tissue specific patterns of TLRs involved in the female reproductive system of domestic animals in the light of current literature.

2.1 Ovarian Cells

Ovarian tissue mainly includes follicles that are at different stages of development such as primordial, primary, secondary, tertiary, and Graafian follicles. These follicles contain the female germ cell, the oocyte, surrounded by different types of cells. The number, size, and the type of these cells change according to the stage of

the follicle. The communication of these cells with each other through their secretions has crucial roles in oocyte maturation and ovulation. Among those, two types of cells, theca interna and granulosa cells, are the main regulators of oocyte development with hormones and molecules they produced (Hirshfield 1991).

2.1.1 TLRs in Follicle Cells

Besides their endocrine function, current studies indicate that both theca and granulosa cells express innate immune system components including TLRs (Fig. 2). Expression of TLRs has been demonstrated in bovine, caprine, murine, and avian granulosa/theca cells. Previous studies, due to the fact that transovarial transmission of pathogens results in egg contamination, focused on the examination of TLRs within granulosa cells in the hen. Woods et al. (2009) demonstrated the presence of TLR2, TLR4, and TLR15 in granulosa cells and that granulosa cells at early stages of differentiation were sensitive for TLR- lipopolysaccharide (LPS) induced apoptosis (Woods et al. 2009). In addition, Kannaki et al. (2010) and Subedi et al. (2007) reported that there was an increase in expression of both TLR4 and TLR5 in the granulosa layer in growing follicles in hen. Apart from this, a recent study indicated that the bovine granulosa cells express all TLR 1-10, and their expression levels changed at different stages of follicle maturation (Xie et al.

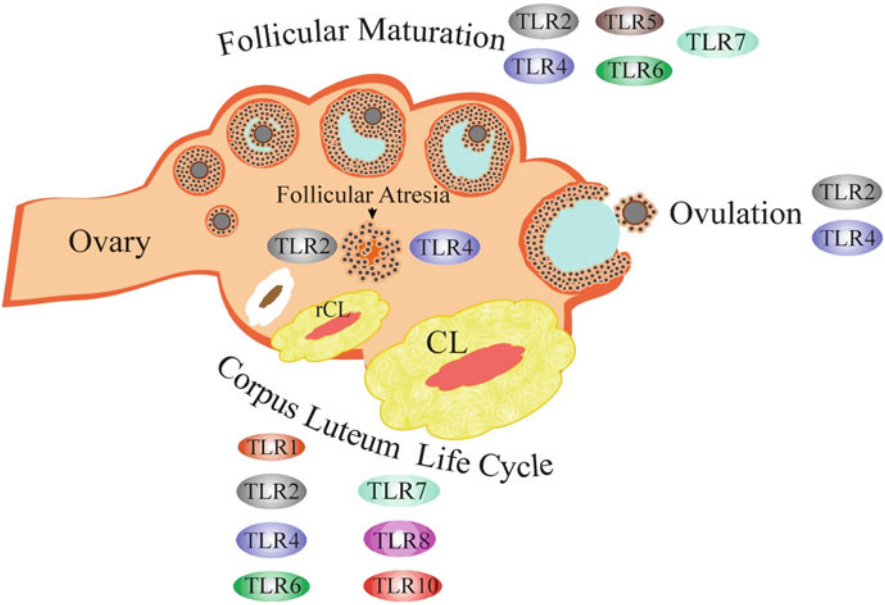

Fig. 2 Schematic model for the differently regulated TLRs in ovarian physiological events such as follicle selection and maturation (Kannaki et al. 2010; Subedi et al. 2007; Xie et al. 2020; Zhu et al. 2016a; Talebi et al. 2018); follicle atresia (Bromfield and Martin Sheldon 2013; Price and Sheldon 2013; Talebi et al. 2018; Woods et al. 2009); ovulation (Liu et al. 2008; Shimada et al. 2006); corpus luteum life cycle (Atli et al. 2018a, b; Gadsby et al. 2017; Lüttgenau et al. 2016) in different domestic animals. rCL indicates regressing corpus luteum

2020). Especially, Xie et al. (2020) indicated that TLR expression in granulosa cells of large follicles was significantly higher than those of small and middle-sized follicles. They also indicated that TLR2/4 in granulosa cells may have specific roles in the ovarian innate immune functions and follicular maturation regulated by FSH. This is a clear evidence for follicle stimulating hormone (FSH)-regulated TLRs involving follicular maturation and steroidogenesis. On the other hand, Zhu et al. (2016a) reported that, in goat, TLRs were not involved in the process of dominant follicle selection, but TLR6 played a role in the development of follicles. Besides follicular maturation and selection, some studies point out the roles of TLR signaling in follicular atresia (Talebi et al. 2018). This was supported by the observation in cattle and hen ovaries that the TLR pathway could be implicated in apoptotic mechanisms in granulosa cells during the follicular phase leading to follicular atresia (Bromfield and Martin Sheldon 2013; Woods et al. 2009). Indeed, it was reported that bacterial PAMPs including LPS and lipoprotein were sensed by bovine granulosa cells from emerged follicles and this was regulated by TLR2 and TLR4 pathways (Price and Sheldon 2013).

An obvious role of TLR signaling in the ovulation process is described by Liu et al. (2008). Briefly, when the follicle reaches the pre-ovulatory stage, LH induced-triggering mechanism starts the expansion of cumulus cell-oocyte complexes (COCs) and rupture of the follicle (known as ovulation) which is considered a sterile pro-inflammation process. Shimada et al. (2006) indicated that cumulus cells release factors including Prostaglandin-Endoperoxide Synthase 2 (PTGS2/COX-2), Interleukin 6 (IL6), Tumor Necrosis Factor Alpha (TNF-a) as well as other cytokines and chemokines which cause degradation of polymeric hyaluronan (HA) into HA fragments during the expansion process of COCs. Those fragments of HA activate TLR2 and TLR4 signaling and then key components of the complex TLR signaling pathway (cluster of differentiation 14 (CD14), Lymphocyte antigen 96 (Ly96), Myeloid differentiation primary response 88 (Myd88), Toll-Like Receptor Adaptor Molecule 1 (Ticam1), TIR Domain Containing Adaptor Protein (Tirap) and Toll-interacting protein (Tollip), interleukin-1 receptor-associated kinases [Iraks], TNFα receptor-associated factors [Trafs] and interferon regulatory factors [Irfs]) are induced in the ovulation process.

2.1.2 TLRs in Luteal Cells

Following the rupture of Graafian follicle, remaining cells (theca interna and granulosa cells) transform into luteal cells which are the main hormone production part of the CL (Wiltbank et al. 2012). Similar to their roles in follicular maturation and ovulation, recent studies indicate roles of TLRs in the developing and regressing CLs (Fig. 2 (Atli et al. 2018a, b; Gadsby et al. 2017; Lüttgenau et al. 2016). It was reported that although TLR2 expression levels only increased in the regression stage of bovine CL, TLR4 expression showed changes between the developing and mature stages of the bovine CL (Atli et al. 2018b). TLR expression in an ovine CL study has revealed that there is a specific expression pattern for each TLR during the luteolysis phase of the CL cycle (Atli et al. 2018a). For example, the expression of TLR2 is upregulated in both the early and late stages of luteolysis. Upregulation of TLR4 is

detectable at late stages of the luteolysis. Expressions of TLR7 and TLR8 significantly increase after functional luteolysis in the regression phase of CL. On the other side, luteolysis downregulates TLR10 expression in the ovine CL. This study indicated a change in the cellular localization of TLR at different stages of the CL cycle. It has also been reported that during the active progesterone secretion phase, TLR2, TLR4, and TLR7 were detected mainly at endothelial cells and very weakly at the luteal cell, but after the induction of luteolysis, all luteal cells expressed TLR2, TLR4, and TLR7 prominently (Atli et al. 2018a). Similarly, Gadsby et al. (2017) indicated roles for TLR1, 2, 4, and 6 in the regressed stage of luteal phase in the bovine CL. Lüttgenau et al. (2016) emphasized the stimulatory effect of LPS on TLR2 and TLR4 expressions in bovine CL. They showed TLR involvement in the deleterious effects of LPS on CL function which may result in an increase in some pro-inflammatory cytokines resulting in decreasing fertility. Apart from the estrous cycle and inflammation, embryonic interferon tau which is produced by the trophoblast in the uterus during the early pregnancy causes an increase in TLR7 and TLR8 and a decrease in TLR10 in ovine CL (Atli et al. 2018a). According to those results, besides LPS induction, involvement of TLRs in the luteolysis and in the establishment and maintenance of pregnancy occur through induction of their endogenous ligands.

2.2 Oviduct Cells

The oviduct (fallopian tube) connects uterus and ovary. Its epithelial cells are an active part of the oviduct and provide an optimal microenvironment for allogeneic spermatozoa storage, capacitation, fertilization, and semi-allogeneic early embryonic development via inhibition of pro-inflammatory response without blocking the effective immune responses against pathogens (Hunter 2012). Therefore, immune regulation in oviduct is equally essential both for the maintenance of physiological functions and prevention of ascending infections from the uterus. Estradiol, progesterone, and luteinizing hormone (LH) have roles to maintain immunologic homeostasis of the oviduct (Pineda 2003). The previous reports indicated that TLRs may play an essential role in oviductal innate immunity. Expressions of TLRs were described in cow, mouse, rabbit, and hen oviduct cells (Ozoe et al. 2009; Shimada et al. 2008). TLRs have been shown to play roles in the reproductive processes and immune defense of oviduct (Kowsar et al. 2013; Shimada et al. 2008). Results from Ozoe et al. (2009) suggest that the hen oviduct cell express at least 6 types of TLRs and among those, TLR4 had a greater expression response to LPS and in turn upregulates cellular functions to synthesize cytokines. In mice, Shimada et al. (2008) indicated that the blocking TLR2/TLR4 with antibodies in cumulus-oocyte complex in oviduct reduces sperm capacitation and fertilization. Similarly, the bovine oviduct epithelium expresses TLR2/TLR4 and their expression levels are regulated by ovarian hormones and luteinizing hormone (LH) (Kowsar et al. 2013). They also demonstrated that a low dose of LPS induces pro-inflammatory status by stimulation of pathway components (TLR-4, Nuclear Factor kappa B (NF-κB),

Cyclooxygenase (COX)-2, Interleukin (IL) 1 beta (IL1-b), and TNF-a), but higher LPS dose switches over toward anti-inflammatory status by stimulation of the components of another pathway (TLR-2, IL-10, and IL-4). Moreover, in bovine oviduct cells, Morillo et al. (2020) indicated that TLR2-mediated anti-inflammatory response could serve to protect capacitated sperm which fertilizes oocyte in the ampulla. In murine, it was reported that TLR2/TLR3 mediated cytokines play roles in infection-induced fallopian tube scarring which is an important problem for fertility (Derbigny et al. 2005).

2.3 Uterine Cells

There are three important cell types including stromal, glandular, and epithelial cells in the endometrium, which is the most dynamic layer of the uterus. The endometrial cells have dual functions: to protect from foreign pathogens and to allow a suitable environment for the growing allograft embryo/fetus until birth. Therefore, endometrium has a very well-organized immune system regulation and detailed regulation of the immune components in the uterus has been the subject of many researches (Taylor and Gomel 2008). Among those, TLR system is described as the first line defender to protect uterus against invading pathogens. Expression and localization of TLRs in all endometrial cells as being physiological regulator or defender against infection agents/components were reported in bovine, equine, rabbit, ovine, and canine (Kannaki et al. 2011). Recent research has provided great insight into TLR signaling during both physiological (Fig. 3) and pathological events such as pregnancy recognition, endometritis, etc. in domestic animal uterus.

2.3.1 TLRs in Endometrial Physiology

Following the understanding of endogenous ligands for TLRs, more recent studies have focused on the physiological roles of TLRs in the endometrium such as ovary-driven hormonal regulation of the estrous cycle, maternal recognition of pregnancy, and sperm–endometrium interaction. Moreover, by enlightening the roles of TLRs in endometrial physiology, these studies allow us to better understand organization of immune regulation of the endometrium including capacitation of spermatozoa, fertilization, the maternal recognition of pregnancy, and the protection of semi-allograft fetus from the maternal immune system. The presence of TLRs in the endometrium which is influenced by ovarian hormones or embryonic secretions is demonstrated in many domestic animal species including ruminant, pig, dog, and horse. Ruiz-Gonzalez et al. (2015) point to TLR expression profiles detected in both uterine epithelia and stroma as well as differential abundance of ovine TLR1-TLR9 due to reproductive statuses such as the day of the estrous cycle (TLR2, TLR3, TLR7, and TLR8) and day of pregnancy (TLR1-TLR3, TLR5-TLR7, and TLR9) in ewes. Like those, it is clearly indicated that TLRs especially TLR4 in the equine endometrium is differentially regulated under the influence of both ovarian steroids and early pregnancy (Atli et al. 2010; Atli and Kose 2019). In dogs, TLRs including TLR1–TLR7 and TLR9 are detected in the endometrium during the estrous cycle

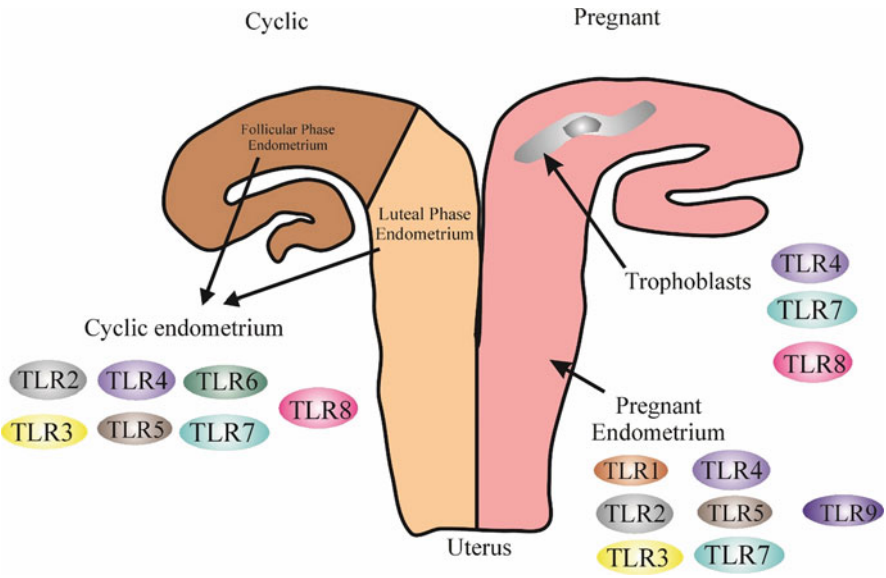

Fig. 3 Schematic model for the differently regulated TLRs in uterine physiological events such as cyclic endometrium including both follicular and luteal phases (Atli et al. 2010; Atli and Kose 2019; Ruiz-Gonzalez et al. 2015; Silva et al. 2010, 2012; Yoo et al. 2019); pregnant endometrium (Ansari et al. 2015; Atli et al. 2010; Ezz et al. 2019; Ruiz-Gonzalez et al. 2015); and trophoblast cells (Kaya et al. 2017; Ruiz-González et al. 2015) in different domestic animals

and specifically, the expression profiles of TLR2 and 4 change at both follicular and luteal phases (Silva et al. 2012). In pigs, all TLRs (TLR1-TLR10) and their intracellular component, MYD88, are expressed in the endometrium in both the estrous cycle and pregnancy. In addition, it was also reported that progesterone induces endometrial expressions of TLR4, TLR5, TLR6, TLR7, and TLR8 (Yoo et al. 2019). Differences in expression of TLRs due to influences by endogenous secretions may be in favor of the establishment of pregnancy but may also be linked to the occurrence of endometrial diseases. Therefore, further studies should focus on those relationships and their balance.

More descriptive and excellent studies about TLR signaling in maternal recognition of pregnancy have been published by Drs. Spencer and Bazer in ruminants. These researchers emphasize that secretion of embryonic interferon tau (IFNT) is regulated by communications among exosomes, ovine endogenous Jaagsiekte retroviruses (enJSRV), and TLR signaling. Their study indicates that there is an increase in TLR signaling mediator expressions including CD14, CD68, Interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor (TRAF6), Interferon Regulatory Factor 6 (IRF6), and IRF7 in trophectoderm cells during the early pregnancy. Moreover, when synthesis of TLR7 and TLR8 proteins was blocked by morpholino antisense oligonucleotides (MAOs) injected into the uterine lumen, developmentally retarded embryos with fewer binucleated cells (BNCs) that

produce less interferon tau (IFNT) are observed (Burns et al. 2016; Ruiz-González et al. 2015; Ruiz-Gonzalez et al. 2015). Similar to those, recent studies have indicated that TLR signaling not only regulates the local environment of the uterus, but also reaches in peripheral blood leucocyte, thymus, lymph node, liver, etc. during early pregnancy (Kaya et al. 2017; Kurar et al. 2012; Gao et al. 2021; Li et al. 2020a; Wu et al. 2021; Zhang et al. 2021). In guide of these facts, thorough understanding of the mechanism of innate immunity under the perspective of TLR signaling may also contribute to prevention of early embryonic loss in domestic animals.

In addition, within the perspective of sperm and endometrium relation, a recent paper (Ezz et al. 2019) showed that sperm attachment to bovine endometrial epithelial cells (BEECs) triggers uterine local innate immunity by regulating TLR2/4 signal transduction on downstream targets (p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinases (JNKs)). These observations suggest that there should be a correct TLR signaling between sperm and endometrial cells before the fertilization. Moreover, these results clearly indicate that the TLR signaling in the uterus plays a critical role in both the selection and capacitation of sperm.

2.3.2 TLRs in Endometrial Pathology

TLRs play critical roles in innate immunity by regulating antimicrobial responses in endometrial tissue. A detailed study by Davies et al. (2008) clearly indicates that bovine endometrium expresses all TLRs 1 to 10 (epithelial cells express TLRs 1 to 7 and 9, stromal cells express TLRs 1 to 4, 6, 7, 9, and 10). This study also shows that TLRs have roles in epithelial cells secretion of prostaglandin E2 in response to bacterial PAMPs (Davies et al. 2008). Moreover, a strong correlation between TLRs expression pattern, specifically TLR4, and postpartum uterine infections is also pointed by Herath et al. (2009). On the other hand, comprehensive analysis of TLR signaling in both epithelial and stromal cells of endometrium in *E.coli*-induced bovine endometritis has just been completed (Ding et al. 2020; Li et al. 2020b). Li et al. (2020b) emphasized that TLR2/4-MyD88/p38 MAPK promotes Prostaglandin E2 (PGE2) synthesis in *E. coli*-infected endometrial tissue damage. This report draws attention that there is a detrimental effect of TLR2/4 signaling on the endometrial epithelial cells which increases endometrial tissue damage by inducing PGE2 synthesis. Moreover, Ding et al. (2020) point that LPS also induces a strong TLR signaling pathway in endometrial stromal cells which causes postpartum persistent endometritis when the epithelial cells are disrupted.

Endometritis-regulated TLR expression was also reported in the other domestic animals such as equine, canine, rabbit besides bovine endometritis models. In the equine post-breeding endometritis model, roles of TLR2 and TLR4 have been investigated after *E.coli* inoculation into the healthy endometrium. This study revealed that expression of TLR4 was elevated at 3 h, but TLR2 remained constant in 12 h after *E.coli* inoculation (Marth et al. 2016). These results indicate critical roles for TLR signaling in post-breeding endometritis in equine endometrium. In canine, Silva et al. (2010) indicated that there is a detrimental role for TLR2 and TLR4 by inducing prostaglandin synthesis pathway, especially at the level of

COX-2 in cystic endometrial hyperplasia–pyometra complex. Similar to this report, Chen et al. (2014) emphasized that intravenous LPS challenge upregulates the expression of TLR2 and 4 in uterine body and horn in rabbit. All these results clearly show the roles of TLRs in microorganism toxins-induced endometritis in domestic animal species. As a result of TLR signaling in endometritis, the evaluation of tissue secretions including prostaglandins, chemokines, and other pro-inflammatory products may be used to understand underlying mechanisms of the disease, to make a differential diagnosis, and to follow the course of the disease.

2.4 Cervical Cells

The cervix is covered by cervical epithelial cells and divides into two parts: (1) ectocervix is proximal face of the vagina, (2) the endocervix is distal to the uterus and should be sterile. Like the uterus, the cervix has unique immunological features. It should give an immune response essential for protection against infections and allow an immunological tolerance to commensal microorganisms, allogeneic sperm, as well as the semi-allogeneic fetuses. Dysregulation of these immune responses can lead to ascending infections, including metritis, and preterm labor (De Tomasi et al. 2019). Previous studies indicate the presence of TLRs in human cervical tissue; however, the estrus cycle, pregnancy, and inflammation-related characteristics of TLRs are still poorly understood in domestic animals. In an in vitro study, it was indicated that estradiol activated TLR2 and TLR4 signaling pathways which altered the cytokine responses of human cervical cells (Lashkari and Anumba 2017). Similarly, another study in humans reported that cervical epithelial cells expressed mRNA of TLR3, TLR9, and TLR7, but had only a weak signal for TLR8 which suggested that these receptors could play a role in regulating the pro-inflammatory cytokine and antiviral environment of the lower female reproductive tract (Andersen et al. 2006). Moreover, Peng et al. (2020) indicated that cervical remodeling and preterm labor were also regulated by TLR4/NF-κB pathway (Peng et al. 2020). During the whole duration of pregnancy, mice cervix was evaluated and they demonstrated the presence of TLR2, TLR3, TLR4, and TLR9 expression in cervix and there was an upregulation of TLR2, 3, and 4 at the later stages of pregnancy (Gonzalez et al. 2007). According to these results, we could assume that TLRs may have similar roles with humans in domestic animal cervical epithelial cells, however, that warrants further research.

2.5 Vaginal Epithelial Cells

The vaginal mucosa, mainly vaginal epithelial cells, is exposed to microorganisms from the external environment, especially at mating and delivery. Localization of the vagina, adjacent to anus, also allows for external environmental contaminations. Moreover, ascending infections from the vagina during the pregnancy is the most common route of uterine infection leading to preterm birth. Therefore, the immune

system of the vagina should be well developed (Takeuchi et al. 2013). Roles of TLR to protect against bacterial and viral pathogens are described in human and murine studies. Soboll et al. (2006) indicate that mouse vaginal tissue expresses TLR1–9 mRNA, with TLR4 and 5 being the most highly expressed TLRs in the vagina. On the contrary, Fazeli et al. (2005) showed that TLR4 expression was absent in human vagina tissue. Although this difference for TLR4 might be due to differences in species or techniques, these studies clearly indicate the presence of TLRs in vaginal epithelial cells. This was also supported with the use of TLR agonists that induce transcriptional and translational expression of pro-inflammatory cytokines in vaginal cells (Joseph et al. 2012). Although their roles were emphasized in vaginal health status for transporting environmental pathogen to the upper reproductive tract which causes postpartum metritis and breeding-induced endometritis or endometrial hyperplasia–pyometra complex in domestic animals (Genís et al. 2017), as in the case of the cervix, no detailed studies are present that evaluate TLR expressions in the vaginal cells.

2.6 Trophoblast Cells

The trophoblast, a part of the embryo, could be described as the most dynamic part among the reproductive tract/organ cells. It differentiates into specific cells to generate an effective placental barrier between mother and fetus. Considering semi-allogeneic fetus in the uterus and the potential of maternal immunity, immunological features of trophoblast should be more sophisticated (Roberts et al. 1999). Many studies have reported that trophoblasts are able to recognize and respond to both viral and bacterial pathogens through TLR signaling. Previous reports (Holmlund et al. 2002; Svinarich et al. 1996) indicated that challenge with zymosan or LPS of the trophoblast caused an increase in IL-6, IL-8, nitric oxide, and granulocyte colony stimulating factor which have potent antimicrobial properties by activation of TLR-2 and TLR-4 signaling, respectively. In mare, Schöniger et al. (2018) pointed to the expression of TLR 2, 4, and 6 in trophoblasts and allantois epithelium. Similarly, cellular localization of TLRs (not TLR1, TLR9) in embryonic trophoblast was reported by Kaya et al. (2017). Among expressed TLRs, a limited number of trophoblasts express TLR4 which indicates a unique expression profile for ovine trophoblast. In addition, Ansari et al. (2015) showed that trophoblast cells expressed TLR4 to modulate immune tolerance in pig uterus during pregnancy. According to these results, based on the well described roles of TLR signaling in human studies (Abrahams et al. 2005; Koga et al. 2009), we could suggest that TLR signaling in the trophoblast in domestic animals not only regulates the maternal immune system in favor of pregnancy, but also protects it from pathogens.

3 Roles of TLR in Male Reproductive Cells

The mammalian testis has a special immune environment due to its exceptional immune privilege and effective local innate immune properties. This specialized immune environment of the testis protects the germ cells from the harmful effects of the systemic immune system. Breakdown of immune homeostasis in the testis may give rise to orchitis, an etiological factor of male infertility. The mechanisms underlying this privilege have been examined for a long time. Increasing evidence indicates that both a systemic immune tolerance and local immunosuppressive milieu are participated in maintaining the immune privilege of the testes (Zhao et al. 2014).

Mammalian testes are composed of two compartments: the seminiferous tubule compartment and the interstitial compartment. The former is further divided into three areas: the convoluted seminiferous tubule, which is lined with germ cells that give rise to spermatozoa, the intermediate region, which is lined by tall Sertoli cells developing a prominent valve structure that controls the flow of intratubular fluid, and the straight seminiferous tubule, which has an epithelial lining that consists of low cuboidal epithelium connecting to the rete testis. The interstitial region contains Leydig cells, lymphatic and blood vessels, nerves. Leydig cells are the major source of androgens or testosterone in males. The interstitial compartment also has macrophages, which may be involved in the differentiation of spermatogonial stem cells (DeFalco et al. 2015; Leeson and Cookson 1974; Mori 1980).

3.1 Sertoli Cells

When fully differentiated, the Sertoli cell is an irregularly shaped, columnar cell, which extends from the basement membrane of the seminiferous epithelium to the tubular lumen. The Sertoli cells are adherent to the basal lamina, which is consisted of an extracellular matrix that serves to separate them from the interstitial compartment (such as heparan sulfate, collagen, and laminin) that maintains the structural integrity of the seminiferous tubules. The Sertoli cell is the key somatic cell playing an essential role in the modulation of spermatogenesis and for the establishment of the rate of sperm production in sexually mature animals. The main functions of these cells are (1) providing nutritional and structural support to the developing germ cells; (2) phagocytosis of degenerating germ cells and residual bodies; (3) formation of the blood–testis barrier; (4) establishment of a localized immune-privileged environment; (5) generation and release of regulatory factors (Foley 2001; Johnson et al. 2008; Walker and Cheng 2005).

3.1.1 TLRs in Sertoli Cells

Several TLRs are expressed in Sertoli cells and have important physiological functions in the testicular immune system. Of these, TLR2 and TLR4 have been observed in mouse Sertoli cells and have been reported to be activated by their specific ligands (Riccioli et al. 2006). Rat Sertoli cells also express TLR4 (Özbek

et al. 2020). TLR2–TLR6 trigger innate immune responses in mouse Sertoli cells and induce the expression of type 1 IFNs and major pro-inflammatory cytokines. In addition, after stimulation of mouse Sertoli cells with TLR2-TLR5 ligands (Fig. 4), they produce inflammatory cytokines such as IL-1α, IL-6, and interferon-α, and -β (Wu et al. 2008). Some studies suggest that nucleic acid sensors trigger the innate immune responses in Sertoli cells because Sertoli cells constitutively have certain levels of the cytosolic DNA and RNA sensors (Zhu et al. 2013). TLR3 stimulates innate antiviral responses in mouse Sertoli cells after inducing with polyinosinic-polycytidylic acid (poly (I:C)), a synthetic dsRNA analog. In addition, activation of TLR3 by poly (I:C), particularly supported phagocytosis of apoptotic germ cells by Sertoli cells. This phagocytosis is a carefully regulated process involving the upregulation of scavenger receptors (Starace et al. 2008; Wu et al. 2008).

Necrotic and apoptotic spermatogenic cells that release heat shock proteins (HSP) and high mobility group box 1 (HMGB1) stimulate an innate immune reaction in the cells of Sertoli, causing testicular inflammation and dysfunction (Nistal et al. 2002). Phagocytosis of testicular defective germ cells by Sertoli cells has the ability to induce autoimmune responses against autoantigens of spermatogenic cells, resulting in the development of autoimmune orchitis (Pelletier et al. 2009). The development of autoimmune orchitis involves the generation of TLR-imitated innate immune responses through endogenous ligands from damaged germ cells. This immune response initiated by TLR is also modulated negatively by the Growth arrest-specific 6 (Gas6)/Tyro3, Axl, and Mertk (TAM) system (Sun et al. 2010). The TAM recipient, knockout mice increase TLR3 and TLR4 stimulating in Sertoli cells, overexpressing pro-inflammatory cytokines and type-1 IFNs despite problems with TLR ligands. On the contrary, Gas6 inhibits the TLR-mediated cytokine secretion. TLR stimulation is suppressed by the Gas6/TAM system because both SOCS1 and SOCS3 have the ability to suppress TLR signaling by triggering cytokine signaling-1 and -3 (SOCS1 and SOCS3) suppressors (Liew et al. 2005). A wide range of microorganisms is able to stimulate multiple TLRs in Sertoli cells to generate high levels of pro-inflammatory cytokines (Dejucq and Jégou 2001).

3.2 Leydig Cells

Leydig cells account for more than 75% of testicular interstitial cells. The major function of Leydig cells is the generation of androgens, mostly testosterone. Androgens play a vital role in normal spermatogenesis and also function in multiple organs besides the testis. Leydig cells also are involved in modulating the testicular immunity and the innate defense against viral infection. Leydig cells were first characterized in 1850 by Franz Leydig as testicular interstitial cells containing large, round lipid droplets. Electron microscopic studies showed that these cells also have abundant smooth endoplasmic reticulum, crystals of Reinke, and mitochondria with tubular cristae. In mammals, at least two types of Leydig cells, adult Leydig cells, and fetal Leydig cells, sequentially develop in the adult and fetal testis, respectively. Fetal and adult Leydig cells are different in ultrastructure,

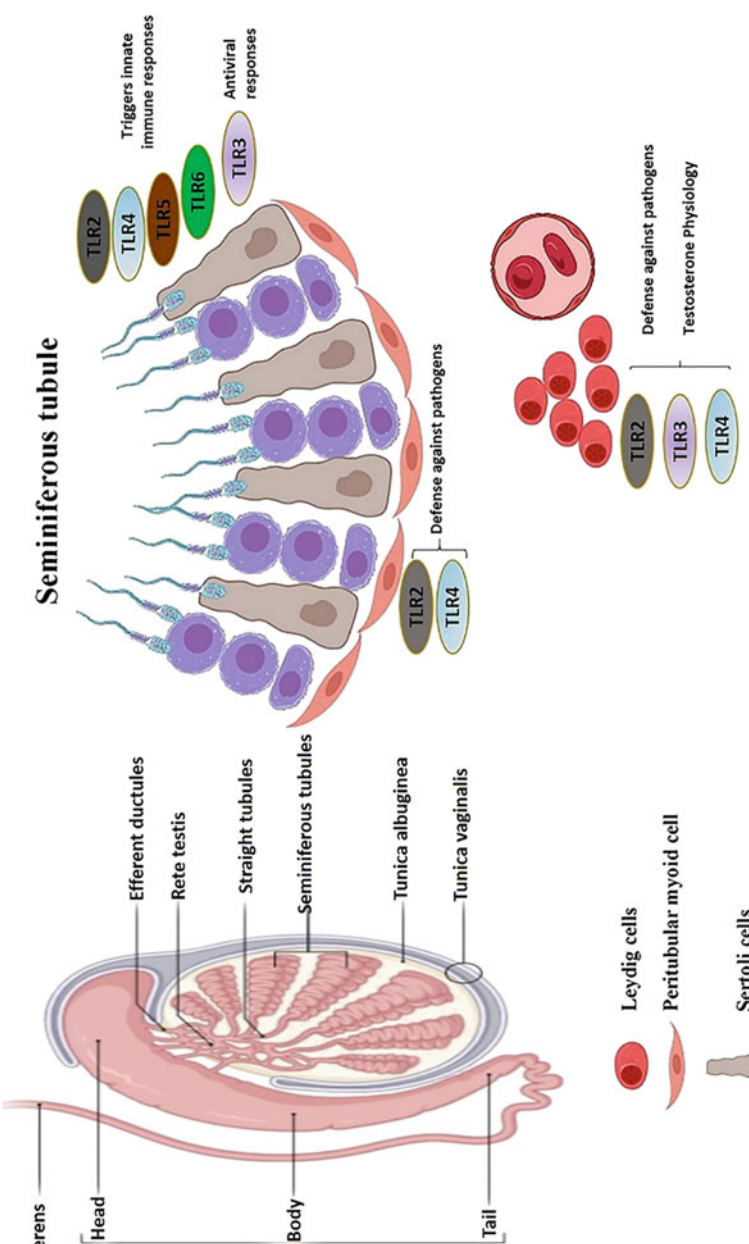

Fig. 4 Schematic of critical TLRs involved in testicular defense systems and testosterone production. TLR2, TLR3, TLR4, TLR5, and TLR6 are expressed in Sertoli cells and have an important role in antiviral and innate immune responses (Özbek et al. 2020; Riccioli et al. 2006; Wu et al. 2008). TLR2 and TLR4 are expressed in both Leydig cell and peritubular myoid cell, they recognize and defend against pathogens (Mayer et al. 2016; Özbek et al. 2020; Schaefer et al. 2005; Shang et al. 2011). TLR-induced cytokines affect testosterone production in Leydig cells (Ding et al. 2016; Samir et al. 2017; Shang et al. 2011)

structure, topography, capacity for androgen synthesis, life span, response to antiandrogens, and mechanism of modulation by pituitary growth factors and gonadotropins (Habert et al. 2001; Sengupta et al. 2020; Shima 2019).

3.2.1 TLRs in Leydig Cells

Leydig cells, like the other testicular cells, are involved in the testicular antiviral defense system. They constitutively express interferon (IFN)-inducible antiviral proteins after challenge with viral antigens. In particular, mouse and rat Leydig cells show stronger antiviral immune responses than human Leydig cells (Le Tortorec et al. 2008). This may explain why broad-spectrum various viruses can infect and cause pathological conditions in the human testis, whereas it does not occur naturally in the mouse testis. Furthermore, testicular damage in wild-type mice with experimentally induced viruses has not been successful. Studies indicated that Leydig cells have various PRRs that detect viruses and trigger innate antiviral immune responses (Theam et al. 2020). Functional TLR2, TLR3, and TLR4 have abundantly been demonstrated in mouse Leydig cells (Shang et al. 2011). Rat Leydig cells also express TLR4 (Özbek et al. 2020). Poly (I:C) and LPS can activate TLR3 and TLR4 in Leydig cells respectively, thereby stimulating the expression of type 1 IFNs and major pro-inflammatory cytokines. In particular, activation of TLR3 and TLR4 inhibits androgen production in Leydig cells (Fig. 4), suggesting that TLR-induced innate immune responses perturb testicular physiology via alteration in testicular testosterone concentration (Shang et al. 2011). Decreased testosterone levels should be caused by the TLR-induced high level of IL-6 and TNF-α because these cytokines suppress testosterone production (Ding et al. 2016; Samir et al. 2017). In particular, TAM receptors negatively modulate TLR-induced innate immune signaling pathways in Leydig cells (Rothlin et al. 2015). Both TAM receptors and their ligand Gas6 are highly found in the mouse testis (Wang et al. 2005). Gas6 is only expressed in Leydig cells, whereas TAM receptors are expressed in Sertoli and Leydig cells. Studies revealed that TAM receptors knockout mice develop autoimmune orchitis. This evidence supports that the Gas6/TAM signaling pathway is pivotal for maintaining the immune privilege status of mouse testis (Zhang et al., 2013). The mechanisms underlying the role of the Gas6/TAM signaling in maintaining testicular immune privilege status can be explained (1) Gas6 eases the phagocytic removal of apoptotic spermatogenic cells via the activation of TAM receptors preventing the release of spermatogenic cell antigens (Xiong et al. 2008); (2) TAM receptors promoted central immune tolerance to autoantigens of germ cell because Mer and Axl knockout mice are prone to autoimmune orchitis (Li et al. 2015); and (3) the suppression of innate immune responses by the Gas6/TAM signaling to promote testicular immune privilege status (Izuka et al. 2020).

3.3 Myoid Peritubular Cells

Myoid peritubular cells (MPCs) surround the seminiferous tubules and establish a wall promoting the tubule's integrity. MPCs comprise contractile elements that

facilitate the transport of the immotile spermatozoa into the epididymis (Maekawa et al. 1996). MPCs synthase the components of the basal lamina. In rodents, only a single layer of MPCs surrounds the tubular wall, whereas several layers of MPCs construct the walls of the seminiferous tubules in humans. The role of MPCs in modulating spermatogenesis is to a great extent unexplored. Previous research has revealed that MPCs are able to modulate spermatogenesis and testis development through secreted biological factors (Verhoeven et al. 2000). In addition, MPCs are reported to express androgen receptors and mediate androgen actions on fetal Sertoli cell proliferation (Scott et al. 2007). Based on their structure and localization, MPCs are considered to be involved in the maintenance of the testicular immune privilege status. A function of MPCs in testicular inflammation has emerged, particularly in experimental autoimmune orchitis. MPCs secrete several cytokines, including leukemia inhibitory factors, MCP-1, and transforming growth factor β-2 (TGFβ-2). TNF-α receptors 1 and 2 also are observed in human MPCs (Schell et al. 2008).

3.3.1 TLRs in Myoid Peritubular Cells

TLRs were expressed in human myoid peritubular cells (Mayer et al. 2016). It became evident that ligands like lipopolysaccharide (LPS) or Pam3CysSerLys4 (PAM) can activate TLR2/4 on myoid peritubular cells (Fig. 4). Moreover, TLR2/4 was also targeted by the small ECM molecule biglycan in the same way as previously expressed in macrophages (Schaefer et al. 2005). Biglycan-induced TLR signaling stimulated an immune response including pro-inflammatory cytokine generation and releasing (Schaefer et al. 2005; Zeng-Brouwers et al. 2014). Furthermore, some studies indicated that human myoid peritubular cells express purinergic receptors (P2RX4 and P2RX7), which are functionally linked to TLRs, with P2RX4 being the prevalent ATP-gated ion channel (Walenta et al. 2018).

3.4 Sperm Cells

Fertilization can be achieved by a healthy spermatozoon, and the life span of spermatozoa relies on environmental conditions. Although the mature sperm cell is presumed to lack endocytosis (Gadella and Evans 2011) and thereby is not related to establishing an endosomal response to pathogens, recently, some studies have reported that sperm can exhibit immune cell-like functions. In this regard, some family members of the receptor are reported to be expressed on sperm, and upon activation, sperm establish a chemotaxis response to attain the ovulated oocyte throughout the fertilization process (Shimada et al. 2008).

Regarding the exposure of sperm to microbes during testicular development, and following maturation, transit, and storage in the epididymis (Hinton et al. 1996), results in negative effects on sperm function such as motility and acrosome reaction, and ultrastructural damage (Diemer et al. 2003; El-Mulla et al. 1996). Accordingly, it is urgent for spermatozoa to have a functional defense to keep spermatozoa protected from pathogens throughout production and storage in the male as well as in the

female reproductive tract after ejaculation. However, the functional role of toll-like receptors in male germ cells in spermatozoa has remained elusive.

3.4.1 TLRs in Sperm Cells

Since each part of the male reproductive tract has a certain role in the production, storage, or emission of spermatozoa and also varies in its exposure to pathogenic organisms, steady-state expression of TLRs (1-10) in all parts of the reproductive tract may equally provide defense of spermatozoa against microbial attack (Saeidi et al. 2014). Also, despite the fact that the innate immune function relies on immune cells, TLR4 and TLR2 are demonstrated to be expressed on sperm (Fig. 5), and specifically recognizes bacterial factors (such as lipopolysaccharide and lipopeptide) (Okazaki 2009). The ejaculated spermatozoa of chicken express TLR-2, TLR-5, TLR-7, TLR-15, and TLR-21 that avian sperm may offer the ability of the innate host defense because spermatozoa itself survives in the sperm storage over a long time. This is consistent with that of chicken sperm which two species also express TLR2, TLR3, TLR4, TLR5, and TLR15 (Das et al. 2011; Kannaki et al. 2017), this may explain the involvement of innate immune receptors for the recognition of various pathogens in spermatozoa, including a gram-positive (TLR2), gram-negative bacteria (LPS by TLR4), and viruses (dsRNA by TLR3). In regard to viral response, TLR3 is shown to be expressed in spermatogonia and spermatocytes that activation by its ligand may mediate antiviral responses (Wang et al. 2012). Moreover, TLR9 has individually detected in the acrosomal region of mice spermatozoa (Fig. 5) and has the ability to recognize viral pathogens (Mihara et al. 2010). Confounding with the current result, the presence of TLR9 is shown to be localized in post acrosomal region and be part of defensive response with transmembrane glycoprotein (Aitken et al. 2020).

In rats, more specifically some TLRs were found to be expressed in testicular sperm whereas other TLRs demonstrated regional localization on epididymal spermatozoa during epididymal transit although TLR9 was found on cauda sperm. Therefore, TLRs may ensure protection in the epithelial lining of the epididymis and spermatozoa (Palladino et al. 2008). Spermatozoa of male cats have also TLR

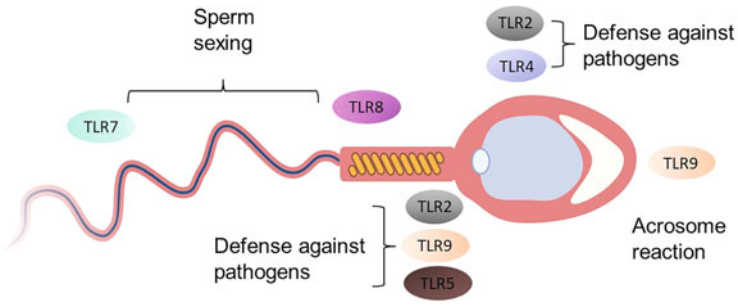

Fig. 5 Putative role model for the critical TLRs expressed in sperm cell involved in defense against pathogens (Akthar et al. 2020; Fujita et al. 2011; Mihara et al. 2010; Saeidi et al. 2014) acrosome reaction (Sahnoun et al. 2017) and sperm sexing (Ren et al. 2021; Umehara et al. 2019, 2020)

protein expression during their transit through the epididymis that principal piece of the spermatozoa in the epididymal segments indicated positive immunoreactivity for the TLR2, 4, 5, and 9 proteins, and in the caput epididymis, TLR2, TLR5, and TLR9 exhibited intense or average immunoreactivity in the neck region of the spermatozoa (Liman et al. 2019). We demonstrated the dynamic immunostaining of TLR4 in spermatogenic cells. In similar line with that of transition, although we did not detect TLR4 expression in both gonocytes and spermatogonia, it appears to be weakly expressed in spermatocytes on postnatal days 20, 50, and 70, and steadily increase in spermatids and spermatozoon on days 50 and 70 compared to 5 and 20 in pubertal days (Özbek et al. 2020). Taken into consideration the role of the epididymis in sperm maturation and storage, the results may imply the idea that spermatozoa are preserved during their functional life.

It is shown that TLRs are associated with male infertility (Fig. 5); TLR3 is constitutively expressed by germ cells that activation of TLR3 leads to upregulation of pro-inflammatory cytokines and tumor necrosis factor-α, through activation of nuclear factor-κB. Given that TLR3 may impair male germ cell homeostasis, TLR3 stimulation by immunostimulant caused apoptosis of mouse spermatogonial stem cell (SSC). This is concurrent with the previous finding that the endotoxin-induced TLR expression pathway in human sperm induces apoptosis and diminishes motility (Fujita et al. 2011), thus undermining fertilization. It is also proposed that induction of TLR expression in spermatozoa may lead to the production of ROS and acrosome reaction while also disruption of TLRs expression is linked with unexplained recurrent spontaneous abortion (Sahnoun et al. 2017).

In fact, in somatic cells, TLR7/8 is essentially a part of the innate immune system engaged in recognizing and fighting viral infections (Heil et al. 2004). It was assumed that TLR7/8 can be activated in response to viral infection either of the female or male reproductive tracts. In a recent study, the role TLR7/8 was speculated to slow down sperm motility (Umehara et al. 2019) that may be subjected to viruses throughout passage via male or female reproductive tracts. In this regard, ligand activation of TLR7/8 impedes the mobility of the X chromosome carrying sperm but not Y sperm, thus enabling the sexing of sperm. This has recently corroborated with that in sexing of goat spermatozoa which the X chromosome encoded TLR7/8 affects motility regulating ATP levels and mitochondrial activity (Ren et al. 2021). However, when two datasets of RNA sequencing from mouse spermatogenesis were analyzed (Soumillon et al. 2013; Zuo et al. 2016), the transcripts of Tlr7/8 are shown to be at low levels regardless of the developmental stage of a sperm cell (pre-meiotic to post-meiotic) (Navarro-Costa et al. 2020). Considering that TLRs accomplish intracellular signaling responses that induce biological roles, activation of TLR in sperm results in dysfunctional sperm mitochondria and impaired motility through classical myeloid differentiation factor 88 (MyD88)-dependent pathway (Zhu et al. 2016b).

Although the limited number of studies which mostly based on expression levels and localization examines the functional role of TLR in sperm cells, it can be speculated that TLRs may provide potential protection against invading pathogens.

4 TLR Polymorphism and Reproduction in Domestic Animals

Polymorphism in TLR genes and their association with disease of economic importance such as repeat breeder and mastitis have been established. In future these polymorphisms could be used as molecular markers for selecting animals in the development of immune-genetically superior stocks.

General health status of livestock is a major issue for an optimum reproduction as infectious diseases negatively affect fertility parameters (Gonda et al. 2007; Kostoulas et al. 2006; Ozsvari et al. 2020). There are some relationships between polymorphism of TLR genes and mastitis in sheep and cattle (Zhang et al. 2009). More recent studies have also indicated that certain polymorphisms in TLR2 gene can affect milk somatic cell count in goats (Ogorevc et al. 2019; Ruiz-Rodriguez et al. 2017). Cows in large Hungarian dairy herds with positive ELISA test results for Mycobacterium avium subsp. paratuberculosis (MAP) had a 23.2 longer service period and 33.8 day longer calving interval compared to ELISA negative cows on average (Ozsvari et al. 2020). Infectious diseases not only decline fertility but also cause economic losses due to costs arising from treatment or eradication programs (Kirkeby et al. 2016). Therefore, breeding livestock resistant to certain infectious diseases would contribute to enhance reproduction ability and reduce treatment costs.

Certain mutations in TLR1 gene (Ser150Gly and Val220Met) and TLR2 (Phe670Leu) have been associated with a hyposensitivity of mononuclear dendritic cells to MAP ligands (Bhide et al. 2009). Koets et al. (2010) estimated for the SNP TLR2 1903 T > C an odd ratio of 1.7 among MAP-infected cows with CT and CC genotypes compared to those with TT genotypes. In accordance with these findings they observed that upon stimulation with MAP monocytes from TT animals showed a 17- and 8.9-fold increase of IL1b and IL12p40 transcripts, respectively, compared to monocytes of TC genotype. Fisher et al. (2011) detected 244 SNPs on TLR genes 1–10 by next generation sequencing. Using a case–control study six of these SNPs showed suggestive associations. Two of these SNPs remained significant even after locus-specific correction. Mutations in the TLR genes may lead to altered recognition of pathogen-associated molecular structures.

More direct studies have also been performed to investigate the association between the TLR polymorphism and reproductive parameters. Shimizu et al. (2017) examined the association of an A-G polymorphism the 4525th nucleotide in intron 1 and a T-C polymorphism at the 1397th nucleotide in exon 3 of TLR4 gene with several reproductive parameters in Holstein cows. The number of artificial insemination in the animals with the T/C genotype in the TLR4 exon3 was found to be lower than that in animals with the C/C genotype (1.6 ± 0.2 and 2.2 ± 0.2, respectively). The days open in the animals with TLR4 exon3 polymorphisms were shorter for the T/C cows compared to those for the C/C genotype (100.7 ± 6.9 and 136.6 ± 9.0 days). Jecminkova et al. (2018) observed Czech Fleckvieh cows with GC genotype for TLR4 226C > G showed a shorter calving interval compared to homozygote ones. Bjelka and Novák (2020) examined TLR1, TLR2, and TLR6 genes for polymorphism in Czech Red Pied cattle population and detected 16 single

nucleotide polymorphisms in total. They observed relationships between certain variants of TLR genes and some reproductive and fitness traits. Maternal calving ease was associated with TLR1 798C>T, TLR2 1044 T>C and TLR6 990 G>A, while TLR2 1313 G>A TLR6 865 G>C and 990G>A polymorphism were associated with calving ease. Production longevity was associated with TLR1 798C>T, and TLR1 1762 G>A, while TLR2 1044 T>C was associated with calf vitality index. Similarly, El-Domany et al. (2019) investigated the association between AluI polymorphism at the third exon of TLR4 and some fertility parameters in Holstein cows raised in Egypt. Among 400 cows they detected three genotypes of AA, AB, and BB which significantly affect reproductive parameters. They observed that the cows with BB genotype showed shorter age at first refreshening (AA: 28.61 ± 0.62; BB: 25.57 ± 0.96), calving interval (AA: 504.21 ± 6.94; BB:358.49 ± 5.60) and days open period (AA: 189.82 ± 10.16; BB: 156.08 ± 11.15). The cows with BB genotype needed a reduced number of services per conception (AA: 6.52 ± 0.10; BB:2.41 ± 014).

According to those results, we could suggest that investigation of TLR polymorphism and reproductive parameters in domestic animals is a promising subject in further studies to understand fertility and genetic polymorphism.

5 Concluding Remarks

This chapter has provided detailed information on the role of TLRs in reproduction of bovine, ovine, equine, canine, avian, and murine species. We now know that TLRs are not only involved in pathogens invasion but also play roles in critical processes of reproduction events such as oocyte/sperm interaction, follicular maturation, spermatozoa capacitation, ovulation, corpus luteum life cycle, early pregnancy establishment and maintenance, and labor. On the other side, some regulatory mechanisms of TLRs on reproductive events remain unclear and need to be evaluated in future studies. In addition, current studies clearly indicate that there is a relation between TLR gene polymorphism and reproduction. Therefore, the discovery of underlying mechanism of the TLR system in domestic animal reproduction might provide an optimal conception to research in reproductive physiology in the species studied.

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Toll-Like Receptors in Adaptive Immunity

Vijay Kumar

Contents

Abstract

The immune (innate and adaptive) system has evolved to protect the host from any danger present in the surrounding outer environment (microbes and associated MAMPs or PAMPs, xenobiotics, and allergens) and dangers originated within the host called danger or damage-associated molecular patterns (DAMPs) and recognizing and clearing the cells dying due to apoptosis. It also helps to lower the tissue damage during trauma and initiates the healing process. The pattern recognition receptors (PRRs) play a crucial role in recognizing

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_543

different PAMPs or MAMPs and DAMPs to initiate the pro-inflammatory immune response to clear them. Toll-like receptors (TLRs) are first recognized PRRs and their discovery proved milestone in the field of immunology as it filled the gap between the first recognition of the pathogen by the immune system and the initiation of the appropriate immune response required to clear the infection by innate immune cells (macrophages, neutrophils, dendritic cells or DCs, and mast cells). However, in addition to their expression by innate immune cells and controlling their function, TLRs are also expressed by adaptive immune cells. We have identified 10 TLRs (TLR1-TLR10) in humans and 12 TLRs (TLR1-TLR13) in laboratory mice till date as TLR10 in mice is present only as a defective pseudogene. The present chapter starts with the introduction of innate immunity, timing of TLR evolution, and the evolution of adaptive immune system and its receptors (T cell receptors or TCRs and B cell receptors or BCRs). The next section describes the role of TLRs in the innate immune function and signaling involved in the generation of inflammation. The subsequent sections describe the expression and function of different TLRs in murine and human adaptive immune cells (B cells and different types of T cells, including $CD4^+$T cells, $CD8^+$T cells, $CD4^+CD25^+T_{regs}$, and $CD8^+CD25^+T_{regs}$, etc.). The modulation of TLRs expressed on T and B cells has a great potential to develop different vaccine candidates, adjuvants, immunotherapies to target various microbial infections, including current COVID-19 pandemic, cancers, and autoimmune and autoinflammatory diseases.

Keywords

Adaptive immunity · B cells · Innate immunity · PRRs · T cells · TLRs

1 Introduction

The immune system is suggested to evolve to protect the host from the surrounding pathogens and other potential molecules external to the human body. However, the concept of damage/death or danger-associated molecular patterns (DAMPs) and process of apoptosis during embryonic development indicates that at least the innate immune system evolved to recognize molecules foreign to the human body as well as self-molecules (proteins, genetic material, etc.) that have lost their functions or cellular location. Recently, the innate immune system activation (epithelium-mediated phagocytosis of dead cells through phosphatidylserine (PS)-mediated target recognition) at blastula stage (the earliest stage of embryonic development) of zebra fish and mouse embryo has been explored (Hoijman et al. 2021). Thus activation of the innate immune system (phagocytosis) at this stage facilitates the error correction process and relevant to the robustness of the embryonic development and the survival of the embryo. Also, macrophage progenitors develop in the yolk sac during embryonic hematopoiesis and tissue macrophages (microglia, Kupffer cells, gut, heart, lung alveolar, peritoneal, skin, and splenic macrophages)

in many organs are of early embryonic origin according to fate map studies (McGrath et al. 2015; Ginhoux et al. 2010; Yona et al. 2013; Epelman et al. 2014; Bertrand et al. 2005; Theret et al. 2019). The yolk sac macrophage progenitors traffic to the embryo at designated time period (embryonic day 8.5 (E8.5)) to E14.5 during development through blood stream (Stremmel et al. 2018). This embryonic migration of macrophage progenitors peaks at E10.5 and decreases after E12.5 days. The details of embryonic development of macrophages and as an innate immunoregulatory cells have been described somewhere else (Epelman et al. 2014; Kumar 2019a). Hence, epithelial cells and macrophages help to clear the apoptotic cells generated during embryonic development. However, the recognition of these DAMPs and microbe or pathogen-associated molecular patterns (MAMPs or PAMPs) play a significant role in the generation of potent innate immune response and the clearance of apoptotic cells, pathogens, and other potential inflammogens. Toll-like receptors (TLRs) are one of several pattern recognition receptors (PRRs) taking part in this crucial process and the regulation of the innate immune response.

TLRs have evolved approximately 600 million years ago (MYA) in eumetazoan ancestors and the Toll gene was first identified in the *Drosophila melanogaster* or the common fruit fly, which plays a crucial role in the dorso-ventral patterning of the body during embryonic development (Anderson et al. 1985; Voogdt and van Putten 2016). Later studies showed its role in antifungal immune response in the *D. melanogaster* through the antimicrobial peptide (AMP) production (Lemaitre et al. 1996). On the other hand, the adaptive immune system has been shown to be evolved first in the jawless gnathostomes or agnathans as variable lymphocyte receptors (VLRs) that recognize antigens (Ags) similar to the B cell receptors (BCRs) and T cell receptors (TCRs) of gnathostomes (jawed vertebrates). The VLRs of jawless gnathostomes have highly diverse leucine-rich repeats (LRRs) sandwiched between amino and carboxy terminal LRRs (Pancer et al. 2004). However, the lymphocytes of jawless vertebrates serving as corresponding adaptive immune cells of jawed vertebrates having VLRs lack recombinatorial Ag receptors present in all jawed vertebrates. Thus, VLR gene rearrangement to generate diversity for an anticipatory immune system depends on diverse LRR cassettes available for insertion into an incomplete germline encode VLR gene (Pancer et al. 2004). Hence, individual lymphocyte from jawless vertebrate has uniquely rearranged VLR gene in a monoallelic manner. Further studies have shown the evolution of two different antigen recognition modes in jawless and jawed vertebrates through rearranged lymphocyte receptors (Alder et al. 2005). Hence, we can assume that the adaptive immune system has first developed in the jawless vertebrates that works differently from jawed, including mammalian adaptive immune system (Sutoh and Kasahara 2016).

The jawed vertebrates also produce other humoral factors called antibodies (Abs) from their B cells maturing into Ab secreting cells (ASCs) or plasma cells, which are absent in jawless vertebrates (Cooper and Alder 2006). However, a most recent study has indicated the presence of an ancient BCR-like molecule called CgIgR in the Pacific oyster (an invertebrate) *Crassostrea gigas* (*C. gigas*) that contains an immunoreceptor tyrosine-based activation motif (ITAM) in cytoplasmic tail (Sun

et al. 2020). The CgIgR recognizes many bacteria through its five extracellular domains and formed dimers that activated recruited CgSyk to promote CgERK phosphorylation (Sun et al. 2020). This event through inducing CgH3K4me2 produces Ig domain-containing proteins (CgICP-2 and CgLRRIG-1), which facilitate phagocytosis. Hence, BCR-like ancient molecules started to evolve in invertebrates and their activation produces Ig domain-containing proteins (CgICPs), which facilitate phagocytosis, like Ab-dependent phagocytosis among vertebrates (Tay et al. 2019; Kumar 2020a). Hence, the evolution of BCR-like molecules responsible for BCR-mediated Ab generation and associated response in jawed vertebrates started to evolve in invertebrates (molluscs). Of note, potential B and T cells of vertebrates forming adaptive immune memory have still not been identified in invertebrates. Hence, further studies are needed to solve the mystery of evolution of adaptive immunity. In every scenario, the major function of both innate immune PRRs, including TLRs and adaptive immune cell receptors (TCRs and BCRs) is to recognize Ags to protect the host from potential danger coming from outside or generated within irrespective of their evolutionary development.

TLRs are the first recognized PRRs in the animal kingdom, which are present both on the outer cell membrane and in the cytosolic organelles (endosome, endolysosomes, lysosomes, phagosomes, and phagolysosomes). The discovery of TLRs (TLR4) in humans in 1997 has filled the gap between the first entry of the pathogen into the animal host and its first recognition by the immune cells (Kumar 2018a; Medzhitov et al. 1997). The detailed pattern of TLR expression by different immune cells, their role in the innate immune response generation, and in the pathogenesis of inflammation are described somewhere else (Kumar 2018a; Takeuchi and Akira 2010; Suresh and Mosser 2013). Adaptive immune response comes in action as a support to the innate immune response in the later stages of infections or chronic inflammatory conditions, including tumors via regulating the innate immune response through generating regulatory and protective adaptive immunity comprising of adaptive immune cells (different T cells and B cells) depending on the need and the immunologic status of the host. Mainly T cells are comprised of helper T (Th), cytotoxic T, and the regulatory T (T_{regs}) cells. On the other hand, B cells lead to the generation of the antibody immune response against antigens in a T cell-dependent or independent manner depending on the type of antigen, host's immunological status, and the disease condition. However, very limited information is available describing the role of TLRs in the regulation of adaptive immune cells homeostasis and the generation of the adaptive immune response. The major aim of the chapter is to describe the role of TLRs in controlling the adaptive immune response mediated by T cell and B cell-based immunity.

2 TLRs in Innate Immunity and Inflammatory Signaling

A new era in the innate immunity was about to start with the discovery of Toll proteins in the *D. melanogaster* as an AMP (Drosomycin) activating protein against fungal pathogens (Lemaitre et al. 1996). In 1991, Gay and Keith showed that

cytoplasmic domain of Toll protein of *Drosophila* was related to interlekin-1receptor (IL-1R) of humans (Gay and Keith 1991). Later in 1997, human homolog of Toll protein was identified in the laboratory of Charles A Janeway Junior, which is now called Toll-like receptor 4 (TLR-4) that recognizes Gram-negative bacterial lipopolysaccharide (LPS) (Medzhitov et al. 1997). The human Toll protein is also a type I transmembrane protein with an extracellular domain having a leucine-rich repeat (LRR) domain, and a cytoplasmic domain homologous to the cytoplasmic domain of the human IL-1R (Medzhitov et al. 1997). Both, Drosophila Toll and the IL-1 receptor signaling pathways activate NF-κB transcription factor (Medzhitov et al. 1997; Gay and Keith 1991). The identification of human Toll protein proved a milestone in the field of immunology and led to the development of concept of PRRs-mediated innate immune response and its regulation (Medzhitov and Janeway 1997). Till date 12 functional TLRs have been discovered in mammals (laboratory mice) and humans have 10 functional TLRs (TLR1-TLR10) (Kumar 2018a; Kawai and Akira 2011). The TLR10 in mice is a defective pseudogene (Jiang et al. 2016). The details of TLRs expression on different innate immune cells, including macrophages, neutrophils, dendritic cells (DCs), mast cells, endothelial cells, platelets, natural killer or NK cells have been discussed somewhere else (Kumar 2018a).

Of these 13 mammalian TLRs, TLR1, TLR2 (lipoteichoic acid or LTA, peptidoglycan or PGN), TLR4 (LPS), TLR5 (bacterial flagellin), TLR6, TLR10 (humans), and probably TLR11 and TLR12 of mice are expressed on the cell surface and recognize both MAMPs/PAMPs and DAMPs (Table 1) (Kumar 2018a). However, DCs, endothelial cells, and epithelial cell also express TLR2 and TLR4 intracellularly (Kumar 2018a; Hornef et al. 2003; Uronen-Hansson et al. 2004; Shuang et al. 2007). On the other hand, TLR3 (dsRNA), TLR7 (ssRNA), TLR8, TLR9 (CpG DNA or dsDNA), and TLR13 (mice) are specifically expressed intracellularly in endoplasmic reticulum (ER), lysosomes, endosomes, endolysosomes, phagosomes, and phagolysosomes (Table 1) (Blasius and Beutler 2010). Of note, these intracellular TLRs only get activated inside the acidic environment of the endolysosomes upon recognizing their corresponding ligands as mentioned in Table 1. This is because of the treatment with chloroquine, bafilomycin A (a macrolide antibiotic that inhibits vacuolar H^+ ATPase (V-ATPase)-dependent acidification in the lysosomes) or ammonium chloride (NH_4Cl) (Häcker et al. 1998; Yoshimori et al. 1991). The recognition of the corresponding ligands by specific TLRs activates the downstream signaling through activating myeloid differentiation primary response 88 (MyD88) or MyD88-independent but TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling pathways or both (Fig. 1).

For example, TLR4 activation stimulates both MyD88-dependent and TRIF-dependent NF-κB activation, whereas TLR3 activation involves TRIF and *TNF receptor associated factor (*TRAF)-dependent type 1 interferon (IFN) activation, TLR9 activation involves MyD88-dependent NF-κB and Interferon regulatory factor 3 (IRF3)-dependent type 1 IFN activation, TLR7/8 activation involves My-D88 activation-based IRF7 activation causing type 1 IFN activation and IFN-inducible/stimulated genes (ISGs) (Fig. 1) (Blasius and Beutler 2010; Kumar 2019b, 2020b).

Table 1 Different TLRs, their location, ligands, and source of ligands

TLRs	TLR localization	Ligands	Origin of ligands
TLR1	Plasma membrane	Triacyl lipopeptide Soluble factors	Bacteria and mycobacteria
TLR2	Plasma membrane and phagosomes	Peptidoglycan (PGN), Lipoteichoic acid (LTA), Lipoproteins or lipopeptides, lipoarabinomannan, A phenol-soluble modulin, Glycoinositolphosp-holipids, glycolipids, porins, zymosan, atypical LPS, Hsp60, Hsp70, Hsp96, (High mobility group box protein 1), S100s HMG-B1, Eosinophil-derived neurotoxin (EDN), an alarmin	Gram +ve bacteria, mycobacteria, Fungi, host-derived DAMPs
TLR3	Endosomes and Endolysosomes	dsRNA	Viruses
TLR4	Plasma membrane and phagosomes	LPS, Taxol or paclitaxel, HMG-B1, Hsp60, Hsp70, Hsp22, Hsp96, Type III repeat extra domain A of fibronectin, Hsp70-like protein 1 (HSP70L1), hyaluronic acids, Polysaccharide fragments of heparin sulphate, Fibrinogen Saturated FAs, Fetuin-A, S100	Gram negative bacteria, Plant, Respiratory syncytial virus (RSV), Mouse mammary tumor virus (MMTV), host-deived DAMPs
TLR5	Plasma membrane	Flagellin , HMGB1	Bacteria, Host-derived DAMP/alarmin
TLR6/ TLR4	Plasma membrane and Phagosomes	Di-acyl lipopeptides Zymosan GPI anchor	Mycoplasma Fungi *Trypanosoma cruzi*
TLR7	Endosomes, Lysosomes, Endolysosome	ssRNA, Loxoribine, Bropirimine	Viruses, synthetic compounds
TLR8	Endolysosome	ssRNA	Viruses
TLR9	Endosomes, Lysosomes, Endolysosomes, and Phagosomes	CpG oligodeoxyneucleotide (ODN), Hemozoin pigment, Genomic DNA, Histones	Bacteria and viruses (HSV), Malaria, Host-derived DAMP
TLR10	Endolysosome	HIV-1-gp41, LPS	HIV-1, *Borrelia Burgdorferi, H. pylori*, and *L. monocytogene-derived* PAMPs
TLR11	Endolysosome	Profilin-like protein flagellin FliC Jain et al. (2011)	*T. gondii* *Salmonella and E. coli*
TLR12	Endolysosome	Profilin-like protein	*Toxoplasma gondii*
TLR13	Endosomes and Endolysosome	23s ribosomal RNA	Bacteria

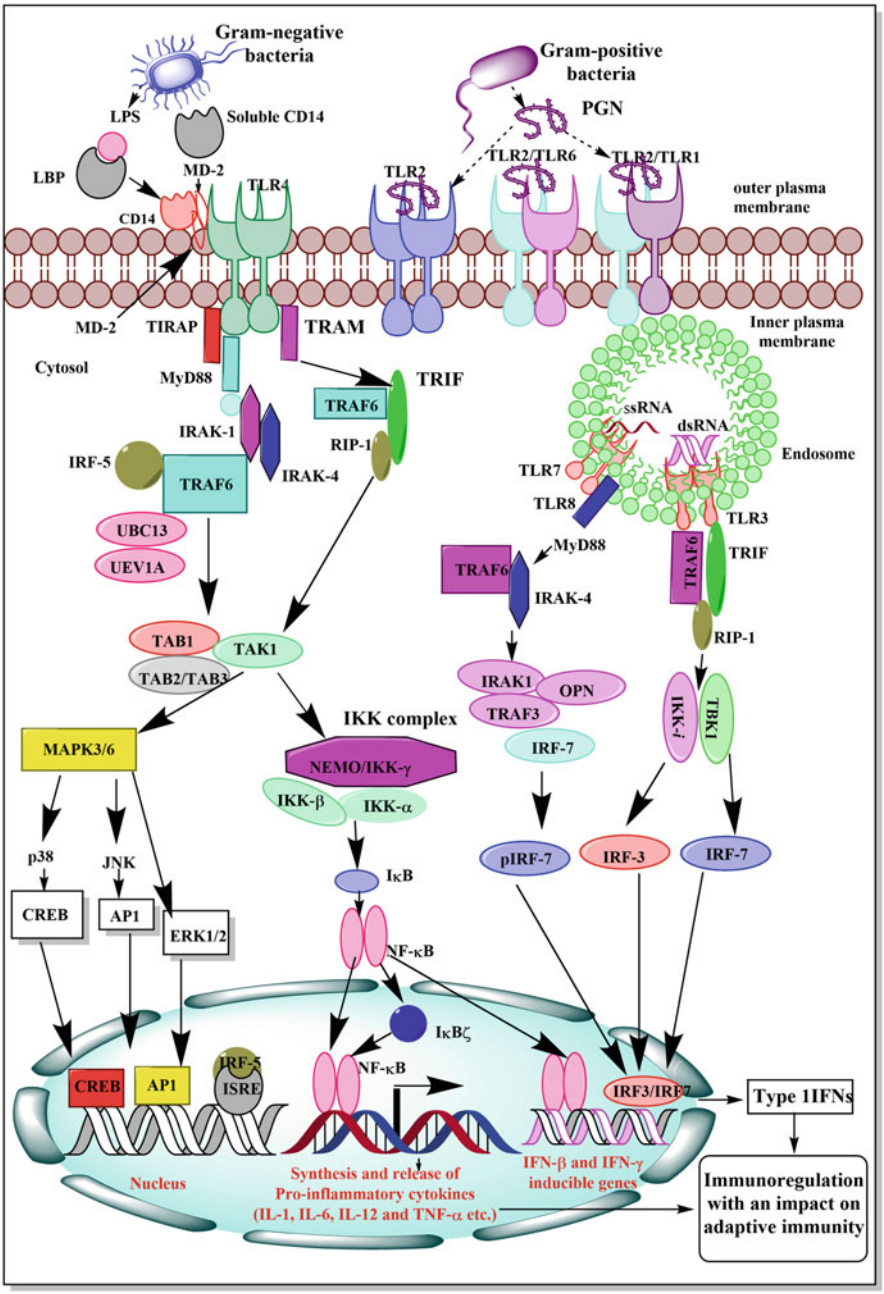

Fig. 1 Signaling pathways activated by stimulation of different TLRs upon ligation of their corresponding ligands. For example, recognition of Gram-negative bacteria or LPS by TLR4 leads to the activation of downstream signaling pathways through the activation of MyD88-dependent and -independent manner to activate NF-κB causing the transcription and translation of pro-inflammatory genes (cytokines and chemokines) as well as the generation of type 1 IFNs.

The TLR2 activation in human monocytes in MyD88-dependent manner activates type 1 IFN production via IRF3 and NF-κB activation driven by TRAF family member-associated NF-kappa-B activator (TANK)-binding kinase 1 (TBK1) and transforming growth factor beta-activated kinase 1 (TAK1 or mitogen-activated protein kinase kinase kinase 7/MAP3K7)-IKKβ (Oosenbrug et al. 2020). However, mature human macrophages do not produce TLR2-dependent type 1 IFNs. The detailed description of different TLR signaling pathways is beyond the scope of the present chapter and the author has described it in detail somewhere else (Blasius and Beutler 2010; Kumar 2019b). Thus, the activation of different TLRs upon recognizing corresponding ligands stimulates NF-κB-dependent pro-inflammatory cytokines (TNF-α, IL-1α, IL-6, IL-12, and IL-8), chemokines, reactive oxygen species (ROS) release, and different IRF3-dependent type 1 IFNs release, and activation of different IFN-inducible genes that along with removing pathogens or DAMPs also affects adaptive immune cells or adaptive immune response (Fig. 1).

3 TLRs in Adaptive Immunity

The adaptive immune response plays a crucial role in developing a pathogen or antigen specific immune response that comprises different B and T cells expressing BCRs and TCRs. For example, in the current corona virus disease-2019 or COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), the T and B cell (antibodies)-based adaptive immune response plays a crucial role in protecting the host as the protective innate immune response is hijacked by the virus (Kumar 2020c, d, e). Along with expressing antigen recognizing receptors (TCR and BCR), adaptive immune cells also express different TLRs.

3.1 TLRs in B Cells

B cells are crucial immune cells of adaptive immune system that along with serving as antigen presenting cells (APCs) also generate different categories of Abs or Igs (IgGs, IgAs, IgM, IgD, and IgE) specific to different antigens. Their numerical and functional dysregulation predisposes the host to several infections (recurrent pyogenic infections, like *Streptococcus pneumoniae* in Bruton's X-linked agammaglobulinemia (XLA) patients) and autoimmune diseases (rheumatoid arthritis or RA, systemic lupus erythematosus or SLE, myasthenia gravis, Hashimoto's disease, etc.). BCRs are crucial for their immunological function. However, B cells also express TLRs, which play a crucial role in their immune function, including Ab

Fig. 1 (continued) Also, the activation of intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) induces the generation of type 1 IFNs that regulate immune response, including the adaptive immunity. Details are mentioned in the text

production, class switching, and antigen presentation. For example, in teleost rainbow trout the TLR activation is crucial for activating IgM^+ B cells that get abrogated through inhibiting both MyD88 and TRIF-dependent TLR signaling (Soleto et al. 2020; Simón et al. 2019).

3.1.1 TLR Expression Pattern and Their Role in Mice B Cells

Murine B cells express TLR4 and its activation plays a crucial role in their proliferation, different cytokine release, enhances Ag presentation, upregulates their activation markers (HLA-DR, CD25, CD80, and CD86), class switch recombination (CSR), and Ab secretion (Bekeredjian-Ding and Jego 2009; Månsson et al. 2006; Agrawal and Gupta 2011) (Fig. 2). Both $TLR4^{-/-}$ and $MyD88^{-/-}$ mice show an impaired Ab production (IgM and IgG) (Pasare and Medzhitov 2005; Schnare et al. 2001) (Fig. 2). However, $MyD88^{-/-}$ mice produce comparable IgE levels upon

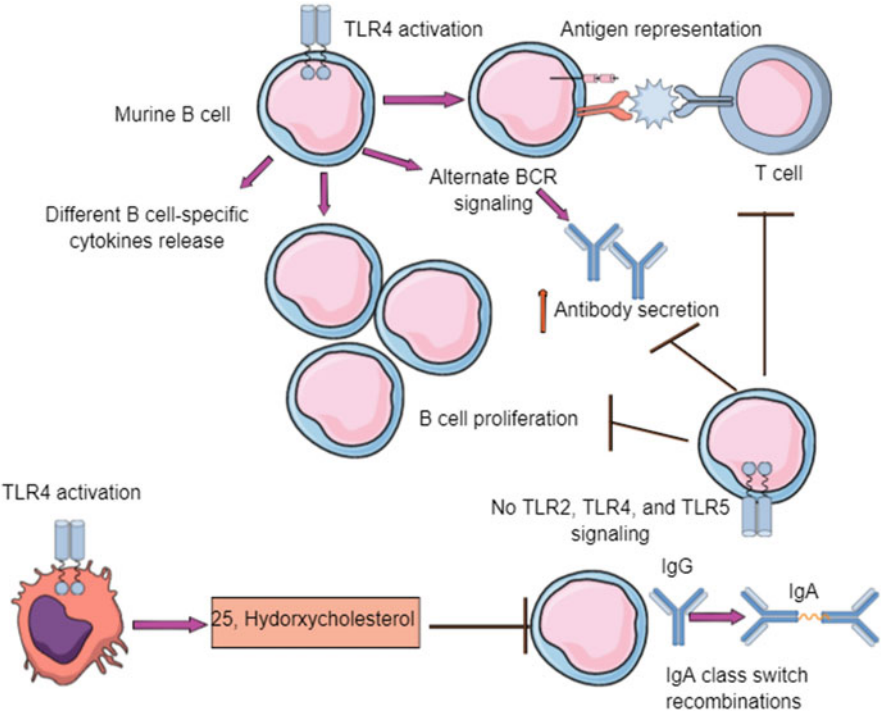

Fig. 2 TLR activation in murine B cells and impact on B cells immune response. The TLR4 activation on murine B cells induces the release of different B cell-specific cytokines, their proliferation, activates alternate BCR signaling to produce increased antibodies, and increases their antigen presenting capacity to activate T cell-based immune response. The absence of TLR2, TLR4, and TLR5 signaling in murine B cells blocks their proliferation, antigen presenting property and antibody production. On the other hand, the TLR4 activation in murine macrophages induces the 25-hydroxycholesterol production that blocks the IgA class switch recombination. See text for details

human serum albumin and LPS (HAS-LPS) challenge with alum immunization to WT mice. Similarly, the stimulation of MyD88$^{-/-}$ B cells with flagellin (a TLR5 ligand) impairs IgM and IgG1 production but without any impairment in IgG3 production as compared to WT mice (Pasare and Medzhitov 2005). However, MyD88$^{-/-}$ mice upon challenge with flagellin show a defective production of all, IgM, IgG1, and IgG3, indicating that TLR of other immune cells (DCs and macrophages) are required for the IgG3 production instead of B cell TLRs. Hence, the activation of B cell-specific TLRs is crucial for the optimal Ab response in response to the T cell-dependent Ag as mice specifically lacking TLRs and MyD88 produce significantly low level of Ag-specific Ab response upon treatment with HAS-LPS. The CD40-CD40L/CD154 interaction is crucial in isotype switching. The normal serum levels of IgA in MyD88$^{-/-}$ mice indicate that TLR signaling on B cells might also be dispensable for IgA production, otherwise TLR signaling on innate immune cells (intestinal epithelial cells or IECs) promotes B cell recruitment to the intestine and increases fecal IgA levels (Shang et al. 2008). However, 25-hydroxycholesterol produced by macrophages in response to the TLR4 activation suppresses IgA class switching in B cells and decreases serum IgA levels (Fig. 2) (Bauman et al. 2009). Hence, TLR activation on other immune cells may impact IgA production but TLRs expressed on B cells don't.

The memory B cells produce higher level of cytokines than naïve B cells upon stimulation with different TLR ligands (Agrawal and Gupta 2011). The TLR2 activation in resting murine B cells synergizes with CD40 signaling induced by CD40L or CD154 in a BCR signaling-independent manner and augments their activation, proliferation, and differentiation (Jain et al. 2011). An increased activation-induced cytidine deaminase (AID) expression and CSR also takes place. This synergistic mechanism of B cell activation abolishes their ability to uptake Ags, stimulate T cells, and Ca^{2+} flux. On the other hand, the synergistic activation of TLR2 and CD86 on resting murine B cells also increases their activation and proliferation and differentiates them into marginal zone precursor B cells in a shorter time window and increase in IgG production also occurs (Jain et al. 2013). In contrast to the CD40 and TLR2 signaling-mediated activated B cells, these B cells (TLR2 and CD86 activated) show an increased Ag uptake.

The TLR4 signaling through MyD88 activation in murine B cells also abolishes the BCR activation-mediated Bruton's tyrosine kinase (Btk) recruitment and the need for phosphoinositide 3-kinase (PI3K) for extracellular signal-regulated kinase (Erk) phosphorylation (Dye et al. 2007). TLR4 activation also enhances mitogen-activated protein kinase/ERK kinase (MEK) phosphorylation and degrades IκBα to induce the alternate BCR signaling pathway in the presence of LPS (Dye et al. 2007). Hence, TLR4 activation in murine B cells activates an alternate BCR signaling pathway without the involvement of classical BCR signalosome (comprising of kinases spleen tyrosine kinase (Syk), Src family kinase Lyn, and Btk, the guanine exchange factor Vav, and the adaptor proteins Grb2 and B cell linker or BLNK)-dependent signaling pathway (Fig. 2) (Dye et al. 2007; Woyach et al. 2012). This enhances their function as crucial adaptive immune cells for Ab production (Fig. 2). However, rough LPS is 100 times more potent than the smooth LPS to activate

murine TLR4 in B cells and associated immune response (Minguet et al. 2008). The LPS-mediated long-term primary CSR-associated Ab generation and memory-like Ab-based response involves binding of lipid A moiety of LPS to the TLR4 and polysaccharide moiety engages BCR that triggers Ca^{2+} flux in the B cells (Pone et al. 2015). The BCR cross-linking also synergizes LPS-TLR4 interaction with TLR1// TLR2 interaction with Pam3CSK4 to induce CSR but TLR4-LPS interaction synergization is absent or very weak with TLR7-R-848 or TLR9-CpG interaction (Pone et al. 2015). When there is no BCR cross-linking TLR7 and TLR9 activation with their corresponding ligands (R-848 and CpG) almost abrogate CSR-induced IgG1, IgG2a, IgG2b, IgG3a, and/or IgA production without affecting B cell proliferation and IgM expression (Pone et al. 2015). The CSR inhibition in response to TLR7 or TLR9 activation alone is associated with the reduced AID expression or IgH germline I_H-S-C_H transcription. Also, murine B cells do not show CSR or plasma cell differentiation when co-stimulated with LPS and CD154 (a CD40 ligand or CD40L). Hence, different TLR ligands and stage of antigenic stimulation of BCRs impacts the phenotype and function of B cells (Ab production, CSR, Plasma cell formation, etc.).

The simultaneous BCR and TLR4 activation elevates the B cell activation additively. On the other hand, the co-engagement of TLR4 and BCR with the Ag-coupled LPS synergistically activates B cell-mediated immune response (Minguet et al. 2008). The immunization of mice with synthetic nanoparticles containing a potential Ag, TLR4, and TLR7 agonists (monophosphoryl lipid A or MPL and R837) induces the synergistic increase in Ag-specific, neutralizing antibodies (NAbs) in comparison with the immunization with only one TLR agonist (Kasturi et al. 2011). This approach also enhances the persistence of GCs and plasma cells, which survive more than 1.5 years in lymph nodes (LNs). However, double stimulation of TLRs (TLR4 and TLR7) in the presence of Ag does not enhance the life span of early-short lived plasma cells in comparison with the stimulation with single TLR ligand (Kasturi et al. 2011). This immunization strategy also induces the early progression of B cells toward memory B cell formation. Along with B cell TLRs, the TLRs expressed on DCs and helper T cells also play a significant role in the associated Ab response (Kasturi et al. 2011). This strategy is of potential use in humans for developing potent vaccine candidates for influenza virus infection and current COVID-19 pandemic after careful further studies.

The synthetic TLR4 (1Z105, a substituted pyrimido[5,4-b]indole specific for the TLR4-MD2 complex) and TLR7 (1V270, a phospholipid conjugate) ligands work through activating MyD88-dependent downstream signaling pathway activating NF-κB during influenza virus vaccination in mice to induce Ab-based immune response that is very rapid, highly sustained, and broadly protective (Goff et al. 2015, 2017). Another study has indicated that the liposome coated TLR4 and TLR7 agonists (2B182C and 1V270) are more potent than 1Z105 and 1V205 combination in eliciting a protective Ab-based immune response (greater diversity in B cell clonotypes of Ig heavy chain (IGH) genes in the draining LNs and broadly specific antibodies against hemagglutinin (HA) antigen) in mice with a great safety profile (Sato-Kaneko et al. 2020). The combined use of TLR4 and TLR7 agonists decreases

the dose of individual TLR ligand and thus the potential of adverse reactions or immune response associated with their higher dose individually. The TLR1/2 activation in murine B cells alters the stimulatory action of TLR4 activation on B cell immune response (suppresses IgG1 production but enhances IgG2a production) despite increasing their viability (Lee and Park 2018). TLR4 and TLR2 deficient mice exhibit a significantly decreased B cell-mediated immune (IgM and IgG production) response upon immunization with *Salmonella typhi* porins (Cervantes-Barragán et al. 2009). On the other hand, the B cell stimulatory potential (IgG2b production, germline γ2b transcript (GLTγ2b), and surface IgG2b expression) of TLR4 agonist or LPS further increases in the presence of Nod2 activator (muramyl dipeptide or MDP) (Lee et al. 2019).

The TLR9 stimulation in B cell with its ligand (CpG-DNA or ODN) contained within a virus particle requires MyD88 activation for generating T cell-dependent (TD) GC formation and associated Ab response (Hou et al. 2011). However, the stimulation with soluble TLR9 agonist used as an adjuvant for a protein Ag requires MyD88 activation in DCs but not in B cells to enhance TD-dependent Ab response irrespective of the inherent immunogenicity of the Ag (Hou et al. 2011). For example, MyD88$^{-/-}$ mice show a considerably reduced Ag-specific IgM and IgG Abs responses against T cell-dependent Ags and exhibit a total loss of IgG2-dependent Ab response (Pasare and Medzhitov 2005). Also, naïve MyD88$^{-/-}$ mice show a decreased serum level of IgM, IgG1, IgG2c, and IgG3 as compared to wild type (WT) mice despite having normal B cell numbers (Pasare and Medzhitov 2005). The immunization of mice with CpG-DNA or oligodeoxynucleotide (ODN) (a TLR9 agonist)-linked with protein Ag increases the Ag-specific CSR-based Ab production (Eckl-Dorna and Batista 2009). However, B cells do not acquire CpG-ODN-Ag conjugate through micropinocytosis as seen in DCs, instead use their BCRs (as BCRs are known to induce non-specific endocytosis) (Eckl-Dorna and Batista 2009; Song et al. 1995). Thereafter the intrinsic TLR9 stimulation through CpG-DNA or CpG-ODN enhances the Ag-specific B cell proliferation and differentiation to give rise extra follicular plasma cells (Eckl-Dorna and Batista 2009). However, the use of this strategy of conjugating the potent TLR agonist with a potential antigens in humans should also be explored to design potent vaccine candidates to generate a potent but safe and regulated immune response against different infectious diseases needing vaccination, including the current COVID-19 pandemic.

Recently, the conjugation of Class R ('restricted', palindromic) ODNs, which are at least 10 times less potent TLR9 antagonists/inhibitors with BCR Ag enhances (10 times) their TLR9 activation potential in non-autoreactive B cells of mice similar to the Class B ("broadly reactive," linear) ODNs (Goeken et al. 2010). This effect is specific for TLR9-induced B cell cycling and apoptosis inhibition, without much increase in the IL-6 production that still requires 10 times more Class R ODNs. Hence, this strategy may be further studied in SLE patients as a therapeutic approach. The TLR9 activation through its agonist called M6-395 in B cells augments their proliferation more potently than TLR4 activation, but it abrogates the LPS and PamCS3K-mediated increase in IgA, IgG1, and IgG3 production (Park

et al. 2012). Hence, M6-395 is a strong polyclonal activator for B cells but does not favor Ig isotype switching. TLR7 is also crucial for GC formation as mice lacking it do not form GC-B cells during retrovirus (Friend virus) infection (Browne 2011). These TLR7-defiecient mice also have an attenuated $CD4^+$T cell-based immune response without any defect in $CD8^+$T cell immune response. TLR7 activation in murine activated B cells in the presence of IL-4 through Ag receptors or CD38 (a cyclic ADP ribose hydrolase) induces CSR and IgG1 production without the involvement of Btk (Tsukamoto et al. 2009). Similar, immune response among B cells has been observed upon stimulation of TLR7 with loxoribine and BCR. TLR7 acts as a receptor for 8-mercaptoguanosine (8-SGuo) and induces AID and B lymphocyte-induced maturation protein-1 (Blimp-1, a transcription factor (encoded by PR domain zinc finger protein 1 (prdm1) gene) crucial for Ab producing plasma cell differentiation, Ab production, and plasma cell longevity) expression in B cells to induce μ to γ1 CSR at DNA level to shift IgG1 production from IgM (Tsukamoto et al. 2009; Shapiro-Shelef et al. 2003; Shapiro-Shelef et al. 2005). This process may also require the presence of IL-4. Another study has indicated that the TLR4 stimulation and MyD88 signaling in B cells are required for GC formation via strongly inducing Blimp-1 (Pasare and Medzhitov 2005).

3.1.2 TLRs Expression Pattern and Function in Human B Cells

Human B cells responsive to TLR ligands or signaling exhibit higher somatic hypermutation (SHM), shorter CDR3 regions or segments, and less negative charges (Simchoni and Cunningham-Rundles 2015). The induction of long positive charge CDR3 segment in B cells upon TLR stimulation suggests the production of autoreactive Abs (Simchoni and Cunningham-Rundles 2015). This was suggested as binding of TLR stimulated culture supernatant of the B cells to the human epithelial type 2 (Hep2) cells, whereas CD40/IL-21 stimulated cell didn't. Hence, human B cells are selective to TLR stimulation and have distinct phenotypic and genetic characters. For example TLR9 (CpG-ODN) and TLR7 (imidazoquinoline compound) stimulation expands IgM^+ memory and plasma cell lineage committed B cells, whereas CD4-/IL-21 stimulation favors proliferation of memory and naive B cells (Simchoni and Cunningham-Rundles 2015). For example, a defective TLR7 and TLR9 signaling in B cells and plasmacytoid DCs (pDCs, which produce type 1 IFNs, including IFN-α) is associated with common variable immunodeficiency (CVID) (Yu et al. 2009, 2012). Another study has shown that despite their molecular differences, the TLR7 and TLR9 agonists induce same genes in human purified B cells responsible for cytokine and chemokine production, co-stimulatory molecule expression, anti-apoptotic genes (BCL2L1), genes (CD72 and IL-21R) essential for their proliferation and differentiation (Hanten et al. 2008). The combined stimulation of TLR2 and TLR3 with Pam3CSK4 and Poly I:C enhances the B cell activation and boosts the immunological immune response (Ab generation and allogenic T cell immune response) by the protein-based vaccination (Fig. 3) (Weir et al. 2017).

Naïve human B cells express low levels of TLRs than innate immune cells, but their level increases in active and memory B cells (Browne 2012). Although human newborns express adult levels of TLRs and CD40, their response to different TLR

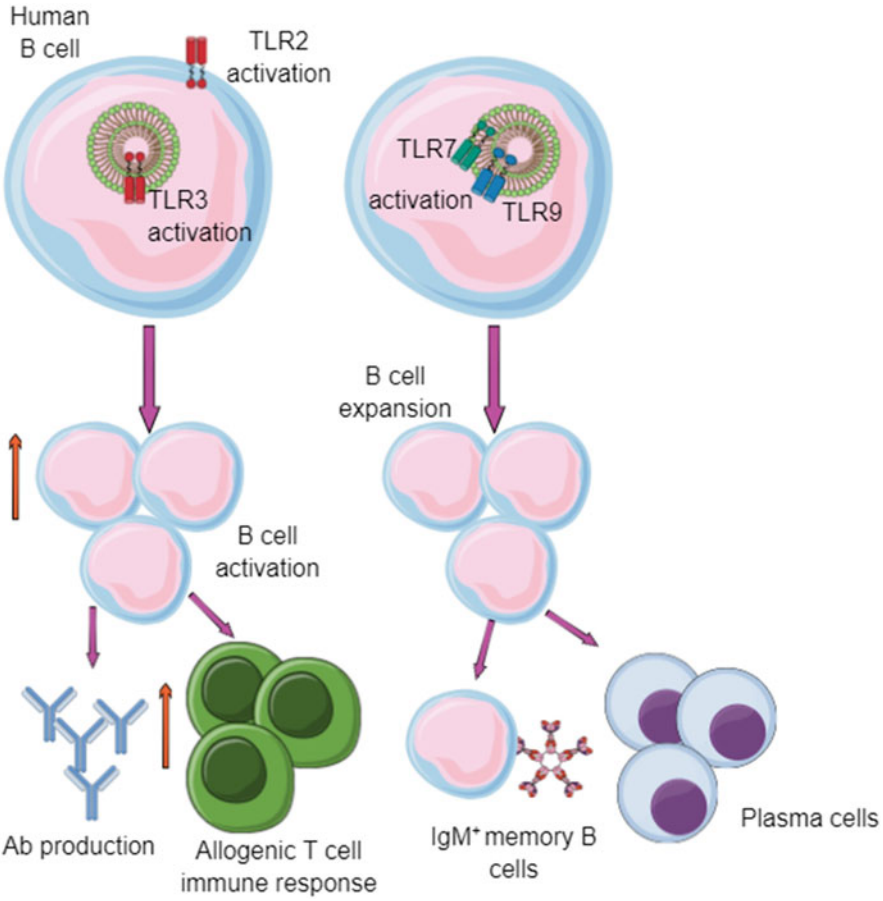

Fig. 3 TLR signaling and human B cells. The activation of TLR2 and TLR3 in human B cells increases their activation, Ab production, and allogenic T cell immune response. On the other hand, the activation of intracellular TLR7 and TLR9 in human B cells increases their expansion, formation of IgM^+ memory B cell formation, and plasma cell formation. Kindly see text for details

ligands varies. For example, neonatal naïve B cells have an impaired B cell response in response to the TLR2 and TLR7 stimulation but produce an increased amount of cytokines in response to the TLR9 stimulation (Pettengill et al. 2016). Also, very few newborn naïve B cells showed CSR to produce IgG in response to the TLR9 activation. Hence, strategies based on TLR-based vaccinations to immunize neonates should be taken care depending on these findings as it may suggest the development of protective Abs in only some neonates but not in all. B cells highly express TLR1, TLR2, TLR6, TLR7, TLR9, and TLR10 without any significant difference between naïve, GC, and memory B cells (Månsson et al. 2006; Agrawal and Gupta 2011; Hornung et al. 2002). The TLR2 activation produces low levels of GM-CSF and G-CSF in human B cells (Agrawal and Gupta 2011). The stimulation

of $TLR2^+$ human B cells isolated from peripheral blood and spleen with TLR2 agonist induces germinal center (GC) formation as indicated by the upregulation of globotriaosylceramide Gb3/CD77 (a molecule that defines a B lymphocyte maturation pathway, specific for GC) and the increased chemokine secretion (Ganley-Leal et al. 2006; Mangeney et al. 1991). On the other hand, activated $TLR2^+$ tonsillar B cells secrete IgM upon stimulation with TLR2 ligands. Human B cells do not express TLR4 and remain unresponsive to its ligands, including LPS (Bekeredjian-Ding and Jego 2009). However, human $CD138^+$ plasma cells express TLR3, TLR4, and TLR8, and their activation promotes Ab secretion (Dorner et al. 2009).

Human B cell expresses TLR2, TLR1, TLR7, TLR9, and TLR10 (Bekeredjian-Ding and Jego 2009; Hornung et al. 2002). The TLR2-mediated modulation of B cell function in humans requires sensitization through BCR cross-linking with anti-immunoglobulin or surface protein A (SpA) from *Staphylococcus aureus* (Bekeredjian-Ding et al. 2007; Ruprecht and Lanzavecchia 2006). The stimulation of human B cell TLR2 does not produce IgM, unlike TLR7 and TLR9 stimulation. The TLR3 stimulation by the viral dsRNA in human B cells of respiratory mucosal surfaces, including tonsils triggers NF-κB-dependent Ig CSR (indicated by germline transcription of downstream CH genes and AID expression) independent of T cell activation (Xu et al. 2008). Also, the TLR3 expressing DCs release B cell activating factor of TNF-α family (BAFF) that further enhances IgG and IgA production. Hence, the acute infection with respiratory viruses with dsRNA as a genetic material can initiate a frequent TLR3 activation-dependent Ab response that is further enhanced by BAFF without the help of T cells. However, exposure to the cold temperature or weather decreases the BAFF production by DCs or human lung epithelial cells in response to the dsRNA or TLR3 ligands, including polyinosinic: polycytidylic acid (poly I:C) that decreases the potency of induction of TLR3-induced B cell activation and the Ab production (Yoshino et al. 2020). The BAFF production involves IFN-β production and the activation of Janus-associate kinase/signal transducer and activator of transduction (JAK/STAT) pathway in human lung epithelial cells. BAFF is crucial for Ab response against West Nile virus (WNV, a ssRNA virus) (Giordano et al. 2020).

The TLR7 stimulation with resiquimod (a TLR7 ligand) in human naïve B cells induces IgM production with a lesser extent to IgG, along with IL-6 and IL-10 (Glaum et al. 2009). However, naïve human B cells proliferate weakly in response to the resiquimod stimulation, but the addition of IL-10 and IL-2 increases their proliferation and Ig production. This response has also been seen in B cells isolated from X-linked hyper-IgM syndrome patients in the presence of resiquimod in combination with IL-2 and IL-10 (Glaum et al. 2009). The TLR7 stimulation with resiquimod in human naïve B cells also induces Ig CSR as indicated by AID and Iγ1-Cμ circle transcripts expression (Glaum et al. 2009). Hence, resiquimod, a TLR7 ligand is a potent stimulator of B cell immune response in humans in the absence of B cell receptor cross-linking and CD40-CD40L interaction that can be used as potential adjuvant for vaccine development. This study also indicates that infections activating TLR7 may predispose the host to develop autoimmunity through activating B cells mediated Abs cross-reactive to host cell proteins.

TLR9 and TLR7 expression decrease in CpG-ODN stimulated B cells (Hornung et al. 2002). The decrease in TLR9 and TLR7 expression on B cells in the presence of CpG-ODN may be strategy to decrease their pro-inflammatory action and the production of type 1 IFN production that supports autoreactive B cells development seen in the patients with systemic lupus erythematosus (SLE) (Hamilton et al. 2018). For example, an increase in the TLR7 expression and activation in $CD19^{+}$B cells in kidneys of patients with IgA nephropathy (IgAN) increases the galactose deficient-IgA1 (Gd-IgA1) production and facilitates the inflammation and kidney damage (Zheng et al. 2020). However, in the presence of IFN-α the CpG-ODN-mediated $CD27^{-}$ naïve human B cells induce their phenotypic differentiation to the plasma cells indicated by CD38 expression upregulation and IgM production, along with IL1-β, IL-6, IL-10, and TNF-α synthesis and release (Giordani et al. 2009). Hence, the presence of IFN-α amplifies the TLR9-mediated effect of CpG-ODN on B cell function and differentiation into Ab producing plasma cells may serve as crucial factor to predispose host to autoimmune diseases under diverse conditions, including hepatitis C virus (HCV) infected patients treated with IFNs and SLE (Silva 2012; Choubey and Moudgil 2011).

The TLR9 stimulation of human B cells generates plasma cells from memory B cells and prolongs the naïve B cells survival (Fig. 3) (Capolunghi et al. 2008). Of note, $CD24^{bright}CD27^{+}$ memory B cells comprise 30–50% of the total B cells in the healthy adults, which include IgM^{+} memory B cells (express IgM and IgD) and switched memory B cells (have different IgG and IgA isotypes). The TLR9 stimulation also induces the differentiation of immature or transitional B cells, which are $CD24^{bright}CD38^{bright}CD27^{-}$ (also called newly formed B cells or NF-B cells) and express higher levels of TLR9 than mature and IgM^{+} memory B cells (Capolunghi et al. 2008; Giltiay et al. 2019). The mature naïve B cells are $CD24^{+}CD38^{+}CD27^{-}$ and memory B cells lack CD38 and express CD24 and CD27. Like adult transitional B cells, 26% of the fetal cord blood-derived transitional B cells upon stimulation with TLR9 ligand also proliferate and differentiate into $CD27^{bright}$ IgM^{+} plasma cells (Capolunghi et al. 2008). A larger population of transitional B cells transform into $CD24^{bright}CD38^{-}CD27^{+}$ memory B cells and smaller population changes into phenotypically identical mature-naive B cells. Of note, fetal cord blood-derived B cells stimulated with TLR9 agonist CpG produce both IgM and IgG, but no IgA. These IgM^{+} memory B cells and transitional B cells both produce anti-polysaccharide IgM Abs in the presence of CpG that protect against *Streptococcus pneumoniae* infection (Capolunghi et al. 2008). The splenectomized adult humans have higher levels of circulating transitional B cells than healthy adults with spleen and produce anti-pneumococcal polysaccharide (PnPS) IgM (Capolunghi et al. 2008). Hence, both IgM^{+} memory B cells and transitional B cells produce Abs upon stimulation with TLR9 agonist or CpG stimulation. TLR9 stimulation of B cells induces AID expression and CSR, and Blimp-1 expression indicates commitment to develop into plasma cells (Capolunghi et al. 2008).

The stimulation of naïve human B cells with TLR9 agonist (CpG-ODN 2006) directly induces their proliferation and Ag presenting function as indicated by an increase in the HLA-DR, CD40, and CD80 expression (Jiang et al. 2007). However,

expanded mature naïve B cells do not mature into $CD27^+$ or IgG^+ memory B cells, instead develop into IgM^+ B cells or plasma cells secreting IgM. Hence, exposure of TLR9 agonist in humans may induce naïve B cells into potent APCs, which present Ags to T cells for cell-mediated immunity (CMI) and IgM secreting plasma cells. MGN1703 (lefitolimod), another TLR9 agonist also increases B cell differentiation and activates other immune cells (NK cells, DCs, and T cells) to release IFNs in LNs in humans (Schleimann et al. 2019). An increase in AID expression and SHM also occurs in B cells in response to MGN1703 indicating the different isotypes of IgG production. Another study has indicated that the CpG-DNA recognition by B cells activates Ab production and CSR by initiating germline C(γ)1, C(γ)2, and C(γ)3 gene transcription through a TLR9-dependent NF-κB activation of innate immune signaling pathway, which cooperates with IL-10 through STAT3 signaling and IFN-responsive genes (He et al. 2004). This indicates that TLR9-based vaccine candidates have potential to use in immunotherapy for different infectious diseases and cancers. For example, CpG-based adjuvants activating TLR9 have shown a great potency in DNA/protein prime boost vaccine against Brugia malayi (Bm-Myo) in BALB/c mice (Gupta et al. 2019). However, the TLR9-mediated over activation of B cells may lead to autoimmune diseases. For example, the drug chloroquine inhibits this TLR9-based pathway and therefore has been used in patients with SLE, an IgG-mediated autoimmune disease.

The BAFF production by neighboring DCs in response to the IFN-α further enhances Ab production independent of T cells involvement. Hence, host- or pathogen-derived CpG DNA may exacerbate the autoimmune or autoinflammatory diseases via stimulating the autoreactive B cells to switch from IgM production to the more pathogenic IgG production. The TLR9-mediated activation of B cell activation, proliferation, and associated immune response (Ab and cytokine production) is inhibited by the binding of complement receptor type 1 (CR1 or CD35) to its natural, complement component C3-derived ligand (Mácsik-Valent et al. 2019). However, it does not inhibit TLR7-mediated B cell activation and associated immune response, but CR1 occupancy on B cells inhibits the synergistic action of TLR7 and TLR9 activation on human B cells. Hence, occupancy of CR1 expressed on B cells by its natural ligands during microbial infections or other inflammatory conditions prevents their overactivation and the production of cross-reactive Abs in response to the TLR7 and TLR9 activation to maintain immune homeostasis.

The dysregulation of TLR expression and function in B cells has been found to be associated with different B cell malignancies, including multiple myeloma (MM), chronic lymphocytic leukemia (CLL) (Isaza-Correa et al. 2014). B cells isolated from patients of Waldenstrom macroglobulinemia (WM, a rare cancer associated with clonal B cell infiltration in the bone marrow leading to the formation of a compartment of cancerous plasma cells secreting monoclonal IgM paraprotein) upon TLR7 activation with R848 (a synthetic TLR7 agonist) fail to show corresponding changes in the transcriptional regulators, indicating a defective TLR7 signaling that uncouples the plasma cell differentiation programing (Shrimpton et al. 2020). Further studies are warranted in the direction. For example, a novel inducible B cell subpopulation ($CD11a^{hi}Fc\gamma RIII^{hi}$) has been identified that produces IFN-γ in

response to TLR ligands and pathogens (*Listeria monocytogenes*, *Escherichia coli*, vesicular stomatitis virus or VSV) in mice (Bao et al. 2014). This population of B cells has been called IFN-γ producing innate B cells. Thus, the number and activation of these IFN-γ producing innate B cells should be investigated in autoimmune and autoinflammatory diseases, including SLE. Hence, different TLRs play a crucial role in the B cell-specific immune response (Ab generation and cytokine release), and their impaired expression and activation may lead to autoimmune diseases, immunodeficiency, and cancers. Thus, studying TLRs in the context of B cells may open avenues for developing different vaccine candidates and adjuvants for infections, including current pandemic COVID-19 and cancers.

3.2 TLRs in T Cells

The inflammatory immune response along with modulating innate immune cells function and properties also impacts adaptive immune cells, including B cells and T cells through releasing various immune mediators (cytokines, chemokines, IFNs, etc.) and generating different DAMPs (Moro-García et al. 2018). Even these T cells (regulatory T cells or T_{regs}) keep in record for the inflammatory immune response as an immune memory and loss many activation-induced changes to suppress their enhanced immunosuppressive function overtime for future to protect the host from a generalized state of immunosuppression that may result from otherwise repeated exposure to same inflammatory condition (van der Veeken et al. 2016). PRRs expressed on innate immune cells recognize these DAMPs and further increase the potency of the pro-inflammatory immune response. However, as described earlier that one type of these PRRs called TLRs are expressed by B cells and modulate or impact their immunological function. Similarly, T cells also express different TLRs and their activation to produce different cytokines (IL-17, IL-22, IL-4, IL-10, IL-13, TGF-β, TNF-α, and IL-9) significantly impacts the immune response associated with inflammation (Kumar 2018a; Kabelitz 2007). Hence, T cells can recognize both PAMPs/MAMPs and DAMPs depending on their characteristic, phenotype, pattern of TLR expression, and location to modulate the inflammatory immune response.

3.2.1 TLRs Expression and Function in Mice T Cells

Naïve murine $CD4^+$T cells express low levels of TLR2 that increases after their stimulation (Fig. 4) (Karim et al. 2017). The *Shigella dysenteriae* porin promotes TLR2 expression on CD3-stimulated $CD4^+$T cells of B6 mice (Biswas et al. 2009). The synergistic effect of TCR and TLR2 stimulation in mice naïve $CD4^+$T cells increases their differentiation to pro-inflammatory Th1 cells secreting IL-2, IFN-γ, TNF-α, IFN-γ-inducible chemokines [IFN-γ induced protein 10 (IP-10 or CXCL10) and monokine-induced by IFN-γ (MIG or CXCL9)] in vitro (Fig. 4) (Karim et al. 2017; Biswas et al. 2009). The TLR2 activation-induced MyD88-dependent signaling pathway in $CD8^+$T cells increases their survival, clonal expansion, and differentiation into long-lived memory T cells during vaccinia virus infection (Fig. 4) (Quigley et al. 2009). This TLR2-dependent MyD88 activation-mediated $CD8^+$T

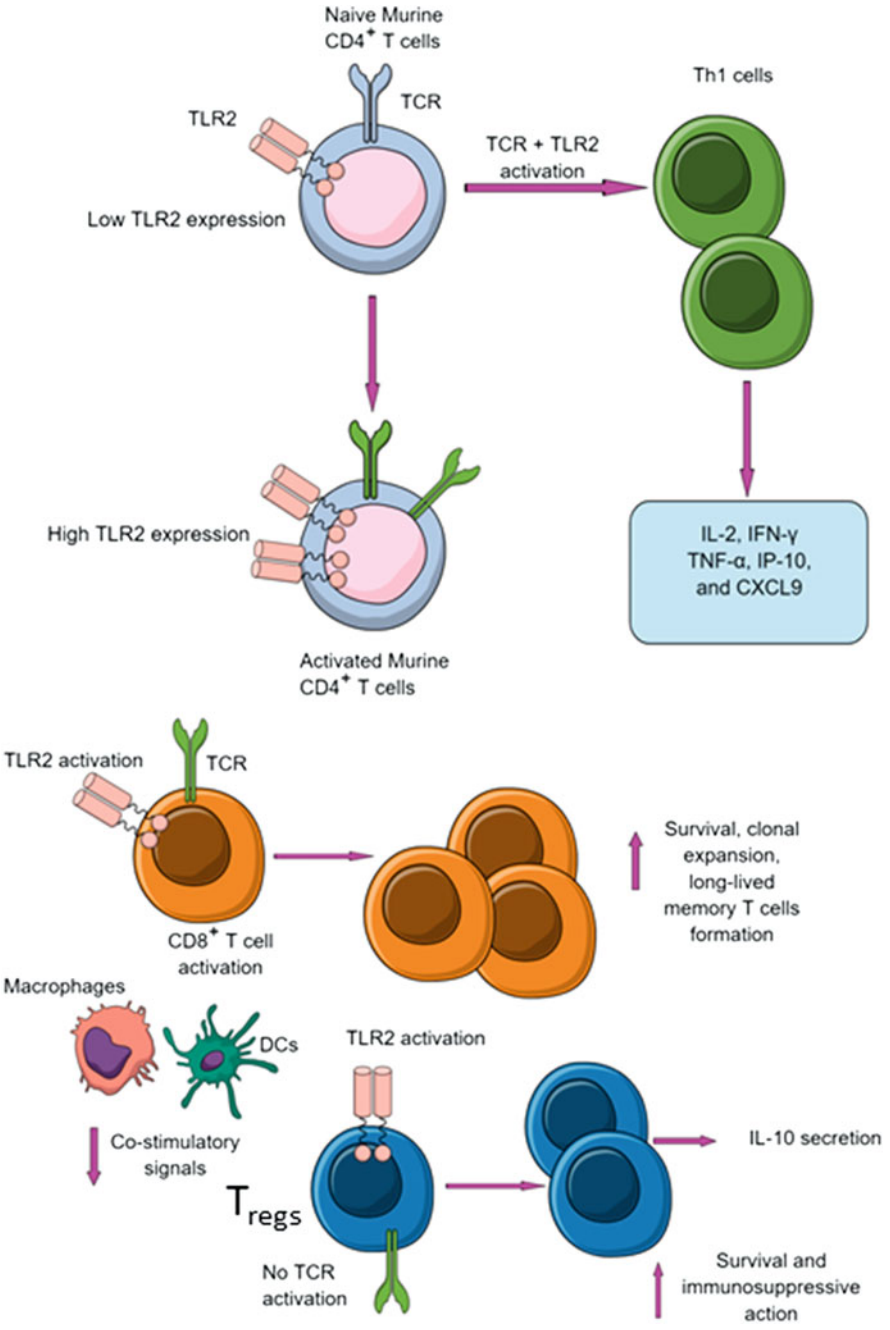

Fig. 4 Role of TLRs in mouse T cells. Naïve murine $CD4^+$T cells show low expression of TLR2. However, the TCR activation increases the TLR2 expression in activated $CD4^+$T cells. The combined activation of TLR2 and TCR polarizes $CD4^+$T cells to the pro-inflammatory Th1 phenotype, which produces higher levels of IL-2, IFN-γ, TNF-α, IP-10, and CXCL9. On the other hand, the TLR2 activation in murine $CD8^+$T cells increases their survival, clonal expansion,

cell survival, proliferation, and memory cell formation depends on PI3K and Akt activation in response to the suboptimal TCR stimulation and reduces the need for co-stimulatory signals from APCs, including macrophages and DCs, and threshold for optimal Ag-induced T cell activation (Fig. 4) (Mercier et al. 2009; Cottalorda et al. 2006). The Akt activation controls memory T cell formation in vivo. The MyD88 activation downstream to TLR signaling plays a crucial role in the initial antiviral $CD8^+$T cells-based immune response, including their expansion (Zhao et al. 2009). Thus, activating MyD88 activation via TLR2-specific ligands or through other means on $CD8^+$T cells may prove beneficial to promote and sustain anti-vaccinia virus immune response. Further studies have indicated that the vaccinia virus-vectored vaccines (VACV) boost the $CD8^+$T cells-based immune response via their intrinsic MyD88-dependent signaling pathway independent of TCR activation or the activation of MyD88-dependent inflammatory environment (Hu et al. 2014, 2017).

The TLR2 co-stimulation on murine $CD8^+$T cells stimulated with anti-CD3 Abs shows an increased IFN-γ production (Lee et al. 2009). Also, the TLR2 expression on *Listeria monocytogenes* specific $CD8^+$T memory cells do not need a pre-stimulation of CD3 and are constitutively expressed on them (Lee et al. 2009). The TLR2 stimulation of $CD8^+$T memory cells also increases their proliferation and expansion induced by IL-7 and they also increase IFN-γ (Cottalorda et al. 2009). Hence, TLR2 signaling on naïve $CD8^+$T cells and memory $CD8^+$T cells plays a crucial role in their maintenance, survival, proliferation, and function. The direct ligation of TLR3 ligand on murine $CD8^+$T cells in vitro increases the CD69 expression, indicating their activation and the adoptive transfer of these activated T cells in the naive mice following vaccination show their superior expansion as compared to their naïve counterparts (Salem et al. 2009). The $CD4^+$T cell-specific MyD88 activation during intracellular bacterial infection induces IFN-γ production and activates hematopoietic stem and progenitor cell activation (Zhang et al. 2013). The MyD88 signaling pathway activation on donor $CD4^+$T cells increases the severity of graft versus host disease (GVHD) in a mouse model of allogeneic hematopoietic stem cell transplantation (allo-SCT) due to the increased survival and proliferation toward TH1, Tc1, and Th17 cells (Matsuoka et al. 2020). On the other hand, host MyD88 signaling protects against acute allogenic GVHD after bone marrow transplantation (BMT) (Xing et al. 2019). The TLR2 stimulation on T_{regs} enhances their survival and disseminating fungal infection caused by *Candida albicans* (candidiasis) induces immunosuppression through TLR2 signaling inducing IL-10 release from them (Fig. 4) (Netea et al. 2004). TLR2 stimulation on T_{regs} via MyD88 signaling pathway increases their survival and immunosuppressive action and increases their proliferation when acts as co-stimulatory receptor with TCR activation (Sutmuller et al. 2006). However, co-stimulation of TLR2 with TCR

Fig. 4 (continued) and induces long-lived memory T cell formation. The TLR2 activation in T_{regs} in the absence of TCR signaling increases the IL-10 production, their survival, and immunosuppressive action. Kindly see text for details

in T_{regs} inhibits their immunosuppressive action. Hence, only activation of TLR2 in T_{regs} increases their immunosuppressive function whereas their stimulation as co-stimulation receptor suppresses immunosuppressive role (Fig. 4). Hence, regulating TLR2 signaling on T cells has bright future in different immunological conditions, including infections, cancers, autoimmunity, and GVHD.

Naïve $CD4^+CD45RB^{high}$ T cells isolated from C57/BL6 (B6) mice express TLR1, TLR2, TLR3, TLR6, TLR7, and TLR8, but not TLR4, TLR5, and TLR9 (Cottalorda et al. 2006; Caramalho et al. 2003; Sobek et al. 2004). However, memory and effector $CD4^+CD45RB^{low}$ T cells and $CD25^+T_{regs}$ express TLR4 and TLR5 (Caramalho et al. 2003). The TLR4 expression in $CD4^+CD45RB^{low}$ T cells and $CD25^+T_{regs}$ of B6 mice is regulated by macrophage migration inhibitory factor (MIF) via regulating suboptimal TCR//CD3-mediated activation and enabling them to sense their microenvironment (Alibashe-Ahmed et al. 2019). However, another study has indicated that activated $CD4^+$T cells express TLR3 and TLR9, but not TLR-2 and TLR-4 in BALB/c mice (Gelman et al. 2004). The TLR3 and TLR9 activation in $CD4^+$T cells in these mice increases their survival upon CD3 stimulation due to the upregulated B cell lymphoma-extra-large ($BCL\text{-}_{XL}$) expression (Gelman et al. 2004). The CpG-mediated TLR9 signaling pathway activation through MyD88 and PI3K plays a crucial role in the $CD4^+$T cell proliferation, prevents their anergy, and induces T-cell-dependent Ab immune response generation (Gelman et al. 2006). The PI3K activation requires an Src-homology domain 2 (SH2) binding motif in MyD88 Toll-like or IL-1receptor (TIR) domain. The deficiency of TIR-domain in the MyD88 abrogates the PI3K interaction with MyD88 that impairs Akt or protein kinase B activation, glycogen-synthase kinase 3 (GSK3) activation, and IL-2 production upon TLR9 stimulation in $CD4^+$T cells (Gelman et al. 2006).

On the other hand, MyD88 death domain (DD) is crucial for NF-κB activation and cell survival. Thus, like CD28-dependent comitogenic signals, MyD88-dependent PI3K activation in $CD4^+$T cells is crucial for their survival and NF-κB activation for their proliferation downstream to the TLR9 activation along with T cell-dependent B cell response. The CpG-dependent TLR9 co-stimulation of $CD4^+$T cells abrogates the immunosuppressive action of T_{regs} and also, TLR9 activation on T_{regs} inhibits their immunosuppressive function (LaRosa et al. 2007). Furthermore, the adjuvant effects of TLRs (TLR3 and TLR9) stimulation on $CD4^+$T effector cells are indirectly controlled by T_{regs} during antigen stimulation, including ovalbumin (OVA) (Olivier et al. 2011). This suggests that in the presence of any kind of TLR agonist the Ag-specific $CD4^+$T cells are under the negative control of T_{regs} as depletion of T_{regs} during OVA immunization in the presence of TLR9 agonist significantly increases the frequencies of OVA-specific IFN-γ-producing $CD4^+$ T cells and IFN-γ secretion (Olivier et al. 2011). The lack of type I IFN signaling strongly inhibits the generation of $CD4^+$ T-cell responses by B-OVA injected with TLR9 agonist and IFN 1-independent OVA-specific $CD4^+$ T-cell responses induced due to the TLR9 activation are also negatively affected by T_{regs} (Olivier et al. 2011). Thus the substantial decrease in the inflammatory environment due to the lack of

type 1 IFNs does not decrease the immunosuppressive function of T_{regs} on Ag-specific $CD4^+$T cells.

Unlike $CD4^+$T cells, the $CD8^+$T cells are not controlled by T_{regs} during Ag-stimulation with TLR3 or TLR9 agonists as adjuvants. The treatment of mice with TLR3 and TLR9 agonists increases the activity of both $CD4^+$T cells and T_{regs} without affecting the immunosuppressive activity of T_{regs} (Olivier et al. 2011). The study has shown that the TLR9 stimulation with the CpG alone is inefficient in activating $CD4^+FoxP3^+T_{regs}$ (Olivier et al. 2011). Hence, mouse TLRs expression on different subsets of T cells depends on their species like some mice species do not express TLR4 (C3H//Hej and C57BL/10ScCr species are endotoxin tolerant mice) or they are defective and are endotoxin tolerant (Moeller et al. 1978; Qureshi et al. 1999). The TLR2 engagement on $CD8^+$T cells enhances their effector function via lowering the threshold for co-stimulatory signals delivered by APCs (Cottalorda et al. 2006). The stimulation of TLR2 and TLR1 on activated murine T_{regs} (iT_{regs} or induced T_{regs}) increases the glycolysis and their proliferation (Gerriets et al. 2016). However, they loss their immunosuppressive characteristics due to the mTORC1 activation. The forkhead box P3 (FoxP3) transcription factor of T_{regs} opposes the PI3K-Akt-mTORC1 signaling, reduces glycolysis and anabolic metabolic pathways (lactate production and pentose-phosphate-pathway flux), but increases the catabolic and oxidative pathways (pyruvate and palmitate oxidation) (Gerriets et al. 2016). However, the glutamine oxidation remains unaltered. The author has described the details of T cell immunometabolism, including T_{regs} and conventional T cells somewhere else (Kumar 2018b). Thus TLR1 and TLR2 activation in iT_{regs} increases their glycolysis, which decreases FoxP3 expression, inhibiting their immunosuppressive function that plays a significant role in inflammatory bowel disease (IBD) immunopathogenesis in mice. Hence, it will be novel to extrapolate these findings in human cases of IBD or Crohn's disease (CD).

The *Schistosoma japonicum* infection increases the TLR7 expression in the mesenteric lymph node (MLN) $CD4^+$ and $CD8^+$T cells that induces IFN-γ production (Qu et al. 2019). The TLR7 stimulation in murine $CD8^+$ T cells enhances glycolysis through MyD88 and protein kinase B (AKT)-mammalian target of rapamycin (mTOR) signaling, and downstream IRF-4 transcription factor to increase their effector functions (Li et al. 2019a). The MyD88 activation downstream of TLR (TLR2, TLR4, TLR3, and TLR9) signaling in $CD4^+$T cells is involved in experimental colitis and IBD, and sustaining chronic intestinal inflammation through increasing their survival and proliferation (Tomita et al. 2008; Fukata et al. 2008). The MyD88 signaling promotes Th17 cells differentiation from $CD4^+$T cells and induces their proliferation by linking IL-1 and IL-23 signaling, and sustaining mTOR signaling (Chang et al. 2013). The MyD88 signaling inhibition in T cells has shown the amelioration of experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis (MS), an autoimmune disease in humans (Chang et al. 2013; Zheng et al. 2019). Studies have shown that the MyD88 signaling in naïve $CD4^+$T cells is essential for generating Th1 and Th17 cells via suppressing the immunosuppressive action of T_{regs} (Schenten et al. 2014). The TLR3, TLR7, and TLR9-mediated MyD88-dependent downstream signaling in

T cells is required for the Th17 cells generation and associated immune response during vaccination for fungal infections (Wang et al. 2016). T cells lacking MyD88 or in the absence of TLR stimuli activating it die due to increased caspase-3 (CASP3)-mediated apoptosis upon activation. Hence, TLR signaling via MyD88 regulates $CD4^+$T cell survival during contraction phase (Wang et al. 2016). Also, the T cell intrinsic MyD88 signaling is crucial for vaccine-induced IL-$17A^+$ $CD8^+$ T cells (Tc17) against lethal fungal pneumonia in mice (Nanjappa et al. 2015). The Tc17 cells are crucial for vaccine-induced immunity against lethal fungal pneumonia in mice lacking $CD4^+$T cells along with cancer and autoimmunity (Nanjappa et al. 2012; Liang et al. 2015). These Tc17 ($CD8^+$T cells secreting IL-17) cells are long-lasting cells and do not exhibit a plasticity toward IFN-γ producing $CD8^+$T cells (Nanjappa et al. 2017). The IFN-γ^+ $CD8^+$T cell (Tc1) response remains intact in $MyD88^{-/-}$ mice upon vaccine challenge against lethal fungal pneumonia, indicating that MyD88 signaling is not crucial for them. The MyD88 signaling through AKT and mTOR signaling increases the Tc17 cell proliferation through TLR2 activation (Nanjappa et al. 2015). Hence, translating these findings depending on the type of TLR expression in human T cells may open new avenues to fight against cancers, infections, and T cells-dependent autoimmune diseases.

3.2.2 TLRs Expression and Function in Human T Cells

Human T ($CD4^+$ and $CD8^+$) cells isolated from peripheral blood and spleen at ≥95% purity have shown the mRNA expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9, and $CD4^+$T cells upregulate the IFN-γ, IL-8, and IL-10 expression upon exposure to flagellin (a TLR5 ligand) and R8-48 (a TLR7/8 ligand) (Fig. 5) (Hornung et al. 2002; Zarember and Godowski 2002; Caron et al. 2005). Naïve human $CD4^+$T cells express TLR2 after stimulation with anti-TCR Ab and IFN-α and produce cytokines upon treatment with TLR2 ligand (Komai-Koma et al. 2004). The $CD4^+CD45RO^+$ memory T cells isolated from peripheral blood constitutively express TLR2 and produce IFN-γ upon TLR2 stimulation that also stimulates IFN-γ production from $CD45RO^+$T cells in the presence of IL-2 or IL-15 (Komai-Koma et al. 2004). Hence, TLR2 serves as a co-stimulatory receptor molecule that participates in the antigenic-T cell development and memory T cell maintenance after antigenic-TCR stimulation (Fig. 5). The TLR2 stimulation on human T_{regs} abrogates their immunosuppressive function through downregulating p27(Kip1) and the restoration of Akt phosphorylation (Fig. 5) (Oberg et al. 2010). Hence, pretreatment of T_{regs} in humans with TLR2 agonists reverses their immunosuppressive action on responding T cells.

The treatment with TLR2 agonist (Pam3CSK4) induces a significant increase in $CD8^+Foxp3^+$ T_{regs} expressing intracellular IL-10 and granzyme B (GzB) and inhibits *Dermatophagoides pteronyssinus* (dust mite)-induced IL-4 release during an allergen-specific immunotherapy (Tsai et al. 2010). The $CD8^+CD25^+FoxP3^+T_{regs}$ directly suppress $CD4^+$T cells proliferation through cell contact. Hence, TLR2 agonist induces immunosuppressive effect on both, $CD4^+$ and $CD8^+T_{regs}$. Thus, TLR2 agonist may serve as good adjuvant against mite allergies. TLR6, TLR8, and TLR10 mRNA in $CD4^+$T cells are either present at a very low level or are absent and

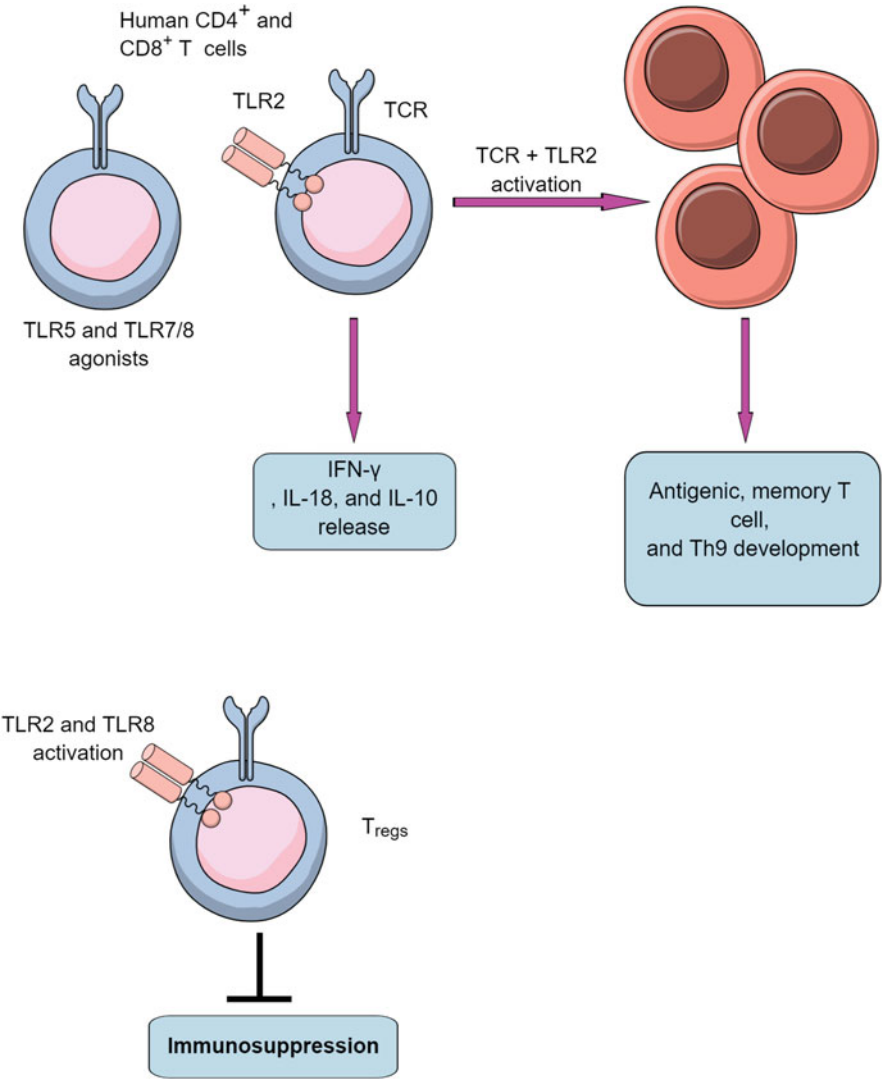

Fig. 5 TLRs in human T cells. The activation of TLR2, TLR5, and TLR7/TLR8 in human $CD4^+$ and $CD8^+$T cells increases the IFN-γ, IL-18, and IL-10 production. The activation of TLR2 as a co-stimulatory receptor induces the antigenic and memory T cell development as well as Th9 development that secretes IL-9. On the other hand, the TLR2 and TLR8 activation in human T_{regs} inhibits their immunosuppression function. Details are discussed in text

vary from donor to donor (Caron et al. 2005). The $CD4^+$T cells did not show this upregulation upon exposure to TLR3 (Poly I:C) and TLR4 ligands (LPS) (Caron et al. 2005). The $CD4^+CD45RO^+$ memory T cells respond more efficiently than $CD4^+CD45RA^+$ naive T cells to different TLR ligands (LPS, flagellin, poly I:C, and R-848) (Caron et al. 2005). Also, among memory T cells $CCR7^-$ cells (effector

memory T cells) are potent toward TLR stimulation than $CCR7^+$ central memory T cells. $CD4^+$ and $CD8^+$T cells isolated from human tonsils express TLR1, TLR2, TLR5, TLR9, and TLR10 predominantly (Mansson et al. 2006). However, tonsillar $CD4^+$T cells show an increased expression of TLR1 and TLR9, and $CD8^+$T cells express TLR4 and TLR3 at higher levels. Human neonatal $CD8^+$T cell expresses TLR2 and TLR5, and their stimulation exerts an additive effect on IFN-γ production (McCarron and Reen 2009). The neonatal $CD4^+$T cells, which are traditionally believed to have low potential to secrete Th1 cytokines in response to the polyclonal or Ag-specific stimuli show an increased pro-inflammatory Th1 immune response (IFN-γ and TNF-α production) and IL-2 production upon TLR2 co-stimulation with anti-CD3 stimulation (Sinnott et al. 2016). However, these naïve neonatal $CD4^+$T cells produce less IL-10. Hence, TLR2 co-stimulation enhances immune function of neonatal $CD4^+$T cells in response to their cognate antigen stimulation, even in the presence of corresponding APCs, including DCs, macrophages, and B cells.

The $CD8^+$T cells isolated from patients with group A streptococcal (GAS) tonsillitis show a higher TLR2, TLR3, and TLR5 expression than healthy controls and their $CD4^+$T cells show a decrease in TLR9 expression (Mansson et al. 2006). Other studies have also shown the TLR3 expression on $CD8^+$T effector cells constitutively (Tabiasco et al. 2006; Ngoi et al. 2008). The TLR3 activation in $CD8^+$T effector cells increases IFN-γ production during both TCR-dependent and independent stimulation. The TLR3 stimulation of chimeric antigen receptor modified T cells (CAR-T) increases the IL-2 and IFN-γ production along with increasing their lytic action against tumor or cancer cells (Di et al. 2019). Hence, the TLR3 activation on $CD8^+$T cells during viral infection (dsRNA) and different cancers may increase their antiviral and anti-tumor cytotoxic or cytolytic action. Of note, TLR3 activation does not involve MyDD8-dependent downstream signaling and the NF-κB activation, instead uses TOLL-IL-1R (TIR)-domain-containing adapter-inducing interferon-β (TRIF) as its downstream adaptor molecule to activate NF-κB and IRF3 activation-dependent cytokine and type 1 IFN production (Fig. 1) (Kumar 2019b; Yamamoto et al. 2003; Chattopadhyay and Sen 2014; Siednienko et al. 2011). The MyD88 adaptor molecule acts as a negative regulator of TLR3 signaling by inhibiting c-Jun N-terminal kinase (c-JNK) activation, IKK-ε activation, and inhibits IFN-β and regulated on activation T cell expressed and secreted (RANTES or CCL5) (Siednienko et al. 2011; Johnson et al. 2008). Along with MyD88, MyD88-adaptor like (Mal), an activator of TLR2/TLR4 signaling also inhibits TLR3/TRIF signaling and inhibits TLR-3-induced type 1 IFN production via associating and forming a complex with IRF7 (Siednienko et al. 2010). However, Mal does not affect the TLR3/TRIF-dependent IL-6 and TNF-α production.

The stimulation of TLR2 expressed on naïve $CD4^+$T cells shows an increased IL-9 mRNA expression and transdifferentiation into Th9 subtype of helper T (Th) cells as indicated by the increased expression of basic leucine zipper transcription factor ATF-like or B cell activating transcription factor (BATF) and PU.1 (a transcription factor (TF) that in humans is encoded by SPI1 gene), which positively regulates Th9 differentiation (Fig. 5) (Karim et al. 2017). Th9 cells promote protective IFN-γ release through releasing IL-9 during mycobacterial

tuberculosis (TB) to induce protective cell-mediated immunity (Alvarez et al. 2013). The TLR2 engagement on $CD4^+$T cells during TCR stimulation increases the population of mycobacterial tuberculosis (MTB) Ag-specific T cells, which exert a protective action through releasing IFN-γ (Reba et al. 2014). Hence, TLR2 signaling on $CD4^+$T cells exerts a protective action during TB. The $CD4^+$T effector cells of intraocular TB show a decreased TLR2 and TLR9 expression that increases pro-inflammatory immune response (increased IL-17a and IFN-γ production) and a decreased TGF-β and IL-10 production (Sharma et al. 2018). However, in the tumor microenvironment the tumor cell-released autophagosomes (TRAPs) have heat shock protein 90α (HSP90α) on their surface that stimulates TLR2 signaling on $CD4^+$T cells to release IL-6 in a MyD88-NF-κB-dependent manner (Chen et al. 2019; Zhou et al. 2016). This IL-6 in an autocrine manner stimulates IL-10 and IL-21 production in response to the STAT3 activation and inhibits $CD4^+$ and $CD8^+$T cell effector function that creates a tumor promoting immunosuppressive environment. Hence, TLR2 activation in $CD4^+$T cells creates a tumor promoting microenvironment. However, chimeric antigen receptor (CAR) T cells show an increased anti-tumor action upon co-stimulation with TLR2 ligands against refractory or relapsed B cell acute lymphoblastic leukemia (Lai et al. 2018). The third generation anti-CD19 CAR T cells with TLR2 domain are under clinical trial for refractory or relapsed (r/r) B cell non-non-Hodgkin's lymphoma (B-NHL) (George et al. 2020).

$CD25^{high}CD4^+$ human regulatory T cells (T_{regs}) and $CD4^+$T cells express TLR8 and TLR5 mRNA, but naïve $CD25^-CD4^+$ T_{regs} do not express TLR8 but express TLR5 (Peng et al. 2005; Crellin et al. 2005). TLR5 stimulation of T_{regs} increases their immunosuppressive function and FoxP3 expression. The co-stimulation of effector $CD4^+$T cells with anti-CD3 Ab and flagellin (a TLR5 ligand) enhances their proliferation and IL-2 secretion (Crellin et al. 2005). The TLR8 stimulation in $CD25^{high}$ T_{regs} reverts their immunosuppressive function and adoptive transfer of TLR8-stimualted $CD25^{high}CD4^+T_{regs}$ enhances anti-tumor immune function in vivo (Peng et al. 2005). The TLR8 stimulation in human T_{regs} inhibits glycolysis and glucose uptake that reverses their immunosuppressive function and enhances their anti-tumor function (Fig. 5) (Li et al. 2019b). For example, the adoptive transfer of TLR8 stimulated T_{regs} enhances their anti-tumor action in vivo in melanoma model. On the other hand, the $CD4^+$T cell co-cultured with ovarian cancer cells (SKOV3) upon stimulation with TLR8 ligand ssRNA 40 shows increased glycolysis, promotes $CD4^+$ $CD25^+$ $Foxp3^+$ T cells differentiation, and enhances the naïve $CD4^+$ T cells suppression (Shang et al. 2020). Hence, specific TLR8 stimulation in T_{regs} may have a potential for anti-tumor immunotherapy or immune cell-based therapeutics. While TLR8 activation on tumor cells may prevent tumor-induced T cell senescence in both naïve and tumor-specific T cells that enhances their anti-tumor effect (Ye et al. 2014). Hence, both tumor cell and T_{regs}-specific TLR8 activation may have great potential for cancer therapeutics.

TLR10 is also expressed by naïve human T_{regs} that decreases in the effector T_{regs} (Bell et al. 2007). The TLR10 expression on T_{regs} is governed by the cooperative FoxP3 and NF-AT complex, and TLR10 expression in T_{regs} increases upon TCR ligation in a Ca^{2+}-dependent manner (Bell et al. 2007). TLR10 has unique anti-

inflammatory properties when expressed with TLR2 or other TLRs excluding TLR1 and also acts as pro-inflammatory TLR by forming TLR10 homodimer or TLR10/TLR1 heterodimer (Fore et al. 2020; Su et al. 2020). The human $CD4^{+}CD25^{+}T_{regs}$ or effector Th1 and Th2 cells do not highly express TLR9 under normal condition, but it increases in T_{regs} synthesizing and releasing IL-10 in vitro that highly depends on 1α25VitaminD3 or calcitriol (Urry et al. 2009). The vitamin D3 or calcitriol intake increases IL-10 release from T_{regs} and TLR9 expression. The calcitriol stimulated T_{regs} producing IL-10 upon stimulation with TLR9 ligand (CpG-ODN) show a decreased IL-10 and IFN-γ production indicating the loss of their immunoregulatory function (Urry et al. 2009). However, they show increased IL-4 synthesis upon TLR9 stimulation. Thus, TLR9 ligation in human calcitriol-induced IL-10 producing T_{regs} has a great potential for cancer and infection immunotherapy or vaccine design. Further studies are required in the direction. Hence, TLRs play a crucial role in the T cell function and their transdifferentiation into different subtypes, which determine the disease phenotype and outcome.

4 Conclusion

TLRs discovery in the early twentieth century revolutionized the field of immunology by filling the gap between the recognition of pathogens by immune cells and the initiation of the pro-inflammatory innate immune response to clear them. This discovery led to the evolution of the concept of pattern recognition receptors (PRRs) responsible for different PAMPs/MAMPs and DAMPs recognition by innate immune cells. However, besides controlling the generation of pro-inflammatory innate immune response, these TLRs are also expressed by the cells of adaptive immunity and regulate their growth, development, and function under diverse condition, including infection, autoimmunity, and cancer. Hence, TLRs also regulate the immune homeostasis among adaptive immune cells and disruption of their expression and function may predispose to the host different autoinflammatory or autoimmune diseases, cancers, and various infections. Hence, adaptive immune cells-based targeting of specific TLRs may have novel therapeutic approach to different immunological conditions, including the current COVID-19 pandemic. For example, the generation of long-term Ab-mediated immunity or T cell-mediated immune response against the SAR-CoV2 infection is the need of time, which can fight the parent SARS-CoV2 along with mutants. Thus, studying TLRs in the context of adaptive immune cells and associated immune response has a bright future for generating different adaptive immune cell-specific immunomodulators and vaccines for different immunological diseases involving specific B and T cells of the adaptive immunity. Also, various host-derived negative regulators of TLR signaling have been identified that can be used for target overactivation of TLRs (Kumar 2020b). Hence, depending on the disease stage the modulation of cell-specific TLR signaling has a great potential to target adaptive immune response in different immunological conditions. Thus, studying TLRs in the context of adaptive immunity is as crucial as innate immunity.

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Intracellular TLRs of Mast Cells in Innate and Acquired Immunity

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_540

Abstract

Mast cells (MCs) distribute to interface tissues with environment, such as skin, airway, and gut mucosa, thereby functioning as the sentinel against invading allergens and pathogens. To respond to and exclude these external substances promptly, MCs possess granules containing inflammatory mediators, including heparin, proteases, tumor necrosis factor, and histamine, and produce these mediators as a consequence of degranulation within minutes of activation. As a delayed response to external substances, MCs de novo synthesize inflammatory mediators, such as cytokines and chemokines, by sensing pathogen- and damage-associated molecular patterns through their pattern recognition receptors, including Toll-like receptors (TLRs). A substantial number of studies have reported immune responses by MCs through surface TLR signaling, particularly TLR2 and TLR4. However, less attention has been paid to immune responses through nucleic acid-recognizing intracellular TLRs. Among intracellular TLRs, human and rodent MCs express TLR3, TLR7, and TLR9, but not TLR8. Some virus infections modulate intracellular TLR expression in MCs. MC-derived mediators, such as histamine, cysteinyl leukotrienes, LL-37, and the granulocyte-macrophage colony-stimulating factor, have also been reported to modulate intracellular TLR expression in an autocrine and/or paracrine fashion. Synthetic ligands for intracellular TLRs and some viruses are sensed by intracellular TLRs of MCs, leading to the production of inflammatory cytokines and chemokines including type I interferons. These MC responses initiate and facilitate innate responses and the subsequent recruitment of additional innate effector cells. MCs also associate with the regulation of adaptive immunity. In this overview, the expression of intracellular TLRs in MCs and the recognition of pathogens, including viruses, by intracellular TLRs in MCs were critically evaluated.

Keywords

Acquired immunity · Innate immunity · Mast cells · TLR3 · TLR7 · TLR9 · Toll-like receptors · Virus

1 Introduction

Mast cells (MCs) are phenotypically defined as cells expressing the high-affinity receptor for immunoglobulin E (IgE) (FcεRI) and the stem cell factor receptor (c-kit). MCs are, therefore, the central player in IgE-mediated allergic responses. Upon pathogen invasion, MCs directly kill pathogens through complement activation and antimicrobial peptide (AMP) release (Thoma-Uszynski et al. 2001; Muller-Eberhard 1986). In addition to the direct effects against pathogens, MCs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through their pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene I

(RIG-1)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), followed by the activation of innate and acquired immune responses, independently of IgE (Sandig and Bulfone-Paus 2012; St John and Abraham 2013; Marshall et al. 2003b).

MC progenitors derived from multipotential progenitors egress from the bone marrow and reach several peripheral tissues, where they accomplish terminal differentiation and proliferation. MCs preferentially distribute to interface tissues with environment, such as skin, airway, and gut mucosa, to serve as the sentinel against invading allergens and pathogens. Upon differentiation and acquirement of their residency in each tissue, MCs are affected by microenvironments, thereby leading to the functional and immunocytological heterogeneity to adapt to each tissue environment where they are distributed (Dudeck et al. 2019; Cildir et al. 2017). Rodent MCs are categorized into connective tissue MCs (CTMCs) and mucosal MCs (MMCs) in accordance with predominant proteoglycan contents in their granules, heparin, and chondroitin. Human MCs are categorized into tryptase- and chymase-positive MCs (MC_{TC}) and tryptase-positive MCs (MC_T). In contrast to rodent MCs, both human MC subsets contain heparin and chondroitin as proteoglycan in their granules. Accordingly, MC_{TC} and MC_T are discriminated based on the protease contents and electron microscopic structural difference of their granules. Rodent CTMCs and MMCs are presumed to correspond to human MC_{TC} and MC_T, respectively. CTMCs and MC_{TC} are constitutively distributed in the skin, gut lamina propria, and pulmonary perivascular tissues, whereas MMCs and MC_T are distributed in the mucosal epithelium of the gut and lungs (Igawa and Di Nardo 2017; Irani et al. 1986).

TLRs, the most thoroughly studied PRRs, are crucial for host defense as they trigger proper immune responses against invading pathogens (Kawai and Akira 2008). Mice lacking myeloid differentiation primary response 88 (MyD88) and Toll/interleukin (IL)-1 receptor domain-containing adaptor-inducing interferon (IFN)-β (TRIF), the major adaptor molecules of TLR signaling, cannot survive to weaning age without antibiotic administration (Hoebe et al. 2003). Similarly, TLR polymorphism results in susceptibility to specific pathogens (Mukherjee et al. 2019). To date, 10 human (TLR1–TLR10) and 12 murine (TLR1–TLR9 and TLR11–TLR13) TLR homologs have been identified. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 (only in humans) are expressed on the cell surface plasma membrane and recognize microbial cell structural components. TLR3, TLR7, TLR8, TLR9, and TLR13 (only in mice) are basically expressed intracellularly and recognize nucleic acids (Kawasaki and Kawai 2014). Similarly, the heterodimers of TLR11 and TLR12 (only in mice) are expressed intracellularly but recognize profilin from the parasite *Toxoplasma gondii*. Profilin is a low-molecular-weight actin-binding protein essential for *T. gondii* invasion into host cells (Koblansky et al. 2013).

Regarding the association between MCs and TLRs, a substantial number of studies have elucidated immune responses through surface TLRs, particularly TLR2 and TLR4. However, less attention has been paid to immune responses through nucleic acid-recognizing intracellular TLRs. Hence, the expression and

function of *intracellular* TLRs of MCs have been comprehensively summarized in the chapter.

2 Expression of Intracellular TLRs in MCs

It has been recently reported that embryonic skin MCs are derived from the yolk sac (Gentek et al. 2018), as is the case for macrophages and epidermal Langerhans cells (Hoeffel et al. 2012), implying a diversity of MC maturation in each tissue. Additionally, MCs exhibit much greater heterogeneity across tissues than conventionally estimated. Thus, TLR expression in MCs would greatly differ according to tissue and age. With this in mind, intracellular TLR expression in rodent and human MCs is comprehensively summarized in the following sections and Table 1. Among intracellular TLRs, TLR11 to TLR13 expression in MCs has not been investigated yet. Thus, expression of TLR3 and TLR7 to TLR9 in MCs is described in this section.

2.1 Rodent MCs

Rat and murine peritoneal MCs expressed TLR3, TLR7, and TLR9 mRNA. TLR3 was detected by flow cytometry in both surface plasma membrane and intracellular compartments (Agier et al. 2016; Orinska et al. 2005; Xie et al. 2018), whereas TLR7 and TLR9 were predominantly detected in intracellular compartments (Agier et al. 2016; Witczak et al. 2014). TLR8 expression was not analyzed.

Murine bone marrow-derived MCs (BMMCs) expressed TLR3 at the mRNA and protein levels (Matsushima et al. 2004; Orinska et al. 2005). In line with peritoneal MCs, TLR3 was detected by flow cytometry in their surface plasma membrane and intracellular compartments (Orinska et al. 2005). A culture system was established by which a large number of fetal skin-derived MCs (FSMCs) can be yielded (Yamada et al. 2003). Of note is that FSMCs share many characteristics with CTMCs. FSMCs exhibited much higher TLR3 mRNA expression than that of BMMCs. BMMCs expressed marginal TLR7 mRNA and no TLR9 mRNA, whereas FSMCs expressed strong TLR7 mRNA and marginal TLR9 mRNA. TLR8 expression was not analyzed in this study (Matsushima et al. 2004). Consistent with TLR7 expression in FSMCs, murine dermal MCs strongly expressed TLR7 (Heib et al. 2007). P815 cells, a mouse mastocytoma cell line, also expressed TLR3, TLR7, and TLR9 at the mRNA and protein levels (Yang et al. 2009).

2.2 Human MCs

TLR expression of ex vivo skin MCs isolated from human skin was analyzed in two reports using real-time polymerase chain reaction and Deep Cap Analysis of Gene Expression sequencing, respectively. Kulka et al. demonstrated that skin MCs

Table 1 Intracellular TLR expression in MCs in the published studies

Reference	Host	Type of MCs	Methods	TLR3	TLR7	TLR8	TLR9
Agier et al. (2016)	Rat	Peritoneal MCs	qPCR/ FC	+	+	N.D.	+
Kulka et al. (2004)	Human	PB $CD34^+$ cell-derived MCs	PCR/ WB	+	+	−	+
Kulka et al. (2004)	Human	LAD–2	PCR/ WB	+	+	−	+
Kulka et al. (2004)	Human	HMC-1	PCR/ WB	+	+	+	+
Yoshioka et al. (2007)	Human	LAD–2	PCR/ WB	N.D.	N.D.	N.D.	+
Matsushima et al. (2004)	Mouse	BMMC	PCR	Low	Low	N.D.	Low
Matsushima et al. (2004)	Mouse	FSMC	PCR	+	+	N.D.	+
Yang et al. (2009)	Mouse	P815	PCR/ FC/IF	+	+	N.D.	+
Orinska et al. (2005)	Mouse	Peritoneal MCs	FC	+	N.D.	N.D.	N.D.
Orinska et al. (2005)	Mouse	BMMC	FC	+	N.D.	N.D.	N.D.
Yoshioka et al. (2007)	Human	LAD–2	WB	N.D.	N.D.	N.D.	+
Witczak et al. (2014)	Rat	Peritoneal MCs	WB	N.D.	+	N.D.	N.D.
Kulka et al. (2004)	Human	Skin MCs	PCR	+	+	−	+
Kulka et al. (2004)	Human	Lung MCs	PCR	+	+	−	−
Motakis et al. (2014)	Human	Skin MCs	CAGE	+	+	N.D.	−
Fig. 1 in this review	Human	Skin MCs	IF	+	+	N.D.	+

N.D. Not done, *qPCR* Quantitative RT-PCR, *FC* Flow cytometry, *WB* Western blotting, *IF* Immunofluorescence, *CAGE* Cap Analysis of Gene Expression

strongly expressed TLR3 and TLR9, weakly expressed TLR7, and did not express TLR8 (Kulka and Befus 2003). Motakis et al. demonstrated that skin MCs expressed TLR3 and TLR7. TLR8 expression was not analyzed. Interestingly, TLR9 expression was not detected in this study (Motakis et al. 2014). Thus, TLR3 and TLR7, but not TLR8, were seemingly expressed in human dermal MCs. TLR9 expression was quite conflicting. Accordingly, this study has sought to determine the expression of TLR9, as well as TLR3 and TLR7, in normal human skin samples at protein levels. Neurofibroma skin samples were utilized as a positive control because MCs are enriched in neurofibroma, and they facilitate tumor promotion (Staser et al. 2010).

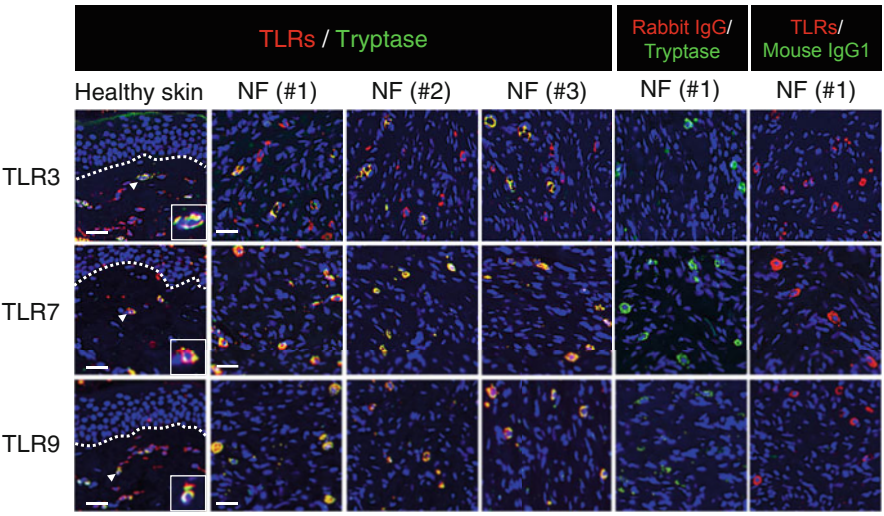

Fig. 1 TLR3, TLR7, and TLR9 expression in MCs of healthy skin and neurofibroma. Immunohistochemistry was performed using formalin-fixed, paraffin-embedded human skin samples in 5 μm sections. The sections were dried, dewaxed, and then rehydrated. Following antigen retrieval in citrate buffer (pH 6.0) and blocking with 5% goat serum for 1 h at room temperature, the sections were incubated overnight at 4°C with mouse anti-tryptase (1:200; abcam), rabbit anti-TLR3 (1:200; abcam), rabbit anti-TLR7 (1:200; Novus), rabbit anti-TLR9 (1:200; abcam), and each isotype-matched control IgG. After washing, the sections were incubated for 3 h at room temperature with Alexa Fluor 488- and Alexa Fluor 555-conjugated anti-mouse and rabbit IgG, respectively. All samples were mounted with VECTASHIELD Mounting Medium supplemented with DAPI (H-1200; Vector Lab). Immunofluorescent images were obtained using a Biorevo BZ-9000 fluorescence microscope (Keyence). MCs in neurofibroma were apparently larger in size than those in healthy skin. MCs expressed TLR3, TLR7, and TLR9 in both healthy skin and neurofibroma. The small panels on the bottom right in healthy skin showed magnified images indicated by arrowheads. Scale bar = 30 μm

Tryptase-positive skin MCs in a healthy individual and patients with neurofibroma clearly expressed TLR9, as well as TLR3 and TLR7 (Fig. 1). Ex vivo lung MCs isolated from adult lung samples strongly expressed TLR3 and TLR7 mRNA but not TLR8 and TLR9 mRNA (Kulka and Befus 2003). Collectively, skin MCs expressed TLR3, TLR7, and TLR9 at the mRNA and protein levels, whereas lung MCs expressed TLR3 and TLR7, but not TLR9, at the mRNA levels, suggesting MC diversity across tissues.

Human peripheral blood (PB) $CD34^+$ cell-derived MCs and laboratory of Allergic Disease MC line (LAD)-2 expressed TLR3, TLR7, and TLR9, but not TLR8, at the mRNA and protein levels. Human MC line 1 (HMC-1) also expressed TLR3, TLR7, and TLR9. In sharp contrast with other MCs, HMC-1 strongly expressed TLR8 at the mRNA and protein levels (Kulka et al. 2004).

Taken together, MCs expressed TLR3, TLR7, and TLR9 but not TLR8. MCs could be the sole cells capable of detecting PAMPs and DAMPs at the infection site in a steady-state condition. For instance, in non-inflamed skin and lung epithelium,

MCs are the sole TLR7-expressing cells capable of detecting single-stranded RNA (ssRNA) derived from viruses (Sha et al. 2004; Ritter et al. 2005).

3 Expressional Alterations of Intracellular TLRs in MCs by Various Stimuli

Some viruses modulate intracellular TLR expression in MCs. Vesicular stomatitis virus (VSV) infection upregulated TLR3 mRNA expression in LAD-2 without inducing degranulation (Tsutsui-Takeuchi et al. 2015). Dengue virus infection in human cord blood (CB)-derived MCs upregulated TLR3 and TLR7 mRNA expression (Brown et al. 2012). Influenza A virus infection in P815 cells upregulated the mRNA expression of TLR3 and TRIF (Meng et al. 2016). Sendai virus infection in human PB-derived MCs upregulated the mRNA expression of TLR3, as well as MDA5 and RIG-1 (Lappalainen et al. 2013).

Histamine, the major mediator released by activated MCs, amplified TLR3 expression and production of monocyte chemoattractant protein (MCP)-1 and IL-13 in HMC-1 and P815 cells at the mRNA and protein levels through the H1 receptor by activating phosphatidylinositol 3-kinase (PI3K)/Akt and Erk1/2/mitogen-activated protein kinase (MAPK). The effect of TLR7 and TLR9 expression was not analyzed (Xie et al. 2018).

Cysteinyl leukotrienes (CysLTs), including LTC4, LTD4, and LTE4, are biosynthesized from arachidonic acid in leukocytes, particularly in MCs, eosinophils, basophils, neutrophils, and monocytes. CysLTs induce strong bronchoconstriction; enhanced vascular permeability, mucin secretion, and activation and recruitment of eosinophils; and enhanced airway reactivity and remodeling, thereby exacerbating asthma (Back et al. 2011). LTE4 itself has been recently reported to activate MCs through the CysLT1 receptor. LTC4 downregulated TLR7 mRNA expression, as well as surface TLRs in human PB $CD34^+$ cell-derived MCs through the CysLT1 receptor (Karpov et al. 2018). LTD4 also downregulated TLR3 and TLR7 mRNA expression (Karpov et al. 2018).

A mature AMP, LL-37, is cleaved from the sole human cathelicidin, hCAP18 (human cationic antibacterial protein of 18 kDa), by proteinase 3 (Sorensen et al. 2001), exerting direct antibacterial effects against Gram-positive and Gram-negative bacteria. Upon bacterial exposure to MCs, MCs form MC extracellular traps consisting of DNA, histones, and granule proteins, such as tryptase and LL-37, to kill bacteria (von Kockritz-Blickwede et al. 2008). LL-37 upregulated the protein expression of TLR3, TLR7, and TLR9 (Agier et al. 2018a) and of NOD1, NOD2, and RIG-1, which were constitutively expressed in rat peritoneal MCs (Agier et al. 2018b). Additionally, LL-37 induced the activation and degranulation of MCs. Although three receptors for LL-37 have been identified to date, including G protein-coupled formyl peptide receptor 2 (FPR2) (De et al. 2000), the $P2X_7$ receptor ($P2X_7$-R) (Tomasinsig et al. 2008), and the epidermal growth factor receptor (EGFR) (Tokumaru et al. 2005), LL-37-induced MC activation and degranulation were independent of these three known LL-37 receptors. Instead, a G protein-

coupled Mas-related gene X2 (MrgX2) has been identified as the responsible LL-37 receptor in LAD-2. Internalized LL-37 interacted with MrgX2 in the perinuclear region and then triggered their activation and degranulation (Subramanian et al. 2011; Yu et al. 2017; Murakami et al. 2018). That is an open question whether LL-37-induced upregulation of TLR expression and the production of cytokines and chemokines are mediated by MrgX2.

The granulocyte-macrophage colony-stimulating factor (GM-CSF) has been reported to upregulate TLR2, TLR4, and TLR9 expression in neutrophils (O'Mahony et al. 2008; Hayashi et al. 2003). Human CB-derived MCs produce GM-CSF after exposure to bacterial peptidoglycan (McCurdy et al. 2003) and IgE (Gilchrest et al. 2003). HMC-1 can also produce GM-CSF in response to a calcium ionophore (Zhao et al. 2004). GM-CSF upregulated TLR3 and TLR7, but not TLR9, at the mRNA and protein levels in P815 cells (Yang et al. 2009). In addition to GM-CSF, IFN-β has been reported to upregulate TLR3 mRNA expression and melanoma differentiation-associated gene 5 (MDA5) and RIG-1 in human PB $CD34^+$ cell-derived MCs (Lappalainen et al. 2013). There might be other inflammatory cytokines that modulate TLR expression in MCs.

Periplaneta americana allergen 7 (Per a 7), one of the major allergens of the American cockroach, is deeply associated with the development and exacerbation of perennial rhinitis and asthma. Per a 7 is tropomyosin of *P. americana*. Tropomyosin is a pan-allergen that strongly cross-reacts with food and inhalant allergens originated from invertebrates (Asturias et al. 1999; Santos et al. 1999). Per a 7 downregulated TLR9 expression and IL-12 production at the mRNA and protein levels in P815 cells by activating ERK and PI3K/Akt signaling (Yang et al. 2012).

Collectively, TLR expression in MCs is modulated by both endogenous and exogenous molecules in an autocrine and paracrine fashion (summarized in the Table 2). Research on the modulation of MC TLRs might enable a better understanding of the involvement of MCs in the regulation of innate and acquired immunity.

4 Intracellular TLR Signaling in MCs

As described, MCs express TLR3, TLR7, and TLR9 as intracellular TLRs. In this section, intracellular TLR signaling in MCs is summarized.

4.1 TLR3 Overview

TLR3 senses double-stranded RNA (dsRNA), which is longer than 30 bp, and its synthetic analog polyinosinic-polycytidylic acid [poly(I:C)]. The RNA virus is divided into a dsRNA virus, the positive-strand ssRNA virus, and the negative-strand ssRNA virus. Although a retrovirus is categorized into positive-strand ssRNA, its genomic RNA is transiently translated into a complementary DNA strand by reverse transcriptase, in sharp contrast with other RNA viruses. Non-self dsRNA

Table 2 Modulation of intracellular TLR expression in MCs in the published studies

Reference	Host	Type of MCs	Methods	Stimuli	TLR3	TLR7	TLR9
Tsutsui-Takeuchi et al. (2015)	Human	LAD–2	qPCR/WB	VSV	Up	N.D.	N.D.
Brown et al. (2012)	Human	CB-derived MCs	qPCR	Dengue virus	Up	Up	N.D.
Meng et al. (2016)	Mouse	P815	FC/WB	Influenza A virus	Up	N.D.	N.D.
Lappalainen et al. (2013)	Human	PB $CD34^+$ cell-derived MCs	qPCR	Sendai virus	Up	N.D.	N.D.
Xie et al. (2018)	Human	HMC-1	qPCR/FC	Histamine	Up	N.D.	N.D.
Xie et al. (2018)	Mouse	P815	qPCR/FC	Histamine	Up	N.D.	N.D.
Karpov et al. (2018)	Human	PB $CD34^+$ cell-derived MCs	qPCR	LTC4	Stable	Down	N.D.
Karpov et al. (2018)	Human	PB $CD34^+$ cell-derived MCs	qPCR	LTD4	Down	Down	N.D.
Agier et al. (2018a, b)	Rat	Peritoneal MCs	qPCR/FC	LL-37	Up	Up	Up
Yang et al. (2009)	Mouse	P815	qPCR/FC	GM-CSF	Up	Up	Stable
Lappalainen et al. (2013)	Human	PB $CD34^+$ cell-derived MCs	qPCR	IFN-β	Up	N.D.	N.D.
Yang et al. (2012)	Mouse	P815	qPCR/FC	Per a 7	N.D.	N.D.	Down

N.D. Not done, *qPCR* Quantitative RT-PCR, *FC* Flow cytometry, *WB* Western blotting, *IF* Immunofluorescence

is derived from the dsRNA virus and positive-strand ssRNA and dsDNA virus, but not the negative-strand ssRNA virus, as intermediate replication products (Weber et al. 2006). Thus, viral nucleic acid could be recognized by TLR3, jumping the viral species barrier.

All TLR signaling, including TLR3 signaling, activates nuclear factor-κB (NF-κB) and MAPKs, thereby leading to the production of proinflammatory cytokines. One exception of TLR3 signaling is that TLR3 absolutely signals through the TRIF adapter protein, whereas other TLRs signal through the MyD88 adapter protein. TRIF associates with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and receptor-interacting protein 1 (RIP1) and then activates NF-κB and MAPK, followed by proinflammatory cytokine production. By contrast, a complex

of TRIF, TRAF6, and RIP3 associates with TRAF3 and then with phosphorylates IFN regulatory factor 3 (IRF3), followed by IFN-β production.

4.2 TLR3 Stimulation in MCs

4.2.1 Rodent MCs

Poly(I:C) did not induce the degranulation of BMMCs (Orinska et al. 2005), FSMCs (Matsushima et al. 2004), and rat peritoneal MCs (Witczak et al. 2020).

Poly(I:C)-stimulated BMMCs induced the mRNA expression of IFN-β, a 15 kDa IFN-stimulated gene, IP10 (CXCL10), and RANTES (CCL5) (Orinska et al. 2005), and protein production of macrophage inflammatory protein (MIP)-1β and RANTES (CCL5), but not IL-13, IL-6, and MCP-1 (CCL2) (Orinska et al. 2005), in contrast to histamine-stimulated human MCs (see Sect. 3). By contrast, another group described that BMMCs did not produce IFN-α, IFN-β, TNF-α, and IL-6 at the protein level in response to poly(I:C) (Keck et al. 2011).

As described in Sect. 2.1, FSMCs (CTMCs) expressed much higher TLR3 mRNA than BMMCs did (Matsushima et al. 2004). Poly(I:C)-stimulated FSMCs produced a substantial amount of protein of TNF-α, IL-6, MIP-1α, MIP-1β, and RANTES, whereas those of poly(I:C)-stimulated BMMCs were quite marginal. Both BMMCs and FSMCs did not produce IL-13 in response to poly(I:C) (Matsushima et al. 2004). Taken together, in contrast to CTMC-like FSMCs, it is controversial whether BMMCs produce type I IFNs and inflammatory cytokines/chemokines in response to poly(I:C).

Intraperitoneal injection of poly(I:C) upregulated the expression of major histocompatibility complex (MHC) class II, CD80, CD28, and complement receptors of murine peritoneal MCs and the recruitment of effector $CD8^+$ T cells (Orinska et al. 2005). In addition, poly(I:C)-treated rat peritoneal MCs upregulated MHC class I expression and induced the de novo synthesis of type I IFNs (IFN-α and IFN-β), proinflammatory lipid mediators (CysLTs), cytokines (TNF-α and IL-1β), and chemokines [MIP-1α (CCL3) and IL-8 (CXCL8)] (Witczak et al. 2020).

Collectively, poly(I:C) triggers the production of IFN-β and IFN-inducible proteins and chemokines recruiting effector $CD8^+$ T cells. In addition, poly(I:C)-treated MCs express MHC class I and II and costimulatory molecules. Thus, TLR3 stimulation by poly(I:C) exerts direct antiviral immunity and triggers the generation and accumulation of virus-antigen-specific cytotoxic T cells independently of MC degranulation (Orinska et al. 2005).

4.2.2 Human MCs

Poly(I:C) itself did not induce degranulation, and it did not influence the IgE-mediated degranulation of human PB $CD34^+$ cell-derived MCs (Kulka et al. 2004). Integrin-mediated MC adhesion to extracellular matrix has been known to facilitate IgE-mediated responses. Poly(I:C)-stimulated LAD-1 MCs decreased the expression of β1 integrin (CD29), the fibronectin receptor, thereby suppressing IgE-mediated MC degranulation (Kulka and Metcalfe 2006).

The stimulation of intracellular TLR3, but not plasma membrane-bound TLR3, with poly(I:C) upregulated the mRNA expression of type I IFNs (IFN-α and IFN-β) and TNF-α in human PB $CD34^+$ cell-derived MCs (Lappalainen et al. 2013). In another study, in which the same MCs were utilized, TLR3 stimulation with poly(I:C) induced the protein production of IFN-α and CysLTs but not IFN-β, TNF-α, GM-CSF, and IL-5 (Kulka et al. 2004). This discrepancy may be caused by the culture period of $CD34^+$ progenitor cells and the method of determining cytokine expression. Therefore, an analysis using ex vivo MCs will be required and informative.

4.3 TLR7 Overview

TLR7 preferentially senses guanosine- and uridine-rich ssRNA and imidazoquinoline compounds, such as resiquimod (R848) and imiquimod (IMQ), and guanine analogs, such as loxoribine (Diebold et al. 2004; Heil et al. 2004; Lund et al. 2004). Human TLR8, but not murine TLR8, also senses ssRNA, although human MCs do not express TLR8 (Jurk et al. 2002). TLR7 even recognizes some small interfering RNAs (siRNAs) (Hornung et al. 2005). Non-self ssRNA is derived from ssRNA viruses, such as HIV, influenza virus, and VSV, and phagosomal bacteria, such as *B. burgdorferi* and group B streptococcus (Mancuso et al. 2009; Petzke et al. 2009).

In sharp contrast to TLR3 signaling, TLR7 signaling completely depends on MyD88. TLR7 stimulation in macrophages, conventional dendritic cells (cDCs), and plasmacytoid DCs (pDCs), in which TLR7 and TLR9 are strongly expressed, recruits MyD88 and IL-1 receptor-associated kinase 4 (IRAK4). Activated IRAK4 dissociated from MyD88 forms a complex with TRAF6, followed by the activation of the TAK1 complex, leading to the activation of NF-κB and MAPKs and subsequent proinflammatory cytokine production. pDCs constitutively express IRF7, which interacts with MyD88, followed by the formation of a complex with IRAK4 and TRAF6, leading to the phosphorylation of IRF7 and subsequent type I IFN production. MCs appear not to have IRF7. By contrast, MCs have a TLR7 signaling pathway required for proinflammatory cytokine production (Sandig et al. 2013; Avila et al. 2012).

4.4 TLR7 Stimulation in MCs

Similarly to poly(I:C) stimulation, R848 did not induce the degranulation of BMMCs and FSMCs (Matsushima et al. 2004). FSMCs produced a large amount of cytokines, such as TNF-α and IL-6, and chemokines, such as MIP-1α, MIP-2, and RANTES, in response to R848 (Matsushima et al. 2004). Although BMMCs could produce these cytokines and chemokines, their amount was much lower than that from FSMCs (Matsushima et al. 2004). The stimulation of rat mature peritoneal MCs with R848 led to the synthesis of CysLTs and IFN-β and impaired anti-IgE-

mediated histamine release (Witczak et al. 2014). The responsible pathway for IFN-β synthesis was not clear in this study. Another work demonstrated that the stimulation of rat peritoneal MCs with R848 led to upregulation of FcεRI expression and production of type I IFNs (IFN-α and IFN-β), TNF, chemokines such as MIP-1α (CCL3) and IL-8 (CXCL8), and proinflammatory lipid mediators (Agier et al. 2021).

Daily topical application of synthetic a TLR7 ligand, IMQ, to murine skin is now widely utilized to induce psoriasis-like dermatitis (van der Fits et al. 2009). In this IMQ-induced psoriasis-like dermatitis model, dermal MC-derived TNF-α and IL-1β initiated skin inflammation by recognizing IMQ, because MCs are the sole TLR7-expressing cells in steady-state skin (see Sect. 2). In addition, TLR7-stimulated dermal MCs facilitated epidermal Langerhans cell migration to draining lymph nodes (dLNs), which was partially mediated by dermal MC-derived IL-1β (Heib et al. 2007). Additionally, topical application of a synthetic TLR7 ligand, IMQ or R848, to murine ear three times weekly for 4 weeks induced systemic lupus erythematosus (SLE)-like autoimmune responses, including elevated autoantibody levels against dsDNA and multiple organ inflammation, such as glomerulonephritis, hepatitis, carditis, and photosensitivity. The expression of IFN-α and Mx1, the IFN-α-inducible gene, was upregulated in the organs of IMQ-treated mice. Although these autoimmune responses were mediated by pDCs, TLR7-expressing dermal MCs seemed to initiate a set of responses by triggering pDC migration into the skin (Yokogawa et al. 2014). Indeed, topical IMQ application to murine skin induced the protein synthesis of TNF-α, IL-1β, and MCP-1 (CCL2) in a TLR7-dependent fashion. Moreover, in vitro stimulation of BMMCs by IMQ also induced MCP-1 production. Collectively, TLR7-expressing dermal MCs produced MCP-1 in response to topical IMQ application, followed by pDC recruitment into the skin and subsequent type I IFN production (Drobits et al. 2012).

Taken together, TLR7 stimulation of MCs triggered proinflammatory cytokines, such as TNF-α and IL-1β, but not type I IFNs. Alternatively, MC-derived MCP-1 recruited pDCs and subsequent type I IFN production. These responses activate innate and adaptive immune responses, resulting in virus clearance. By contrast, the systemic administration of a potent TLR7 ligand, SM360320, which mimics systemic virus infection, transiently induced anorexia and hypothermia. These symptoms were triggered by MCs and TLR7/MyD88-mediated TNF-α but not IL-1β and IL-6 (Hayashi et al. 2008).

In addition to triggering inflammation through MC TLR7, MCs may suppress excess inflammation through TLR7. Repeated subcutaneous administration of a weak agonistic TLR7 ligand reduced MC-driven neutrophil migration in dextran sodium sulfate-induced colitis and thioglycolate-induced peritonitis with unknown underlying mechanisms (Hayashi et al. 2012).

4.5 TLR9 Overview

TLR9 efficiently senses ssDNA containing unmethylated CpG dinucleotides (CpG motifs), commonly present in viral and bacterial DNA, resulting in robust type I IFN

production. CpG motifs are four times less abundant in mammalian genomic DNA than in viral and bacterial DNA, and CpG motifs in mammalian genomic DNA are typically methylated. These methylated mammalian CpG motifs are poorly recognized by TLR9 (Hemmi et al. 2000; Lund et al. 2003; Muller et al. 2008). TLR9 also senses synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG ODN). Non-self unmethylated CpG motifs are derived from dsDNA viruses, such as mouse cytomegalovirus (CMV) and herpes simplex virus (HSV) 1 and 2 (Lund et al. 2003; Krug et al. 2004a, b). Although TLR9 had been originally identified to recognize bacterial DNA (Hemmi et al. 2000), MCs did not produce type I IFNs upon exposure to Gram-positive and Gram-negative bacteria because bacteria were firmly bound at the MC cell surface, thereby not internalizing in order to recognize TLR9 (Dietrich et al. 2010).

Almost all DNA viruses are dsDNA viruses, except for parvovirus and hepadnavirus. Therefore, dsDNA must be digested into short ssDNA fragments to recognize TLR9. In pDCs, virus replication is not required for the induction of type I IFNs. Viruses undergo endocytosis and are transported to the lysosomal compartments, where virions are subsequently degraded into ssDNA fragments by DNase II, which is the lysosomal DNase (Kawai and Akira 2008). Lysosomal DNase II is also required for bacterial DNA recognition by TLR9 of bone marrow-derived DCs (Chan et al. 2015). Regarding TLR9 signaling, please refer to Sect. 4.3.

4.6 TLR9 Stimulation in MCs

Similarly to poly(I:C) and R848 stimulation, CpG ODN did not induce the degranulation of BMMCs and FSMCs (Matsushima et al. 2004; Zhu and Marshall 2001). FSMCs produced a large amount of cytokines, such as TNF-α and IL-6, and chemokines, such as MIP-1α, MIP-2, and RANTES, in response to CpG ODN (Matsushima et al. 2004). Although BMMCs could produce these cytokines and chemokines, the amount was much lower than that from FSMCs (Matsushima et al. 2004; Zhu and Marshall 2001). *Escherichia coli*-derived bacterial DNA also induced IL-6 production in BMMCs (Zhu and Marshall 2001). BMMCs did not produce IFN-γ, GM-CSF, IL-12p40, and IL-4 in response to CpG ODN (Zhu and Marshall 2001). However, natural killer (NK) cells cocultured with CpG ODN-stimulated BMMCs amplified IFN-γ production independently of MC-derived TNF-α and dependently of OX40 signaling (Vosskuhl et al. 2010), suggesting that the interactions between MCs and NK cells facilitate virus clearance.

In a murine in vivo study, intraperitoneal CpG ODN administration suppressed the increase of the peribronchial MC number and of their growth factors, such as IL-4 and IL-9, in a repeated OVA exposure model, a model of chronic asthma. However, it was unclear whether this effect was through TLR9 due to undecided TLR9 expression in murine peribronchial MCs (Ikeda et al. 2003). NC/Nga mice kept in conventional conditions developed atopic dermatitis-like skin inflammation accompanied by increased serum IgE levels, infiltration of MCs and eosinophils, and Th2 cytokines (Vestergaard et al. 2000). Topical CpG ODN application ameliorated

skin inflammation and decreased serum IgE levels, infiltration of MCs and eosinophils, and Th2 cytokines but increased Th1 cytokines and regulatory T cells (Inoue and Aramaki 2007). Given the TLR9 expression in FSMCs, these responses were likely in part mediated by MC TLR9. Collectively, CpG ODN administration in mice improved the condition of Th2-mediated diseases through TLR9 signaling, including in MCs.

5 Role of Intracellular TLRs of MCs on Innate Immunity

As described earlier, MCs could produce cytokines, such as TNF-α, IL-6, type I IFNs, and IL-1β, chemokines, such as MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), IL-8 (CXCL8), and IP10 (CXCL10), and lipid mediator CysLTs upon stimulation of their intracellular TLRs without degranulation. These inflammatory mediators are crucial for host defense against invading pathogens. Type I IFNs directly kill the virus, whereas others recruit innate immune cells to the infection site (Cardamone et al. 2016). TNF-α, IL-8 (CXCL8), and IP10 (CXCL10) recruit neutrophils. RANTES (CCL5) and LTC4 recruit eosinophils. Of note is that MCs are the only immune cells that constitutively stored pre-synthesized TNF-α in their granules and thus immediately released such TNF-α upon degranulation (Gordon and Galli 1991). Given that MCs stimulated their intracellular TLRs and did not lead to degranulation, MCs produced newly synthesized, but not pre-synthesized, TNF-α through intracellular TLR signaling upon virus infection. TNF-α from MCs is critical for virus clearance. HSV is recognized by intracellular TLRs, such as TLR3 and TLR9, and surface TLRs, such as TLR2 and TLR4 (Kawamura et al. 2014). HSV-2 did not induce degranulation in murine MCs. Intradermal injection of HSV-2 into MC-deficient $Kit^{W/W\text{-}v}$ led to increased clinical severity and mortality with an elevated virus titer in the infected sites. Intradermal reconstitution with BMMCs from wild-type mice, but not TNF- or IL-6-deficient mice, restored HSV-2-induced high mortality, suggesting that MC-derived TNF-α and IL-6 facilitate the clearance of skin-invading HSV-2 (Aoki et al. 2013). In the following subsections, pathogens recognized by intracellular TLRs of MCs and the subsequent triggering innate immune responses are summarized.

5.1 Viruses

The MC cell line and in vitro-generated MCs can respond to a variety of viruses. Similarly, MCs in virus-infected mice alter their phenotype and trigger antiviral immune responses (Marshall et al. 2003a, 2019). In several inflammatory diseases including asthma, MCs are not only increased the number but also activated at the inflammation sites, implying MC interaction with invading pathogens or their products. Similarly, in the LNs of HIV-infected individuals, MCs increased in number, suggesting that activated MCs were recruited during chronic HIV infection in vivo (Paiva et al. 1996). In dengue virus-infected mice, MCs promote the

accumulation of NK and NKT cells to the infected skin sites and dLNs, thereby enhancing viral clearance (St John et al. 2011; Portales-Cervantes et al. 2017).

Regardless of the importance of virus recognition by intracellular TLRs, there are a few published reports with direct evidence that intracellular TLRs of MCs sense viral nucleic acid by using TLR-deficient mice or cellular TLR depletion. The influenza A virus (a negative-strand ssRNA virus) productively infected P815 cells, followed by the production of TNF-α, IL-6, IFN-γ, and MCP-1 (CCL2) through TLR3 (Meng et al. 2016). The Newcastle disease virus (a negative-strand ssRNA virus) infection in BMMCs induced TLR3 phosphorylation, followed by IFN-β production and c-kit downregulation; chemokine production, such as IP10 (CXCL10) and RANTES (CCL5); and upregulation of costimulatory molecules (Orinska et al. 2005). Given that RANTES (CCL5) selectively recruit NK, NKT, and T cells, virus-infected MCs appear to facilitate antiviral immune responses through release of a panel of mediators. Dengue virus (a positive-strand ssRNA virus) infection in MC-like line rat basophilic leukemia-2H3 cells induced TNF-α production through TLR3 in coordination with MDA5 and RIG-1. However, the production of IFN-α and IP10 (CXCL10) depends on MDA5 and RIG-1 but not TLR3 (St John et al. 2011).

Intraperitoneal injection of CMV (a dsDNA virus) into mice activated peritoneal MCs and induced degranulation. CMV-induced MC degranulation was prevented in TRIF- and TLR3-deficient mice, but not MyD88-deficient mice, suggesting a TLR3/TRIF-dependent MC activation (Becker et al. 2015). However, CMV-induced MC degranulation in BMMCs generated from TLR3-deficient mice was comparable to those in BMMCs generated from wild-type mice, indicating that CMV-induced MC degranulation was not mediated by MC TLR3 (Becker et al. 2015).

Intravenous administration of VSV (a negative-strand ssRNA virus) into mice led to the production of IFN-β, MCP-1 (CCL2), MIP-1β (CCL4), and IP10 (CXCL10). The production of these inflammatory mediators was impaired in MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, but not in TLR3-deficient mice, suggesting that VSV was recognized by MDA5 and RIG-1 of murine MCs (Fukuda et al. 2013). By contrast, VSV replication in LAD-2 was enhanced with TLR3 siRNA treatment and MDA5 and RIG-1 siRNA treatment (Tsutsui-Takeuchi et al. 2015). Thus, MC TLR3 does not recognize VSV, but it indirectly regulates viral replication in MCs.

MCs are abundantly present in mucosa where HIV infection occurs. After HIV exposure to the mucosa, HIV primarily infects epithelial Langerhans cells, a type of antigen-presenting cells (APCs) expressing the HIV receptor, CD4 and CCR5 (Ahmed et al. 2015; Kawamura et al. 2005). MCs could also express CD4 and CCR5. Therefore, a population of MCs in circulation, human CB-derived MCs, and tissue MCs are susceptible to M-tropic HIV, but not T-tropic HIV, subsequently being a reservoir of persistent HIV infection (Li et al. 2001; Bannert et al. 2001; Sundstrom et al. 2007). Productive HIV infection in MCs is corroborated by the evidence that HIV infection in MCs is inhibited by the pretreatment of MCs with an anti-CCR5 monoclonal antibody and that p24 antigen is detected and increased in MCs after HIV exposure. Given that stimulation of TLR3 and TLR7 reverses HIV latency in various types of HIV-infected cells (Macedo et al. 2018;

Alvarez-Carbonell et al. 2017; Cheng et al. 2018), stimulation of these intracellular TLRs in MCs with synthetic agonists may be promising to reduce the latently infected cells.

5.2 SARS-CoV-2

The global pandemic of coronavirus disease 2019 (COVID-19) caused by severe respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is still uncontrolled. Human lung MCs barely express angiotensin-converting enzyme 2 (ACE2), the cell entry receptor for SARS-CoV-2, suggesting that MCs are unable to recognize it directly (Gebremeskel et al. 2021). However, SARS-CoV-2 is an ssRNA virus. Thus, ssRNA derived from SARS-CoV-2 could be recognized by TLR7 expressed by human lung MCs. As described above, human lung MCs do not express TLR8.

MC-specific proteases such as chymase, β-tryptase, and carboxypeptidase (CPA)-3 were elevated in serum from patients with SARS-CoV-2 infection. Moreover, expression of MC protease genes, *TPSB2* and *TPSAB1*, which encode for α- and β-tryptase, respectively, was also elevated in lung tissues from patients with SARS-CoV-2 infection (Gebremeskel et al. 2021). These data suggest that MCs are activated during SARS-CoV-2 infection. MC activation may be facilitated by proinflammatory cytokines released by SARS-CoV-2-infected bronchial epithelial cells and fibroblasts. SARS-CoV-2 has been known to induce IL-1 production by MCs with unknown underlying mechanisms (Conti et al. 2020a, b). IL-1 amplifies the production of other proinflammatory cytokines and proinflammatory arachidonic acid, such as prostaglandins and thromboxane A2, leading to the onset of cytokine storm, followed by severe inflammatory with respiratory distress and death. Taken together, MCs are not the center of SARS-CoV-2 infection. However, MCs are certainly involved in the severe inflammation observed in patients with SARS-CoV-2 infection.

5.3 Bacteria, Fungi, and Parasites

Murine MCs are crucial for bacterial clearance in an acute septic peritonitis model. MC-mediated bacterial clearance was facilitated by MC-derived TNF-α and subsequent neutrophil recruitment in coordination with their complement system (Echtenacher et al. 1996; Malaviya et al. 1996; Prodeus et al. 1997). Murine MC-derived TNF-α and GM-CSF promoted activation, phagocytosis, and generation of reactive oxygen species and suppressed apoptosis of neutrophils (Doener et al. 2013). It seems that MC-mediated bacterial clearance primarily depended on TNF-α and neutrophil recruitment. Upon *Listeria monocytogenes* (Gram-positive bacteria) and *Salmonella typhimurium* (Gram-negative bacteria) exposure to murine peritoneal macrophages, macrophages recognized bacteria through both surface and intracellular TLRs, thereby leading to the production of proinflammatory cytokines and type I IFNs. By contrast, murine peritoneal MCs recognized bacteria by surface

TLRs, but not intracellular TLRs, thereby leading to the production of only proinflammatory cytokines, but not type I IFNs. This reflected the inability to internalize and translocate bacteria into endolysosomal compartments (Dietrich et al. 2010). MCs could internalize and translocate VSV and then produce type I IFN via RIG-1. These data suggest a difference in TLR responsiveness against bacteria between types of immune cells. Additionally, intracellular TLRs of MCs appear not to participate in sensing bacteria.

Murine MCs recognize the *Candida albicans* yeast or hyphae through a C-type lectin receptor, Dectin-1, and TLR2 (Pinke et al. 2016; Piliponsky and Romani 2018). However, the involvement of intracellular TLRs of MCs in fungal recognition has not been elucidated so far. In consideration of the recognition of fungal RNA from *C. albicans* or *Saccharomyces cerevisiae* through TLR7 by BMDCs and BM-derived macrophages (Biondo et al. 2012), it is possible that MC TLR7 could also recognize fungal RNA.

MCs have long been assumed as a sentinel in host defense against parasites (Mukai et al. 2016). MCs could exert direct cytotoxicity on helminths by secreting serine proteases, such as chymase and tryptase (Mukai et al. 2018; Vukman et al. 2016; Hepworth et al. 2012). MCP-1 (CCL2) enhances intestinal epithelial barrier permeability, leading to increased luminal flow and subsequent parasite expulsion (Vukman et al. 2016). MCs participate in host defense against helminth infection by facilitating the Th2 condition in coordination with Th2 cells, group 2 innate lymphoid cells, and eosinophils (Anthony et al. 2007; Harris and Loke 2017). PAMPs from invading protozoa, helminths, and arthropods could stimulate both surface and intracellular TLRs (Ashour 2015). However, the involvement of intracellular TLRs of MCs in parasitic recognition has not been elucidated so far, as is the case with fungus recognition.

6 Role of Intracellular TLRs of MCs on Acquired Immunity

As described earlier, MCs initiate and facilitate innate responses and subsequent recruitment of additional innate effector cells. MCs also associate with the regulation of adaptive immunity. The activation of MCs through FcεRI cross-linking leads to the upregulation of very late antigen 4 integrins and lymphocyte function-associated antigen-1, facilitating direct contact with dermal DCs through a synapse formation (Carroll-Portillo et al. 2015). In this situation, MCs transfer internalized MC-specific antigens to DCs, leading to T-cell activation (Carroll-Portillo et al. 2015). Conversely, dermal DCs transfer plasma membrane containing MHCII to MCs through immunological synapse-like contacts known as trogocytosis, leading to the facilitation of Th1 polarization (Dudeck et al. 2017; Miyake et al. 2017). This also allows MCs to be APCs. Collectively, these bidirectional antigen transfers between MCs and DCs affect the T-cell priming and activation. In addition to DC-derived MHCII, HMC-1 (Love et al. 1996) and human PB $CD34^+$ cell-derived MCs (Suurmond et al. 2013) upregulate endogenous MHCII in response to IFN-γ. Ex vivo human skin-derived MCs also upregulate MHCII in response to IFN-γ, acquire and present

antigens, and prime T cells (Lotfi-Emran et al. 2018). MCs may prime T cells in dLNs, because murine MCs could migrate to dLNs in a contact hypersensitivity model (Wang et al. 1998) and an ultraviolet irradiation model (Byrne et al. 2008). Taken together, MCs could acquire antigen directly or from DCs, migrate to dLNs, and then prime T cells.

MC-derived TNF-α, which is produced through TLR3, TLR7, and TLR9 in MCs, induces E-selectin expression on blood vessels, leading to the accumulation of multiple DC subsets within tissues (Shelburne et al. 2009). MC-derived TNF-α also matures DCs (Dudeck et al. 2011) and promotes dermal DC migration to dLNs in a murine contact hypersensitivity models (Otsuka et al. 2011; Suto et al. 2006). TLR stimulation leads to not only the production of inflammatory cytokines and chemokines but also the induction of costimulatory molecules on the plasma membrane, resulting in T-cell activation. The modulation of the expression of costimulatory molecules on MCs after the stimulation of intracellular TLRs of MCs has been largely unknown.

7 Clinical Implications of Intracellular TLRs of MCs

7.1 Chloroquine and Hydroxychloroquine

The immature form of intracellular TLRs translocates from the endoplasmic reticulum to late endosome or early lysosome (Latz et al. 2004). Its acidic lysosomal environment is required for the maturation of intracellular TLRs and binding of ligands to mature intracellular TLRs (Hacker et al. 1998).

The antimalarial agents, chloroquine (CQ) and hydroxychloroquine (HCQ), have been widely used for autoimmune diseases, such as SLE and rheumatoid arthritis. CQ and HCQ have been assumed to increase the pH in late endosome or early lysosome, thereby inhibiting the maturation of intracellular TLRs and ligand binding to mature intracellular TLRs, leading to the impaired production of inflammatory cytokines (Fox 1993; Kuznik et al. 2011). HCQ interferes with the lysosome function of MCs. In vitro incubation of human PB $CD34^+$ cell-derived MCs with HCQ did not alter the tryptase intracellular amount but impaired its enzymatic activity, resulting in the accumulation of non-functional tryptase in MC granules (Espinosa et al. 2018). HCQ also decreased the production of IL-8 and GM-CSF but not MIP-1β by MCs (Espinosa et al. 2018). HCQ-induced interference of intracellular TLR signaling in MCs may cause this decreased cytokine production. In addition, oral HCQ administration to MRL/lpr mice, a murine model for SLE, decreased the dermal MC number (Shimomatsu et al. 2016). HCQ, therefore, alters the MC biology and function directly and indirectly by modulating the lysosomal environment of MCs and subsequent intracellular TLR signaling.

7.2 Imiquimod

The small synthetic immune response modifier, IMQ, has been approved to treat some cutaneous malignant neoplasms, including actinic keratosis (AK). IMQ is recognized mainly by TLR7, followed by the activation of the transcription factor NF-κB and the production of inflammatory cytokines, such as type I IFNs (Torres et al. 2007). Comparing skin infiltrates between responding and non-responding AK lesions against topical application of a 5% IMQ cream, MCs (Oyama et al. 2017) and pDCs (Ogawa et al. 2014) increased in number in the responded AK lesions, suggesting that the IMQ-triggered production of type I IFNs and other inflammatory cytokines through TLR7 in MCs and pDCs is crucial for the cure of AK by the 5% IMQ cream.

8 Summary and Future Perspectives

Human skin MCs express TLR3, TLR7, and TLR9, whereas human lung MCs express TLR3 and TLR7, but not TLR9, suggesting that MCs are heterogeneous across tissues. The expression of intracellular TLRs in some MC cell lines is different from those in human ex vivo MCs. Therefore, studies should pay attention to the selection of cell lines in experiments. Some virus infection and MC-derived mediators, such as histamine, CysLTs, LL-37, and GM-CSF, modulate intracellular TLR expression in MCs. Thus, intracellular TLR signaling in MCs is strictly regulated by MC activation. TLR3, TLR7, and TLR9 in MCs are functional because each synthetic ligand triggers their signaling. However, upon virus infection, it is still unclear whether these intracellular TLRs of MCs participate in the recognition of viral nucleic acids because there were a few published reports in terms of direct evidence that intracellular TLRs of MCs sense viral nucleic acid from TLR-deficient mice or cellular TLR depletion. Viral nucleic acids are recognized by not only TLRs but also RIG-1 and MDA5, which are also expressed in MCs. For example, VSV is recognized by RIG-1 and MDA5 but not TLR3 of murine MCs (Fukuda et al. 2013). MCs may not be assumed as a central player in antiviral responses. However, MC-derived TNF-α and IL-6 are exclusively essential for clearance of skin-invading HSV-2 in mice (Aoki et al. 2013, 2016). Considering these situations, exploration of the responsible intracellular PRRs in MCs for each virus recognition is important and necessary for drug development and subsequent clinical application.

Acknowledgement We thank all the colleagues of our department, in particular, the former colleagues who were engaged in MC research: Rui Aoki, Hironori Matsushima, Nobuo Yamada, and Hiroyuki Matsue.

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TLR10 and Its Role in Immunity

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Contents

Abstract

Toll like receptors (TLRs) are the most studied pattern recognition receptors (PRRs) as they connect the innate to the acquired immune response. To date, there are ten human TLRs which are expressed either on the plasma membrane or on the endosomes. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are plasma membrane TLRs that recognise extracellular components of pathogens, whereas TLR3, TLR7, TLR8 and TLR9 are located on endosomes where they recognise

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_541

foreign nucleic acids. Of these TLRs, TLR10 is the latest human TLR to be discovered and its function and ligands are still unclear. TLR10 is the only known member of TLR family that can elicit anti-inflammatory effect. TLR10 can inhibit other TLRs by competing with stimulatory TLRs, dimerising with TLR1, TLR2 and TLR6, and by inducing PI3K/Akt to produce IL-1Ra. There is controversy on the function of TLR10 as an anti-inflammatory TLR as initial studies on TLR10 revealed it to promote inflammation. Herein, we review the detailed functions of TLR10 in immunity and give an account of how and when TLR10 can act on both sides of the inflammatory spectrum.

Keywords

Anti-inflammatory · Immunity · TLR10

1 Introduction

Microbial products are recognised by the innate immune system via germ-line encoded pattern recognition receptors (PPRs) (Bourke et al. 2003; Hasan et al. 2004; Fore et al. 2020). PRRs include Toll Like Receptors (TLRs), Node Like Receptors (NLRs) and RIG-1 like receptors and amongst these PRRs, TLRs are the most studied (Regan et al. 2013). PRRs are mainly found on immune cells and to a lesser extent on non-immune cells like trophoblasts and epithelial cells. TLRs are type 1 transmembrane glycoproteins that are characterised by extracellular, transmembrane and cytoplasmic domains (Medzhitov 2001; Hasan et al. 2005; Fore et al. 2020). The extracellular domain, rich in leucine repeats, is for microbial pattern recognition, whereas the intracellular domain is for signal transduction. TLRs and interleukine-1 receptor (IL-1R) have the same cytoplasmic domain (Medzhitov 2001; Akira 2003; Nagase et al. 2003; Hasan et al. 2004; Takeda and Akira 2005; Opsal et al. 2006; Fore et al. 2020). To date, there are 13 characterised TLRs, of which TLR1 through TLR10 are found in humans, and TLR1-TLR9, TLR11-TLR13 and a pseudogene for TLR10 are found in mice (Takeda and Akira 2005; Fore et al. 2020). Exogenous stimuli are sensed by TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 which are located on the plasma membrane whereas endogenous stimuli, mainly in the form of nucleic acids, are recognised by endosomal TLRs: TLR3, TLR7, TLR8 and TLR9 (Guan et al. 2010). TLR1, TLR2, TLR4 and TLR6 recognise various microbial structures, whereas more specific recognition is mediated by TLR3, TLR5, TLR7 and TLR8, and TLR9, which recognise ssRNA, flagellin, dsRNA and unmethylated CpG motifs, respectively. Engagement of the TLRs by their respective ligands results in recruitment of either the myeloid differentiation primary response 88 (MyD88) or TIR domain containing activator of TLR (TRIF). Initiation of the MyD88 or TRIF signalling pathways triggers a multifaceted response including production of chemokines that will recruit phagocytes and synthesis of cytokines that will activate an adaptive immune response involving B and T lymphocytes.

TLR10 is the latest TLR to be discovered (Chuang and Ulevitch 2001; Lazarus et al. 2004) and interest on TLR10 research has risen since its discovery. The gene that encodes for TLR10 is on chromosome 4p14, located on the same gene cluster with TLR1 and TLR6 (Chuang and Ulevitch 2001; Lazarus et al. 2004; Hasan et al. 2005; Sun et al. 2005; Kızıldağ et al. 2018). The TLR10 gene consists of three exons in a transcript of 3,270 bases (Lazarus et al. 2004; Sun et al. 2005). Mice, which are the main animal model for in vivo studies, do not have a functional gene for TLR10 (Nie et al. 2018; Fore et al. 2020), even though other vertebrates like sheep (Chang et al. 2009), pigs (Shinkai et al. 2006), horses (Tarlinton et al. 2016) and cattle (Opsal et al. 2006) have the functional gene product (protein). Like other TLRs, TLR10 is a transmembrane protein receptor composed of an extracellular domain rich in leucine repeats for ligand recognition, and an intracellular domain (homologous to IL-1 for signalling) (Sindhu et al. 2018; Zhang et al. 2018). TLR10 is closely related to TLR1 and TLR6 with a similarity index of 50% and 49%, respectively (Chuang and Ulevitch 2001; Sindhu et al. 2018). TLR10 is predominantly expressed in tissues rich in immune cells such as the spleen, lymph nodes and tonsils (Hornung et al. 2002; Nagase et al. 2003; Govindaraj et al. 2010; Fore et al. 2020). To exert its function, TLR10 can either homodimerise or it can heterodimerise with TLR1 or TLR2. As a homodimer, TLR10 regulates the other TLRs by producing anti-inflammatory cytokines, however, the function of individual TLR in TLR10 heterodimerisation remains an unattended focus of research. Certain studies have found that TLR10 acts as an inhibitor of TLR2, whilst others have reported that TLR10 stimulates and amplifies TLR2 activity when heterodimerised with TRL2.

2 Ligands of TLR10

TLR10 can homodimerise (Hasan et al. 2005; Govindaraj et al. 2010) or heterodimerise with either TL1 or TLR2 (Hasan et al. 2005; Chen et al. 2007; Govindaraj et al. 2010; Guan et al. 2010). The association of TLR2 with either TLR1 or TLR6 increases ligand binding efficiency, as well as the discrimination of triacyl and diacyl lipopeptides from bacteria (Hasan et al. 2005). Heterodimerisation of TLRs may result in inhibiting the signalling of the TLR as when TLR1 associates with TLR2, the TLR2 mediated response is inhibited, likewise TLR1 inhibits TLR4 signalling response in endothelial cells (Sun et al. 2005). There is a likely possibility that TLR10 may act as a coreceptor for TLR1 and TLR2, and therefore might share the same family of ligands (Hasan et al. 2005). TLR2 and TLR10 are from the same gene cluster (Regan et al. 2013), and a study by Govindaraj et al. demonstrated through homology modelling that the ligand binding pocket of TLR10 is similar to that of TLR2, suggesting they can recognise the same or overlapping ligands (Govindaraj et al. 2010). Expression of TLR10 mRNA in gastric mucosal increases after infection with *H. pylori* suggests that *H. pylori* is a ligand source for TLR10 (Nagashima et al. 2015). The list of potential and known TLR10 ligands is shown in Table 1.

Table 1 List of identified TLR10 ligands and their mode of action

Proposed ligand/ ligand source	Mode of recognition	Upregulated cytokines/gene activated	Overall response	Reference
Listeria monocytogenes	Internal recognition by TLR10	CCL20 and IL-8	Inflammatory	(Regan et al. 2013)
Listeria monocytogenes	TLR2/TLR10 heterodimer	Activation of NF-κB	Inflammatory	(Regan et al. 2013)
Lipopolysaccharides	TLR2/TLR10 heterodimer	Activation of NF-κB	Inflammatory	(Nagashima et al. 2015)
Pam_3CSK_4	TLR2/TLR10 heterodimer	Computational modelling	Not stated	(Govindaraj et al. 2010)
$PamCysPamSK_4$	TLR1/TLR10 heterodimer, TLR10 homodimer	Computational modelling	Not stated	(Govindaraj et al. 2010)
HIV-1 gp41	TLR10	IL-8 and NF-κB activation	Inflammatory	(Henrick et al. 2019)
Double stranded RNA (dsRNA)	Competition for ligand with TLR3	Inhibition of TLR3 signalling	Immunoregulatory	(Lee et al. 2018)
Borrelia burgdorferi	TLR2/TLR10 heterodimer	Production of IL-1Ra	Anti-inflammatory	(Oosting et al. 2014)

3 Expression and Distribution of TLR10

Lymphoid tissues, including the spleen, thymus, lymph node and tonsils, express high levels of TLR10 mRNA (Chuang and Ulevitch 2001; Opsal et al. 2006). The expression of TLR10 in these lymphoid tissues is restricted to certain types of immune cells, such as B cells and the degree of expression varies from cell-to-cell. In swine, the expression of TLR1 and TLR6 does not vary significantly among tissues, however that of TLR10 is tissue specific (Opsal et al. 2006; Shinkai et al. 2006). Human Treg cells express TLR5, TLR6, TLR8 and TLR10 and the expression of these TLRs on Tregs has a significant function. Transcription of TLR10 on Tregs is regulated by FOXP3 (Bell et al. 2007; Verma et al. 2014) postulating a different operational mechanism of TLR10. Regulation of TLR10 expression on Tregs happens via binding of FOXP3 to the fork head consensus binding element on TLR10 DNA (Bell et al. 2007).

TLR9 and TLR10 are predominantly expressed in B cells. Resting B cells express a significant amount of TLR9 and TLR10, which is upregulated upon activation of B cells (Bernasconi et al. 2003; Bourke et al. 2003; Lazarus et al. 2004; Isnardi et al. 2008). Memory B cells express higher levels of TLR10 than any other subset of B cells (Bernasconi et al. 2003; Isnardi et al. 2008). In human tumour cells, high

expression of TLR10 is associated with immune infiltration especially B cells and is positively correlated with tumour development (Ge et al. 2020). High TLR10 expression levels, which are associated with immune infiltration, might play a significant role in regulating tumour development, as the expression levels of TLR10 are low in advanced stages of breast cancer (Shi et al. 2020). Conversely, monocytes do not express TLR10 (Kadowaki et al. 2001; Hubert et al. 2004) and they have been used as a negative control for TLR10 expression (Bourke et al. 2003), and on the contrary, hypoxia was found to increase expression of TLR10 on monocytes (Kim et al. 2010). The differentiation of monocytes into $CD11c^+$ immature dendritic cells (DCs), pre-plasmacytoid DCs (Bourke et al. 2003) and mature plasmacytoid DCs results in low detectable levels of TLR10 (Hornung et al. 2002; Hubert et al. 2004). Infiltration of eosinophils is associated with the pathology of high inflammatory lung diseases like asthma, hence TLRs which are highly expressed in eosinophils might be linked with inflammation. TLR1, TLR7, TLR9 and TLR10 are highly expressed on eosinophils compared to the other human TLRs. Neutrophils express nearly all the identified human TLRs reflecting the important role of neutrophils as first-line effector cells (Nagase et al. 2003). In addition, macrophages (Smith et al. 2014), epithelial cells, gastric mucosa (Nagashima et al. 2015), fallopian tubes (Hart et al. 2009), eyes (Mohammed et al. 2011), prostate epithelial cells (Fan et al. 2019) express TLR10.

4 TLR10 Signalling

The C-terminal end of TLR10 contains a highly conserved sequence of 13 amino acids which is likely required for downstream signalling (Chuang and Ulevitch 2001). The TIR domain of TLR10 directly interacts with MyD88 but not TRAM, TIRAP or TRIF. This was shown using the chimera CD4TLR10 that consists of a CD4 extracellular domain, TLR10 transmembrane domain and TIR domain. MyD88 blocked signalling activity of CD4TLR10, whilst that of CD4TLR4 was partially blocked and that of CD4TLR3 was not affected (Hasan et al. 2005). The signalling pathway via the MyD88 adaptor protein is shown in Fig. 1. Specific TLR ligands induce different signalling pathways, however the CD4TLR chimera can activate all downstream pathways, therefore a robust tool for assessing the overall signalling capacity of a given TLR (e.g. TRL10) (Hasan et al. 2004). Mutation of the TIR domain affects the signalling activity of TLRs mainly due to inability to recruit adaptor proteins, resulting in no signalling. The TIR domain of TLRs has a conserved amino acid; those that recruit MyD88 the conserved amino acid is proline and for TLR3 which exclusively recruit TRIF is alanine (Akira 2003).

CD4TLR10 chimera strongly induces induction of AP-1 and IL-4 compared to other CD4TLRs. Signalling via the TLR10 TIR domain also results in the activation of NFκB, TNF-α, IL-6 and ENA-78. Production of ENA-78 which is a neutrophil chemoattractant through TLR10 signalling is cell type specific. Of the cells which express TLR10, neutrophils secrete ENA-78 but B cells do not. ENA-78 is a neutrophil chemoattractant which belongs to the IL-8 chemokine family (Hasan

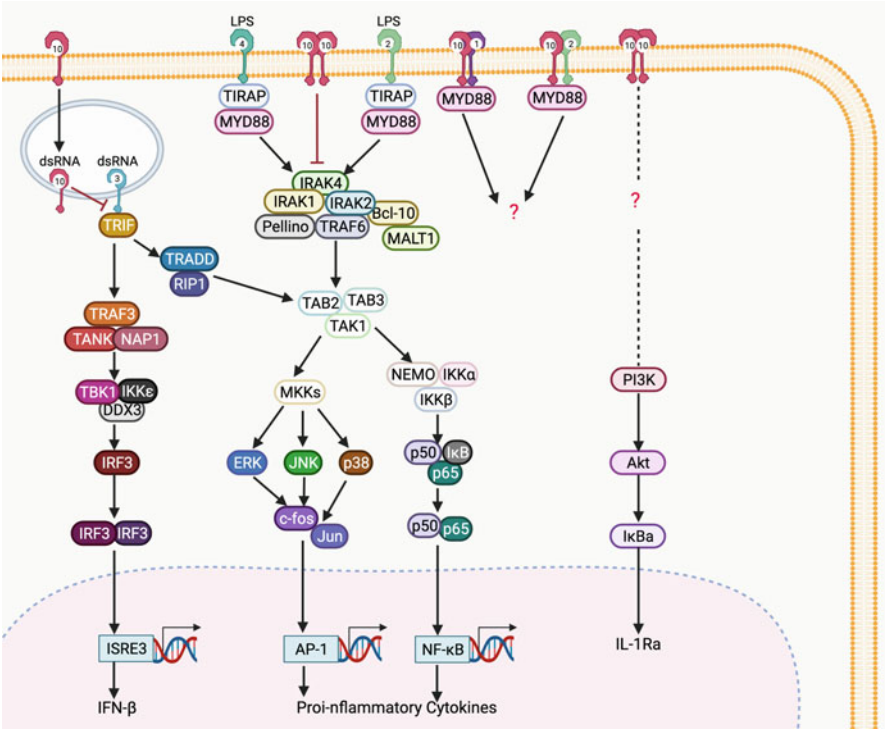

Fig. 1 Signalling pathways inhibited by TLR10. To exert its function, TLR10 can homodimerise or heterodimerise with other TLRs. Further, TLR10 can compete for ligands with TLR2 and TLR4 resulting in inhibition of their signalling pathways. TLR10 can translocate to the endosomes and compete for dsRNA with TLR3 causing inhibition of the TLR3 signalling. As a homodimer, TLR10 can induce production of the anti-inflammatory cytokine, IL-1Ra via the PI3K/Akt pathway

et al. 2004). Guan et al. used the CD4TLR10 fusion protein, and in contrast to the above findings, the authors reported failure of the fusion protein to induce NF-κB activation (Guan et al. 2010). In line with this study, Hess et al. used TLR10 monoclonal antibodies on isolated human monocytes that caused a decrease in the production of pro-inflammatory cytokines. Furthermore, TLR10 antibody-mediated engagement resulted in reduced phosphorylation of several TLR signalling pathways (Hess et al. 2017a).

5 Functions of TLR10 in Immunity

5.1 Inflammatory

Induction of TLR10 requires active viral replication and de novo protein synthesis. The robust induction of pro-inflammatory cytokine expression as well as antiviral IFNs (such as type I and type III IFNs) occurred via TLR10 signalling, which is activated by functional RNA-protein complex of influenza virus (Lee et al. 2014). Besides, TLR10 level is more pronounced following infection with highly pathogenic avian influenza H5N1 virus compared to a low pathogenic H1N1 virus. The levels of TLR10 expression are significantly upregulated in HIV-1 infection, as HIV-1 gp41 acts as a ligand for TLR10 in human macrophages and MECs, leading to IL-8 induction and NF-ĸB activation. TLR10 and TLR1 likely form heterodimers, as TLR10 depletion by siRNA-mediated knockdown negatively affects the HIV-1 proviral integration. TLR10 inhibition also significantly lowered the level of IL-8 production in cells treated with gp41, p17 and p24 (Henrick et al. 2019). This is important because IL-8 is an immune cell chemoattractant.

The TLR2/TLR10 complex can recognise the tetra-acyl lipid A region on *H. pylori* lipopolysaccharide structure resulting in activation of NF-ĸB (Nagashima et al. 2015). Live *H. pylori* induces high upregulation compared to bacteria-free culture supernatants (Pachathundikandi and Backert 2016). TLR2/TLR10 heterodimer induced more NF-ĸB activation in intestinal epithelial cells and macrophages compared to TLR2 following *Listeria monocytogenes* infection (Regan et al. 2013). Surprisingly, as a homodimer TLR10 failed to upregulate the pro-inflammatory cytokine, IL-1β following *H. pylori* infection, even though a pronounced upregulation of TNF-α was noted (Pachathundikandi and Backert 2016). The inability of TLR10 to downregulate TNF-α was also noted by Van Le and Kim Young, who showed that TLR10 can downregulate IL-8, IL-1β and CCL20 (Bizzintino et al. 2011). Pathogenic organisms that replicate in the cytoplasm may induce intracellular TLR10 to produce pro-inflammatory effect after recognition of a PAMP associated with bacterial or viral infection (Mourao-Sa et al. 2013).

5.2 Anti-Inflammatory

TLR10 is the only known member of TLR family that can elicit anti-inflammatory effect (Boutens et al. 2018). The proposed molecular mechanisms are: (1) competition for ligands with stimulatory TLRs; (2) competition for dimerisation with TLR1, TLR2 or TLR6; and (3) TLR10-specific direct production of anti-inflammatory cytokine IL-1Ra induced by PI3K/Akt. IL-1Ra which is an antagonist of IL-1R can inhibit the generation of T17 lymphocytes and their subsequent pro-inflammatory cytokines (Jiménez-Dalmaroni et al. 2016), as TLR2-mediated cytokine production was induced by blocking TLR10 using specific inhibitory antibodies. Monoclonal antibodies against TLR10 induced the secretion of pro-inflammatory cytokines following *H. pylori* infection, whereas TLR4

monoclonal antibodies dampened these cytokines' production (Neuper et al. 2020). Additionally, patients with loss-of-function mutations in TLR10 showed upregulation of TLR2-mediated cytokine production (Oosting et al. 2014). Knockdown of TLR2 resulted in downregulation of phospho-NF-κB P65, IL-6 and IL-8, whereas knockdown of TLR10 resulted in their upregulation (Fan et al. 2019). Thus, there is a special mechanism employed by TLR10 to exert its inhibitory function.

TLR10 induces apoptosis via activation of caspase-3 supporting the function of TLR10 as an anti-inflammatory TLR because NF-ƙB is a transcriptional factor that inhibits apoptosis (Torices et al. 2016). In response to gram-positive bacterial peptidoglycan, TLR10 activates apoptosis resulting in reduced chemokine secretion and NF-ƙB activity (Mulla et al. 2013). This finding is supported by Guan et al. who stated that TLR10 did not activate the MyD88 pathway that activates NF-ƙB (Guan et al. 2010). On the contrary, Hasan et al. demonstrated that TLR10 was able to trigger NF-ƙB activity via MyD88 (Hasan et al. 2005). Jiang et al. proposed that to act as an anti-inflammatory agent, TLR10 functions as a homodimer, as observed in their experiment that the replacement of TLR10 extracellular domain with CD4 resulted in a receptor that had full suppressive characteristics (Jiang et al. 2016).

Mainly expressed on B cells, TLR10 also has a functional role as an intrinsic suppressor of B cell. Hess et al. demonstrated that antibody-mediated engagement of TLR10 on primary human B cells reduced B cell proliferation, cytokine production and signal transduction. TLR10 transgenic mice also have shown diminished antibody response when induced by T-independent or T-dependent antigen. Although the exact pathway of this phenomenon is not yet known, there are several proposed mechanisms which exploit the synergy of B cell receptor, TLRs and TNF receptors in B cells and involve PKD, BTK, TRAF5 and Syk protein pathway (Hess et al. 2017b). A study of rheumatoid arthritis (RA) concluded that elevated expression of TLR10 in B cell subsets is correlated with the progression of RA. B cell subsets that comparatively express less TLR10, such as $CD27^{+}$ memory B cells and naïve B cells, tend to be more inflammatory and worsen RA. On the other hand, $CD27^{-}IgD^{-}$ B cells which express higher TLR10 have immunosuppressive properties (Zhang et al. 2018).

5.3 In Trained Innate Immune Memory

Every organism has the capability to adapt to environmental stimuli, including induced enhancement to repeated responses against potentially harmful microorganisms. This characteristic enables one to respond faster and more efficiently to an antigen after its previous exposition. Formation of antigen-specific memory T cells and B cells in adaptive immune response is an example of this particular feature (Černý and Střížˇ 2019). However, it has been discovered that certain infections might also induce significant immune response to unrelated infections, and this is due to the modulation of host macrophages (Sugawara and Nikaido 2014). For example, children who had BCG scar and positive tuberculin

reaction have better survival rate against malaria and other major infections (Roth et al. 2005).

This unique ability of innate immune cells is not only beneficial for protection against microbial agents but can be also against chronic inflammatory diseases in which innate immune cells play an important role, such as in rheumatoid arthritis and systemic lupus erythematous. The characteristics of trained innate immune cells are mostly increased cytokine production, changes in cellular metabolism (mainly increased glycolysis and lactate production) and epigenetic reprogramming. Trained immunity can initiate a disease, maintain or worsen its symptoms (Arts et al. 2018).

As TLR10 is the only TLR that has anti-inflammatory properties, it may also play a role in the induction of trained immunity. According to a research by Mourits et al., TLR10 does affect the induction of BCG-induced trained immunity in vitro. However, its role in vivo is still an open debate, as genetic variation in TLR10 gene does not affect the induction of trained immunity by BCG vaccination (Mourits et al. 2019). TLR10 expression levels are increased 2 weeks after BCG vaccination. Engagement of the TLR10 receptor in ex vivo shows an increase in IL-1Ra after vaccination than before vaccination, signifying a role of TLR10 in trained immunity (Rodgers and Milling 2020). We postulate that the role of TLR10 in trained innate immune memory may depend on the condition of TLR10 stimulation, or may have little involvement, hence further research is needed.

6 Mutations in TLR10 and Effects on Its Functions

Mutations in the TLR10 gene are associated with either increased inflammation or reduced inflammation. The overall response is highly dependent upon the location and type of mutation. Some of these mutations result in a less functional, unfunctional or highly responsive gene. A TLR10 variant where an isoleucine, a hydrophobic amino acid, on position 437 is substituted by threonine, a polar amino acid, resulting in the inability of TLR10 to downregulate the transcriptional activity of NF-ϰB (Torices et al. 2016). Chronic inflammation is one of the biological processes that contribute to prostate cancer aetiology. Two TLR10 SNPs, rs11096955 and rs11096957, are associated with lower risk of prostate cancer (Stevens et al. 2008; Fore et al. 2020). SNP TLR10 rs1004195 is associated with the development of Immunoglobulin A Nephropathy (IgAN) among Korean children (Park et al. 2011). Other inflammatory diseases that are associated with TLR10 SNP include asthma (Lazarus et al. 2004; Kormann et al. 2008; Törmänen et al. 2017, 2018) *H. pylori* (Tongtawee et al. 2018), hashimotos (Cho et al. 2014), colorectal cancer (Kopp et al. 2018) and chronic sarcoidosis (Veltkamp et al. 2012).

7 Animal Models for TLR10

The major hindrance in TLR10 studies is the lack of a functional mouse homologue because most ligands for TLRs were identified by using mutant or genetically deficient mouse models (Hasan et al. 2005). A complete human TLR10 homologue is available in rats hence knockout technology should be conducted in rats to fully elucidate the functions of TLR10 (Hubert et al. 2004; Verma et al. 2014). The lack of a functional gene in mice can be complemented by generating human TLR10 transgenic mice as done by (Oosting et al. 2014; Boutens et al. 2018).

8 Conclusion

TLR10 is a plasma membrane receptor that mainly functions as an anti-inflammatory PRR. To exert its function, TLR10 can compete for ligands with other TLRs such as TLR2 and TLR3 or by dimerising with either TLR1, TLR2 or TLR3, or by activating the production of the anti-inflammatory cytokine, IL-1Ra. Other research has shown TLR10 to be an inflammatory receptor. The controversy in function of TLR10 may be attributed to the lack of a known natural ligand for TLR10. This may also be as a result of the complex mechanism by which TLR10 exerts its function. Understanding the mechanism by which TLR10 exerts its functions and identifying specific natural ligands for TLR10 is crucial in answering these controversies. It is of importance to know if TLR10 exert its functions via signalling pathway or without, as in the case of IL-1Ra.

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Toll-Like Receptors in Stem/Progenitor Cells

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_539

Abstract

One of the bridges that control the cross-talk between the innate and adaptive immune systems is toll-like receptors (TLRs). TLRs interact with molecules shared and maintained by the source pathogens, but also with endogenous molecules derived from injured tissues (damage/danger-associated molecular patterns – DAMPs). This is likely why some kinds of stem/progenitor cells (SCs) have been found to express TLRs. The role of TLRs in regulating basal motility, proliferation, processes of differentiation, self-renewal, and immunomodulation has been demonstrated in these cells. In this book chapter, we will discuss the many different functions assumed by the TLRs in SCs, pointing out that, depending on the context and the type of ligands they perceive, they may have different effects. In addition, the role of TLR in SC's response to specific tissue damage and in reparative processes will be addressed, as well as how the discovery of molecules mediating TLR signaling's differential function may be decisive for the development of new therapeutic strategies. Given the available studies on TLRs in SCs, the significance of TLRs in sensing an injury to stem/progenitor cells and evaluating their action and reparative activity, which depends on the circumstances, will be discussed here. It could also be possible that SCs used in therapy could theoretically be exposed to TLR ligands, which could modulate their in vivo therapeutic potential. In this context, we need to better understand the mechanisms of action of TLRs on SCs and learn how to regulate these receptors and their downstream pathways in a precise way in order to modulate SC proliferation, survival, migration, and differentiation in the pathological environment. In this way, cell therapy may be strengthened and made safer in the future.

Keywords

Adult renal stem/progenitor cells · Hematopoietic stem cells · Mesenchymal stem cells · Neuronal progenitor cells · Renal progenitor cells · TLR ligands · Toll like receptors

1 Introduction

Toll-like receptors (TLRs) are single-pass membrane-spanning receptors without catalytic activity. TLRs are part of the most large family of pattern recognition receptor (PRR) (Tsan and Gao 2004) that play a key role in the innate immune system defense against hostile microorganisms. They recognize the damage/danger-associated molecular patterns (DAMPS) that are endogenous molecules derived from injured tissues (Tsan and Gao 2004).

Toll-like receptors are type I membrane proteins expressed by immune and non-immune cells (i.e., monocytes, macrophages, endothelial cells) in the plasma or intracellular membrane (endosomes). The recognition of endogenous ligands by TLRs plays a key role in the regulation of inflammation, both in pathological and physiological conditions. The classified endogenous ligands are various, but among the most important we mention the heat shock protein (HSP) 60, HSP, heparan sulfate, hyaluronan, extra domain A fibronectin, uric acid, oxidized LDL, intracellular components of fragmented cells, myeloid-related proteins 8 and 14, eosinophil-derived neurotoxin, and human defensin-3. These ligands are referred to as "warning signs" because they are accessible to TLRs in contexts related to damage, injury, or non-infectious threats (DelaRosa et al. 2012).

It is clear that all types of DAMPs interact with TLRs, and there are increasing evidences that showed that DAMPs are different from pathogen associated molecular patterns (PAMPs) in spite of the fact that molecular mechanisms are similar. Compared to PAMPs, DAMPs bind receptors in different sites (Hodgkinson et al. 2008) and with different action (Midwood and Piccinini 2010; Schaefer 2014). In addition, studies showed differences in the downstream pathways and outcomes of TLR signaling (Taylor et al. 2007; Midwood and Piccinini 2010; Schaefer 2014).

The first TLR was discovered more than 30 years ago in *Drosophila melanogaster* in the TOLL gene. To date, 13 classes of human TLRs with distinct polymorphic forms have been recognized. There are two groups of TLRs: TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11, TLR12, and TLR13 are located on the cell surface, and TLR3, TLR7, TLR8, and TLR9 are localized to the endosomal/lysosomal compartment. Differently to the others, TLR10 was thought to be an orphan receptor in humans but much recent studies have identified its ligands and its activation exerts anti-inflammatory action. Of note, mice only have TLR10 pseudogene and do not express TLR10 (Oosting et al. 2014; Jiang et al. 2016; Henrick et al. 2019; Kumar 2020).

TLRs function is essential in critical conditions and this can explain why they are localized both in animals and plants (Akira 2003; Beutler 2004; Shigeoka et al.

2007). They recognized a broad group of molecules, PAMPs, that are situated on the surface of bacteria, virus, and fungus (Medzhitov et al. 1997; Takeda et al. 2003).

PAMP molecules are related to pattern recognition molecules (PRMs), situated on the surface of immune cells (Wardle 2007), that comprise TLRs. Differently to PRRs, PRM refers to a greater group of elements of innate system (McGuinness et al. 2003). Along with immune cells (macrophage, neutrophil, dendritic and NK cells), non-immune cells, including epithelial cells, adipocytes, and neurons also express TLRs (Ballak et al. 2015; Vijay 2018; Igata et al. 2019).

After recognizing PAMPs, TLRs trigger immune response and intracellular signaling pathways that lead to the induction of inflammatory cytokine genes (Akira 2003; Kopp and Medzhitov 2003; Takeda et al. 2003; Tsan and Gao 2004). Moreover, activation of the TLRs leads also to the development of antigen specific adaptive immunity, unrolling an important role in regulating the cross-talk within the two immune responses (Medzhitov 2001; Beutler et al. 2003; Takeda et al. 2003; Iwasaki and Medzhitov 2004).

Furthermore, TLRs have been discovered on different type of stem/progenitor cells (SC), in which they contribute to activity of basal motility, self-renewal, differentiation, and immunomodulation.

In this chapter, we report the distinct role of TLR in SC, with peculiar attention on SC's plastic activity in response to specific ligands. TLRs have been demonstrated to be critical in the reparative processes. Besides, several studies demonstrated that TLRs have a role in the course of reparative processes performed by the SC, coherent with the TLR importance for the proper constitution of dorsoventral patterning for growth in *D. melanogaster* (Hashimoto et al. 1988). We want to portray and treat the properties of TLRs during reparative processes unrolled by various tissue stem/progenitor cells, prospecting new therapeutic strategies.

2 TLR and Stromal Mesenchymal Cells

Mesenchymal stromal cells (MSC) were first described more than 30 years ago, and the term MSC was coined by Caplan, during the first clinical application (Chen et al. 2015; Wu et al. 2018). Up to now, MSC have been identified and propagated from many different sources, including bone marrow (BM-MSC), adipose tissue (AT-MSC), peripheral blood, umbilical cord blood (UCB-MSC), and Wharton jelly (WJ-MSC) (Caplan 1991; Raicevic et al. 2011). The most widely studied sources of stromal cells are autologous bone marrow and adipose tissue derived MSCs.

Often, stromal cells are erroneously defined as mesenchymal stem cells. Adult and perinatal MSCs have recently led to evidence supporting similar, but not identical, behaviors and properties in most if not all the human MSCs (Chen et al. 2015; Wu et al. 2018).

MSCs have been revealed high expression of TLR levels, mainly allocated on cell surface. TLRs modulate MSC proliferative, immunomodulatory, and migratory and differentiation potential (Raicevic et al. 2010, 2011).

Different studies have been showed the implication of TLR expression and transduction in MSC from different sources; BM-MSC are the most characterized, while AT and UCB showed limited evidence (Sangiorgi and Panepucci 2016).

To date, it is known that human BM-MSC, AT-MSC, and UCB-MSC express high levels of TLR3 and TLR4, and low levels of TLR1, TLR2, TLR5, TLR6, and TLR9 (Shirjang et al. 2017). In nearly all the MSCs studied, an expression deficiency of TLR7, TLR8, and TLR10 has been shown. In WJ-MSC, a limited expression of the TLR3 isoform has been described, and this receptor seems to be non-functional since its ligation did not lead to cytokine secretion.

This particular expression has been associated with poor immunogenic phenotype and ineffective response to lipopolysaccharides (LPS) stimulation in WJ-MSC (Raicevic et al. 2011; Najar et al. 2017).

Researchers have been investigating about the likely connection between TLR signaling and MSC anti-inflammatory and immune-modulatory properties (Nurmenniemi et al. 2010).

2.1 TLRs in Immunomodulatory Properties of MSC

Among the characteristics of MSCs, there is immunoregulatory capacity, relevant to immune response and valorized in clinical applications. These properties have been largely characterized. Human MSCs can modify inflammatory status and might affect various effector cells, both lymphoid cells (T, B, and NK cells) and myeloid components (monocytes, dendritic cells) (Liotta et al. 2008; Opitz et al. 2009; Giuliani et al. 2014; Castro-Manrreza and Montesinos 2015). The effect of MSC has been attributed principally to cell-to-cell connections and the secretion of soluble factors, including transforming growth factor- (TGF-) β 1, hepatocyte growth factor (HGF), prostaglandin E2, interleukin-(IL-) 10, indoleamine 2,3-dioxygenase (IDO), interferon (IFN-) γ, and nitric oxide (NO), following activation in reaction to inflammation (Liotta et al. 2008; Raicevic et al. 2010; Shirjang et al. 2017) (Fig. 1).

Studies have been concentrated largely on antiproliferative effect of T cell, with different and often contrasting results. In AT-MSCs, immunomodulatory factors are not expressed in constitutive way, but these cells can release inhibitory factors following activation (Delarosa et al. 2009). Furthermore, the immunomodulatory characteristics of AT-MSC are not conditioned by TLR activation (Lombardo et al. 2009). Nearly a decade ago, a study demonstrated that TLR3 and TLR4 ligation increases the immunomodulatory properties in BM-MSC (Opitz et al. 2009). Further investigations indicated that Notch signaling and upregulation of delta-like 1 (DL1) improve Treg induction, in TLR3 and TLR4-activated MSCs (Rashedi et al. 2017). On the contrary, other investigations demonstrated that the ligation of TLR3 or TLR4 on BM-MSC influenced in a negative way the T-cell proliferation by interfering with the Jagged-1 expression and, thus, inhibiting its signaling to the Notch receptor (Liotta et al. 2008).

The influence that the inflammatory environment has on BM-MSC may explain these different effects. However, rapid and low level exposure of MSC to TLR4

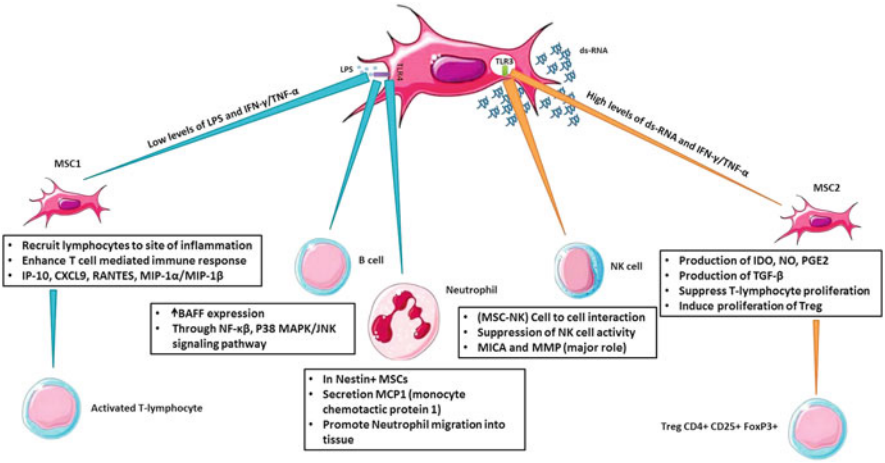

Fig. 1 The schematic illustration of TLRs activation on the surface of MSCs (different sources) on the immune cells TLR-priming MSCs can start different signaling pathways in different stages of inflammatory milieu and in various types of immune cells, depending on the nature of receptors, ligands, and cell sources. Similarly, according to the hypothesis recommended by Waterman et al. MSCs and, consequently, monocytes can polarize into two distinct phenotypes, depending on the concentrations of two inflammatory mediators such as TNF-α and IFN-γ. At the low levels of these mediators and LPS (TLR4 specific ligands), MSCs convert into pro-inflammatory phenotype to induce T-lymphocytes responses and recruit inflammatory cells to sites of damage by releasing chemokines, including MIP-1α, MIP-1β, RANTES, CXCL9, and IP-10. TGF-β and IL-6 are constitutively expressed by MSCs. At the high levels of TNF-α, IFN-γ, ds-RNA (TLR3 specific ligand), and TGF-β 1 MSCs switch to MSC2 phenotype in favor of the development of Treg. In consequence, it appears that the insufficient levels of TNF-α and IFN-γ enhance the pro-inflammatory phenotypes of MSCs. Thus, all of the molecular events of MSCs can occur in the different stages of inflammation in order to have appropriate functions. MSC2 contributes to promoting host immune responses, leading to creating a loop for preventing tissue injury and inducing tissue repair. Reprinted by permission from Elsevier Inc.: Elsevier Inc., Cell Immunol. 2017 May;315:1–10. doi: https://doi.org/10.1016/j.cellimm.2016.12.005. "Toll-like receptors as a key regulator of mesenchymal stem cell function: An up-to-date review" Solmaz Shirjang, Behzad Mansoori, Saeed Solali, Majid Farshdousti Hagh, Karim Shamsasenjan © 2016 Published by Elsevier Inc

agonists polarizes MSCs toward a pro-inflammatory phenotype that is essential for the rapid response to the damage. TLR4-triggered MSC generate collagen deposits, the expression of pro-inflammatory mediators, and inversion of the suppressive mechanisms of the T-cell.

Conversely, the exposition on TLR3 seems to induce MSC toward an immunosuppressive phenotype, fundamental for repairing injury through anti-inflammatory responses. Furthermore, it was demonstrated that stimulation of TLR9 by DSP30, that is a CpG oligodeoxynucleotide (CpG ODN), increases the proliferation and the suppressive strength of BM-MSC, preserving them from TLR4 stimulation by LPS and limiting the capacity of MSC to contain the proliferation of the T lymphocytes (Sangiorgi et al. 2016). Thus, MSCs have been described as transiting to pro- or anti-

inflammatory phenotypes, based on the type of TLR forms expressed on their surface (TLR3 or 4) or which ligands they can perceive (Waterman et al. 2010). Instead, WJ-MSCs do not respond to TLR4 or TLR3 binding; this effect could be due to factors involved in the modulation of the immune system (i.e., HGF) that could be overexpressed or by the expression of non-functional TLRs (Raicevic et al. 2011).

These data have allowed to consider MSCs for clinical use, especially for their immunomodulatory action which can give promise outcomes in therapeutic field (Delarosa et al. 2009). If inflammation is present, the beneficial activity of MSCs may be reduced or cancelled (Raicevic et al. 2010). Therefore, WJ-MSCs, as already mentioned, low expressing the TLR3, are considered an interesting source of cells with efficient immunomodulatory characteristics (Shirjang et al. 2017).

Although it is clear that to increase the immunomodulatory activity of various MSCs, it could be used for the silencing of some forms of TLR, it is necessary to further investigate the molecular mechanisms and the effects on MSCs triggered by TLR before using them in the clinical setting.

2.2 TLRs in Differentiation Capacity of MSC

The differentiation capacity of various MSCs to diverse tissue phenotypes has been widely cited and frequently described as a mechanism depending on age (Shirjang et al. 2017). Nevertheless, last investigations show the key role of TLR molecules in MSC maturation toward various cell phenotypes. It has been demonstrated that the activation of TLRs influences MSC maturation into osteocytes.

Osteoblastic maturation has been described as a biological process activating TLR2, TLR3, and TLR4 (Hwa Cho et al. 2006; Pevsner-Fischer et al. 2007; Liotta et al. 2008; Lombardo et al. 2009; Herzmann et al. 2017).

Furthermore, AT-MSC proliferation can be reduced by CpG oligodeoxynucleotides activating TLR9 with consequent improvement of osteocyte differentiation (Hwa Cho et al. 2006).

The TLR9 agonist CpG-ODN that has a phosphorothioate backbone (PTO-Cp-G-ODN) has been reported to antagonize the bone morphogenetic proteins (BMP)-induced Smad [SMA ("small" worm phenotype) and MAD family ("Mothers Against Decapentaplegic")] signaling in a TLR9-independent way, therefore repressing the AT- and UCB-MSCs maturation in osteoblasts (Delarosa et al. 2009). Moreover, the TLR9 expression is reduced during osteogenic differentiation (Nurmenniemi et al. 2010). On the contrary, LPS, which is TLR4 agonist, or flagellin, TLR5 agonist, induces osteogenic differentiation in UCB-MSCs (Berk et al. 2009); anyway, these results must be confirmed by more investigations.

Presently, there are no data about the involvement of TLR in adipogenic differentiation; and it is necessary to further study the role of TLR2 in chondrogenic maturation since the relative data are incoherent (Fig. 2).

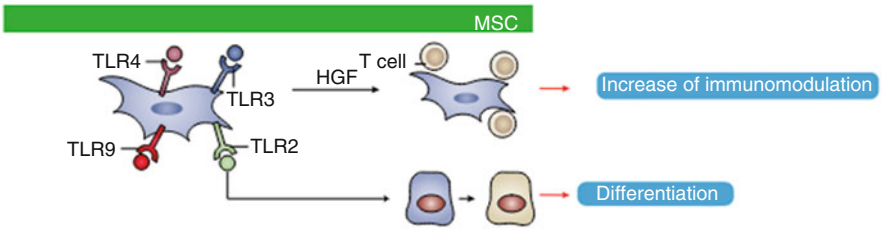

Fig. 2 In MSCs, TLR3 and TLR4 triggering induces an immunomodulation increase, while TLR2 is involved in differentiation processes. Reprinted and adapted by permission from: Hindawi: Hindawi Stem Cells International, 2019 Volume 2019, Article ID 6795845, 12 pages. "Role of Toll-Like Receptors in Actuating Stem/Progenitor Cell Repair Mechanisms: Different Functions in Different Cells," Fabio Sallustio et al.© 2019 Stem Cell International

2.3 TLRs in Migration of MSCs

The MSCs' function includes the capacity to migrate to ischemic, inflammatory, or mechanical damage sites or growth of the tumor site (Kholodenko et al. 2013). Research has revealed the TLR3 as the leading mediator for migration responses, using various TLR agonists as chemotactic agents to control the impact of TLR stimulation on MSC migration (Tomchuck et al. 2008). Migration, however, appears to be highly dependent on exposure period because, after a 1 h incubation, TLR3 and TLR4 promoted migration, while TLR repressed migration and invasion after 24 h incubation with the same chemotactic agents (Liotta et al. 2008; Waterman et al. 2010).

In addition, inhibition of the expression of TLR3 and TLR4 with knock-down plasmids cut by half unprimed MSC migration potential (Tomchuck et al. 2008). The treatment of transfected cells by LPS or poly(I:C) has however led to an increased migration compared to unstimulated controls (Waterman et al. 2010). TLR9 activation was also shown to aid MSC delivery to target tissues, via an MMP-13 mediated mechanism (Nurmenniemi et al. 2010), as well as the role that TLR3 and TLR4 play.

The numerous answers of MSCs are once again supportive for TLR controlling these cells via complex and still unknown molecular mechanisms, but according to the even minor changes in the environment.

3 TLRs in Dental Mesenchymal Stem Cells

In the last 20 years, biological and clinical interest in MSCs has increased due to their potential in tissue regeneration and cell and gene therapy.

Stem cells are undifferentiated cells showing a long-term self-renewal ability, with a capability of replicating for several times, maintaining an undifferentiated state; nevertheless, MSCs, following specific stimuli, are capable of differentiating toward different cell types (Bluteau et al. 2008).

Table 1 Conditions to assess the human MSC stemness

1.	Adhesion to plastic TCP		
2.	Phenotype[a]	Positive (≥95%)	Negative (≤2%)
		CD105	CD45
		CD73	CD34
		CD90	CD 14 o CD11b
			CD79α o CD19
			HLA-DR
3.	In vitro differentiation capacity in osteoblasts, adipocytes, and chondrocytes		

[a]A multicolor analysis is recommended for evaluation of co-expression

Stem cells can be divided into embryonic stem cells (ESC) and adult stem cells (ASC). ESCs arise from the inner part of the blastocyst. They are defined as pluripotent stem cells because they are able to differentiate towards the adult cells. Conversely, ASCs are multipotent because their differentiation potential is limited to particular cell lines. ASCs are characterized by an intense paracrine activity, able to induce strong biological effects on the surrounding cells, such as the induction of cell proliferation and differentiation, as well as the activation of reparative and regenerative mechanisms (Paduano et al. 2017).

MSCs are multipotent cells, with a fibroblast-like morphology, capable of self-renewing, and of originating tissues such as bone, cartilage, muscle, stromal cells, tendons, and connective tissue. The last decade, the Mesenchymal and Tissue Stem Cell Committee of the "International Society for Cellular Therapy (ISCT)" has suggested the minimum criteria for the characterization of human MSCs. According to ISCT, there are three mandatory conditions to assess the cell stemness (Table 1).

Bone marrow was initially considered the main source of MSC. Over the years, however, several scholars have shown that MSCs are present in different human tissues such as blood, umbilical cord, placenta, fat, heart, brain, skin, muscles, liver, and teeth (Egusa et al. 2012).

3.1 Introduction on Dental Derived MSCs

Dental pulp is a specialized connective tissue located within the living teeth. Within the pulp structure, we can distinguish four different layers: an outermost layer consisting of odontoblasts able of generating dentin and dentin-like matrix; a second layer, called "cell-free zone," poor in cells but rich in collagen fibers; a third layer, called "cell-rich zone," made up of progenitor cells and undifferentiated cells, some of which are stem cells; an innermost layer that represents the pulp living area, rich in blood vessels and nerves. The stem cells and undifferentiated cells of the "cell-rich zone" can migrate to various districts where, under several different stimuli, they can generate new differentiated cells and regenerate specialized tissues (d'Aquino et al. 2007).

Dental pulp of third molars is considered an easily accessible source of MSC. In 2000, Gronthos and colleagues first isolated dental pulp stem cells (DPSCs), on the basis of their clonogenic capacity and high proliferative rate (Gronthos et al. 2000).

All the dental derived MSCs have widely demonstrated multipotency and a versatile ability to regenerate different dental and periodontal tissues, in vitro and in vivo. They also showed positivity to the specific markers: STRO-1, CD13, CD24, CD29, CD44, CD73, CD90, CD105, CD106, CD146, Oct4, Nanog and β2 integrin, but they were negative for CD14, CD34, CD45, and HLA-DR. The persistence of CD45 negativity and CD34 positivity demonstrates that DPSCs do not derive from a hematopoietic source, but are of mesenchymal origin (Barry and Murphy 2004; d'Aquino et al. 2007).

DPSCs are a heterogeneous population, and the different markers previously listed can be expressed in subpopulations of the same cells. They can survive in culture for long periods and be maintained for several passages: no clear signs of senescence have been observed up to more than 80 passages. Furthermore, they can be cryopreserved for long periods without losing their multipotency (Tatullo et al. 2015b).

Their differentiation into odontoblasts has been widely shown. Furthermore, DPSCs can also differentiate into adipocytes and neurons by exhibiting both morphology and their respective gene markers. Furthermore, their chondrogenic and myogenic differentiation was observed in vitro. The plasticity and multipotency of DPSCs can be related to the origins of the dental pulp, consisting of both ectodermal and mesodermal components and cells deriving from the neural crest (d'Aquino et al. 2007).

3.2 Focus on Novel and Promising Dental Derived MSCs

In 2013, a new population of mesenchymal stem cells has been isolated from human periapical cysts. Marrelli, Paduano, and Tatullo first demonstrated the existence within periapical inflamed tissues of residing cells showing the MSCs characteristics, termed MSCs "human Periapical Cyst-Mesenchymal Stem Cells" (hPCy-MSCs). These are isolated from human periapical cysts and are able to express stem cell-like properties (Marrelli et al. 2013).

Furthermore, hPCy-MSCs, under appropriate pro-neurogenic stimulation, acquire neuronal morphology and significantly over-express different neural markers, both at the protein level and at the primary transcript level (Tatullo et al. 2017).

hPCy-MSC express CD13, CD29, CD44, CD73, CD90, CD105, STRO-1, and CD146 and do not express hematopoietic markers such as CD45. Furthermore, research groups have demonstrated the central role of the CD146 cell adhesion receptor in influencing the properties of hPCy-MSCs. CD146 MSCs exhibit better proliferative and clonogenic potential, showing also increased levels of the KLF4 stemness gene marker (Paduano et al. 2016).

Tatullo has reported that hPCy-MSCs are able to grow in osteogenic media, where they seem to be capable of differentiating into osteoblast/odontoblast-like cells: however, hPCy-MSCs are naively oriented towards osteogenesis, while the DPSCs are oriented towards dentinogenesis (Tatullo et al. 2015a).

Commonly, after the osteogenic induction in specific media, the expression of bone-specific genes is induced, such as osteopontin (OPN), osteocalcin (OSC), alkaline phosphatase and dentine matrix protein 1 (DMP-1). With the aim to further confirm the multi-lineage differentiation capacity of hPCys-MSCs, the team of Tatullo's Lab demonstrated that hPCys-MSCs were able to effectively differentiate into neuron-like cells: their work highlighted reliable results by immunofluorescence, western blotting, and flow cytometry assays. Interestingly, basal expression levels of neuronal- and astrocyte-specific proteins (β-III tubulin and GFAP) were observed in hPCys-MSCs to be similar to those in DPSCs (Marrelli et al. 2015).

3.3 TLRs and Dental Derived MSCs

Multipotent stromal cells (MSCs) of various origins have been shown to express functional TLRs in specific patterns, making them selectively sensitive to microbial compounds. Triggered TLRs can modulate proliferation, migration, differentiation, immunosuppression, and potential in MSCs. The specific pattern of expression of TLRs varies according to the tissue of origin of the MSCs, which may have an implication on the therapeutic potential of MSCs during transplantation in inflammatory environments in vivo (Fawzy-El-Sayed et al. 2016).

Several investigations were carried out on dental MSCs, related to the expression of TLRs. First studies reported that TLRs 2, 3, and 4 were found in MSCs derived from dental follicle (Chatzivasileiou et al. 2013).

These first results stimulated researchers to increase their studies on this matter; periodontal ligament stem cells express TLRs 1, 2, 3, 4, 5, 6, 8, 9, and 10: in these cells, TLRs-mediated nuclear factor κB pathway has been demonstrated to affect the osteogenic differentiation of these cells (Li et al. 2014).

On the other hand, the gingival mesenchymal stem/progenitor cells G-MSCs express TLRs 1, 2, 3, 4, 5, 6, 7, and 10 (Fawzy El-Sayed and Dörfer 2016), while the well-known DPSCs express TLRs 1 and 10 (Fawzy El-Sayed et al. 2016).

Thus, the latest studies have clearly demonstrated that dental stem cells have the ability to express several TLRs, which seem to be involved in several regulations of biological pathways. Specifically, mesenchymal stem cells seem to interact with their cellular environment via their toll-like receptors, inducing the production of co-factors leading to pro- or anti-inflammatory immune responses. Furthermore, dental MSCs can communicate with cells of the immune system, interacting with macrophages through the direct activity of indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE-2). Dental MSCs acting on macrophages are able to push their polarization towards the M2 phenotype: the expression of IDO is induced by the stimulation of INF-γ or by the ligands that interact with the toll-like receptor TLR3 and TLR4, well represented on dental MSCs membrane. This interesting

binding activates a protein kinase that triggers a signal cascade leading to the production of IDO enzyme. This interaction also allows the decrease in the amount of TNF-α, IL-1α, and IL-6 and the increase in the production of IL-10 by macrophages. In a recent study, stem/progenitor cells derived from the gingival papilla (G-MSC) were studied to understand how TLRs could regulate the immune response. In particular, all TLR agonists induced pro-inflammatory cytokines, with the exception of the TLR3 agonist, which significantly promoted the anti-inflammatory response.

This TLR-mediated immunomodulation could also affect their therapeutic potential in vivo, thus also highlighting the presence of two different TLR-induced pro-inflammatory and anti-inflammatory phenotypes of G-MSCs (Mekhemar et al. 2018).

A study carried out in 2020 on G-MSCs has pushed the research on G-MSCs TLR3 receptors; their activation allowed to maintain the phenotype of the mesenchymal stem/progenitor cell, also guiding the differentiation, allowing a move away from the undifferentiated pluripotent cell phenotype. This type of modulation could also influence the potential therapeutic applications of G-MSCs (Mekhemar et al. 2020).

In a study published in 2020, the expression profile of TLRs related to apical papilla stem cells (SCAP) was defined for the first time, both in the presence of inflammatory processes and in the absence. This TLRs expression profile is essential for understanding the efficacy of SCAP cells towards inflammatory agents, particularly during periodontitis and periimplantitis promoted by bacteria infections. In fact, during regenerative procedures, SCAPs, similarly to other stem cells, could interact with the inflammatory microenvironment through the TLRs. The dental MSCs generally can express TLRs among 1 and 10; conversely, an inflammatory microenvironment locally regulates the expression of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9 and locally reduces the expression of TLR3, TLR7, TLR8, and TLR10 (Fehrmann et al. 2020).

Alveolar bone proper-derived mesenchymal stem/progenitor cells (AB-MSC) can locally interact with alveolar osteoblasts (OB) through signals detected by TLRs expressed on their surfaces; this co-stimulation is strategically used in direct transplantations to treat alveolar defects, periodontal minus, or peri-implant damages: the specific molecular patterns associated with pathogens (PAMPs) locally interact with the molecules associated to damaged tissues (DAMPs), and the TLRs are involved in such interaction.

To date, the interaction between the receptors and the possible ligands has not been studied in detail. Somehow, a general model has been proposed by research community: in this proposed model, the cellular response to ligands is by all the subsets of TLRs expressed in the cell. In fact, the co-transfection of different TLRs in dental cell models has shown their ability to inhibit or increase the recognition of different ligands. In the oral environment, the expression of the different types of TLRs allows dental stem cells to properly reply to different stimuli: such cells, in fact, can recognize different ligands present in the extracellular matrix. More in detail, the expression of TLR1 and TLR2 could favor the recognition of lipoproteins,

while the expression of TLR7 may increase the cell ability to recognize viral pathogens, thus reducing the oral tissues to viral infections.

Interestingly, studies have highlighted how tissue damages could increase the expression of TLR2 in AB-MSC and OB: TLR2 is usually linked to heat shock proteins (HSPs) released during cellular stress and acting as chaperones during both wound healing and tissue remodeling. The upregulated expression of TLR4 may also increase the sensitivity to HSPs, as well as to components of the extracellular matrix, including fibrinogen, fibronectin, heparan sulfate, and hyaluronic acid, which are released during tissue damage and have direct impact on the healing phases. Finally, the expression of TLR7 and TLR8 also allows a stronger cell response to tissue damage in oral bone tissues and related OBs (Fawzy El-Sayed et al. 2017).

The most investigated dental stem cells are surely DPSCs: the TLRs expression profile of human DPSC in presence and absence of inflammatory processes was described for the first time in 2016. DPSCs show considerable regenerative potential in vivo*:* interestingly, during the dental pulp regeneration, DPSCs interact with the local environment via toll-like receptors (TLRs). In basal conditions, DPSCs express on their surface all the TLRs (1-10) in different percentage; on the other hand, in the presence of inflammatory processes, an upregulation of the expression of the TLRs 2, 3, 4, 5, and 8 was highlighted, while a downregulation and cancellation/abolition of TLR6 have also been reported (Table 2) (Fawzy El-Sayed et al. 2016).

The interaction among stem cell research, regenerative and reparative dentistry, and tissue engineering has highly increased the overall interest of several research groups towards biomedical applications involving such topics. Dental stem cells have been investigated in several studies focused on microRNAs (miRNAs), a class of small non-coding RNAs that play a crucial role in the regulation of DPSC phenotypes. Interestingly, the overexpression of specific miRNAs increased the proliferation of DPSC and inhibited the differentiation of DPSC. On the contrary, studies reported that TLR4 expression negatively impacted on the levels of specific miRNAs acting as critical regulators of DPSCs behavior (Sun et al. 2017).

The overlapping among dental stem cells, extracellular matrix, and TLRs expression seems to be well demonstrated. Despite only few studies have focused on this cross-talk, the preliminary considerations support this research field also in the next years. The main advantages will be related to the biological reply of dental stem cells in pathological conditions and in regenerative/reparative procedures. The next steps will be aimed to better characterize these receptors, so to modulate their activity in a safe and well-controlled way.

4 TLRs and Hematopoietic Stem Cells

In patients with cancer, such as multiple myeloma or leukemia, hematopoietic stem cells (HSCs) are by far the most studied and infused of all stem cells. Since HSCs are the capstone of the blood hierarchy, they can regenerate the entire hematolymphoid system and this makes a powerful tool for blood disorders (Park et al. 2015), TLR2

Table 2 TLR expression and pathway involved in oral MSCs

Oral MSCs	TLR expression	Pathway	References
G-MSC	TLR3	TLR3 binding agonists promotes pro-inflammatory cytokine production, binding agonists promote anti-inflammatory response.	Mekhemar et al. (2018)
G-MSC	TLR3	Activated TLR3 allows the maintenance of stemness while also promoting differentiation.	Mekhemar et al. (2020)
SCAP	TLR1-10	TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9 respond positively to inflammatory stimuli by activating specific signaling pathways for each ligand. While TLR3, TLR7, TLR8, and TLR10 are downregulated in the inflammatory environment.	Fehrmann et al. (2020)
DPSC	TLR1-10	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7 TLR8, TLR9, and TLR10 respond positively to inflammatory stimuli by activating specific signaling pathways for each ligand. TLR6 is not present in an inflammatory environment.	Fawzy El-Sayed et al. (2016)
AB-MSC	TLR1, TLR2, TLR4, TLR7, TLR 8, TLR10	TLR1,2 favor the recognition of lipoproteins; TLR7 favors the recognition of viral pathogens; TLR2, TLR4 in the presence of tissue lesions favor wound healing and tissue remodeling; TLR7 and 8 trigger specific responses related to cell damage.	Fawzy El-Sayed et al. (2017)

and TLR4 were expressed by Early HSC. During infection, components within the microbial environment stimulate dormant stem cells, which differentiate into immature myeloid cells, and rapidly replenish the innate immune system. Signaling in granulocyte and macrophage progenitors by myeloid differentiation primary response 88 (Myd88) downstream enables proliferation and maturation without the need for growth factors. In HSC, LPS was effectively recognized by the TLR4/MD-2 complex interacting with the CD14 coreceptor. In addition, typical lymphoid progenitors are preferentially oriented towards differentiation of dendritic cells (Nagai et al. 2006) (Fig. 3). Interestingly, murine short-term HSC has been shown to be more effective in generating cytokines than mature immune cells in response to TLR ligand stimulation (Zhao et al. 2014).

It has been shown that TLR expression on myeloid cells senses bacterial products, causing myelopoiesis. LPS exposure, either directly through cell-intrinsic TLR signaling or indirectly through upregulation of myeloid-derived inflammatory cytokines, has been shown to trigger HSCs. Chronic in vivo treatment with LPS increases in HSC cycling and myeloid differentiation, resulting in a lack of repopulation activity in transplantation experiments (Song et al. 2002; Esplin et al. 2011). Indeed, during bacterial infection or LPS treatment, the TLR4/Sca-1 axis contributes

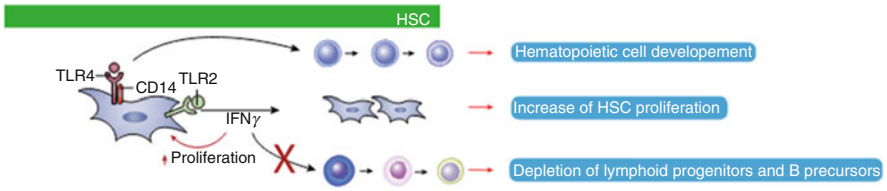

Fig. 3 TLR4 and TLR2 play a major role in influencing the cell biology of HSCs. TLR4 triggers hematopoietic cell development. TLR2 induces an increase in HSC proliferation, avoiding depletion of lymphoid progenitors and B cell precursors. Reprinted and adapted by permission from: Hindawi: Hindawi Stem Cells International, 2019 Volume 2019, Article ID 6795845, 12 pages. "Role of Toll-Like Receptors in Actuating Stem/Progenitor Cell Repair Mechanisms: Different Functions in Different Cells," Fabio Sallustio et al.© 2019 Stem Cell International

to granulopoiesis beginning with HSC (Shi et al. 2013). Notably, as shown by chronic low-dose LPS disruption in human HSC and B-lineage progenitors, the duration and entity of the stimulation can affect cell lymphopoiesis. Increased numbers of proliferating HSC paired with a higher IFN-γ protein level indicate a possible local source of this cytokine. This leads to lymphoid progenitors and B precursors being depleted (Liu et al. 2015) (Fig. 3).

Histamine also plays an important role in HSC expansion in addition to the LPS, hampering cycling depletion of MB-HSC (Chen et al. 2017b). LPS in vivo stimulation directly induces HSC proliferation via TLR4 interaction; however, HSC self-renewal and repopulation activity are weakened by prolonged LPS exposure. Although initial activation of TLR4 in HSC may therefore be beneficial for counteracting systemic infection, prolonged signaling of TLR4 may have deleterious effects and lead to inflammation-related dysfunction (Takizawa et al. 2017). Systemic exposure to the TLR2 agonist, however, results in a loss of self-renewal of HSC in the bone marrow. At least in part, these effects have been shown to be mediated by the granulocyte colony-stimulating factor and tumor necrosis factor-alpha (Herman et al. 2016).

In conclusion, these studies support a TLR signaling-mediated mechanism in which HSCs sense non-self PAMPs, enabling them to respond quickly to infections in order to replenish the hematopoietic system; however, prolonged exposure can affect self-renewal and differentiation resulting in the exhaustion of the HSC pool.

5 Possible Role of TLRs in Neurodevelopment and Neurodevelopmental Psychiatric Conditions

Even though TLRs have extensively been investigated as molecular elements with potentially critical importance to the immune response, relatively few studies have been conducted on the role of this family of molecules in major psychiatric disorders. Nonetheless, emerging evidence has prompted the importance of TLRs in specific psychiatric condition due to the established relationship between the immune response, neurodevelopment, and neuropathophysiology of such

conditions. Within this perspective, prototypical examples are represented by schizophrenia, major depression disorder, and autism spectrum disorders, whose biological bases recognize a clear involvement of an altered immune response directly and/or indirectly related with dysfunctional neurodevelopment. Not surprisingly, recent studies have revealed a possible role of TLRs in all these disorders.

5.1 TLRs: Developmental Tools that Regulate Neuronal Morphogenesis

TLRs, well-known players of the innate immune response, have been discovered in the last decade as key players of molecular mechanisms relevant to neuronal plasticity and neurogenesis during brain development. In higher eukaryotic animals, brain is considered an immune-privileged organ because of the presence of the blood brain barrier that isolates brain cells from peripheral immune system cells and exogenous pathogens. In addition, brain has its own specialized resident population of phagocytic cells, the microglia, that is responsible for local inflammatory response. Thus, the fact that neurons retain low expression levels of TLRs, not sufficient for triggering innate immune responses, suggests that they might be implicated in biological processes different from immune response.

5.2 TLRs Expression During Brain Development

Studies have shown that expression of TLRs varies according to developmental stage. This has importantly contributed to support further investigations of TLRs role in developmental processes.

TLR2, TLR1, and TLR6 are expressed early postnatally (Okun et al. 2010a; Kaul et al. 2012). TLR4 expression gradually increases during early embryonic stages and remains stably high during adulthood (Lathia et al. 2008; Kaul et al. 2012). TLR5 maintains stable expression levels through the whole developmental process (Kaul et al. 2012), while TLR3 expression reaches its highest levels in the early period of cortical development (Lathia et al. 2008), then declines to maintain low expression levels in the adulthood. TLR7 expression levels in the developing mouse transiently increase at the time of birth and gradually decline (Kaul et al. 2012). TLR8 brain expression can be detected from early embryonic stages, then increases until dramatically declining postnatally and remaining low in the adult brain (Ma et al. 2006a, b; Kaul et al. 2012).

Finally, TLR9 expression constantly increases during late embryogenesis and postnatal stages until adult levels are reached and remain stable (Kaul et al. 2012).

Spatial information on the expression of genes coding for these receptor proteins across brain development is still poor. TLR2 is expressed on cells in areas of the adult brain associated with the generation of new neurons, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Rolls et al. 2007; Okun et al. 2011). Further examination

revealed that both TLR2 and TLR4 are expressed on adult neural progenitor cells (NPCs) (Covacu et al. 2009).

TLRs signaling involves 4 Toll/IL-1 receptor (TIR) adaptor proteins, namely, MyD88, Mal, Trif, TRAM, and SARM (O'Neill and Bowie 2007). Interestingly, these adaptor-signaling proteins also exhibit specific and distinct expression patterns during brain development. In particular, TRIF mRNA levels gradually increase after birth, then decrease (Kaul et al. 2012). SARM protein expression levels gradually increase in the brain, peaking during the most significant period for neuronal proliferation, while after birth its expression dramatically decreases (Kim et al. 2007). Data are rather inconsistent and inconclusive as for MyD88, as one study reported that MyD88 expression is relatively constant following birth (Kaul et al. 2012) while a second study found that its levels decrease during development (Okun et al. 2011). As a whole, regardless for specific direction of expression patterns suggested by studies, variation of TLR family genes across neurodevelopment highlights possible roles of these proteins in such a complex biological process and neurogenesis.

5.3 TLRs and Neurogenesis

Neurogenesis occurs robustly during embryogenesis and gradually diminishes after birth and during adulthood (Duan et al. 2008), consisting of multiple steps, whose proper articulation is essential to the correct brain development: (1) during proliferation, neuronal progenitor cells (NPCs) give rise to transiently amplifying cells and proliferating progenitors cells tightly associated with astrocytes and vascular structures; (2) during differentiation, transiently amplifying cells differentiate into immature neurons; (3) during migration, immature neurons migrate a short distance into hippocampal granular cell layer and olfactory bulb; (4) finally, immature neurons extend their projecting axon and dendrites; and (5) functional integration of newly formed neurons into existing neuronal networks occurs (Suh et al. 2009). In mammals, neurogenesis occurs in the SVZ of the lateral ventricles (Sakamoto et al. 2014). In adult rodents, neurogenesis is restricted to the SGZ of the hippocampal DG and the sub-ependymal zone (SEZ) lining the lateral ventricles. In humans, recent studies have shown that neurogenesis also occurs in the striatum (Ernst et al. 2014). NPCs can self-renew and differentiate into all types of neural cells (neurons, astrocytes, and oligodentrocytes) (Gage 2000).

Studies indicate that TLR2, TLR3, and TLR4 have distinct effect on NPCs proliferation. In particular, reports suggest TLR3 negatively regulates embryonic NPCs proliferation, correlated with diminished TLR3 expression during development (Lathia et al. 2008). TLR3-deficient mice have increased hippocampal and DG volumes and enhanced hippocampal neurogenesis (Okun et al. 2011). TLR2 deficiency impairs hippocampal neurogenesis, whereas TLR4 downregulates neuronal proliferation (Rolls et al. 2007). So far, the impact of TLR5, 7, 8, or 9 on NPCs proliferation has not been elucidated yet.

Consistently with changes in expression, effects of TLR2 on NPCs differentiation vary according to the stage of neurodevelopment. TLR2 embryonic deficiency does not affect differentiation of embryonic NPCs. Whereas in the adult brain loss of TLR2 promotes astrocytic rather than neuronal fate in differentiating NPCs (Rolls et al. 2007). TLR4 deficiency enhances neuronal differentiation (Rolls et al. 2007). These cells, however, do not survive, suggesting that additional surviving signals are required to successfully conclude the neurogenesis process. A similar effect is produced in MyD88-deficient mice, implying that MyD88 may mediate TLR4 signaling during neurogenesis (Gage 2000). Finally, deficiency of TLR7 and TLR8 results in abnormal neuronal differentiation and maturation (Liu et al. 2013; Chen et al. 2017a; Hung et al. 2018).

TLRs-mediated signaling in axonal growth during neuronal differentiation is currently poorly explored. Some studies have shown that TLR7 and TLR3 negatively affect axonal growth (Cameron et al. 2007; Liu et al. 2013; Chen et al. 2017a; Hung et al. 2018), while further reports have suggested activation of TLR3, TLR7, and TLR8 to downregulate dendritic outgrowth (Liu et al. 2013; Chen et al. 2017a; Hung et al. 2018).

5.4 TLRs and Brain Repair

Neurogenesis continues in the hippocampal DG and SVZ throughout adulthood for cognition or repair of lost neurons following injury or disease (Parent et al. 1997; Duan et al. 2008). New evidence has emerged showing that TLRs may be involved in brain repair after stroke by neurogenesis modulation. In particular, a recent study in TLR4-deficient mice that underwent experimental ischemia showed that deficiency of this receptor keeps cells in less differentiated stages, with an accumulation of pre-neuroblasts unable to migrate through their predefined pathway (Palma-Tortosa et al. 2019). Other results indicate that TLR2 receptor can enhance adult neurogenesis from neural stem cells in the hippocampal DG after cerebral ischemia through promoting proliferation and neural differentiation of neural stem cells and survival of newborn neurons (Seong et al. 2018). Therefore, these results support TLRs may play a role in future strategy to treat brain injury.

5.5 Toll-Like Receptors and Psychiatric Disorders

There is increasing evidence of association between the innate immune system and psychiatric disorders. Such an association may, at least in part, lay on the previously highlighted role of TLRs (Larsen et al. 2007; Okun et al. 2010b) especially in the case for psychiatric disorder whose pathophysiology has established bases in neurodevelopment, including schizophrenia (SCZ), major depression disorder (MDD), or autism. Consistently, experiments on animals have suggested that intrauterine infection through TLR activation might impair neurodevelopment, synaptic

plasticity, and perinatal brain damage that trigger psychiatric-like phenotypes in other experimental contexts (Larsen et al. 2007).

5.6 TLR and Schizophrenia

Clinical and preclinical studies have demonstrated an important role of neuroinflammation in the pathogenesis of SCZ (Barichello et al. 2020). In literature there is evidence of microglial activation and increased levels of cytokines and chemokines in post-mortem brain samples from patients with SCZ, as well as in fetal and adult brains of offspring subjected to maternal immune activation during fetal life (Garay et al. 2013; Feigenson et al. 2014; Réus et al. 2017; Barichello et al. 2020). Importantly to the topic of the current work, the activation of TLRs by PAMPs initiates an intracellular kinase cascade by inducing the translocation of NF-κB transcription factor, which leads to the production of a variety of inflammatory mediators and cytokines and finally to the activation of microglia (Anderson 2000).

TLR3 can signal through a TIR-domain-containing adapter inducing interferon-β (TRIF-dependent pathway that recruits the TNF receptor-associated factor-3 (TRAF-3) (Barichello et al. 2020) with the activation of interferon regulatory factor-3 (IRF-3) and IRF-7. This pathway generates the production of IFN-α or IFN-β (Barichello et al. 2020). In another pathway, TLR-3 activates proteins such as TRIF, AP1, and NF-κB, inducing the expression of pro-inflammatory cytokine genes (Barichello et al. 2020).

TLR4, CD14, and myeloid differentiation protein-2 (MD-2) form a complex that recruits the MyD88 adapter-like (Mal) and the TIR domain-containing adaptor protein (TIRAP). Mal/TIRAP recruits myeloid differentiation primary response gene 88 (MYD88) adaptor. The MyD88 adaptor molecule connects with the serine/threonine kinase IL-1 receptor-associated protein triggering the phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK-1) and IRAK-2 and the recruitment of TNF receptor-associated factor-6 (TRAF-6) adaptor (Barichello et al. 2020). TRAF-6 activates inhibitory IκB kinases and mitogen-activated protein kinases (MAPKs), resulting in NF-κB and activator protein-1 (AP-1) transcription factor activation and production of cytokines. The TLR4 complex also takes on TRIF-related adaptor molecules that interact with TRIF adaptor and activate the interferon regulatory factor-3 (IRF-3) transcription factor (Barichello et al. 2020).

Notably, studies reported the post-mortem cerebellum of human subjects with SCZ displays an increase of TLR-4, MyD88, and IκBα expression and a reduced NF-κB activity. Furthermore, in schizophrenic patients, post-mortem prefrontal cortex TLR4, MyD88, and IκBα protein levels were lower, while nuclear transcription NF-κB activity was increased as compared to controls (MacDowell et al. 2017).

Not surprisingly, expression and activity levels of TLR and TLR-related genes are reported to be affected by antipsychotic medication that is used to treat schizophrenia and other psychotic disorders. With this regard, Garcia-Bueno and collaborators (García-Bueno et al. 2016) evaluated the effect of treatment with

antipsychotics on post-mortem brain gene expression in patients with schizophrenia and discovered that the group treated with antipsychotics presented higher levels of TLR4, MyD88 protein, and MyD88 mRNA compared to healthy controls. Furthermore, an MDA decrease was observed in the antipsychotic-free group compared to the control and the antipsychotic treatment groups, but the antipsychotic-free group showed higher levels of NF-κB protein compared with the control group (García-Bueno et al. 2016). As a whole, data available on this topic indicate that TLR family proteins may play a role in the pathophysiology of schizophrenia especially via their established impact on molecular cascades involved in neuroimmune inflammatory response.

5.7 TLR and Major Depression

MDD is a serious mental illness that affects 300 million people worldwide (WHO 2020). A subset of depressed patients shows inflammation signs as indicated by increased level of interleukin 6 (IL-6), IL-1ß, C-reactive protein (CRP), tumor necrosis factor (TNF), and IL-1 receptor antagonist (IL-1Ra) in blood and cerebrospinal fluid (Dowlati et al. 2010; Goldsmith et al. 2016; Enache et al. 2019). Preclinical studies showed how TLRs play a key role in mediating stress-induced inflammatory responses associated with depression-like behavior (Figueroa-Hall et al. 2020). In a recent study, transcriptome analyses revealed that mice subjected to repeated-social defeat stress (R-SDS) exhibited elevated levels of TLR2 and TLR4-specific damage-associated molecular patterns (DAMPs) in the medial prefrontal cortex, and increased TLR2 and TLR4 mRNA expression in microglia. R-SDS-induced microglial activation was associated with social avoidance, behavior not detectable in TLR2/TLR4 double knockout mice (Nie et al. 2018). Consistent with these data, blockade of TLR2 and TLR4, with a TLR2/TLR4 antagonist OxPAPC, prevented hippocampal pro-inflammatory responses after an immune challenge with lipopolysaccharides (Weber et al. 2013).

Several studies have investigated the role of TLRs in major depression disorder (MDD) by focusing on TLR4 expression, TLR-related proteins, cytokine induction in peripheral blood mononuclear cells (PBMCs), monocytes, and post-mortem tissue from depressed patients (Figueroa-Hall et al. 2020). Hung and collaborators analyzed TLRs and IL-6 mRNA expression in PBMCs, monocytes, and whole blood cells of patients with MDD before and after antidepressant treatments with selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), norepinephrine–dopamine reuptake inhibitors (NDRIs), and tricyclic antidepressants. Rather significantly, TLRs mRNA levels were differentially expressed in (MDD) sample compared to healthy controls and TLR4 was found to be an independent risk factor predicting MDD clinical severity (Hung et al. 2014). TLR mRNA levels decreased after 4 weeks of treatment with SSRIs or SNRIs, indicating a TLR-mediated anti-inflammatory role for antidepressants (Hung et al. 2017). Moreover, studies exploring the association between subscales of the Hamilton Depression Rating Scale (HAMD-17) and peripheral TLR4 mRNA

in MDD subjects showed that signs of anxiety and weight loss in HAMD-17 were predictive of TLR4 mRNA levels (Wu et al. 2015).

5.8 TLR and Other Psychiatric Disorders

While differences in immune mechanisms in Tourette patients have recently been described (Weidinger et al. 2014), a significant lower receptor expression of TLR4 after lipopolysaccharide LPS stimulation was found in the patient with this severe psychiatric disorder, suggesting TLR family proteins may play a role in the pathophysiology of the disorder via an altered immune response. Quite consistently, in the work by Weidinger and collaborators, Tourette patients had higher levels of lipopolysaccharide-binding protein (sCD14) in the unstimulated condition versus LPS stimulation group suggesting that an impaired activation of the innate immune response in TS, especially in regard to bacterial infection might be implicated.

Research was also conducted on the association between impairment of innate immunity and autism spectrum disease (ASD). In particular, a working group isolated peripheral blood monocytes from children with ASD and from age-matched controls and stimulated these cell cultures in vitro with distinct toll-like receptor (TLR) ligands. After in vitro challenge with TLR ligands, they observed a marked increase in pro-inflammatory IL-1β, IL-6, and TNFα responses following TLR 2, and IL-1β response following TLR4 stimulation in monocyte cultures from children with ASD as compared to controls. In particular, data indicated a possible differential innate immune response to TLR 2, 4, and 9 from monocyte cell cultures derived from ASD children when compared to healthy controls and suggested that an underlying dysfunction in monocyte pathogen recognition and/or TLR signaling pathways may be altered in young children with ASD (Enstrom et al. 2010).

6 TLRs and Renal Stem/Progenitor Cells

Recently, resident adult renal progenitor cells (ARPCs) have been isolated from both human kidney tubules and glomeruli (Bussolati et al. 2005; Sagrinati et al. 2006; Sallustio et al. 2010; Procino et al. 2011; Angelotti et al. 2012). These two cell populations share surface markers, CD24, CD133, and Pax2, a transcription factor contained in undifferentiated mesenchyme, and their profiles of gene expression are similar (Bussolati et al. 2005; Sagrinati et al. 2006; Sallustio et al. 2010; Procino et al. 2011; Angelotti et al. 2012).

To date, studies by other groups (Bussolati et al. 2008; Angelotti et al. 2012;) and Sallustio's research group (Sallustio et al. 2010, 2015, 2017) indicate that, due to their multipotent differentiation capacity and their reparative properties, both tubular and glomerular ARPCs may be an alternative source of cellular therapy for kidney diseases (Sallustio et al. 2015, 2017). These cells have been shown to regenerate tubular cells and enhance renal function once injected into acute or chronic renal

injury models (Bussolati et al. 2005, 2008; Sagrinati et al. 2006; Angelotti et al. 2012). Additional studies support the contribution of ARPCs in patients with acute or chronic tubular damage to the repair of damaged renal parenchyma (Loverre et al. 2008).

The expression of TLRs in several tissues has been demonstrated by several publications, but the significance of these receptors in ARPCs is novel. Initial studies centered on TLR expression and function in renal tissue provided that the TLRs respond to PAMPs and DAMPs. The function of TLR2 in chronic renal injury, characterized by inflammation, apoptosis, and fibrosis, was elucidated by Leemans et al. (Leemans et al. 2009). They found that, in the first step of obstructive nephropathy, TLR2 is involved in the renal inflammatory response, but not in the development of renal fibrosis and subsequent progressive injury (Leemans et al. 2009). Sallustio's group has shown for the first time that TLR2 is upregulated in ARPCs and is responsible for activating them to facilitate renal repair after kidney injury (Sallustio et al. 2010). TLR2 could serve as a sensor for tissue damage. Indeed, in response to TLR2 stimulation, ARPCs secrete monocyte chemoattractant protein-1 (MCP-1) and C3 via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation, as well as pro-inflammatory cytokines (IL-6 and IL-8) (Sallustio et al. 2010) (Fig. 5). As confirmed by preclinical studies in a rat model of glycerol-induced acute kidney injury, the development of these cytokines and chemokines may be useful for renal repair processes, where IL-6 has been shown to induce tubular regeneration and defend against further injury (Homsi et al. 2002; Nechemia-Arbely et al. 2008). The C3, IL-8, and MCP-1 cleavage fragments play important roles in mobilizing and modulating the trafficking of SC (Widera et al. 2004; Sallustio et al. 2015). In addition, ARPCs increased their proliferation rate upon TLR2 stimulation in order to increase the pool of resident cells and avoid depletion (Sallustio et al. 2010).

Moreover, by preventing cisplatin-induced apoptosis in renal proximal tubular epithelial cells, TLR2 activation on resident tARPCs induces reparative processes (RPTECs). Tubular ARPCs have been shown to produce and secrete inhibin-A and decorin (both as protein and as microvesicle-shuttled mRNA) involved in the tubular cell regenerative process after RPTEC damage and upon TLR2 activation. In the presence of TLR2-blocking agents, all these regenerative processes can be null. Interestingly, in related preclinical environments, glomerular ARPCs have been shown to be unable to cause tubular cell regeneration (Sallustio et al. 2017) (Fig. 4).

These data emphasize the importance of TLR2 in the mediation of tARPCs' reparative properties.

Also microRNA (miRNAs) can mediate TLR2 overexpression in ARPCs. miRNAs are major stem cell fate and behavior regulators and control several target genes. The low level of miR-1,225-5p was shown to induce high TLR2 expression and regulate other essential genes, such as Paired box 8 (PAX-8), IL-8, bone morphogenetic protein receptor type II (BMPR2), IGF1, inhibin-A, cyclin D1, and WNT1, all involved in ARPC regenerative processes, among several miRNAs differentially modulated in tARPCs relative to RPTECs (Sallustio et al. 2010, 2013; Rinkevich et al. 2014).

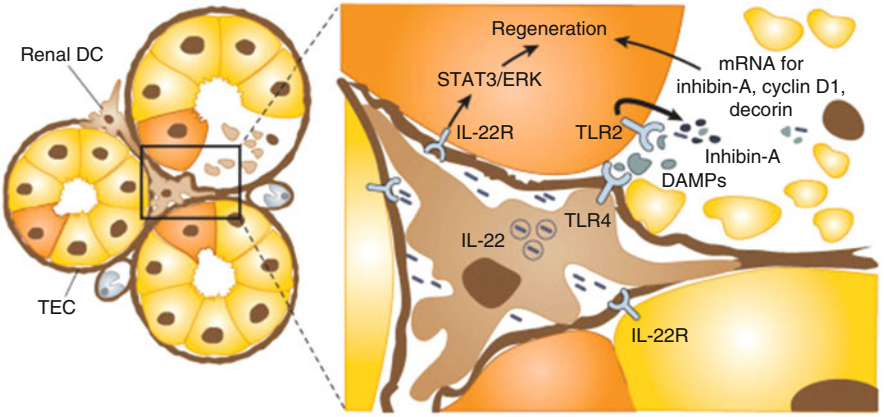

Fig. 4 Regenerative mechanism driven by toll-like receptors on renal progenitors and dendritic cells. Acute kidney injury generally causes apoptosis of tubular epithelial cells (TECs, yellow). TEC death, in turn, leads to the relief of damage-associated molecular patterns (DAMPs) into the extracellular space, where they can activate toll-like receptors (TLRs) on contiguous cells that survive the triggering insult. Renal tubular progenitors (orange) have a high capacity to survive injuries and can drive regeneration by TLR2 activation at their surface leading to the release of inhibin-A, cyclin D1, and decorin. Secondly, DAMPs capable of agonistic activity on TLR4 on the membrane of renal dendritic cells (DCs) in the interstitial compartment could induce the secretion of interleukin-22 (IL-22), which enhances tubular regeneration via the IL-22R/STAT3/ERK signaling pathway. Reprinted by permission from: Elsevier Inc.: Elsevier Inc., Kidney International, 2013 Mar;83(3):351–353. "What can tubular progenitor cultures teach us about kidney regeneration?", P. Romagnani, HJ. Anders. © 2013 International Society of Nephrology

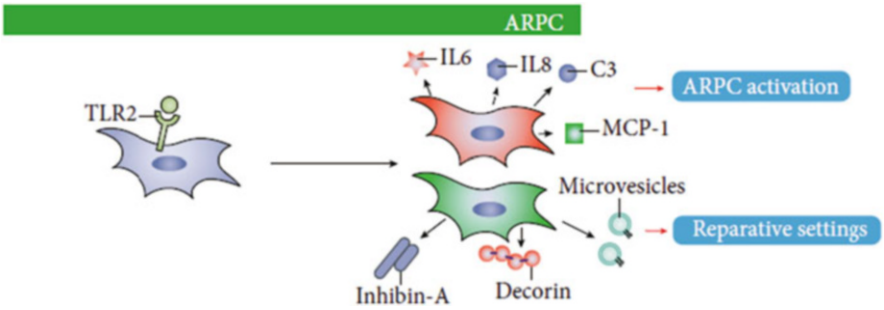

Fig. 5 Among TLRs, TLR2 is strongly upregulated in ARPCs, and it is principally involved in reparative properties of ARPCs. TLR2 is responsible for the secretion of several reparative cytokines and chemokines, including IL-6, IL-8, C3, MCP-1, inhibin-A, and decorin.: Hindawi: Hindawi Stem Cells International, 2019 Volume 2019, Article ID 6795845, 12 pages. "Role of Toll-Like Receptors in Actuating Stem/Progenitor Cell Repair Mechanisms: Different Functions in Different Cells," Fabio Sallustio et al.© 2019 Stem Cell International

Together, the Leemans and our group's findings support the use of ARPCs in the treatment of renal failure. The efficiency of TLR in sensing an injury and establishing the action and reparative operation of stem/progenitor cells, however, depends on the conditions in which cells are located.

Moreover, Sallustio's research group shows that ARPCs can preserve endothelial phenotype by preventing the development of the LPS-induced endothelial-to-mesenchymal transition (EndMT) process. Endothelial dysfunction is an indicator of kidney damage after LPS injection (Sallustio et al. 2019). Endothelial cells (ECs), via the EndMT, contribute to the development of the fibrosis. ARPCs stabilized the EC proliferation rate and inverted the LPS-induced EndMT. Sallustio's group identified the secretion of CXCL6, SAA4, and BPIFA2 antiseptic peptides as the principal mechanism that can counteract the effect of LPS in our model (Sallustio et al. 2019).

The LPS-induced ARPC activation molecular mechanism and whether the distinct activation in these cells could be determined by a difference in TLR4 (i.e., the LPS receptor) expression in ARPCs, ECs, and RPTECs were also investigated. When tested, however, no difference between these cells in receptor expression was found, indicating that the difference in LPS response leading to antifibrotic ARPC effects could be due to the activation of unique signaling pathways (Sallustio et al. 2019) (Fig. 5).

Whether the TLR2, expressed on ARPCs, is involved in the LPS-induced activation of ARPCs along with TLR4 was evaluated. The LPS-induced EndMT process was not reversed by ARPCs pre-treated for anti-TLR4, confirming the role of TLR4 in sensing the LPS and triggering the downstream pathway. Moreover, the blocking of TLR2 did not result in any major functional impact on EndMT regulation by ARPCs.

The activation of TLR4 effectively results in the activation of two different intracellular pathways: the MyD88-dependent and MyD88-independent pathways (Zhao et al. 2014). The MyD88-independent pathway leads, in particular, to the phosphorylation of IRF3 upon activation of TIR-domain-containing adaptor-inducing IFN-b (TRIF). IRF3 activation causes the suppression and promotion of anti-inflammatory or immunoregulatory cytokines by pro-inflammatory cytokines (Esplin et al. 2011). Sallustio's group therefore investigated the intracellular pathways involved in LPS activation of ARPC, which confers antifibrotic effects on them. Interestingly, after LPS stimulation, ARPCs can only activate the MyD88-independent pathway (via IRF3 phosphorylation and increased TRIF expression); this can explain their specific protective effects on the endothelial compartment. Moreover, the MyD88-dependent pathway is activated following LPS stimulation exclusively in ECs.

These results are supported by previous studies reporting that the major players involved in eliciting the functional effects of LPS within ECs are activated through the MyD88-dependent pathway, in particular by (PI3K)/Akt signaling, which regulates the balance between cell viability and inflammation (Song et al. 2002; Wardle 2007; Shi et al. 2013; Liu et al. 2015).

Such data open new perspectives on the treatment of both sepsis- and endotoxemia-induced AKI, suggesting an underestimated role of ARPCs in preventing endothelial dysfunction and novel strategies to protect the endothelial compartment and promote kidney repair.

In addition, recently, Sallustio's research group has demonstrated that ARPCs have also immunomodulatory capacity when triggered for the TLR2. Indeed, they can modulate T regulatory cells and double-negative T cells. They co-cultured ARPCs activated by triggering Toll-like Receptor 2 (TLR2) with human peripheral blood mononuclear cells for 5 days and 15 days and studied their immunomodulatory capacity on T cell subpopulations. The researchers found that activated-ARPCs were able to decrease T cell proliferation but did not affect $CD8^+$and $CD4^+$ T cells. Instead, Tregs and $CD3^+$ $CD4^-$ $CD8^-$ double-negative (DN) T cells decreased after 5 days and increased after 15 days of co-culture (Curci et al. 2020). In addition, they found that PAI1, MCP1, GM-CSF, and CXCL1 were significantly expressed by TLR2-activated ARPCs alone and were upregulated in T cells co-cultured with activated ARPCs.

ARPC immunomodulatory effect is considerable only when triggered by TLR2 agonists such as LTA that are a major constituent of the cell wall of gram-positive bacteria and are important for stimulating innate immune responses to gram-positive bacteria (Sangiorgi and Panepucci 2016). This is something similar to what happens with the MSC, whose immunosuppressive capacity is not constitutive but regulated by inflammatory microenvironment: the quantities and types of inflammatory chemokines differ considerably throughout the beginning and progression of inflammatory diseases and therefore critically affect the triggering of immunoregulation by MSCs, thus controlling ultimately the immunoregulatory effects of these cells (Medzhitov 2001; Kopp and Medzhitov 2003).

If ARPCs perceived the inflammation by means of the LTA binding on TLR2 (Tsan and Gao 2004; Taylor et al. 2007), then the Tregs generation was inhibited both in the short term (5 days) and the long term (15 days). Instead, if ARPCs were not activated by LTA (the TLR2 agonist), they can inhibit Tregs anyway, even if to a lesser extent, in the short term; in contrast, they increased Tregs generation in the long term.

This type of trend is typical of the physiological response to tissue damage. Therefore, ARPCs immunomodulatory properties in response to an inflammatory environment are important since they can lead to regulation of Tregs and DN T cells, which are involved in the balance between immune tolerance and autoimmunity. Considering that many renal diseases are characterized by inflammatory infiltrating T cells, which are mostly DN T cells, further investigations would be useful to more extensively study the contribution of ARPCs in modulating immune system during acute and chronic kidney injury (Curci et al. 2020).

7 TLRs and Placental Stem Cells

In perinatal tissues, and particularly in the placenta, toll-like receptors are also broadly expressed. The presence of TLR on trophoblasts, decidual cells, and amniotic epithelium at the maternal–fetal interface has been measured and related to specific functions (Mitsunari et al. 2006; Koga and Mor 2010).

In a temporal and spatial way, the representation of different TLR types is defined. During the first trimester, for example, TLR6 is not expressed, whereas a late gestational fetus has been shown to be positive for its expression (Abrahams et al. 2004). Villous cytotrophoblasts and extravillous trophoblasts constitutively express TLR2 and TLR4, but not syncytiotrophoblasts (which will form the outer trophoblast layer). Such temporal expression enables a punctual response to microbial contamination that may occur during the 9 months of human pregnancy to be carried out by placental tissues of fetal origin (such as the amniotic membrane). In comparison with fetal tissue, very little is known about the expression of maternal deciduous TLRs.

Recent studies have shown that amniotic epithelial cells (hAEC) express TLR4, indicating their main role in the preservation of amniotic fluid sterility (Ma et al. 2006c). Interestingly, in amniotic fluid, soluble TLR2 forms were identified, interfering with the binding of the respective ligand to TLR2 and downregulating the inflammatory response of the host to bacteria. All in all, these pieces of evidence underline the significance of the TLR system as a sentinel for a wide variety of pathogens that could cause the amniotic fluid inflammatory response (Koga and Mor 2010).

As an important therapeutic strategy in regenerative medicine, placenta-derived stem cells have been proposed as an important treatment approach due to their easy isolation, cellular multipotency, low immune response, and immunomodulatory capacity, as well as the lack of ethical problems (Parolini et al. 2008; Gramignoli 2016). Placental cells of fetal origin have generally been classified into four populations: amnion membrane-isolated hAEC and amniotic mesenchymal stromal cells (hAMSC), human chorionic mesenchymal stromal cells (hCMSC), and human chorionic trophoblastic cells (hCTC) (Parolini et al. 2008; Hass et al. 2011).

Recently, the existence of TLR4 in hAMSC and its role in premature membrane breakup in response to fetal fibronectin has been demonstrated. The expression of TLRs in hAMSC was shown in another interesting study, with a particular interest in immune surveillance during infection and in pro-inflammatory response to activation of TLR2 and TLR6 (Sato et al. 2016). These initial findings indicate hAMSC and its function in pregnancy immunomodulation.

Several TLR family members (TLR5 and TLR6/2 are expressed and functionally active) are expressed by amniotic epithelial cells and respond to multiple TLR ligands (Gillaux et al. 2011).

HAECs produce and secrete pro-inflammatory cytokines, metalloproteinases (MMP-9) after stimulation with TLR6/2 and TRL5 agonists, and activate the NF-κB signaling pathway (Hass et al. 2011; Gillaux et al. 2011; Gramignoli 2016). TLR4 induction, on the other hand, does not result in an inflammatory response but triggers apoptotic processes, which can lead to premature membrane rupture (Gillaux et al. 2011) (Fig. 6). It has been suggested that the response of hAEC in the presence of intrauterine infection depends on the activation of TLR (Gillaux et al. 2011). Further studies are required, however, to determine the role of hAEC in the immune response and their significance as sentinels for a wide variety of pathogens.

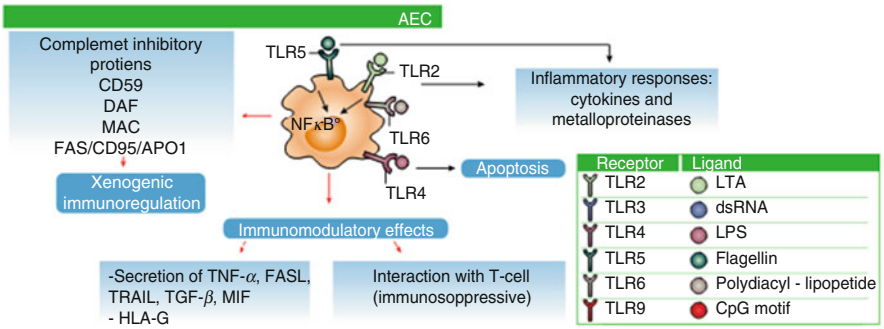

Fig. 6 AEC expresses various TLR family members and responds to multiple TLR ligands that heavily influence the cell behavior. Reprinted and adapted by permission from: Hindawi: Hindawi Stem Cells International, 2019 Volume 2019, Article ID 6795845, 12 pages. "Role of Toll-Like Receptors in Actuating Stem/Progenitor Cell Repair Mechanisms: Different Functions in Different Cells," Fabio Sallustio et al.© 2019 Stem Cell International

HAECs have been reported to have immunomodulatory and anti-inflammatory properties, similar to MSCs, which after an insult may be of particular benefit in regenerative medicine (Skvorak et al. 2013a; Strom et al. 2013). Important effects of xenogeneic immunoregulation (Strom et al. 2013) have been seen in the expression of complement inhibitory proteins, CD59 antigen (decay-accelerating factor), membrane attack complex, and Fas antigen/CD95/APO1. Taken together, the expression and immunomodulatory properties of TLR in hAEC indicate that these cells have the potential to correct inflammatory diseases, and this approach has therefore been considered to be the first allogeneic cell therapy that does not need help for immunosuppression therapy (Hass et al. 2011; Strom and Gramignoli 2016) (Fig. 6). The positive findings obtained in recent preclinical studies on liver disease treatment (Skvorak et al. 2013a, b; Strom et al. 2013; Gramignoli 2016) have indicated the use of hAEC in a variety of acute and chronic disorders, not just liver-related ones.

8 TLRs and Intestinal Stem Cells

At the base of the crypt area of the intestinal epithelium, intestinal stem cells (ISCs) have both the capacity for self-renewal and the ability to differentiate into various types of cells such as Paneth cells, absorptive enterocytes, goblet cells, and enteroendocrine lineages (Chen et al. 2018).

In normal conditions and in response to injury, the regulatory mechanisms that regulate stem cell proliferation are only beginning to be explored. When ISCs replicate by overcoming the usual controls of cell division, they can result in cancer; hence, maintaining a balance between self-renewal and differentiation of ISCs is a hallmark of an intestinal functional niche. An increasing number of signaling

pathways can play important roles in regulating stem cell proliferation, including Wnt, BMP, Hedgehog, and Notch (Umar 2010).

Factors controlling the proliferation and apoptosis of ISCs are still not well known to date. Since ISCs are in contact with microbial ligands, a critical role could be played by immune receptors such as toll-like receptors (Neal et al. 2012). In particular, over-stressed TLR-4 repressed ISC proliferation during enterocolitis and induced apoptosis through upregulated apoptosis modulator p53 (PUMA). For this condition, the TLR4-PUMA axis may therefore be a therapeutic target (Neal et al. 2012). It was also found that TLR-2, TLR-4, and TLR-5 are expressed by putative human colonic stem cells. TLR-4 mediated Wnt signaling in these cells that controls the role of stem cells (Brown et al. 2014).

The effects of microbiota and TLR signaling on ISCs that may affect the regeneration and defense of the damaged mucosal barrier (Kawai and Akira 2007; Hou et al. 2018) are, however, little understood. The protective effect of Lactobacillus reuteri D8 on the integrity of the intestinal mucosa (Hou et al. 2018) has been seen in recent studies. In particular, this lactobacillus triggered the release of IL-22 by lamina propria lymphocytes, which induced ISC proliferation and encouraged the recovery of the intestinal epithelium after damage caused by TNF-alpha (Hou et al. 2018). In addition, while the cross-talk between the whole microbiota and the signaling of TLR/MyD88 on the ISCs is not yet well elucidated, it has been shown that MyD88-/-mice are more prone to colitis caused by acute dextran sodium sulfate (DSS-) and develop a more serious disease (Araki et al. 2005).

In addition, in the crypt-specific core microbiota, LPS (the TLR-4 agonist) is found and can control intestinal epithelium proliferation by inducing necroptosis death of stem cells and enhancing cell differentiation towards the goblet cell lineage. In addition, low and non-toxic LPS concentrations improve tissue damage tolerance after transplantation, increasing parenchymal regeneration. Therefore, after intestinal transplantation, TLR-4 could have a great effect on the regulation of stem cell activity (Naito et al. 2017).

In response to hypoxic stimulus, causing ISC proliferation, TLR4 signaling may also be implicated. Before intestinal insults, such as intestinal transplantation, hypoxic preconditioning can boost ISC activation. The TLR pathway may therefore be a therapeutic target likely to enhance graft survival in the small intestine (Chen et al. 2018).

9 TLRs Differentially Expressed by Different Stem Cells: Implications for Stem Cell-Based Therapy

Many distinct TLR functions emerge in SC from the analyzed data, pointing out that SC can have different roles depending on the context and the type of ligands they can recognize. In addition, we addressed the role of TLR in the SC reaction to a particular tissue damage and in the reparative processes and how the discovery of molecules mediating the differential function of TLR signaling could be decisive for the development of new therapeutic strategies. These factors include new insights for

stem cell-based therapy: it may be feasible to pretreat the SC with a particular TLR ligand. It may make a kind of commitment, for instance, to cytokine production or other differentiation. On the other hand, data on the response of TLR-stimulated cells provide an additional factor to be taken into account in order to achieve success with stem cell therapy.

Results on TLRs in immunomodulatory properties of MSC pose a significant warning for the use of MSCs in clinical application. In fact, if, on the one hand, the immunosuppressive ability of MSCs is a key factor for their therapeutic use (Lombardo et al. 2009), on the other hand, if inflammation is present, the advantage of the use of MSCs may be lost, and MSCs may lose their immunosuppressive roles involved in the eradication of pathogenic agents and in the regulation of the allogeneic reaction (Raicevic et al. 2010). In this scenario, when immunosuppressive properties are required, WJ-MSCs can represent the most attractive tool (Shirjang et al. 2017). While silencing certain TLRs may be a way of optimizing the immunosuppressive impact of MSCs, before paving the way for new immune therapies, the molecular mechanisms and effects of TLR-priming MSCs still need to be thoroughly understood.

In addition, we can also argue that, depending not only on what stimuli it perceives, but also on the form of stem/progenitor cells in which the TLR is expressed and the specificity of the signaling that it can activate, the same TLR could have different effects. For example, the triggering of TLR4 can cause very different effects in different stem/progenitor cells: it induces an increase in immunomodulation in MSCs; it induces hematopoietic cell growth in HSC, proliferation in NPC, and apoptosis in hAEC (Fig. 1). On the contrary, TLR2 appears to have more similar effects: it induces differentiation in MSC, HSC, NPC, and ARPC and induces inflammatory response proliferation and activation in HSC, ARPC, and hAEC (Fig. 1). On the other hand, in some SCs, some TLRs can be expressed specifically and can have definite functions that depend on the affinity of the ligand. TLR5 and TLR6, as previously mentioned, can bind flagellin and diacylated ligands, respectively, and are expressed in hAECs that induce pro-inflammatory cytokines and metalloproteinases (MMP-9) when enabled (Fig. 1).

10 Conclusion

The function and significance of TLRs in sensing an injury by stem/progenitor cells clearly emerges, taking into account the available studies on TLRs in SCs. Depending on the conditions under which the cells are located, TLRs may determine their actions and reparative activity in certain SC types. It could therefore be conceivable that SCs used in therapy could be exposed to TLR ligands, which could in vivo modulate their therapeutic potential (Delarosa et al. 2009). TLR agonists are being used as infectious disease or cancer vaccine adjuvants and as tumor therapeutics. TLR antibodies and TLR signaling pathway inhibitors also have important potential as therapeutics for inflammatory disorders (Dunne et al. 2011).

Some TLR agonists in various diseases have demonstrated therapeutic potential in recent years. As a topical therapy for skin cancer, imiquimod is a TLR7 agonist with demonstrated antitumor activity. Currently approved by the US FDA, several phase 2 clinical trials have shown its protection and effectiveness in other cancer forms, such as in situ carcinoma of the bladder (Donin et al. 2017), intraepithelial neoplasia of the cervix (Koeneman et al. 2017), or cutaneous metastases of breast cancer (Salazar et al. 2017).

TLR9 agonists have recently been suggested as a therapeutic alternative for glioblastoma (CpG oligonucleotide) (Carpentier and Lambert 2017) or asthma (Casale et al. 2015), but there was no additional benefit for patients in phase 2 clinical studies.

In this context, to modulate SC proliferation, survival, migration, and differentiation in the pathological setting, we need to better understand the mechanisms of action of TLRs on SC and learn how to regulate these receptors and their downstream pathways in a very specific way. In this way, cell therapy may be strengthened and made safer in the future.

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TLR Signaling in Brain Immunity

Mengdi Guo, Yun Xu, and Cun-Jin Zhang

Contents

Abstract

Toll-like receptors (TLRs) comprise a group of transmembrane proteins with crucial roles in pathogen recognition, immune responses, and signal transduction. This family represented the first line of immune homeostasis in an evolutionarily conserved manner. Extensive researches in the past two decades had emphasized their structural and functional characteristics under both healthy and pathological conditions. In this review, we summarized the current understanding of TLR signaling in the central nervous system (CNS), which had been viewed as a

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_542

previously “immune-privileged” but now “immune-specialized” area, with major implications for further investigation of pathological nature as well as potential therapeutic manipulation of TLR signaling in various neurological disorders.

Keywords

Central nervous system · Immune signaling pathways · Neurological disorders · Toll-like receptor

Abbreviations

AD	Alzheimer’s disease
Akt	Protein kinase B (PKB)
APC	Antigen-presenting cell
ASC	Adapter protein apoptosis associated speck-like protein containing a CARD
Aβ	Aggregated β amyloid
BBB	Blood–brain barrier
CAM	CNS-associated macrophage
cFN	Cellular fibronectin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CVD	Cerebral vessel disease
CXCL9	C-X-C motif chemokine ligand 9
EAE	Experimental autoimmune encephalomyelitis
ER	Endoplasmic reticulum
FADD	FAS-associated death domain
HSP	Heat shock protein
HSV	Herpes simplex virus
ICH	Intracerebral hemorrhage
IKK-γ	Inhibitor of nuclear factor kappa-B kinase subunit γ
IKK-α	Inhibitor of nuclear factor kappa-B kinase subunit α
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit β
IRAK1	Interleukin 1 receptor associated kinase 1
IRF	Interferon regulatory factor
IRI	Ischemia–reperfusion injury
JNK	The c-Jun N-terminal kinase
LBP	LPS binding protein
LPS	Lipopolysaccharide
MAL	MyD88-adaptor-like
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MKK	Mitogen-activated protein kinase kinase
MS	Multiple sclerosis

mTORC	The mechanistic target of rapamycin
MyD88	Myeloid differentiation factor 88
NEMO	NF-kB essential modulator
NFT	Neurofibrillary tangle
NLRC4	NLR family CARD domain containing 4
NLRP1	NLR family pyrin domain containing 1
NLRP2	NLR family pyrin domain containing 2
NLRP3	NLR family pyrin domain containing 3
NPC	Neural progenitor cell
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood monocyte
PI3K	PI3K/Akt/mTORC1
PRR	Pattern recognition receptor
RIP1	Receptor-interacting protein 1
SARM	Sterile α- and armadillo-motif containing protein
SMOC	Supramolecular organizing center
TAK1	TGF-β-activated kinase 1
TBK1	TANK-binding kinase 1
TGFβ	Transforming growth factor-β
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TRAF6	TNF receptor associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing interferon β
VEGF	Vascular endothelial cell growth factor
VSV	Vesicular stomatitis virus

1 Introduction

Innate immunity constitutes a first-line barrier of immunological assault in nearly all organisms. Host defense mechanism against pathogenic insults can be rapidly mobilized following the recognition of pathogen-associated molecular patterns (PAMPs), largely relying on the pattern recognition receptors (PRRs) located in both the intracellular and extracellular milieus (Fitzgerald and Kagan 2020). Among the best characterized are toll like receptors (TLRs), a class of highly conserved type I transmembrane receptors with 13 distinct subtypes described in mammalian and 10 functional members in human (TLR1–TLR10) (Nishimura and Naito 2005). Each receptor shares a comparable structure organization with a ligands-recognition ectodomain and a cytoplasmic TIR domain, while cognate ligands such as microbe components or endogenous molecules can directly initiate TLR dimers formation and subsequent signaling cascades through MyD88/TRIF (MyD88, Myeloid differentiation factor 88; TRIF, TIR-domain-containing adaptor inducing interferon)

dependent pathways (Brown et al. 2011). Accumulating researches have demonstrated the biological functions, distribution profiles, and signal transduction network of TLRs, thus extending our knowledge about pathological mechanisms amenable to next-generation therapeutics.

Considering the limited regenerative capacity and physiological "segregation" from periphery immune system, mammal brain should employ specialized immune strategies to manage immune surveillance and responses to pathogenic or traumatic stimulation (Rivest 2009). Broad expression of immune molecules including TLRs has been widely confirmed not only in brain intrinsic cell types, but also in invading leukocytes under pathological status (Greenhalgh et al. 2020). To date, the exact roles of TLRs in brain remain elusive that they may exert either protective or detrimental effects, largely depending on the specific context of neuropathology (Gong et al. 2020; Hammond et al. 2019). Moreover, beyond their classical roles as potent executioners of neuroinflammation, TLRs may also perform non-immune cellular process in brain including neurogenesis, neural progenitor cell (NPC) stemness, memory maintenance, and neurotransmission, which have been fully reviewed elsewhere (Alvarado and Lathia 2016; Garcia Bueno et al. 2016; Ii Timberlake and Dwivedi 2019). It remains a great field of interest in neuroimmunology studies, although TLRs associated inflammatory process has received much attention. Here, we briefly describe the established mechanisms of TLRs signaling in brain immune homeostasis, along with associated neuropathological changes involved in diseases onset and progression.

2 Biology of TLRs in Brain

2.1 Localization of TLRs in Brain

In 2002, Bsibsi et al. reported the first documentation of TLRs distribution profiles in human brains. Cadaveric specimens from control donors and multiple sclerosis patients were examined by semi-quantitative RT-PCR and immunohistochemical analysis, showing the expression of TLRs in normal and inflamed glial cells (Bsibsi et al. 2002). Follow-up studies further defined that TLRs expressed in microglia, CNS-associated macrophages (CAMs), astrocytes, oligodendrocytes, neurons and neural stem cells, although basal enrichment and reactive changes of individual TLR might be cell type and anatomical localization dependent (Jack et al. 2005; Lafon et al. 2006). Tissues at CNS interface such as choroid plexus constitutively expressed TLR1–4 and were thought to be involved in the crosstalk between CNS parenchyma and periphery (Mottahedin et al. 2019). Recent studies proved a TLR2-mediated leukocyte trafficking route in the developing mouse brain, thus revealing the previously unrecognized chemotaxis signature and cytoskeleton remodeling profiles during systematic inflammation induced CNS pathologies (Mottahedin et al. 2019; Stridh et al. 2013). In the brain parenchyma, almost all of the currently identified TLRs could be detected at the mRNA level, with prominent expression of TLR2 and relatively high expression of TLR3, 4, and 6, as well as barely detectable

Table 1 TLR expression in brain intrinsic cell types

Cell type	Species	TLRs
Microglia	Human	TLR1–9
	Mouse	TLR1–9
Astrocyte	Human	TLR 1–5, TLR9
	Mouse	TLR 1–9
Neuron	Human	TLR3
	Mouse	TLR2–4, TLR6–9, TLR11–13
Oligodendrocyte	Human	TLR2
	Mouse	TLR2–4

TLR5, 7, 8, and 9 during homeostasis (Mishra et al. 2006). However, not all of them could be detected at the protein level, partly attributed to the poor specificity and reactivity of antibody and potential post-transcriptional regulatory mechanisms (Lee et al. 2013; Lehnardt 2010). Served as one of the earliest determinants of microbial threats, initial TLR-mediated activation also dynamically regulated their own expression levels under neuropathological conditions – TLR2 and 3 might comply with self-amplified cascades, but TLR4 was subject to negative feedback in cognate ligands stimulated microglia. Astrocytes could upregulate TLR2, 3, and 4 following TLR3 ligation, while ependymal cells and neurofilaments displayed substantial upregulation of TLR7 and 8 in parasite-infected cerebellar and periventricular white matter (Jack et al. 2005; Mishra et al. 2006).

Brain parenchyma intrinsic TLRs exhibited characteristic expression profiles tailored to different cell types (summarized in Table 1). As the only resident immune cells of CNS, microglia constitutively expressed mRNA for TLR1–9, priming for the initiation of innate immune responses and subsequent restoration of brain integrity. Immunostaining of primary cultured microglia showed cell-surface expression of TLR2 and interestingly, intracellular vesicles localized TLR3 and 4. Astrocytes robustly expressed TLR3 but low-level TLR 1, 4, 5, and 9, and virtually absent TLR 2, 6, 7, 8, and 10 in vivo, with cultured astrocytes exclusively expressed TLR3 and 4 on the cellular membrane surface (Bsibsi et al. 2002; Jack et al. 2005). These surprising observations might relate to the differences in the functional properties between microglia and astrocytes (Trudler et al. 2010). Emerging evidences have suggested that non-immune brain cells such as oligodendrocyte and neuronal lineage cells could express TLRs in a relatively restricted manner. TLR2 was documented in oligodendrocyte precursor cells in normal and MS lesion areas. TLR2, 3, and 4 were also detectable during in vitro oligodendrocytes culture, although their functions remained controversial (Lee et al. 2013; Setzu et al. 2006; Sloane et al. 2010). Neuronal TLR2 and 4 could render brain vulnerable to energy deprivation and IFN stimulation-induced cell death (Tang et al. 2007), while TLR3 and 8 enabled neuroinflammation to take account of the neurodegenerative disorders' diversity (Hammond et al. 2019; Ma et al. 2006; Préhaud et al. 2005).

Beside the brain resident cell types, invasive peripheral cells including granulocytes, lymphocytes, monocytes/macrophages, dendritic cells, and even platelets, also contributed to the central TLRs pool through the ruptured blood–

brain barrier (BBB). Recent studies have confirmed TLR4 of platelets and neutrophils as mediators of neutrophil extracellular traps (NETs) formation and subsequently aggravated inflammation in ischemic stroke (S. Kim et al. 2019; Peña-Martínez et al. 2019). TLR2 in CD4+ T cells promoted Th17 responses and that loss of TLR2 dramatically ameliorated the prognosis of EAE (EAE, Experimental Autoimmune Encephalomyelitis) (Nyirenda et al. 2011; Reynolds et al. 2010). In systematic inflammation associated CNS disorders like ammonia-induced brain edema, macrophage and T lymphocyte derived TLR9 were required for the production of proinflammatory cytokine (Manakkat Vijay et al. 2019). Peripheral cells entering the brain parenchyma may also directly affect the functions of brain intrinsic cells and suggested a collaborative network of peripheral-resident cell communications after immunological insults (Greenhalgh et al. 2020).

2.2 Ligand-Recognition Process of TLRs

TLR family members were divided into two categories in terms of their subcellular localization – while plasma membrane localized TLRs (TLR1, 2, 4, 5, and 6) primarily recognized microbial cell surface components, endosomal TLRs (TLR3, 7, 8, 9, and 13) focused on the identification of nucleotide (McGettrick and O'Neill 2010). Mammal brain basically followed the same principles with peripheral immune system in the case of ligands recognition. Specifically, TLR4 detected lipopolysaccharide (LPS), an essential lipid structure of gram-negative bacteria (Poltorak et al. 1998); TLR2/1 and TL2/6 heterodimers identified bacterial lipoproteins (Kang et al. 2009; Ozinsky et al. 2000); TLR5 was generally known as a sensor of protein flagellin (Gewirtz et al. 2001); and endosomal TLR3, TLR7/8, TLR9, and TLR13 recognized dsRNA, ssRNA, unmethylated CpG containing ssDNA and bacterial ribosomal RNA, respectively (Alexopoulou et al. 2001; Diebold et al. 2004) (shown in Table 2). In mammals, TLRs directly interacted

Table 2 Exogenous and endogenous TLR ligands

TLRs	Exogenous ligands	Endogenous ligands
TLR2/1, TLR2/6	Lipoprotein	HMGB1, HSPs, SAA, Aβ, β-defensin 3, histone, eosinophil-derived neurotoxin
TLR3	dsRNA	mRNA
TLR4	LPS	HMGB1, HSPs, S100s, heparin sulfate, fibrinogen, neutrophil elastase, histone, SAA, β-defensin 2, oxidized LDL
TLR5	Flagellin	
TLR7	ssRNA	microRNAs, IgG–ribonucleoprotein complex
TLR8	ssRNA	
TLR9	CpG DNA	mtDNA, HMGB1, IgG–chromatin complex
TLR11/12	Profilin	
TLR13	Ribosomal RNA	

with cognate microbial products through dimerization of the leucine-rich-repeat-containing ectodomains. These ectodomains could either be homodimers (TLR3, 4, 5, 7, 8 and 9) or heterodimers (TLR2/1 and TLR2/6), which forced the intracellular TIR domains to dimerize and elicited subsequent recruitment of intracellular adaptor proteins (Kang and Lee 2011). Damage-associated molecular patterns (DAMPs) liberated under cellular stress were also identified as endogenous ligands of TLRs (see Table 2 for details). The engagement of (HSP) family members, S100 family members (Vogl et al. 2007), high mobility group box 1 (Laird et al. 2014), fibrinogen (Smiley et al. 2001), serum amyloid A (He et al. 2009), mRNA (Karikó et al. 2004), and mitochondrial DNA (Shintani et al. 2013) have been demonstrated to exacerbate inflammatory responses without evidence of an infectious etiology, although the controversial functions of certain TLR in these sterile pathologies remain to be investigated further. Besides, it is noteworthy that a large number of these experiments were carried out with recombinant DAMPs derived from *Escherichia coli*, indicating that the effects of microbial contamination cannot be entirely excluded for the activation of TLRs (Gong et al. 2020).

Several synergistic mechanisms were responsible for the ligand-recognition process of TLRs in multiple cell types (Kang et al. 2009). One of them was the unique biosynthetic trafficking pathway. Unlike plasma membrane localized TLRs, which were synthesized and transported via the canonical secretory pathways, intracellular compartmentalization of endosomal TLRs required several dedicated trafficking chaperones to reach their ultimate destination. The polytopic membrane protein UNC93B1 physically interacted with TLR3, 7, and 9 in the endoplasmic reticulum (ER) and facilitated their proper folding, cleavage and delivery to the endolysosomes (Kim et al. 2008; Pelka et al. 2018). Further investigations provided new mechanisms that Unc93B1 interactions mediated the internalization of TLR7 by recruiting syntenin-1 and restricted the release of TLR9, a process essential for CpG DNA binding and downstream signaling transduction (Majer et al. 2019a, 2019b). As for the CNS, Unc93B1 was a pivotal switch for DAMPs recognition by transforming downstream signaling from stress tolerance to sterile inflammation in neurons, revealing an alternative role of TLR9 in self-original DNA identification (Shintani et al. 2013). Studies of two other ER-localized proteins, gp96 and CNPY3 also demonstrated the importance of protein folding in the ligand-recognition process of TLRs, with the only exception of TLR3 (Liu et al. 2010; Takahashi et al. 2007). Moreover, the LPS binding protein (LBP) promoted the transferring of a single LPS molecule to TLR4-MD2 complex by CD14, thereby allowing the recognition of LPS to occur at picomolar concentrations (Akashi et al. 2000).

2.3 TLR Signaling Transduction

Dimerization of the cytosolic TIR (Toll/interleukin-1 receptor) domains following ligands-TLRs interactions resulted in the activation of signal cascades, which eventually initiated diverse cellular responses including inflammatory gene transcription, oxidative stress, metabolic reprogramming, and cytoskeleton remodeling (Fitzgerald

and Kagan 2020). Numerous studies have confirmed TLR signaling as the vital mediator of brain immunity, although landmark studies revealing the universal mechanisms were predominantly conducted in peripheral immune cells (Henry et al. 2008; Olson and Miller 2004; Tang et al. 2007). Five adaptor proteins, known as myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif containing protein (SARM) were engaged in TLR signaling transduction through the assembly of supramolecular organizing centers (SMOCs) called myddosome and triffosome (O'Neill and Bowie 2007). While MAL and TRAM mainly served as bridging adaptors, MyD88 and TRIF constituted the core of SMOCs and triggered two alternative signaling pathways, respectively: MyD88-dependent and MyD88-independent pathways (Fig. 1).

As the key adaptor protein for all TLRs with the exception of TLR3, MyD88 interacted with the dimerized TIR domains to recruit the serine/threonine kinase IRAK1 (interleukin 1 receptor associated kinase 1) and IRAK4 and constituted the core of the myddosome. Tight package of IRAKs within the myddosome facilitated their latent autophosphorylation ability, and drove the recruitment of the E3 ubiquitin ligase TRAF6 in conjunction with the specific ubiquitin conjugating enzyme Ubc13/Uev1A. The IRAK1/TRAF6 complex then activated TGF-β-activated kinase 1 (TAK1) and several TAK1-binding proteins which induced the phosphorylation of various kinases including IKK-α, IKK-β and NEMO/IKK-γ, MKK3/6 and MKK4/7, resulting in the activation of NF-κB, P38 MAPK, and JNK signaling as well as the subsequent gene transcription involved in inflammation, immune regulation, proliferation, and cell fate determination (O'Neill and Bowie 2007; Zheng et al. 2011). MyD88 also engaged in the nuclear translocation of IRF1, 5, and 7. It was reported that MyD88 formed a cytoplasmic complex with IRF1 in myeloid DCs (Negishi et al. 2006), while IRF5 was fully integrated into the TRAF6 signaling pathway (Takaoka et al. 2005). In the case of signaling by TLR7, 8, and 9, MyD88 also led to the activation of IRF7 through direct interaction, or IRAK4-TRAF6 and TRAF3 dependent pathways (Honda et al. 2004; Kawai et al. 2004; Oganesyan et al. 2006). These mechanisms finally arranged the host defense against virus replication via regulation of type-I IFN expression. Alternative adaptor molecules have been identified in the downstream responses emanating from TLR3 and the TLR4-induced MyD88-independent pathway. TRIF was such an exclusive adaptor with distinct protein interaction motifs that could directly or indirectly recruit the downstream proteins TRAF3/6, TBK1, and RIP1. TRAF3 was crucial for both MyD88 and TRIF signaling to induce the transcription of IFN-β following the activation of TLR3 and 4 but not TLR2, 7, and 9, suggesting the evolutionarily diverged roles of TLR3 and 4 from other TLRs (Doyle et al. 2002; Häcker et al. 2006). The N-termini of TRIF was proposed to engage with TBK1, a central upstream kinase for IRF3 activation in a NAP1 or TRAF3 mediated manner (Oganesyan et al. 2006; Sasai et al. 2005). For NF-κB activation, distinct sites at the N- and C-termini of TRIF provoked two separate pathways, which respectively engaged in the interaction with TRAF6 and RIP1/3 (Jiang et al. 2004). The absence

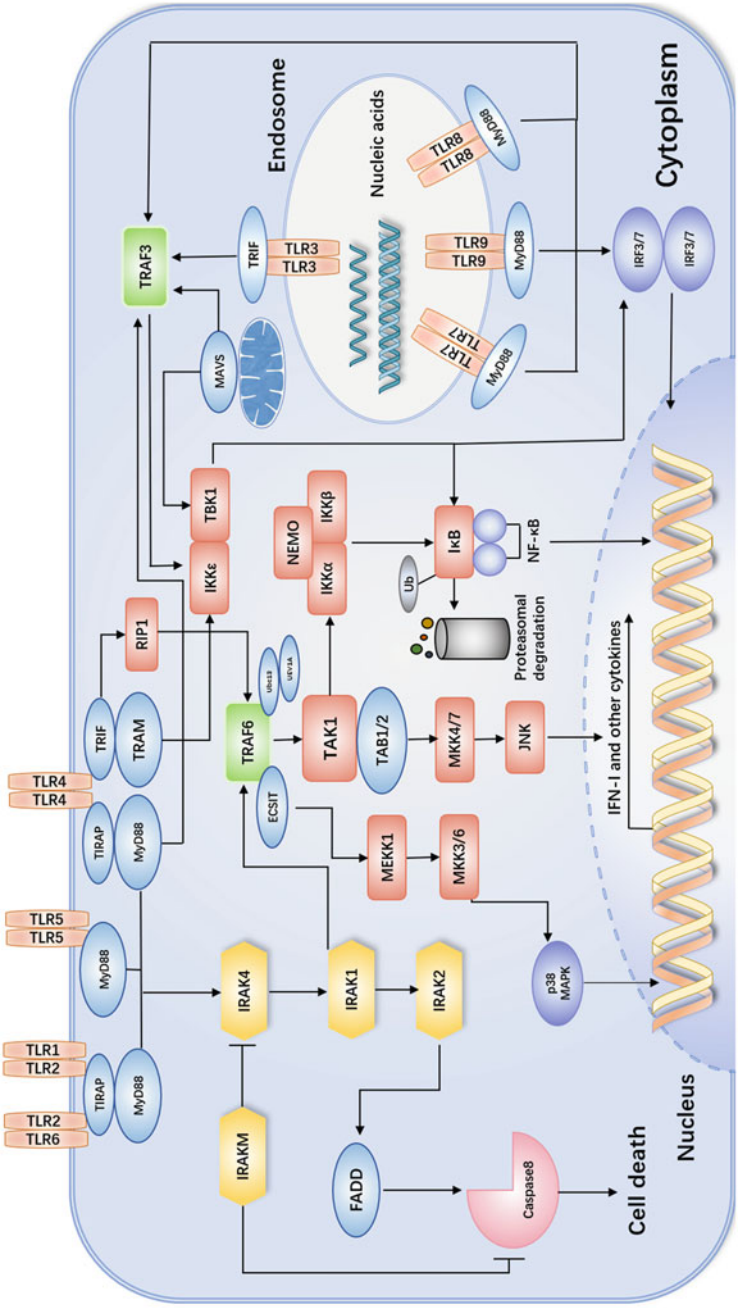

Fig. 1 Simplified schematic diagram of TLR signaling transduction

of RIP1 completely blocked the TLR3-induced NF-κB activation, while RIP3 served as a negative regulator of the TRIF–RIP1–NF-κB pathway (Cusson-Hermance et al. 2005; Meylan et al. 2004). Ligand stimulation of either TLR3 or TLR4 also led to a rapid increase of SARM expression to inhibit TRIF signaling, at least partly via preventing the recruitment of downstream effector proteins (Carty et al. 2006). In addition, TLRs activated a TRIF-RIP1-FADD (FAS-associated death domain) facilitated apoptosis pathway through caspase-8 (Dillon et al. 2014), a molecular switch recently reported to control apoptosis, necroptosis, and pyroptosis (Fritsch et al. 2019).

General principles shaping TLR signaling transduction are widely adopted but mutually distinct in the brain resident cell types, with regard to their respective expression profiles of TLR signal elements. In microglia, a broad spectrum of ligands promoted TLR signaling transduction through both MyD88 dependent and independent pathways, stimulating the phagocytosis of pathogens and aggregated proteins such as amyloid fibers, and the production of a spectrum of inflammatory factors (Olson and Miller 2004). TLR2, 3, and 4 ligation induced strong proinflammatory polarizing response in microglia, characterized by the secretion of high levels of IL-12, TNF-α, IL-6, CXCL-10, and IFN-β (Jack et al. 2005). TLR5 has been established as a modulator of microglial function involved in the PI3K/Akt/mTORC1 pathway (Ifuku et al. 2020), while TLR7 and 9 served as nuclear acid detectors to modify the extent and pattern of neuroinflammation in multiple CNS disorders (Butchi et al. 2010; Lehmann et al. 2012a; Matsuda et al. 2015), indicating their contribution to inflammatory and injurious processes in mammal brain. Astrocytes exhibited more inert properties compared with microglia following TLRs activation, with TLR3 dominating in the comprehensive neuroprotective responses through the secretion of a variety of neuroprotection factors and anti-inflammatory cytokines, such as neurotrophin-4, VEGF and TGF-β (Farina et al. 2005). Indeed, when an agonist for TLR3 (poly I:C) was added into organotypic human brain slice cultures, survival of neurons significantly improved (Bsibsi et al. 2006). Astrocytes also generated a proinflammatory environment via NF-κB, MAPK, and JAK1/STAT1 signaling pathways upon TLR4 activation (Gorina et al. 2011). Convincing evidences also revealed that neurons could express distinct functional TLRs under specific pathophysiological conditions. However, it seemed that neurons were more pronounced for the unique sensitivity to TLRs-mediated activation of JNK pathway to apoptosis, instead of the conventional NF-κB pathway to inflammation as in glial cells (Trudler et al. 2010).

Apart from the conventional intracellular signal cascades, TLR signals were also demonstrated to communicate with other innate immune pathways. The cooperation between TLRs and inflammasome has been extensively investigated in the past two decades. As a cytoplasmic protein complex processed the maturation and secretion of IL-1β and IL-18, functional inflammasome complied with a “two-hit” process (Hanamsagar et al. 2012). TLR signaling initiated transcription and translation of pro-IL-1β, inflammasome sensor NLRP3 and the noncanonical sensor caspase-11 shaped the priming stage of inflammasome activation, finally driving a form of cell death known as pyroptosis. Generally, priming for canonical caspase-1

inflammasomes is mediated by the TLR4-MyD88 axis, whereas priming of noncanonical caspase-11 upon pathogen challenges is mediated by TLR4-TRIF (Patel et al. 2017). Numerous studies have established the cooperative relationship between TLRs and inflammasome in brain disorders. In the pathogenesis of EAE, TLR stimulation led to the formation of IRAKM-caspase-8-ASC complex and the production of noncanonical NLRP3 inflammasome derived IL-1β, resulting in the continuous expansion of chronically activated microglia population (Zhang et al. 2018). NLRP3 inflammasome activated caspase-1 could directly cleave TRIF to negatively regulate TLR-4-TRIF mediated autophagy (Lai et al. 2018). In CNS non-immune cells such as human brain vascular pericytes, no canonical activation of NLRP1, NLRP2, NLRP3, or NLRC4 inflammasomes were also observed, indicating that pericytes might have an important regulatory role in neuroinflammation (Nyúl-Tóth et al. 2017). Interestingly, synergies between multiple TLRs suggested that pairwise activation of distinct TLRs modified the pattern and extent of inflammation and neurodegeneration in the brain, largely depending on the combination of the TLR family members engaged (Rosenberger et al. 2014).

3 Pathophysiological Roles of TLR Signaling in Brain Immunity

3.1 Host Defense Against Pathogen Infection

Brain established host defense mechanisms through triggering immune responses while encountering with biochemically diverse microbial molecules. Archetypical cell types of innate immunity in both brain parenchyma and peripheral immune system had been documented to exhibit dual roles in the fight against pathogen invading process. During viral infection, Wang et al. investigated the involvement of TLR3, a detector for viral double-stranded (ds)RNA on West Nile virus infection. Although enhanced viral load was found in the peripheral blood, TLR3-deficient ($TLR3^{-/-}$) mice had resolution of central nervous system dysfunction and impaired proinflammatory cytokine production compared with their littermate controls, illustrating that Tnfrsf1a signaling was responsible for the blood–brain barrier compromise upon dsRNA mediated TLR3 stimulation (Wang et al. 2004). In HSV-2 infected mice brain, it was found that TNF-α and the IFN-stimulated gene CXCL9 (C-X-C Motif Chemokine Ligand 9) were expressed during infection in either TLR2 or TLR9 dependent manner. TLR2 and TLR9 could synergistically stimulate innate antiviral responses, thereby protecting against HSV-2 replication in the brain (Sørensen et al. 2008). Contrary to the conventional wisdom that pathogen-resistance process was predominantly mediated by glial cells, one study also demonstrated an intrinsic machinery of human neurons to response to viral double-stranded RNA (Préhaud et al. 2005). TLRs are also essential for antibacterial immunity of CNS. In a mice model of *Staphylococcus aureus* craniotomy infection, TLR2 but not TLR9 was important for preventing elevated bacterial burden in the infected brain. Caspase-1 KO mice displayed reduced IL-1β release

coincident with increased *S. aureus* titers, suggestive of pathway cooperativity with TLR signaling (Aldrich et al. 2020). However, another study reported no differences in *S. aureus* titers, with a subsequent study using different *S. aureus* strains confirmed elevated bacterial loads in TLR2-deficient mice (Vidlak et al. 2011). Unlike the subtle phenotypes observed with TLR2, the TIR adaptor MyD88 was more likely to generate protective immune responses (Esen and Kielian 2006). In the face of gram-negative meningeal pathogens, proinflammatory cytokine release was significantly reduced in TLR4 mutant and MyD88-KO microglia, indicating critical roles for both MyD88 and TLR4 dependent signaling pathways in microglial responses toward gram-negative bacteria (S. Liu and Kielian 2009). In parasitic infections, a rodent model of experimental cerebral malaria reported that therapeutic inhibition of TLR8 and 9 by a synthetic antagonist of nucleotide-sensing TLRs prevented deleterious inflammatory responses (Franklin et al. 2011). Neuronal TLR11, 12, and 13 also appeared to be upregulated in a murine model of neurocysticercosis, thereby conferring neurons uncovered innate immune functions against parasites (Mishra et al. 2008). It is important to mention that the activation of TLRs and their downstream signaling pathways are dictated by the context of different pathogens, representing one of the greatest challenges in anti-microbial drug discovery.

3.2 Tissue Injury and Repair Following Sterile Neuroinflammation

Engagement of DAMPs, the endogenous TLR ligands, might contribute to exacerbated neurotoxicity, although enhanced neuroinflammation was also widely considered as a common responsive process to guarantee brain integrity. Studies of ischemia–reperfusion injury (IRI), an experimental procedure that led to vast DAMP releasing provided most of the in vivo evidence for their pathological roles as TLR agonists. An overwhelming majority of past researches focused on the delicate balance between the amplification and resolution of sterile neuroinflammation. Microglia TLR2 was significantly associated with maximal upregulation in ipsilateral hemisphere compared with TLR4 and TLR9 and exhibited detrimental effects with proinflammatory and pro-apoptotic capabilities (Lehnardt et al. 2007). Systemic administration of TLR4, 7, and 9 agonists before cerebral ischemia has been demonstrated to induce robust neuroprotection via upregulation of IFN-associated genes, which supported the conjecture that TLR reprogramming was an endogenous process capable of providing protection against subsequent TLR-mediated ischemic injury (Leung et al. 2012). In the white matter injury models, noncanonical TLR4/TRIF pathway induced persistent activation of FoxO3 downstream of AKT (Srivastava et al. 2018), adapting the persistent engagement of TLR4 that chronically disrupted the repair capacity of OPCs. Endosomal TLRs also served as regulatory nodes of cellular stress responses, which integrated cellular stress signals with inflammation, metabolism, and cell fate determination in a cell-intrinsic manner. Extracellular microRNA let-7b, a 21–22 short noncoding ssRNA derived from damaged neurons could act as an activator of TLR7 in both microglia and neurons

through downstream molecules MyD88 and IRAK4, accumulating the decay of neurons and exacerbate neuroinflammation in Alzheimer's disease (Lehmann et al. 2012b). TLR9 stimulation was reported to increase the AMP/ATP ratio and initiate AMPK signaling pathway, leading to increased hypoxia-tolerance in terminal differentiation neurons without inducing canonical inflammatory response (Shintani et al. 2013). Contrary to the traditional view, recent studies provided evidence for the requirement of proinflammatory activation in myelin debris degradation after myelin injury. MyD88-deficient microglia showed impaired expression of TNF-α after demyelinating injury in mice and zebrafish, which was essential for the myelin debris degradation within phagolysosomal and the formation of new oligodendrocytes (Cunha et al. 2020). These facts challenged the established idea that anti-inflammatory therapies delivering direct benefits to the subsides of sterile neuroinflammation, especially in the later tissue-repair phase. Given the vast diversity of DAMPs, complicated TLR signaling bias and controversial roles of inflammatory mediators in the pathological process of brain injury, the underlying pathological mechanisms behind sterile neuroinflammation should be investigated further to promote brain regeneration and favorable long-term prognosis (Gong et al. 2020).

3.3 Impact of TLRs in Shaping Adaptive Immune Responses

It has been widely accepted that efficient immune responses depended on the coordination between innate and adaptive immunity. Stimulation of TLRs not only caused changes to the activation of myeloid cells in brain immune microenvironment, but also augmented the extent and specificity of adaptive immunity (Dong and Yong 2019). Basically, ligands of TLRs such as LPS were used as immune adjuvant, with an important role of TLR4 mediated pathway independent of MyD88 in the upregulation of MHC class II molecules, and TRIF was responsible for the process via the induction of type-I IFNs (Jain and Pasare 2017). This principal mechanism was engaged in the activation of DCs, the main peripheral APC that migrated to the draining lymph nodes to prime naive T cells, which has been well established in the MS/EAE pathophysiology (Almolda et al. 2011). Microglia could also serve as antigen-presenting cells, and the activation of TLRs was shown to accelerate the endocytosis and phagocytosis of antigen, thus shaping the adaptive immune response in the CNS (Wlodarczyk et al. 2014). A recent study demonstrated that microglia were not infected by the VSV (Vesicular Stomatitis Virus) but cross-presented antigen to antiviral T cells, thus preventing the brain from nasal virus infection (Moseman et al. 2020). After undergoing adoptive transfer of MBP-specific T cells, EAE mice exhibited decreased motility in an MHC II dependent manner, suggesting that the autoreactive T cells could interact with APCs, which might include microglia (Kawakami et al. 2005). However, other studies found that microglia isolated from naive mice could not induce the proliferation of TH1 cells even after stimulation with IFN-γ and LPS, which might be due to the lower expression of MHCII, CD40, CD80, and CD86 at least partly (Mack et al.

2003). Therefore, the hypothesis that microglia were major APCs in the brain parenchyma remained a topic of debate in EAE/MS. In addition to regulating antigen presentation, costimulatory molecules essential for T cell activation were also induced by the activation of TLRs. A functionally diverse set of innate cytokines, for instance type-I IFNs and IL-1 family of cytokines, could be produced followed by the activation of TLRs in the brain immune microenvironment, and cooperate with the T cell priming cytokines to regulate the generation of reactive T cells, finally constituting an inflammatory cytokine regulatory network under neuropathological conditions (Becher et al. 2017). Further studies to explore the roles of TLR family members in brain pathogenesis could foreseeably provide more therapeutic targets for the immune-modifying management of CNS disorders.

4 Immune Function of TLRs and CNS Disorders

4.1 Cerebral Vessel Disease (CVD)

One of the key contributors of secondary brain injury after cerebral vessel disease is neuroinflammation which can be a consequence of innate immune responses. Recent studies have shown the essential role of TLRs in the pathogenesis of cerebral vessel disease. While in ischemic neurons, the activation of TLR2 and TLR4 signaling was engaged in the JNK-AP-1 pathway, promoting their demise (Tang et al. 2007). As for astrocytes, the predominantly detected TLR3 did not influence the outcome of ischemic stroke (Hyakkoku et al. 2010). However, microglia, the resident macrophages of the brain that safeguarded neuronal functions, expressed a host of TLRs which were activated to recruit neutrophils, leukocytes, and monocytes to the focal infarct by a large generation of inflammatory cytokines and chemokines. Both TLR 2 and TLR 4 could exacerbate the detrimental neuro-inflammation after ischemia (Lehnardt et al. 2007; Tang et al. 2007). It has been demonstrated by several clinical studies that increased expression of TLR2/TLR4 was linked with poor outcome as well as higher inflammatory response in patients with ischemic stroke. TLR2/TLR 4 knockout mice also exhibited smaller infarct sizes and improved neurologic test scores (Brea et al. 2011a). Besides, Brea et al. have found a correlation between TLR7 and 8 expression levels and infarct volumes in acute ischemic stroke (Brea et al. 2011b). As for intracerebral hemorrhage (ICH), TLR4 activation was also related with a robust inflammatory response and sustained functional deficits after ICH (Sansing et al. 2011).

Advances in understanding the association between the TLRs and cerebral vessel disease have facilitated a lot in the identification of several novel therapeutic targets for CVD treatment. Both TLR2 and TLR4 were regarded as potential therapeutic targets (Kilic et al. 2008) and arouse a great deal of interest. The blockade of TLR2/TLR4 or their endogenous ligands, such as cFN or HSP60, could be promising anti-inflammatory therapeutic agents targeting secondary brain injury. Ample evidence has indicated that TLR4 receptors might be viable pharmacological targets (Anttila et al. 2017). Specially, considering that diabetes not only increased the risk

of stroke but also impaired the functional recovery, the inhibition of vascular TLR 4 could provide more profound therapeutic benefits for neurobehavioral recovery in diabetes (Abdul et al. 2019). Moreover, it's interesting that TLR 4 signaling can be redirected to induce a neuroprotective response following stroke by LPS preconditioning. This redirection was initiated through suppressed NF-κB activity, enhanced IRF3 activity, and upregulated anti-inflammatory/type I IFN gene expression (Pradillo et al. 2009; Vartanian et al. 2011). Recombinant growth-arrest-specific protein 6(rGas6), another agent attenuating neurological deficits, also inhibited inflammation through TLR/TRAF/NF-κB pathway (Wu et al. 2018). Further research into the mechanisms involved in the TLRs is still needed to provide more therapeutic targets for CVD treatment.

4.2 Alzheimer's Disease (AD)

In the case of neurodegenerative diseases, pathologic engagement of TLRs stimulated by DAMPs contributed to the exacerbated inflammation responses and enhanced neuropathology. Alzheimer's disease (AD) is among the most common neurodegenerative diseases, which can be recognized with the characteristic amyloid plaques composed of abnormally aggregated β amyloid (Aβ) and neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau protein (Yang et al. 2020). Aβ plaques were surrounded and infiltrated by astrocytes and microglia in an activated state, contributing to local neuroinflammation and synapse loss (D Trudler et al. 2010). It was assumed that specific interactions of the receptor complexes of microglia, including TLR2, TLR4, and the co-receptor CD14 with fibrillary Aβ were at the basis of Aβ phagocytosis, which was dependent upon MyD88. Additionally, activation of microglia by TLR2, TLR3, TLR4, TLR7, and TLR9 ligands markedly induced ingestion and/or clearance of Aβ by microglia (Yang et al. 2020), consistent with the fact that mRNA levels of TLR 2, 4, 5, 7, and 9 increased in senile plaque associated microglia, thus indicating that TLRs played important roles in sensing and responding to the presence of Aβ, particularly in microglia. Mounting evidence suggested that the expression of CD14, TLR4, TLR2, and TLR5 increased in animal models of AD or patients with AD (Carty and Bowie 2011; Herrera-Rivero et al. 2019). Increased TLR-3 immunoreactivity and TLR-3 mRNA localized to microglia in AD compared to non-demented cases is demonstrated in the present study, as well as an association of TLR-3 mRNA levels with Aβ and NFTs (Walker et al. 2018). In addition, microRNA let-7, a highly abundant regulator of gene expression in the CNS and significantly increased in the cerebrospinal fluid (CSF) from patients with AD, could induce neurodegeneration by activating TLR-7. These results suggested that the TLR signaling pathways were involved in the clearance of Aβ deposits in the brain and that TLR might be a therapeutic target for Alzheimer's disease (Trudler et al. 2010).

It was shown that APP mouse models with a TLR4 deficiency had an increase in diffuse and fibrillar Aβ deposition and poorer spatial learning, as compared to TLR4 wildtype APP mouse models, in line with the study where AD mouse models had a

TLR2 deficiency (Trudler et al. 2010). An acute (one-time) injection of LPS has been proven to be effective in activating microglia and exacerbating cerebral β-amyloidosis (Wendeln et al. 2018). It was also suggested that the neutralization of TLR5 could prevent the upregulation of TYRO3 and GAS6 which played pivotal roles in limiting inflammatory responses upon TLR stimulation TAM, induced by co-stimulation with Aβ and flagellin for 24 h in THP-1 cells (Herrera-Rivero et al. 2019). Recent studies suggested aggregated Aβ was significantly decreased through AAV-mediated expression of human TLR5 ectodomain (sTLR5) alone or fused to human IgG4 Fc (sTLR5Fc) in AD mouse models (Chakrabarty et al. 2018). Furthermore, exposure of microglia to the TLR9 ligand CpG DNA resulted in clearance of Aβ from microglial cells and ameliorated cognitive deficits in AD mouse models (Chakrabarty et al. 2018). The loss of IRAK4 function was also essential for transduction of TLR signals which results in decreased aggregated Aβ in AD mouse models, promoting Aβ phagocytosis and restoring olfactory behavior (Cameron et al. 2012).

4.3 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is an autoimmune disorder of the CNS characterized by progressive demyelination and pathological inflammation. Immune responses triggered by TLRs were involved in MS pathogenesis. In MS patients, the peripheral blood monocytes (PBMCs) exhibited a lower TLR1 expression but upregulated TLR2 expression (Sloane et al. 2010). In the EAE animal model, TLR2 activation enhanced Th17 proliferation and cytokine production and resulted in tissue damage, while deficiency of TLR2 impaired Th17 responses and ameliorated EAE (Reynolds et al. 2010; Zheng et al. 2019). Tregs were also demonstrated to have a higher level of TLR2 as detected from the blood of MS patients. The functions of TLR3 were different from other TLRs such as TLR2, TLR4, TLR7, and TLR9. Stimulating TLR3 by poly I:C (an agonist for TLR3) suppressed the relapse of demyelination in EAE, indicating that TLR3 activation might be neuroprotective (Gambuzza et al. 2011). The effect of TLR4 activation was similar with TLR2 during the pathogenesis of MS. A decreased NO production and a weakened ability to inhibit Th1 and Th17 proliferation was shown in LPS-pretreated MCSs, an agonist for TLR4 (Vega-Letter et al. 2016), while another study showed that TLR4 damaged the repairment of oligodendrocyte progenitors (Srivastava et al. 2018). As to the astrocytes, knockdown of TLR4 inhibited its ability to product inflammatory mediators. It is noteworthy that a controversial role of TLR4 was discussed. Marta et al. found that TLR4 exacerbated EAE symptoms, yet TLR4-deficient mice had an increase in Th17 proliferation and serum IL-17 level (Gambuzza et al. 2011; Marta et al. 2008, 2009). As for TLR7 and TLR9, different opinions have been put forward on their roles in MS. TLR7 and TLR9 seemed to have positive effects on MS and downregulate the disease severity, since mice deficient in TLR7 or TLR9 were more susceptible to EAE. On the contrary, some researches also indicated that

TLR7 expression increased in the spinal cord of EAE mice, and deficient in TLR9 was not susceptible to EAE (Gambuzza et al. 2011; Prinz et al. 2006).

Microglia-derived IL-1β was necessary in the pathogenesis of MS, and TLR played a part in this progress. TLR stimulation involved in the formation of IRAKM-caspase-8-ASC complex, leading to the activation of caspase-8 and release of IL-1β in microglia. Meanwhile, IL-1β helped to grow the microglia population and increased the production of inflammatory cytokines/chemokines, thereby resulting in neuroinflammation (Zhang et al. 2018). Similarly, Peli1, an E3 ubiquitin ligase, also mediated neuroinflammation in a TLR-dependent manner. The novel TLR signaling pathway mediator TRAF3 was involved in this process. The absence of TRAF3 restored the activation of microglia and enhanced susceptibility to EAE (Xiao et al. 2013). Considering the importance of TLRs signal pathway in MS, TLR agonists/antagonists might become novel therapeutic targets to regulate the unbalanced immune responses in the near future (Gambuzza et al. 2011).

5 Conclusions and Future Perspectives

Given the fact that TLRs play vital roles in the pathogenesis of neuroinflammation, further investigations into the ligands-receptors mediated immune signaling pathways will greatly expand therapeutic options and facilitate corresponding solutions of brain insults. However, current evidence suggests that TLR-triggered responses are still elusive under the background of various brain disorders, which may exert either beneficial or detrimental effects depending on the strength, extent and dynamic cross-regulation of signaling within the family, as well as interactions with other danger-recognition receptors. Moreover, a negligible paradox impeded the utilization of pharmacological intervention is that TLRs could contribute to both pathogen clearance and sterile inflammation by respectively recognizing PAMPs and DAMPs, two processes sharing the same nature of molecular biology but inducing exactly opposite disease phenotypes. The inhibition of TLR signaling during sterile inflammatory diseases such as secondary brain injury following cerebrovascular events or neurodegenerative disorders may increase the risk of infection, while TLR agonists were widely used as adjuvants for the induction of adaptive immunity with a potential risk for the progress of autoimmune diseases. How to avoid these side effects remains great challenges for the clinical application of TLRs-targeting compounds. Finally, although many efforts have been reported to reveal the critical mechanisms underlying TLR signaling transduction within the brain compartment, a better understanding as to how these inflammatory cascades are effectively terminated in a self-restricted manner may uncover novel insights for the manipulation of TLR signaling activity in brain immunity, thus implementing basic findings into clinical practices in a scheme relatively closer to natural physiological state.

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Toll-Like Receptor 4 in Pain: Bridging Molecules-to-Cells-to-Systems

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Contents

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2022_587

Abstract

Pain impacts the lives of billions of people around the world – both directly and indirectly. It is complex and transcends beyond an unpleasant sensory experience to encompass emotional experiences. To date, there are no successful treatments for sufferers of chronic pain. Although opioids do not provide any benefit to chronic pain sufferers, they are still prescribed, often resulting in more complications such as hyperalgesia and dependence. In order to develop effective and safe medications to manage, and perhaps even treat pain, it is important to evaluate novel contributors to pain pathologies. As such, in this chapter we review the role of Toll-like receptor 4, a receptor of the innate immune system, that continues to gain substantial attention in the field of pain research. Positioned in the nexus of the neuro and immune systems, TLR4 may provide one of the missing pieces in understanding the complexities of pain. Here we consider how TLR4 enables a mechanistical understanding of pain as a multidimensional biopsychosocial state from molecules to cells to systems and back again.

Keywords

A20 · Biased signalling · Biopsychosocial pain · Chronic pain · Genetics · GPCRs · Neuroimmunology · Pain · Toll-like receptors · TRPV1

1 Introduction

Pain is complex, occurring at multiple levels from the molecular and cellular to psychological and behavioural, with impact on both the individual and society. Hence a biopsychosocial model of pain is the most compelling (Raja et al. 2020). But the biopsychosocial pain nexus (the interface of these inputs that leads to pain experiences) is largely unexplored from the perspective of the cellular and molecular networks that can connect this multidimensional state. When this important protective physiological response transcends from acute physical pain to chronic pain it encompasses emotional states of helplessness, anxiety and depression. The manifestation and severity of chronic pain in individuals cannot yet be predicted, nor can it

be completely evaluated due to the use of subjective assessments and the dire lack of objective measures. Subjective measures of pain are prone to manipulation and can be completely ineffective in some cases, such as for young children, non-verbal patients or those with neurodegenerative diseases. With chronic pain impacting the quality of life of 1 in 5 individuals worldwide, it is critical to understand the complex molecular mechanisms behind the diverse symptoms of pain. Such a mechanistic understanding will inform a future of precision medicine and precision pain management, evolving us beyond the current empirical practices.

In 2020, the International Association for the Study of Pain revised its definition of pain (Raja et al. 2020) as follows: 'An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage'. This definition is augmented by six additional contextual clarifications of critical importance: pain is a personal experience influenced to varying degrees of biopsychosocial factors. Pain and nociception are different. Pain cannot be inferred solely from activity in sensory neurons. Throughout life, individuals learn the concept of pain. A person's report of pain should be respected. While pain can be adaptive, it may adversely affect function and social and psychological wellbeing. Verbal description is only one of several behaviours to express pain; inability to communicate does not negate the possibility that humans or non-human animals experience pain. This pain definition highlights the complexities that we face in defining what pain is, even in humans with whom we generally can communicate.

Although much is known about nociceptive pathways (the transmission of a noxious stimulus to the brain) resulting in the pain experience (see von Hehn et al. (2012) for review) the transition from acute to chronic pain remains poorly understood. What is clear though is the emerging role of the innate immune system, in particular Toll-like receptors (TLRs), in the detection, creation, transition and maintenance stages that facilitate chronic pain (Lacagnina et al. 2018). In this chapter we will provide an overview of TLR4 in pain; an important member of the TLR family that has been implicated in chronic pain. Importantly, embodied within this greater understanding of the involvement of TLR4 in pain is the ability for us to begin to appreciate the biopsychosocial nature of pain in its full complexity. This step of appreciating the true complexity of pain must not be overlooked across the multidimensionality of pain as it is cultivated and experienced in time (seconds to years), distance (nanometres to metres) and endogenous to exogenous environments.

1.1 Conceptualising Pain as Multidimensional States

We are challenged to address pain in more complex ways, as exemplified by the complexity of the revised IASP pain definition. For pain to be appreciated simultaneously from the top down at a systems level as a biopsychosocial multidimensional state, and from the bottom up as events occurring at the nanoscale in a complex web of molecular and cellular events, we must define mechanisms that can mediate these profound events over vast time and length scales. Much of our research and development focus over the past two decades has been on interrogating the roles

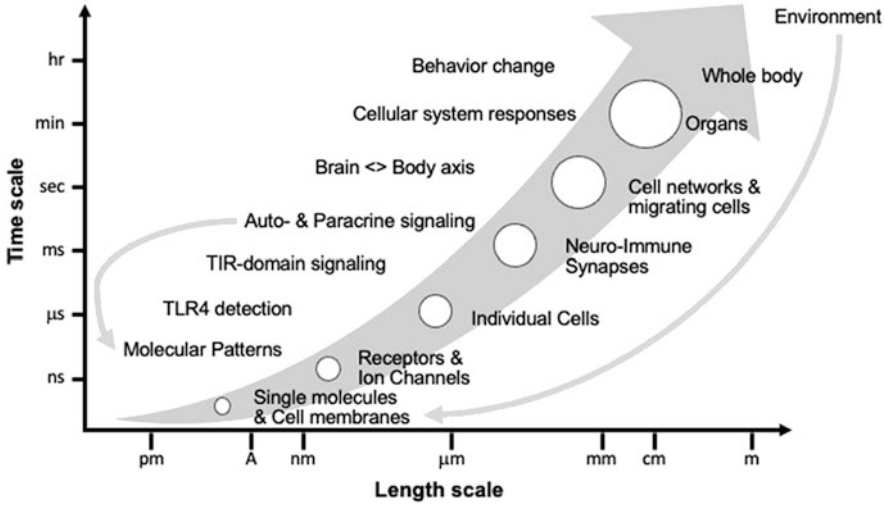

Fig. 1 Viewing pain over a multidimensional scale of time and space

that the innate immune system, and specifically TLR4, might play in this complex multidimensional pain state.

Such challenges of conceptualisation, like we face in understanding pain, have been explored in mathematics and theoretical physics. In these disciplines, models that help us conceptualise complex systems are designed from their foundations to operate in real-world scenarios. This is distinct from the classical Cartesian approaches currently adopted in the field of pain research that necessitate operations between two binary states of homeostasis and an altered state. For the pain field, this would mean only including injured and uninjured states in experimental settings. Or considering only neuronal action potentials in nociceptive fibres as 'pain'. In contrast, the evolved view of biological conditions acknowledges that real-world systems are unstable and continuously compensating to reach some form of new steady state condition, termed allostasis. This establishment of synchrony within complex systems has been set forth in examples like the Kuramoto model (Dattani and Barahona 2017) and dynamical systems models. The real world is dominated by open systems owing to their co-existence with their environment and this is at the foundation of viewing pain as a complex biopsychosocial state. These open systems are constantly changing and adapt to new conditions but do so constrained to some form of inherent order or synchrony related to that specific system. These models force us to connect time and space with defined molecular and cellular mechanisms (Fig. 1). This is crucial for the field to acknowledge when we consider pain.

A field that has embraced this multidimensionality and open systems biological approach is psychoneuroimmunology. Psychoneuroimmunology conceptualises health as a system involving interactions between the body and mind. From a psychoneuroimmunology model perspective becoming sick is a complex molecular pattern-to-cells-to-systems response that is initiated by our innate immune system

(Dantzer and Kelley 2007). Inherently this is a multidimensional opportunity and challenge. Hence, the innate pattern recognition system and receptors like Toll-Like Receptor 4 (TLR4) are key molecular connectors of mind and body (Frank et al. 2015). Our innate immune system is the ultimate integrated surveillance and response system because it is the first responder to the detection of threats from diverse origins. When we get sick with an illness, our innate immune system mounts a protective inflammatory response within minutes of exposure, which helps to control and limit the negative impact of the invading pathogen (Dantzer and Kelley 2007). These events are triggered by our molecular pattern recognition capabilities at the nanoscale, a crucial one being TLR4. The resulting inflammatory response recruits specific molecular systems to mitigate the threat and occurs so rapidly that we are not consciously aware of a change in our health status, even though profound molecular events are occurring in our blood and tissues. These molecular nanoscale responses in turn have an immediate impact on neural activity at macroscales to elicit functionally adaptive behaviours by the organism that prioritise personal health and protect the community (Lasselin 2021). Here psychoneuroimmunology acknowledges that the system can respond to endogenous and exogenous factors, scaling and focusing the response to these as needed (Fig. 1). This allows for the innate immune system to undertake information down sampling at the receptor level as it scales the immune signalling. Moreover, some brain networks are more sensitive to these molecular changes, resulting in specific functional adaptations observed early in discrete immune responses (Wegner et al. 2014; Hutchinson 2014). A crucial and long appreciated adaptation is illness-induced pain states. However, the historic association of these illness events and the modern understanding of TLR4 in hypernociception and complex multidimensional biopsychosocial pain states are only just beginning to be appreciated.

2 The Shared Toll-Like Receptor Language of Sensory Neurons and Immune Cells

As a receptor family, TLRs play a sentinel surveillance role as pattern recognition receptors that can identify molecular patterns as ‘non-self’ or ‘danger’ signals (Buchanan et al. 2010). Within the CNS, these receptors are mainly found in the innate immune system on endothelial cells, microglia, some astrocytes and sensory neurons (Bsibsi et al. 2002; Goethals et al. 2010; Nagyoszi et al. 2010). Whilst neurons have specific, ligand selective receptors for neurotransmitters, TLRs have evolved to unequivocally recognise a vast array of threats via pathogen-associated molecular patterns (PAMPs; microbial pathogens) and danger-associated molecular patterns (DAMPs; cellular signals of danger or stress) (Akira and Takeda 2004). In response to detection, TLRs then coordinate signal transduction to modulate the inflammatory state of the host. Importantly, TLRs are also expressed on peripheral immune cells. As the immune and sensory nervous systems communicate via shared mediators and networks, this places TLRs in the nexus of both neuro and immune systems (neuroimmune interface) where they are perfectly positioned to influence

nociceptive processing and pain (Lacagnina et al. 2018). This is a crucial molecules-to-systems capacity of TLRs enabling a common molecular language to span the biopsychosocial divide. Importantly this influence and communication is bidirectional between neuro and immune systems, making TLRs a critical connector of the biological, psychological and environmental state of the organism. Hence, this multidimensionality of TLRs and the cells that express them enables a molecular understanding of the biopsychosocial pain condition.

2.1 The Molecular Origins of TLR4 in Acute Pain

The illness response is a coordinated set of molecular drivers, to behaviourally presented adaptations, which develop during the course of an infection (Dantzer 2001). During an infection multiple systems are adapted (Kelley et al. 2003) including sensory disturbances such as increased sensitivity to pain (Yirmiya et al. 2006). Experimentally these behaviours and sensory changes can be recreated using either the exogenous bacterial endotoxin or the endogenous-mimicking effector agents like recombinant proinflammatory cytokines, such as Interleukin-1 beta (IL-1β). These data demonstrate that the innate immune response to molecular threat and the generation of molecular mediators like IL-1β is sufficient to change neuroimmune function (Kelley et al. 2003; Dantzer and Kelley 2007). The identification and naming of TLR4 as an innate immune detection system for endotoxin entwines two key threads; that innate immune function and sensory changes are associated with illness.

The specific use of lipopolysaccharide (LPS), the classical TLR4 ligand, to induce hyperalgesia and induction of inflammatory mediators identified the importance of TLR4 in pathology (Wicrtelak et al. 1994). This identification of exogenous agent driven TLR4 pain behaviours led to the implication of endogenously derived factors in nociceptive hypersensitivity. Clinically, systemic LPS results in anatomically dependent altered mechanical sensitivity, which correlates with peripheral immune activation, specifically circulating IL-6 (Wegner et al. 2014). There is a clear role for TLR4 in pain sensitisation as part of an adaptive illness response, likewise, changes in expression can be associated with transition to maladaptive, pathological pain states.

2.2 Why Is the Role of TLR4 in Persistent Pain Important?

De Leo and colleagues were the first to demonstrate that changes in TLR4 expression are important for generating a pathological pain state. They demonstrated TLR4 upregulation in spinal microglia is associated with cytokine expression and establishment of thermal and mechanical sensitivity following spinal nerve ligation, a model of neuropathic pain (Tanga et al. 2005). Importantly both behavioural and spinal molecular changes were attenuated in genetically modified (TLR4 knockout (KO) and point mutation) mice and rats with spinally administered TLR4 antisense

oligodeoxynucleotide (Tanga et al. 2005). Likewise, Yaksh and colleagues show that TLR4 mediates transition to pathological pain in a mouse serum-transferred arthritis model. Like De Leo et al., they correlated spinal microglial reactivity with mechanical hypersensitivity, inhibited by TLR4 genetic manipulation (KO) and antagonism (LPS from Rhodobacter sphaeroides [LPS-RS]). Interestingly, they demonstrated administration of LPS-RS following onset of mechanical hypersensitivity had no behavioural effect, alluding to TLR4 being an important component of generation, but not maintenance, of the molecular pathological pain phenotype (Christianson et al. 2011). This contrasts earlier studies which report LPS-RS attenuation of established chronic constriction injury (CCI)-induced neuropathic pain in rats (Hutchinson et al. 2008). Collectively these studies highlight non-neuronal changes in TLR4 expression influence the inflammatory environment leading to pathological pain. Additionally, changes to neuronal TLR4 expression must also be considered in the context of the molecular pathological pain phenotype. Moreover, it is important to note that TLR4 does not act alone, as blockade of TLR2 has also been reported to attenuate neuropathic pain in rodent models and TLR3 has also been implicated in neuropathic pain (Jurga et al. 2016).

This implication of the nanoscale events of TLR4 in pain needs to then be connected to what TLR4 expressing cell systems are critical to pain. To this end TLR4 expression has been reported in rat trigeminal (TG) and dorsal root ganglia (DRG) by Wadachi and Hargreaves. Upregulation of TLR4 was also observed on neurons within inflamed human dental pulp (Wadachi and Hargreaves 2006). The authors reported co-expression with neuronal-ion channel, transient receptor potential cation channel subfamily V member 1 (TRPV1), an important nociceptive detector of noxious stimuli. The authors later demonstrated that TLR4 antagonist LPS-RS alters the activity of neuronal TRPV1 following activation of TLR4 with LPS (Diogenes et al. 2011). The relationship between TLR4 and TRPV1 is an important neuroimmune interaction discussed in greater detail later in this chapter. TLR4 expression has also been observed in rat and human DRG neurons in a study investigating chemotherapy-induced peripheral neuropathy (CIPN) (Li et al. 2015). TLR4 can therefore be considered more than an initiator of immune signalling, but a receptor with a direct role in nociceptor detection of injury, danger and infection by neurons, underwriting its critical multidimensionality. The numerous examples of TLR4-dependent pathological pain models suggest an evolved response to illness and injury as opposed to nociceptive pathways hijacked by immune infrastructure. As a result, TLRs have been brought forward as key contributors to pain pathology and novel targets for modifying pain processing.

3 With Immense Power Must Come Profound Controls

3.1 Overview of TLR Signalling

TLR4 relays critical information about the presence of danger/damage signals to intracellular adapter proteins critical for initiating a cellular response (please see Fitzgerald and Kagan (2020) for recent review on TLRs). This TLR4 signalling is achieved by a multi-receptor complex, which includes a TLR4 dimer and its co-receptors myeloid differentiation factor 2 (MD-2) and Cluster of differentiation 14 (CD14) (Núñez Miguel et al. 2007). Shimazu and co-workers highlighted the critical role of MD-2 in TLR4 signalling (Shimazu et al. 1999). MD-2 functions by recognising LPS and dimerising TLR4 monomers, which in turn allows for receptor interaction with intracellular adapter proteins. CD14 has been reported to enhance LPS recognition and due to its sensitivity is capable of binding picomolar concentrations of LPS (Gioannini et al. 2004). Highlighting the importance of understanding the contributions of other proteins in TLR4 signalling, and hence potential targets for pain management, is the evidence from Cao et al. demonstrating that CD-14 knockout mice display significantly decreased behavioural sensitivity to pain following L5 nerve transection compared to wild-type injured mice (Cao et al. 2009). The TLR4 multi-protein signalling complex assembly further demonstrates the multidimensionality of the system. Here we see that extracellular environments may be conditioned by paracrine or autocrine pre-signalling events to influence the potential TLR4 signalling capacity. Importantly, this complexity of TLR4's non-membrane bound co-factors, like MD-2, is crucial to incorporate into in vitro systems. For example, the use of non-biologically relevant culture media that does not have the necessary co-factors can result in several orders of magnitude change in the TLR4 ligand responsivity, causing the apparent loss of function and may explain differences in reported functions of TLR4 (Hutchinson et al. 2010).

Once this complex signalling unit is formed following binding of a ligand, TLR4 is the only member of the TLR family capable of activating two major intracellular signalling pathways: the myeloid differentiation primary response 88 (MyD88)-dependent pathway and the TIR-domain-containing adapter-inducing interferon (IFN)-β (TRIF) pathway (also commonly referred to as MyD88-independent). The MyD88 pathway is activated by the recruitment of two adapter proteins MyD88 and Mal (also known as TIR Domain Containing Adaptor Protein [TIRAP]) to TLR4 at the plasma membrane. This results in the rapid activation of transcription factor NF-κB, MAPKs, activator protein-1 (AP-1), and IFN regulatory factor 5 (IRF5) and the ultimately the secretion of proinflammatory cytokines such as interleukin (IL-) 1β, IL-6, tumour necrosis factor (TNF) and chemokines like monocyte chemoattractant protein 1 (MCP-1) and IL-8 (Arthur and Ley 2013; Medzhitov et al. 1998; Takaoka et al. 2005). This complex signalling system can also lead to the production of nitric oxide synthase (iNOS) and anti-inflammatory IL-1R antagonist and IL-10 (Lacagnina et al. 2018). Evidence that the MyD88 pathway is implicated in pain pathophysiology has been presented. Following chronic constriction injury, an increase in MyD88 protein levels is observed in nociceptive pathways

including the DRG, dorsal horn and related signalling components (Lacagnina et al. 2018). In studies where MyD88 signalling is blocked, as in the Liu et al. study with the use of an inhibitory peptide, an attenuation of mechanical allodynia and thermal hyperalgesia was reported (Liu et al. 2017). It should be noted that much of this intracellular signalling knowledge has been derived from classical immune cell studies. Therefore, the heterogeneity in secondary signalling pathways across different cell systems like glia and neurons may be profound. For example, differences in TLR4 signalling capacity have been noted between macrophages and dendritic cells under specific conditions (Tsukamoto et al. 2013).

Parallel or instead of the MyD88 pathway, TRIF signalling can occur once TLR4 has internalised and recruited adapter proteins TRIF and TRAM. This leads to the activation of interferon regulatory factor 3 (IRF3) and the release of type 1 interferons such as beta interferon (IFN-β) and IL-10 (Yamamoto et al. 2003). There is some evidence that the expression of IFNs is implicated in the inhibition of nociceptive transmission (Liu et al. 2016). The activation of the TRIF pathway also results in the activation of NF-κB, albeit delayed (Sakai et al. 2017). The kinetic profile of each signalling event may play a fundamental role in the ultimate physiological response. This complexity of one receptor with multiple downstream potential signalling partners underscores the information down sampling that the TLR4 signalling system can compute at the molecular level. However, we are yet to fully appreciate what determines this molecular computation and how it contributes to pathological pain.

Much of the characterisation of TLR4 signalling has been conducted with LPS. Interestingly, depending on the bacterial species LPS is isolated from, activation of both pathways simultaneously or selective activation of either the MyD88 dependent or TRIF pathway is possible (Stephens et al. 2021). This molecular computation is termed 'biased signalling' and has been well studied in the context of G protein-coupled receptor (GPCR) signalling. As the roles of TLR4's signalling pathways have yet to be fully deciphered, 'biased' LPS provides a valuable tool to further interrogate TLR4 function in pain. Excitingly, the concept of biased signalling presents a tantalising opportunity to pharmacologically modulate TLR4 signalling with biased ligands allowing potential therapies to move beyond complete antagonism to selectively activating/deactivating MyD88-dependent and -independent pathways.

3.2 Regulation of TLR4 Signalling

Innate immune signalling is fundamental in maintaining homeostasis, therefore its regulation at molecular, cell and systems levels is crucial in preventing a detrimental inflammatory response. At the molecular level, TLR4 is critical to the innate immune response, thus, tightly tuned regulation of its signalling is pertinent. As with many aspects of human biology, there is high redundancy in TLR4 regulation, with multiple mechanisms beyond transcription factor control.

3.2.1 TLR4 Epigenetics

Previous research into epigenetic regulation of innate immune responses has focused primarily on epigenetic mechanisms downstream of TLR activation, with less focus on the contribution of epigenetic modifications to variable TLR4 expression in primary tissue, which is somewhat justified based on the general hypomethylation status of the human TLR4 promoter (Xie et al. 2018). Reflecting this, in the sole study to date in the context of pain, TLR4 promoter methylation was not significantly associated with persistent pain after breast cancer surgery (Kringel et al. 2019). Whilst there are many reports of case-control differences in TLR4 epigenetics for other diseases/conditions, a key limitation of these studies has been that they have either not quantified TLR4 expression or not reported on the correlation between epigenetics and expression. Consequently, whilst experimental (e.g., in vitro) modification of TLR4 promoter methylation, histone trimethylation and/or acetylation can alter TLR4 expression (Du et al. 2019; Kim et al. 2016; Takahashi et al. 2011), it remains unclear the extent to which epigenetics determine clinical variability in TLR4 expression in or between individuals (Poole et al. 2020), or between tissues/cell types. The field would advance more quickly if clinical epigenetics and expression were quantified and reported (i.e., their correlation) in tandem.

3.2.2 Post-Transcriptional/Translational Regulation

TLR4 splice variants have been identified in multiple species, some of which may act as negative regulators of TLR4 signalling (Vaure and Liu 2014). In addition, human and rodent TLR4 are targeted by multiple miRNAs, with strong evidence (reporter assay, Western blot and/or qPCR) for effects on TLR4 expression (Huang et al. 2019). Tissue expression of these miRNAs changes in response to TLR4 pathway activation and in different pain states/pathologies, and they may play important negative-feedback roles in the control of inflammation. For example, miR-124, miR-146a and miR-451 target TLR4 directly, as well as other genes in the TLR4 signalling pathway (Wang et al. 2021; Yang et al. 2011; Sun and Zhang 2018; Lu et al. 2015; Taganov et al. 2006; Ma et al. 2014). Their expression changes in response to TLR activation in vitro, and in animal models of inflammatory and neuropathic pain, with the administration of miR-124 and miR-146a mimics able to attenuate pain in those models (Ma et al. 2014; Ponomarev et al. 2011; Taganov et al. 2006; Willemen et al. 2012; Lu et al. 2015; Kynast et al. 2013; Grace et al. 2018; Sun and Zhang 2018).

Glycosylation is a form of co- and post-translational modification that modifies proteins by the addition of specific glycans. We now know that the presence, absence or even the pattern of glycosylation plays a key role in biological processes such as cellular communication, differentiation and intracellular signal transduction (Ohtsubo and Marth 2006). Examples in literature suggest that polysialic acid (PSA), a cell surface glycan, is involved in a number of plasticity-related responses including cellular adaptations to pain (Rutishauser 2008). In 2006, Weber and co-workers reported an increase in PSA neural adhesion molecule expression in the hippocampus of heroin addicts (Weber et al. 2006). At the receptor level, there is

evidence to implicate an important role for glycosylation in regulating TLR4 signalling. For example, da Silva and colleagues have demonstrated that N-linked glycosylation sites are important for TLR4 activity and that removal of glycosylation sites (N-linked) on both TLR4 and its accessory protein MD-2 inhibits LPS-induced activation (da Silva Correia and Ulevitch 2002). In line with this, it has been shown cleavage of sialic acid and endogenous sialidase activity by neuraminidase 1 facilitates signal transduction of TLR4 (Feng et al. 2012; Amith et al. 2010). Furthermore, it has recently been reported that endogenous neuraminidase is critically involved in TLR4 mediated microglial reactivity (Allendorf et al. 2020). Taken together, these studies demonstrate the need to investigate the role of glycosylation in pain as they may provide novel targets for the management of pain and addiction.

Following TLR4 activation at the cell surface, there are also mechanisms in place to regulate signalling at the level of intracellular protein cascades. Here, the predominant mechanisms modify ubiquitin structures. Ubiquitin is a small protein which links together forming chains. These ubiquitin chains are fundamental in linking proteins to form intracellular signalling cascades which facilitate signal transduction. This is regulated by specialised Deubiquitinating enzymes (DUBs), which cleave the ubiquitin chains to prevent further downstream protein interaction, thus arresting signalling (Das et al. 2020). In the context of TLR4, the most characterised DUB is A20 (encoded by *TNFAIP3*).

A20 is recognised as a crucial regulator of numerous inflammatory signalling pathways (TLR4, IL1R, TNFR1 and 2, NLRs, IL-17R, TCR) (Catrysse et al. 2014). A20 is particularly unique owing to its dual enzymatic function both ligating and cleaving ubiquitin motifs (Coornaert et al. 2009). Despite this complex dual function, literature to date suggests it is the K63-linked DUB activity of A20 that regulates TLR4 signalling. Specifically, in the canonical MyD88 dependant pathway A20 binds to adaptor protein TRAF6 and cleaves the K63 ubiquitin chain to prevent further signalling (Boone et al. 2004). The extent of regulation exerted by A20 on the MyD88-independent pathway is less clear.

Beyond its direct regulation of TLR4 signalling, A20 also exerts regulatory action on the mechanisms by which TLR4 exerts a biological effect. For example, A20 regulates the signalling of various cytokines (such as TNF-α and IL-1) produced following TLR4 activation. This is important as it means A20 is strongly positioned to regulate the action of TLR4 signalling at multiple points. As a result, dysfunction of A20 is associated with a range of TLR4- linked pathologies (Ma and Malynn 2012). Endotoxic shock, also known as septic shock, is a well-characterised pathology driven by TLR4. Early work demonstrates that A20 is fundamental in preventing endotoxic shock via a TLR specific mechanism (Boone et al. 2004). Furthermore, TLR4 signalling has been implicated in pathologies of the central nervous system (CNS) which has led to the subsequent exploration of A20 in this domain. It has recently been shown that A20 is critical in the regulation of inflammatory signalling in the CNS and that its ablation is associated with uncontrolled inflammation, including infiltration by peripheral immune cells, and poor prognosis of experimental autoimmune encephalitis (a rodent model of multiple sclerosis)

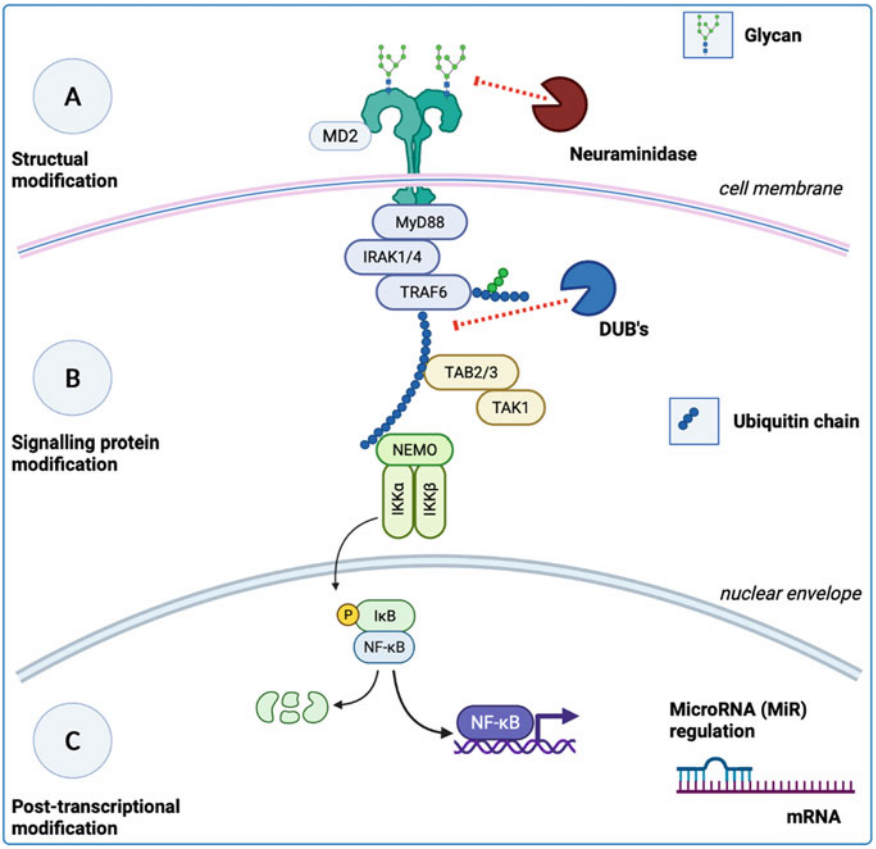

Fig. 2 Post-transcriptional/translational regulation of TLR4 signalling. Schematic illustrating three broad areas of post-transcriptional/translational TLR4 regulation focusing on the MyD88 dependent pathway. (**a**) Modifications to receptor glycan residues at the cell surface serve to regulate signal transduction. (**b**) Intracellular mechanisms such as de-ubiquitination by deubiquitinating enzymes (DUBs) arrest signal transduction by inhibiting intracellular signalling cascades. (**c**) Post-transcriptional regulation of TLR4 signalling occurs through via MicroRNA (MiR) targeting of TLR4 and various proteins involved in its signalling. Created with BioRender.com

(Mohebiany et al. 2020; Voet et al. 2018). Despite extensive literature implicating TLR4 signalling in pain and addiction, the role of A20 is yet to be established here.

In outlining the three predominant post-transcriptional/translational mechanisms by which TLR4 is regulated (Fig. 2), it is apparent there is a gaping hole in our understanding of how immune regulation mediates pain and addiction. By exploring these further, novel therapeutic targets and strategies may be developed, helping to improve the quality of life of individuals impacted by pain.

4 Molecular Interactions Amplify Pain Complexity: TLR Interactions with Other Proteins

4.1 Ion Channels Like TRPV1 Also 'Talk' to TLR4

Transient receptor potential cation channel subfamily V member 1 (TRPV1) has been extensively studied in the context of pain as it allows control of nociceptive activation. TRPV1 is a non-selective neuronal-ion channel first classified in 1997 by Caterina and colleagues as vanilloid receptor 1 (VR1); a receptor for capsaicin, the pungent ingredient found in chilli peppers (Caterina et al. 1997). In addition to capsaicin, TRPV1 is activated by noxious heat, protons and a myriad of exogenous and endogenous chemicals including piperine (black pepper), spider toxins and endocannabinoids (e.g., anandamide) (Liu and Simon 1996; Siemens et al. 2006; Zygmunt et al. 1999). TRPV1 is expressed throughout the nervous system on small and medium diameter, C- and Aδ-fibre nociceptors, spinal cord dorsal horn, hypothalamus, hippocampus, cortex and microglia (Kunert-Keil et al. 2006; Mezey et al. 2000; Szallasi et al. 2007). TRPV1 therefore presents as an ideal multimodal detector for noxious stimuli and an important component of the nociceptive system.

This Cartesian mode of study has now established that activation of TRPV1 on primary afferents results in action potential generation and nociceptive firing, leading to an initial 'spontaneous' pain response followed by a period of hypersensitivity (Caterina and Julius 2001). However, it is now clear that there is a multidimensionality to TRPV1 function as LPS application results in potentiation of capsaicin-induced calcitonin gene related peptide (CGRP) content, correlating with increased CGRP release from excised rat trachea (Hua et al. 1996). Interestingly this effect was blocked by IL-1β and cyclooxygenase (COX) inhibitors (Hua et al. 1996), suggesting a more complex neuroimmune involvement in the system. A headache model also observed an increase in capsaicin-induced behaviours when animals were subjected to a 5 h LPS treatment (Kemper et al. 1998). These studies demonstrate the ability for inflammatory responses to modulate the function of TRPV1.

It is now known that cytokines (IL-6, IL-1β, TNFα) and nerve growth factor (NGF) are associated with TRPV1 upregulation and membrane trafficking, while prostaglandins (PGE2, PI2), bradykinin and NGF sensitise the receptor by initiating receptor phosphorylation and dephosphorylation events via protein kinase A and C (PKA/C), phospholipase C (PLC), Src tyrosine kinase (Src) and phosphoinositide 3-kinase (PI3K) (Cesare et al. 1999; Ebbinghaus et al. 2012; Fang et al. 2015; Hensellek et al. 2007; Stein et al. 2006; Zhang et al. 2005; Moriyama et al. 2005; Numazaki et al. 2003; Stratiievska et al. 2018). This has been complemented by recent studies which have investigated the TRPV1/TLR4 relationship specifically. It is now clear that potentiated TRPV1 responses are created following comparatively short LPS exposure. Short-term activation (15 min) with LPS can potentiate capsaicin responses including increased inward current amplitude and calcium accumulation in TLR4/TRPV1 expressing HEK cells (Min et al. 2014).

These results are replicated in primary cells, with 15 min LPS stimulation of rat trigeminal neurons capable of producing potentiation of capsaicin evoked calcium

accumulation and CGRP release, which can be attenuated by TLR4 antagonist LPS-RS (Diogenes et al. 2011; Ferraz et al. 2011). Primary cells excised from a rat CIPN model reveal chemotherapy agent paclitaxel increases TRPV1 sensitisation in a TLR4 dependent manner (Li et al. 2015). In excised rat DRG neurons, 10 min application of paclitaxel potentiates capsaicin-induced intercellular calcium accumulation, an effect blocked by co-treatment with LPS-RS. Excised DRG neurons from rats with paclitaxel-induced CIPN show potentiated capsaicin-induced calcium accumulation compared to vehicle treated rats. The effect is therefore seen after both acute and long-term paclitaxel exposure (Li et al. 2015). Paclitaxel not only induces primary-nociceptor-specific changes, but spinal changes; ex vivo studies show an increased rate of miniature excitatory post synaptic currents (mEPSCs) to a second application of capsaicin following paclitaxel treatment, an effect blocked by LPS-RS (Li et al. 2015).

Comparable findings are observed in a 2,4,6-trinitrobenzine sulphate (TNBS)-induced colitis model, where capsaicin-induced currents were significantly potentiated in colitis animals, an effect attenuated in TLR4 KO mice (Wu et al. 2019). Interestingly TLR4 KO mice show significantly reduced inward currents compared to WT animals, suggesting activation of TLR4 is not necessary for this functional interaction between the receptors (Wu et al. 2019). The exact mechanism of potentiated TRPV1 responses following short-term TLR4 agonism is yet to be identified, although as above, kinase activity is a strong candidate.

TLR4 mediated increases in PKA and PKC, and calcium dependent increases in phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) following LPS have been reported (Cabral et al. 2015; Kim et al. 2015). Activation of *Src* is reported to last between 5 and 60 min following LPS administration in human lung microvascular epithelial cells, reversed by TLR4 small interfering (si)RNA-induced knockdown (Gong et al. 2008). These kinases were previously referenced for their TRPV1 modulating effects following cellular exposure to proinflammatory mediators. Therefore, they provide outstanding candidates for indirect, rapid, TLR4-dependent TRPV1 sensitisation (Fig. 3).

There is growing evidence to suggest just the presence of TLR4 alone alters TRPV1 mediated responses. It is important to note that multiple studies report co-expression in vivo; in trigeminal ganglia (TG), and DRG in rats and humans (Ferraz et al. 2011; Li et al. 2015; Wadachi and Hargreaves 2006; Wu et al. 2019). In vitro studies co-expressing TRPV1 and TLR4 report altered response amplitude and calcium accumulation in HEK overexpression systems when compared to cells expressing TRPV1 only (Min et al. 2014). One proposed link involves the TIR domain of TLR4, whereby the interaction with TIR prevents desensitisation of and internalisation of TRPV1 (Min et al. 2018). This potential interaction requires further study, as does the proposed mechanism, as studies contradict this theory, suggesting TLR4 may facilitate receptor desensitisation from repeated capsaicin doses (Li et al. 2015). Min et al. acknowledge unidentified intermediary adaptors may be involved in the observed TIR domain potentiation of TRPV1 activity (Min et al. 2018).

One potential intermediary factor is cytoplasmic scaffolding protein A-kinase anchoring protein 79 (AKAP79), which is important to the sensitising effect of PKA

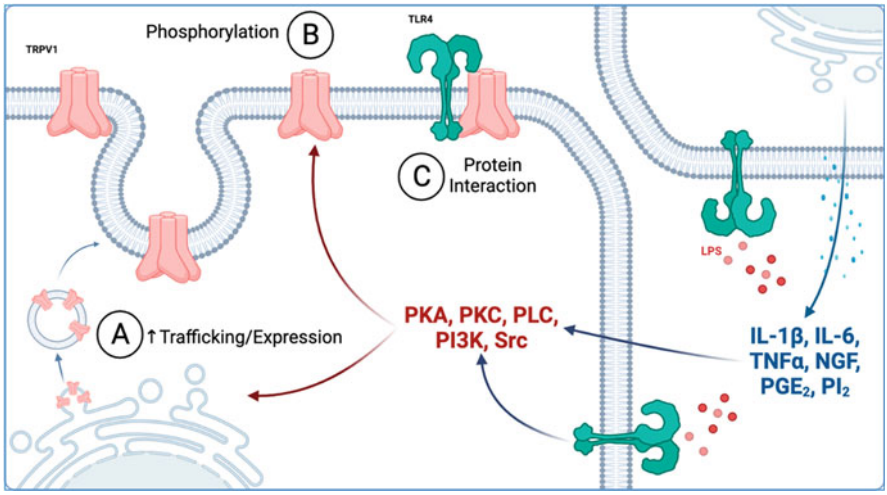

Fig. 3 Proposed mechanisms of direct and indirect TLR4-induced TRPV1 sensitisation. (**a**) LPS-induced increase in enzymatic activity (PKC, PKA, PI3K) results in increased trafficking of TRPV1 to the cell membrane. (**b**) LPS-induced increase in enzymatic activity (Src, PKA, PKC, PLC) alters TRPV1 sensitisation via receptor phosphorylation events. (**c**) Direct interaction proposed between the TIR domain of TLR4 and TRPV1. Created with BioRender.com

and PKC on TRPV1 (Faux and Scott 1997; Jeske et al. 2008, 2009; Zhang et al. 2008). TLR-TRP interactions have been observed previously; co-immunoprecipitation revealed an interaction between TRPA1 and TLR7 which potentiates TRPA1 induced inward current, suggesting a physical interaction (Park et al. 2014). Further investigation of a direct protein–protein link is required. While detection of unmodified protein interactions in tissues remains elusive due to the unreliability of available TLR4 antibodies, advances in protein–protein interaction assays such as bioluminescence resonance energy transfer (BRET) have the potential to improve our understanding using overexpression models (Dimri et al. 2016; McCarthy et al. 2017).

Importantly, Hutchinson et al. demonstrated the human clinical relevance of a TLR4/TRPV1 relationship. In this case, where capsaicin and endotoxin were co-administered to healthy individuals, a potentiation of capsaicin-induced mechanical allodynia and hyperalgesia was observed at 3 h, but not 2 h or directly following intravenous low dose (0.4 ng/kg) LPS (Hutchinson et al. 2013). Interestingly this effect was anatomically variable, observed on the forearm but not the forehead. Further, the timing of potentiation correlated with peak levels of serum IL-6 (Hutchinson et al. 2013). Importantly, the nature of the measurement of mechanical allodynia demonstrates central sensitisation was created by this combined neuro and immune nociceptive challenges. Therefore, the mechanism of action here appears to be one of the inflammatory mediated sensitisation and/or ascending sensitisation events, rather than the rapid changes observed in vitro in primary DRG, TG and HEK293FT cultures. There is significant evidence that the interaction between

TLR4 and TRPV1 has the potential to effect pain outcomes. The next steps need to clarify the events or residues that link the two receptors and identify those that may be important for generation and/or maintenance of pathological pain states.

4.2 GPCRs Do Not Operate in Isolation

GPCRs are the largest and most researched class of cell surface receptors highlighting the important role they play in human physiology and disease. Their widespread expression allows them to influence diverse biological outcomes by transducing a range of extracellular signals to intracellular mediators (G proteins and β-arrestin). Importantly many GPCR ligands have been implicated in the regulation of inflammatory responses through the modulation of immune cell functions such as the production of inflammatory mediators. However, their widespread expression and recognition of diverse ligands is not sufficient to explain their influence on the myriad of physiological and pathophysiological states. It is now appreciated that GPCRs achieve many of their biological actions through 'receptor crosstalk'; a process by which GPCRs can influence the signalling outcomes of other GPCRs and unrelated receptors. This is achieved primarily either via heterologous desensitisation or direct receptor interaction (heteromerisation).

Desensitisation is an important regulatory mechanism which has evolved to prevent the overstimulation of receptors in the presence of continuous agonist stimulation (Lefkowitz et al. 1992; Lefkowitz and Shenoy 2005). When an agonist activated GPCR is, itself, desensitised to prevent further signal transduction, this is described as homologous desensitisation. In the case where the continuous activation of one GPCR results in the desensitisation of another, often inactivated, GPCR or unrelated receptor this is termed as heterologous desensitisation. Heterologous desensitisation can be viewed as an indirect modulation of a third-party receptor signalling system. Heterologous desensitisation is mediated by intracellular signalling mediators such as second messenger-dependent GPCR kinases (GRKs) and β-arrestin regulatory/signalling proteins and/or by altering the expression of receptor proteins. β-arrestin was originally discovered in the context of GPCR signalling regulation; it is responsible for attenuating G protein-dependent GPCR signalling and modulating GPCR endocytosis. However, β-arrestin is now appreciated as a key signalling protein. It functions as a molecular scaffold for pathways including MAPK (Shukla et al. 2011). Beyond its recognition as a GPCR signalling protein, it has been implicated in the negative regulation of TLR-mediated signalling. β-arrestin 2 has been demonstrated to interact with and prevent post-translational modification of TRAF6 (Wang et al. 2006) and IKBα (Gao et al. 2004) and therefore prevent the activation of NF-Kβ in both cases (Fig. 4). Further demonstrating the important regulatory role of β-arrestin 2 on TLR4 signalling, mice lacking β-arrestin 2 were reported to attenuate IL-10, a key cytokine which inhibits the production of proinflammatory cytokines following TLR stimulation (Li et al. 2014). These mice when treated with LPS were shown to be more susceptible to LPS-induced septic shock. Li and co-workers determined β-arrestin 2 negatively regulated TLR4-

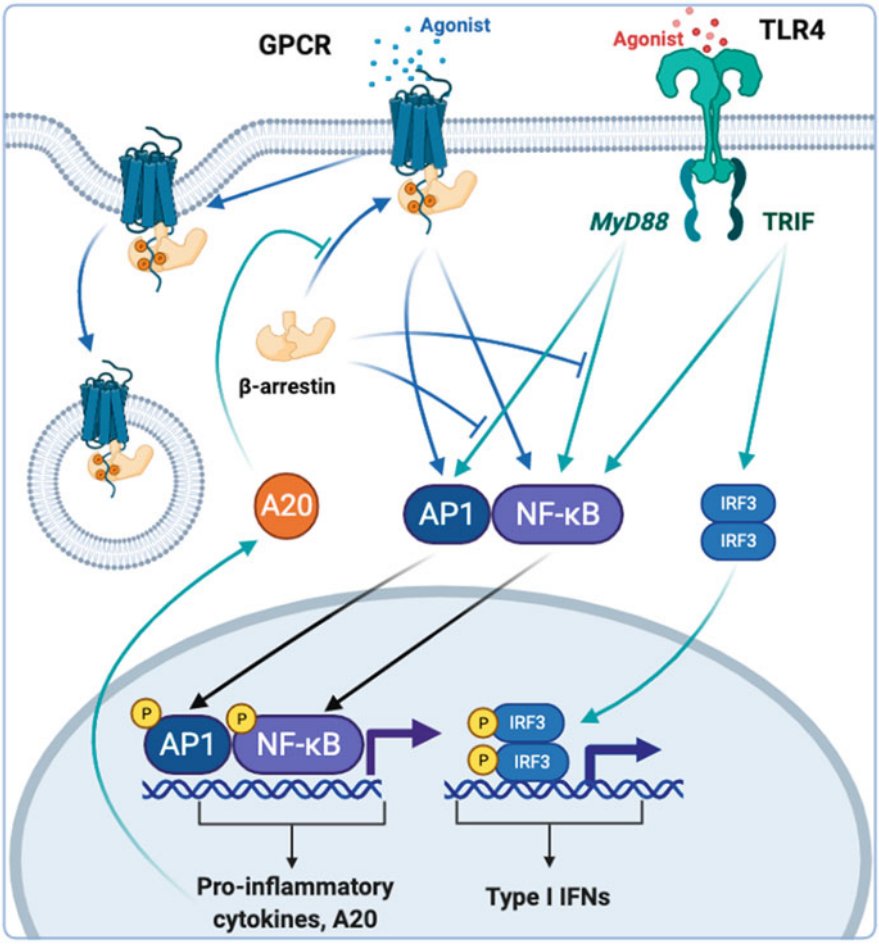

Fig. 4 Proposed mechanisms of direct and indirect TLR4-GPCR interactions. Consequences of TLR4 activation (green arrows) include the generation of proinflammatory cytokines, Type 1 IFNs and activation of inflammatory regulators such as A20. A20 activation results in the regulation of β-arrestin recruitment, and therefore, receptor function and trafficking. Consequences of GPCR signalling proteins and receptor activation (blue arrows) include negative regulation and activation of signalling pathways common to those downstream of TLR4 activation (black arrows). Adapted from 'TLR4/5/7/8 Signalling Cascade', by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

mediated inflammatory responses via regulation of p38 MAPK and resulting IL-10 expression. In this context, β-arrestin 2 can be viewed as an important player in preventing excessive inflammation. Therapies targeting the activity and/or expression of β-arrestin 2 in specific cells critical for the transition from acute to persistent pain may prove to be fruitful in preventing chronic disease.

GPCR heteromerisation is a more direct approach requiring close proximity of receptor proteins. Heteromerisation requires at least two functional receptor units to form a macromolecular complex, either by directly interacting with each other or through a 'bridging' protein. Critically, a heteromer is defined as such if it demonstrates biochemical properties that are different from those of its individual components (Ferré et al. 2009). Although GPCR-TLR heteromers have yet to be definitively identified, this is a very exciting and active area of research.

Many GPCRs have been implicated in pain, with the opioid receptors being one of the most studied in this respect. GPCRs have also been reported to be implicated in the creation of microglial reactive states – a role often attributed to TLRs (Gu et al. 2021). Taken together with their inherent ability to 'collaborate' with other receptors and signalling systems, it is no surprise that GPCR signalling has been implicated in TLR function in the context of pain. It is important to highlight though that this modulation/communication is bidirectional. A 2008 study by Loniewski was the first study to report a role for TLRs in the regulation of GRKs and arrestins in macrophages. This study reported the activation of TLR4 selectively decreased β-arrestin 1 and GRK5/6 protein expression but increased GRK2 protein expression in in vitro studies (Loniewski et al. 2008). Interestingly, it was demonstrated the localisation of the TLRs (plasma membrane or intracellular) as well as choice of signalling pathway (MyD88 dependent or independent) determined which proteins were regulated. This highlights both the precise nature and the complexity in the regulation of signalling pathways. In the next section, we will highlight two GPCRs, chemokine and opioid receptors, and their relationship with TLR4 function and signalling in pain as an example of how the multidimensionality of pain spans receptor families and classes.

4.2.1 Chemokine Receptors Talk to TLR4

Chemokines are a family of small proinflammatory cytokines that transduce their actions via the G protein-coupled chemokine receptors. Although both chemokines and their receptors have been implicated in pain for over 20 years (Oh et al. 2001), their role in the neuroimmune mechanisms responsible for persistent pain is relatively recent (Knerlich-Lukoschus et al. 2011). C-X-C motif receptor 4 (CXCR4) and its ligand CXCL12 have been extensively investigated for their role in the neuromodulation of pathological pain. This discovery has led to investigations into the relationship between these chemokine receptors and TLRs in pain. Early studies of TLR4 and CXCR4 co-localisation and receptor crosstalk provided support for further investigations (Hajishengallis et al. 2008; Triantafilou et al. 2008). Furthermore, a 2016 study reported TLR4 to be co-localised with CXCL12 and CXCR4 in the spinal dorsal horn of rats with ischemia-reperfusion-induced inflammatory pain but not in control animals (Li et al. 2016). This demonstrated that the relationship between TLR4 and CXCR4 receptor protein expression is dynamic, but no conclusions can be made whether co-expression is a result or cause of pain pathology. Interestingly, the attenuation of CXCL12/CXCR4 expression demonstrated a reduction in the sensation of inflammatory pain in a mechanism similar to direct antagonism of TLR4, by the TLR4 antagonist TAK-242 (Li et al. 2016). Although

this was attributed to the downregulation of cytokines IL-1β and TNF-α, there was no investigation of TLR4/CXCR4 heterodimerisation. However, in a 2012 study reporting high mobility group box protein 1 (HMGB1), a nuclear protein and known TLR4 activator, can not only activate CXCR4 but also form a complex with CXCL12 (Schiraldi et al. 2012) the authors concluded that this could not be explained by heterodimersation of TLR4-CXCR4 as the effect was also observed in cells isolated from TLR4 knockout mice. However, it is not implausible to suggest this lack of detection of a heterodimer may be specific to this ligand combination and does not rule out receptor interaction completely. Nevertheless, a sophisticated relationship between the TLR4 and chemokine receptor signalling pathways exists (likely through downstream signalling pathway such as NF-kβ, MAPK and a series of signal transducers and activators of transcription pathways) and may be open for exploitation for novel pain therapies (Fig. 4).

4.2.2 Opioid Receptors and TLR4 Communicate

To date, opioid agonists are considered the gold standard for acute pain management and are well known to act on the G protein-coupled opioid receptors. However, although acute pain is effectively managed by opioid agonists there is little success or patient benefit in managing chronic pain with opioids. Paradoxically, the use of opioids in persistent pain can enhance the sensitisation of neuronal and immune cells resulting in opioid-induced hyperalgesia (Hutchinson et al. 2010). Multiple systems are at play with the complete multidimensional mechanism yet to be fully understood (King et al. 2005; Kovelowski et al. 2000; Ossipov et al. 2003, 2004, 2005). A contemporary neuroimmune hypothesis has immerged with TLR4-induced microglial reactivity and inflammatory signalling negatively impacting the beneficial opioid analgesia pharmacodynamics.

Hutchinson and co-workers have previously reported that the antagonism of TLR4 increases the magnitude and duration of morphine analgesia (Hutchinson et al. 2007). Furthermore, it has been reported in mice lacking TLR4 there was a significant lack of analgesic tolerance to morphine compared to wild-type and MyD88 knockout mice (Thomas et al. 2022; Liu et al. 2011). Together these suggest a role for TLR4 in analgesic tolerance, for which there is also opposing data (Ferrini et al. 2013; Fukagawa et al. 2013; Mattioli et al. 2014). For example, in a study comparing mutant TLR4 to wild-type mice, no difference in morphine induced hyperalgesia was observed (Ferrini et al. 2013). These discrepancies underscore how complex the biopsychosocial state must be, as it is clear that the absence of TLR4 can be compensated by other systems (Thomas et al. 2022). Further refinement of these models capturing principles of the Kuramoto model and the Fröhlich condensate may help reconcile such challenges.

There is much evidence supporting a connection between the opioid receptor and TLR4 signalling systems including their co-expression in several non-neuronal cell types including glia and macrophages (Maduna et al. 2019; Franchi et al. 2012) and receptor crosstalk via common downstream signalling pathways such as MAPK, PKC and NF-Kβ as described earlier. For example, A20 (a key regulator of TLR4 signalling introduced earlier) has been described by Shao and colleagues as in

inhibitor of β-arrestin 2 recruitment to μ opioid receptor (Fig. 4) (Shao et al. 2020). It is now widely accepted that many of the unwanted side effects of opioids are due to the μ opioid engagement of the β-arrestin 2 signalling pathway (Bohn et al. 2000). In this study, it was demonstrated A20 plays a key role in enhancing the analgesic effects of morphine at the μ opioid receptor by interacting with β-arrestin 2 and inhibiting its interaction with the μ opioid receptor (Shao et al. 2020). This type of multidimensionality of TLR4 actions may be exploited as a feasible target for pain management via A20 modulation.

4.2.3 From Single Receptor Systems to Complex Receptor Systems

The complex relationship between opioids and cytokines in the context of pain has been reviewed previously in detail (Thomas et al. 2015). Briefly, there is evidence in literature supporting the hypothesis chemokines can influence the perception of pain and inhibit opioid-induced analgesia via heterologous desensitisation. Szabo and co-workers demonstrated that DAMGO (μ opioid receptor agonist) treatment of rats pre-treated with a CXCR4 receptor ligand (CXCL12) exhibited a dose-dependent reduction in analgesic responses compared to saline pre-treated control rats (Szabo et al. 2002). Taken together with reports that CXCR4 forms a complex with δ opioid receptor (Pello et al. 2008), it is no surprise that heterodimerisation between CXCR4 and μ opioid receptors has been investigated as a possible mechanism for receptor crosstalk. A very recent study by Ma and co-workers has investigated the existence of putative CXCR4 and μ opioid receptor heteromers by developing a bivalent ligand that has the capability to interact with both receptor units simultaneously (Ma et al. 2022). This technique has been reported previously for the investigation of other GPCR heteromers including CXCR4 and μ opioid receptor heteromers (Akgün et al. 2015; Ma et al. 2020). Although this provides some insight into the proximity of the ligand binding sites of the receptors, further demonstration of the effect on receptor heteromer specific signalling profiling and co-internalisation such as provided for the GPCR heteromer 1A-adrenoceptor-CXCR2 would add further weight (Mustafa et al. 2012). It is also important to consider heterodimerisation may only represent one of many mechanisms by which CXCR4 and μ opioid receptors crosstalk. It is likely a more complex relationship between these receptor systems exists. Furthermore, as it has been established that CXCR4 and μ opioid receptors both co-localise with TLR4 in neuroanatomical structures important for pain processing, such as dorsal root ganglion and spinal cord dorsal horn (Li et al. 2016), it is not inconceivable that these receptor protein signalling systems work in concert, regulating each other's signalling outcomes to achieve the complexity seen in chronic pain patients. In order to further our knowledge of pain and the development of effective analgesics, future research approaches should examine the 'holistic signalling system' and not receptor signalling pathways in isolation. Such an approach will enable the true complexity of the biopsychosocial contributors to pain to be appreciated from molecules-to-cells-to-systems. Of course, this raises the issues of nature versus nurture and the profound heritability of pain states. As such, an examination of genomic determinates of health and disease is warranted.

5 Human TLR4 Genetic Polymorphisms

Given the important roles of TLR4 signalling in acute and chronic pain and non-classical opioid pharmacology outlined above, polymorphic variability in *TLR4* has the potential to contribute to interindividual variability in pain and treatment response. Likely reflecting the crucial role of our innate immune system in survival, human TLR genes display relatively little polymorphic variability. For example, only 3 missense or protein truncating polymorphisms with a global minor allele frequency (MAF) greater than 1% have so far been identified in *TLR4* (Howe et al. 2021). The most common of these is rs4986790 (c.896A>G, Asp299Gly), with a global MAF of only 6%, but large inter-population variability in frequency (e.g., 0% in East Asian, but up to 14% in South Asian, populations) (Barratt et al. 2021). It is in strong linkage disequilibrium with the next most common missense SNP, rs4986791 (c.1196C>T, Thr399Ile) (global MAF = 4%). However, functional consequences of the rs49867910-rs4986791 haplotype appear primarily due to rs4986790, which has been associated with reduced TLR4 signalling in vitro, ex vivo and in vivo, without significant effects on expression (Long et al. 2014; Lundberg et al. 2008; Arbour et al. 2000).

Regarding pain specifically, rs4986790 variant genotypes have been associated with lower pain tolerance and higher post-surgical morphine requirements (Barratt et al. 2021), as well as increased risk of endometriosis (Latha et al. 2011). This suggests that, at least in some cases, the TLR4 rs4986790 SNP may result in a dysregulated, rather than simply hypo-responsive, in vivo/clinical phenotype. Other, more common, non-coding TLR4 SNPs (e.g., rs1927911, rs2770150, rs2149356) have been associated with altered immunophenotypes. However, they have not yet been investigated for associations with variability in pain phenotypes specifically.

Though in its relatively early stages, research on TLR-related genetics and pain has so far revealed large inter-population variability in frequencies of TLR gene polymorphisms that may contribute to observed 'interethnic' differences in pain. In addition, it has demonstrated that genotypes associated with increased or decreased TLR function in vitro do not necessarily translate to increased or decreased pain, respectively, in vivo/clinically. This may be due to the penetrance of genetic effects in general likely being influenced by life history and clinical context (Somogyi et al. 2016; Rafiei et al. 2012; Barratt et al. 2021) as well as the complexity of systems in which TLRs function, where both increased or decreased function might lead to dysregulation and pathology. Further research on the combined effects of TLR4 signalling pathway genetic polymorphisms on pain, psychoneuroimmune and drug response phenotypes as part of a 'signalling ecosystem' has the potential to lead to a better understanding of key in vivo system regulators for the identification of new drug targets and greater precision in pain management.

6 Pharmacological Modulation of TLR4 Signalling

6.1 Understanding How Opioid Ligands Modulate TLR4 Function Will Inform Drug Design

Literature suggests that opioid ligands can modulate TLR4 expression. Separate in vitro and ex vivo studies have observed both the up and downregulation of TLR4 protein following morphine exposure (Chen et al. 2021; Franchi et al. 2012). Furthermore, there is evidence that opioids can promote the internalisation of TLR4 from the cell surface (Liang et al. 2016). As discussed earlier, many of these actions can be attributed to receptor crosstalk between the opioid receptors and TLR4. However, others may be due to the direct activation of the TLR4 receptor.

The discovery that the opioid receptor antagonists, naltrexone and naloxone, blocked the biological effects of LPS (Liu et al. 2000; Das et al. 1995; Yirmiya et al. 1994) and literature reporting that opioid agonists may competitively bind and activate TLR4 (Wang et al. 2012; Watkins et al. 2009) have raised interesting questions around the actions of opioid ligands on TLR4 and their own role in compromising their analgesic effects via opioid receptors. Opioids including morphine and morphine-3-glucuronide (M3G) have been reported to non-stereoselectively activate TLR4 signalling (Wang et al. 2012). The opioid-dependent activation of TLR4 results in the release of proinflammatory mediators such as nitric oxide, reactive oxygen species, prostaglandin E2, interleukins, interferons and various chemokines which exacerbate nociception and can lead to hyperalgesia. Despite the (−)-isomers of naltrexone and naloxone displaying the same potencies as (+)- isomers (Wang et al. 2016; Hutchinson et al. 2008), (+)-isomers of opioids may have particular application on TLR4 signalling modulation without effecting the beneficial effect of endogenous (−) opioid isomers on opioid receptors (Wang et al. 2020).

Wang and colleagues have demonstrated that (+)-Naltrexone and (+)-naloxone inhibit the LPS-induced activation of IRF3 and prevent IFN-β (Wang et al. 2016). However, they do not inhibit TLR4 dependent activation of either NF-κB or MAPKs. This suggests (+)-naltrexone and (+)-naloxone are TRIF-IRF3-biased TLR4 antagonists (Wang et al. 2016). In vivo studies also showed (+)-naltrexone and (+)-naloxone decrease opioid, cocaine and methamphetamine-induced dependence and addiction (Wang et al. 2019; Hutchinson et al. 2012; Brown et al. 2018; Hutchinson et al. 2010). Based on this data the TRIF-biased (+)-opioid TLR4 antagonists may be suitable targets for future development for opioid addiction and dependence.

To understand the details of the molecular interactions of (+)-naltrexone and its derivatives with TLR4/MD-2 and extend the ligand-based drug discovery, Zhang and co-workers performed in silico and in vitro assays (Zhang et al. 2018). These studies elucidated the innate immune recognition of the opioid inactive (+)-isomers. The calculated binding free energies of (+)-naltrexone and its derivatives in complex with MD-2 via molecular dynamics simulations correlated well with their

experimental binding affinities and TLR4 antagonistic activities. It indicated that the binding free energies would be an excellent criterion to evaluate the antagonistic activities during rational drug design. Increasing the hydrophobicity of substituted group at N-17 improved its TLR4 antagonistic activity, while charged groups disfavoured the binding with MD-2 (Zhang et al. 2018). This provided molecular insight into the innate immune recognition of opioid inactive (+)-isomers and has the potential to aid the development of new (+)-opioid based TLR4 antagonists.

Although TLR4/MD-2 cannot stereoselectively recognise naltrexone isomers, whether TLR4/MD-2 is enantioselective is still unclear. By linking 2 naltrexone units through a rigid pyrrole spacer, three bivalent ligands ((+)-norbinaltorphimine [(+)-1], (−)-norbinaltorphimine [(−)-1] and the meso isomer of norbinaltorphimine [2]) were formed. Surprisingly, (+)-1 showed ∼25 times better TLR4 antagonist activity than naltrexone in the microglial BV-2 cell line, whereas (−)-1 lost its TLR4 activity (Zhang et al. 2019). The enantioselectivity of norbinaltorphimine was further confirmed in primary microglia, astrocytes and macrophages (Zhang et al. 2019). By rebuilding the binding energy profile, molecular dynamic simulations further uncovered the mechanism of enantioselectivity: the stereochemistry of (+)-1 is derived from the (+)-naltrexone pharmacophore. This is the first report showing enantioselective modulation of the innate immune TLR signalling and is an exciting development for future pain targeted therapies.

6.2 Biased Signalling and the Future of TLR4 Signalling Modulation for Pain Management

As discussed earlier, evidence in literature suggests blockade of TLR4 signalling provides beneficial outcomes in pain pathologies and opioid dependence (Hutchinson et al. 2008; Bettoni et al. 2008). For example, following treatment with TLR4 antagonist FP-1, Bettoni and colleagues reported increased morphine analgesia together with reduced hyperalgesia in mice with painful neuropathy. However, complete antagonism of TLR4 may have detrimental impacts on the body's immunity and inflammation status. There is now increasing evidence supporting the role of the MyD88 pathway in pain pathophysiology (Lacagnina et al. 2018; Liu et al. 2017). Exploiting the potential of TLR4 to signal via two distinct signalling pathways (biased signalling) presents exciting opportunities to intelligently modulate TLR4 signalling and function to manage pain, without impacting its role in immunity and inflammation, recently reviewed (Lin et al. 2021).

Studies investigating the signalling properties of LPS isolated from different bacterial species have highlighted that biased ligands have the capability to selectively activate/deactivate MyD88 or TRIF-dependent signalling pathways (Stephens et al. 2021). Similar to the literature in the GPCR field, where biased signalling and biased ligands have been extensively researched (Kenakin 2011), it is clear that the interaction between a ligand/receptor protein results in the stabilisation of different receptor conformations. Each receptor conformation then either reveals or uncovers specific binding sites for specific intracellular signalling adapter proteins resulting in

the activation/deactivation of the chosen pathway. Although biased ligands have the power to influence the signalling and ultimately biological outcome of TLR4 signalling, it is important to note that the microenvironment of the endosome is very different to that of the plasma membrane (for example, lipid composition and the pH). By promoting unique TLR4 dimer conformations, the microenvironment may also play a role in biased signalling.

Examples of how TLR4 biased signalling can be exploited already exist where TLR4 activators are included as adjuvants in vaccines for their immunostimulatory properties (Mata-Haro et al. 2007; Bowen et al. 2012; Richard et al. 2020). In the study by Mata-Haro and co-workers, it was demonstrated that monophosphoryl lipid A (MPLA), a low-toxic derivative of LPS used as an adjuvant in human vaccines, displays reduced MyD88-dependent signalling activity, but strong TRIF-dependent signalling (Mata-Haro et al. 2007). In a study where the vaccine adjuvant CRX-547 was utilised, a minor structural modification to the carboxyl bioisostere corresponding to the 1-phosphate group on most lipid A types resulted in a TRIF-selective signal (Bowen et al. 2012). Much can be learnt from these examples when identifying and designing TLR4 biased ligands for chronic pain management.

Our understanding of biased signalling has been increased by investigating the impact of the varying length of lipid A chains from LPS isolated from different bacterial species and human pathogens (Maeshima and Fernandez 2013; Stephens et al. 2021). Structure-activity relationship (SAR) studies have provided evidence that structure of the LPS can result in varying degrees of inflammation due to the preferential activation of the signalling pathways (Maeshima and Fernandez 2013). However, it can be argued that an important approach to identify biased ligands is structure-based drug design (SBDD). Molecular dynamics is an important tool, which has the power to move beyond ligand docking to biased receptor structures of TLR4, for predicting the stable interactions between ligands and biased TLR4 receptor states. Unfortunately, due to the lack structural data representing the 'biased' conformations of TLR4 receptor, this is not currently possible.

Therefore, another approach to identifying biased ligands is to screen compound libraries. The downstream factors of TLR4 signalling are essential markers for the screening and discovery of biased modulators of TLR4. The secretion of IFN-β and CXCL10 are often used for the discovery of TRIF-biased TLR4 small molecule modulators as they are only dependent on TLR4-induced TRIF signalling (Bowen et al. 2012; Richard et al. 2020). Given that the internalisation of TLR4 into endosomes is crucial for the activation of TRIF-dependent signalling (Park et al. 2009), TLR4 internalisation can also be used as a specific marker of TRIF-biased signalling. An antibody described by Zanoni and co-workers, SA15-21, is reported to recognise both the monomer and dimer forms of TLR4. Although laborious and not high throughput, this antibody could be potentially used to examine the endocytosis of TLR4 during compound screening (Zanoni et al. 2011). However, among the downstream proteins of TLR4 signalling, no cytokine or chemokine has been found to be strictly dependent on the MyD88 signalling pathway. A lack of tools to identify TLR4-induced MyD88 signalling may explain why there are no reported MyD88-biased small molecule modulators of TLR4 signalling (Lin et al. 2021). This obstacle

can be overcome by utilising proximity-based assays to directly identify the interaction of TLR4 protein with signalling adapter proteins MyD88 or TRIF (Lin et al. 2021). This technology has the capability to identify in real-time, which signalling pathway has been activated. Alternatively, the ubiquitination of IL-1R-associated kinase-1 (IRAK1) is specific to the MyD88-dependent signalling (Conze et al. 2008). Therefore, the measurement of the polyubiquitination of IRAK1 by in-cell Western assay would be an excellent way to screen and identify MyD88-biased small molecule modulators, potentially for novel pain indications in the future.

7 Conclusion

We have provided an overview of TLR4 signalling and regulation in pain. Importantly, this review highlights the need to examine and appreciate the role of TLR4 signalling and regulation as part of a 'signalling ecosystem'. This takes into consideration the many other proteins and signalling pathways which modulate TLR4 signalling or are themselves modulated by TLR4 and its signalling partners. By doing so, only then we may be able to understand the deep and complex mechanisms that result in the biopsychosocial nature of pain. Although the complex interactions may be challenging and take time to decipher fully, it is clear that TLR4 receptor signalling provides multiple mechanisms for modulating the unwanted effects of opioid analgesics and the management of complex pain pathologies. Optimistically, TLR4 may provide one of the missing 'bridges' to the future of precision medicine and pain management.

Acknowledgements This work was supported by the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics (CE140100003), Australian Postgraduate Award, an Australian Research Council Future Fellowship (FT180100565) and the University of Adelaide Davies Livestock Research Centre.

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Toll-Like Receptor 4: A Novel Target to Tackle Drug Addiction?

Jianfeng Liu, Jun-Xu Li, and Ruyan Wu

Contents

Abstract

Drug addiction is a chronic brain disease characterized by compulsive drug-seeking and drug-taking behaviors despite the major negative consequences. Current well-established neuronal underpinnings of drug addiction have promoted the substantial progress in understanding this disorder. However,

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2022_586

non-neuronal mechanisms of drug addiction have long been underestimated. Fortunately, increased evidence indicates that neuroimmune system, especially Toll-like receptor 4 (TLR4) signaling, plays an important role in the different stages of drug addiction. Drugs like opioids, psychostimulants, and alcohol activate TLR4 signaling and enhance the proinflammatory response, which is associated with drug reward-related behaviors. While extensive studies have shown that inhibition of TLR4 attenuated drug-related responses, there are conflicting findings implicating that TLR4 signaling may not be essential to drug addiction. In this chapter, preclinical and clinical studies will be discussed to further evaluate whether TLR4-based neuroimmune pharmacotherapy can be used to treat drug addiction. Furthermore, the possible mechanisms underlying the effects of TLR4 inhibition in modulating drug-related behaviors will also be discussed.

Keywords

Alcohol · Drug addiction · Non-neuronal mechanisms · Opioid · Psychostimulants · Toll-like receptor 4

1 Introduction

Drug addiction is a chronic brain disease characterized by compulsive drug-seeking and drug-taking behaviors despite the major negative consequences (Cheron and Kerchove d'Exaerde 2021). It is one of the leading causes of disability and fatality worldwide today, with a huge annual cost related to crime, reduced work productivity and health care (Nestler and Luscher 2019). Current studies focusing on neuronal adaptations have yielded much progress in the research of drug addiction. For example, it is suggested that molecular, synaptic, and neurocircuitry neuroadaptations combine to promote the transition to drug addiction, which is comprised of increased incentive salience, decreased reward, increased stress, and decreased executive function (Wise and Koob 2014). However, non-neuronal underpinnings of drug addiction have long been underestimated (Kashima and Grueter 2017). Fortunately, a growing body of studies indicate that neuroimmune system plays an important role in the different stages of drug addiction, including binge/intoxication, withdrawal, and relapse (Hutchinson et al. 2012; June et al. 2015; Northcutt et al. 2015).

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PPRs) in the innate immune system which detect and respond both to exogenous pathogen associated molecular patterns (PAMPs) and endogenous danger associated molecular patterns (DAMPs) (Koropatnick et al. 2004; Hennessy et al. 2010; Connolly and O'Neill 2012). Toll-like receptor 4 (TLR4) is one of the TLRs and its activation leads to enhanced production of proinflammatory cytokines and chemokines. In the brain, TLR4 is mainly expressed in glial cells like microglia and astrocytes (Vaure and Liu 2014). Upon recognition of PAMPs or DAMPs, TLR4 signals through two distinct

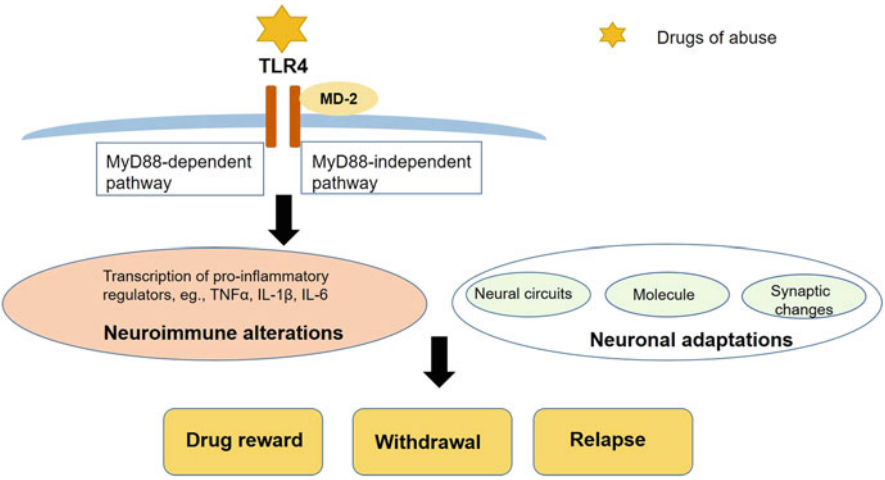

Fig. 1 Schematic representation of the role of toll-like receptor 4 (TLR4) signaling in drug addiction. It should be noted that the non-neuronal mechanism underlying drug addiction is not clear. Drugs of abuse bind to the accessory receptor of TLR4, MD-2, and activate the downstream signaling which comprised of two distinct pathways (MyD88-dependent and MyD88-independent pathways). This activation leads to the transcription of proinflammatory regulators like TNF-α and IL-β and enhances non-neuronal alterations, which subsequently act in concert with neuronal adaptations and contribute to drug reward-related behaviors, withdrawal and relapse

pathways, the myeloid differentiation primary response protein 88 (MyD88)-dependent and MyD88-independent pathway (Kawai and Akira 2007a). In the MyD88-dependent pathway, the signal transduces through activation of Interleukin-1 receptor associated kinases (IRAKs, like IRAK1 and IRAK4) and TNF receptor associated factor 6 (TRAF6), which subsequently promotes the phosphorylation of inhibitors of nuclear factor κB kinases (IKK). The activation in turn leads to the NFκB activation and the production of proinflammatory cytokines and chemokines (Kawai and Akira 2007b). Alternatively, in MyD88-independent pathway, the adaptor protein TRIF, TRAF3 and interferon regulatory factor 3 (IRF3) are involved (Takeda and Akira 2005) (Fig. 1).

TLR4 signaling is suggested to be involved in several neuropsychiatric disorders, including major depressive disorders, neurodegenerative disorders, and impulsive control (Nie et al. 2018; Landreth and Reed-Geaghan 2009; Aurelian et al. 2016; Garcia Bueno et al. 2016; Liu et al. 2019). As drugs of abuse can be considered as "exogenous," it is recognized that drugs of different class activate TLR4 signaling and induce proinflammatory responses. Emerging evidence has suggested the important role of TLR4 signaling in regulating drug addiction (Crews et al. 2017). In this chapter, we will discuss the preclinical and clinical evidence of TLR4 signaling modulation in drug addiction (i.e., opioid, psychostimulants, and alcohol addiction), in order to evaluate whether TLR4-based neuroimmune pharmacotherapy can be used as novel treatment for this disorder. Furthermore, we will also discuss the

possible mechanisms underlying the effects of TLR4 antagonism in regulating drug-related behaviors.

2 Role of TLR4 Signaling in Drug Addiction

2.1 Opioid

Although the major targets of opioids in the brain are opioid receptors, which probably mediate most of the effects of opioids within the CNS, growing evidence has demonstrated that opioids can also interact with TLRs, among which the TLR4 is best studied in opioid addiction. In vitro evidence suggests that the molecular interaction between the opioid system and TLR4 is complex. The opioid antagonist naloxone inhibited the classic TLR4 agonist LPS-induced secretion of IL-β and morphological changes of microglia in mixed brain cell cultures (Das et al. 1995). In contrast, both morphine and fentanyl could activate TLR4 in unstimulated cells, even though the activation level was much lower than that was stimulated by LPS (Hutchinson et al. 2010). Morphine exposure could elevate TLR4 protein and mRNA expression as well as activate TLR4-related signaling pathways in the Nucleus Accumbens (NAc) (Schwarz and Bilbo 2013). Interestingly, morphine and fentanyl could attenuate LPS-induced activation of TLR4 in a non-competitive manner (Hutchinson et al. 2010). These findings suggest that opioids might interact with TLR4 and act as its partial agonists. Besides in vitro reports, many behavioral studies have explored the role of TLR4 in mediating the effects of opioids, including addictive properties (Gabr et al. 2021).

Many pharmacological studies using the TLR4 antagonists such as (+)-naloxone and lipopolysaccharide from Rhodobacter sphaeroides (LPS-RS) have implicated that TLR4 participates in the development of opioid addiction and relapse. (+)-Naloxone blocked morphine-induced conditioned place preference (CPP), remifentanil self-administration, drug-induced reinstatement of heroin-seeking behavior, and dopamine release in the NAc (Hutchinson et al. 2012; Yue et al. 2020). Another study found that microinjection of TLR4 antagonist LPS-RS into the ventral tegmental area (VTA) prevented the conditioning and maintenance, but not expression, of morphine-induced CPP (Chen et al. 2017). In the same study, it was suggested that the STAT3 might mediate the function of TLR4 since LPS-RS prevented morphine-induced activation of STAT3 in the VTA (Chen et al. 2017). Interestingly, microinjection of LPS-RS into the NAc did not affect drug-induced reinstatement of heroin-seeking, suggesting that the NAc might not be the critical brain site where TLR4 regulates opioid addiction (Yue et al. 2020). Consistent with pharmacological findings, global deletion of tlr4 or myd88 gene prevented oxycodone-induced CPP in mice (Hutchinson et al. 2012). Studies that evaluated the effects of ibudilast also provided some implications on the role of TLR4 in opioid addiction. Ibudilast is principally a Phosphodiesterase 4 (PDE4) inhibitor but also exerts antagonist property at TLR4. Moreover, ibudilast could decrease morphine-induced dopamine release in the NAc in rodents (Bland et al. 2009).

Opioid withdrawal has been demonstrated to participate in the development of opioid addiction via a negative reinforcing mechanism (Koob and Volkow 2010). Several pharmacological studies have indicated that TLR4 also regulates opioid withdrawal. The TLR4 antagonist (+)-naloxone could significantly attenuate the μ opioid receptor antagonist (−)-naloxone-precipitated withdrawal behavior in morphine-dependent rats (Hutchinson et al. 2010). The TLR4 antagonist ibudilast reduced spontaneous withdrawal-induced hyperactivity in rats (Hutchinson et al. 2009). In contrast, the genetic deletion of TLR4 genes did not affect opioid withdrawal. Compared to wildtype Balb/c mice, both TLR4-KO and MyD88-KO mice (Balb/c background) showed similar degrees of naloxone-precipitated jumping behavior, an animal model of opioid withdrawal (Liu et al. 2011). A more recent study also reported similar findings that both TLR4 mutant and null mice showed normal morphine withdrawal behaviors (Mattioli et al. 2014). These findings suggest that the tlr4 gene might not be critical for opioid withdrawal. However, it should be noted that global deletion of TLR4 or MyD88 genes may result in changes in many other genes that could compensate for the loss in the function of TLR4 signaling. Therefore, future studies using conditional deletion of TLR4 are required to address the role of the tlr4 gene in the development of opioid addiction.

Nevertheless, not all literature supports the view that TLR4 mediates opioid addiction. Acute injection of (+)-naltrexone did not affect incubated cue-induced heroin-seeking or extended access heroin self-administration. Whereas chronic administration of (+)-naltrexone reduced incubated cue-induced heroin-seeking but did not affect ongoing extended access heroin self-administration (Theberge et al. 2013). One explanation is that TLR4 signaling might only participate in some particular opioid addiction-related behaviors. Furthermore, many factors such as opioid dose, history of drug use, and treatment strategy (i.e., acute or chronic treatment) are essential factors that might dramatically influence the pharmacological effects of TLR4 antagonists on opioid addiction.

In clinical settings, TLR4 antagonist ibudilast was tested for its efficacy in attenuating opioid-related effects. Ibudilast was shown to reduce ratings of drug liking following 15 mg of oxycodone and heroin craving (Metz et al. 2017). Meanwhile, ibudilast also decreased drug breakpoint under the 15 mg but not 30 mg oxycodone condition in a progressive-ratio oxycodone self-administration task, suggesting that ibudilast attenuated, at least to some extent, the reinforcing effects of oxycodone (Metz et al. 2017). On the contrary, ibudilast was unable to consistently affect subjective effect ratings of oxycodone in opioid-dependent volunteers in another study (Cooper et al. 2017). Nevertheless, it decreased ratings of withdrawal symptoms on some SOWS items during detoxification (Cooper et al. 2016).

2.2 Psychostimulants

2.2.1 Cocaine

Cocaine activates innate immune system within the brain through its interaction with TLR4 (Cearley et al. 2011; Coller and Hutchinson 2012), possibly in a region-specific manner (Burkovetskaya et al. 2020). Cocaine docked to the same binding site of MD-2 as the classical TLR4 agonist LPS and increased the proinflammatory responses. This effect is associated with cocaine-induced dopamine release and cocaine reward, an effect that can be blocked by TLR4 antagonist (+)-naloxone (Northcutt et al. 2015). Pretreatment of (+)-naloxone or LPS-RS attenuated cocaine-induced elevation of extracellular dopamine in the NAc, while they alone did not affect the dopamine signaling. Meanwhile, non-TLR4 modulator, neurotensin, did not affect cocaine-induced dopamine elevation, suggesting the specificity to TLR4 receptor. Moreover, pretreatment of TLR4 antagonists blocked the development of cocaine CPP and self-administration, while sparing food-maintained responses (Northcutt et al. 2015). Consistently, TLR4 mutant mice showed less responses to cocaine self-administration and cocaine reward learning, suggesting the importance of TLR4 in cocaine reinforcement (Kashima and Grueter 2017; Northcutt et al. 2015).

However, inconsistent findings suggest that TLR4 may not be crucial for cocaine-related behavioral and neurochemical alterations. Tanda and colleagues found that (+)-naloxone or (+)-naltrexone did not decrease cocaine or heroin-induced dopamine levels in the NAc shell (Tanda et al. 2016). Both antagonists attenuated cocaine or remifentanil self-administration at a higher dose that decreased food-maintained responding as well, suggesting a lack of selectivity on reward behaviors (Tanda et al. 2016). In addition, (+)-naloxone did not interact with cocaine subjective effects in the drug-discrimination studies (Tanda et al. 2016). It is further shown that a TLR4 agonist reactivated microglia, suppressed striatal synaptic strength, and finally decreased cocaine-induced sensitization (Lewitus et al. 2016). These results challenge the current knowledge of TLR4 in cocaine addiction, yet call for further examination and clarification of the role of TLR4 in cocaine-related responses.

A recent clinical study showed that cocaine users had a significant increase in IL-6 compared with control group, demonstrating an activation of the immune system (Moreira et al. 2016). Nonetheless, there are few clinical studies examining the effect of neuroimmune modulators in regulating cocaine addiction. More clinical investigations focusing on the possibility of neuroimmune signaling as novel therapeutic target for cocaine addiction are needed.

2.2.2 Methamphetamine

Methamphetamine (METH) exposure activates glia cells and enhances proinflammatory cytokines release (Goncalves et al. 2008; Loftis et al. 2011; Nakajima et al. 2004). Indeed, METH was shown to bind to MD-2, the key receptor of TLR4 and enhanced CD11b and IL-6 in mRNAs in the VTA (Wang et al. 2019). Increased evidence suggests that modulation of TLR4 can reduce METH-related behavioral and neurochemical effects (Fujita et al. 2012; Narita et al. 2006; Zhang

et al. 2006). TLR4 antagonists (+)-naloxone and LPS-RS reduced METH-induced elevation of dopamine in the NAc (Wang et al. 2019). Ibudilast, AV1013, and minocycline decreased METH-induced behavioral sensitization, drug-primed and cue-induced METH-seeking (Snider et al. 2012; Beardsley et al. 2010), METH-induced conditioned place preference (CPP) (Fujita et al. 2012; Chen et al. 2009) and METH self-administration (Snider et al. 2013). These findings indicate an essential role of glia activation underlying the rewarding effects of METH. Interestingly, it is also implicated that cannabinoids Δ9-tetrahydrocannabinol and cannabidiol might be effective for protection of METH-induced inflammation through modulation of TLR4 and NF-κB signaling (Majdi et al. 2019).

Clinical studies also yielded inspiring results that neuroimmune modulators could be effective against METH-related symptoms. Initially, a case study reported that minocycline significantly improved the psychotic symptoms in METH use disorders (Tanibuchi et al. 2010). Later, in an early-stage study, ibudilast reduced several METH-related subjective effects including High, Good, Stimulated and Like, suggesting its effect in attenuating the reward-associated subjective effects of METH (Worley et al. 2016). Moreover, ibudilast is also shown to improve the attention performance during the early abstinence from METH dependence (Birath et al. 2017). All these results implicated that neuroimmune modulators may have protective effects on METH-related disorders. However, a most recent clinical trial showed that ibudilast did not affect METH abstinence (Heinzerling et al. 2020). This randomized, placebo-controlled trial included 64 patients with METH use disorders for the 12-week ibudilast treatment and urine specimen was collected for drug screen and study assessments (Heinzerling et al. 2020). Ibudilast was well tolerated yet did not alter METH abstinence (Heinzerling et al. 2020). No significant correlation between serum ibudilast levels and METH use during treatment for patients was observed (Heinzerling et al. 2020). These results seem discouraging, yet it is still early to conclude that ibudilast has no effect on METH-related actions. No further assessment on the effect of ibudilast on METH intake or craving was provided. Indeed, a pilot clinical study showed that ibudilast could reduce METH-induced elevation of peripheral markers of inflammation, which may underlie the mechanisms of METH addiction. As such, more research investigating the effects of TLR4 modulation in METH-taking or relapse could add valuable information to the field.

2.2.3 Nicotine

Currently, there are no studies examining the role of TLR4 in nicotine addiction. Although it is suggested that nicotine increased the expression of TLR4 and also upregulated TLR4-related proinflammatory responses in vitro (Yin et al. 2014; Hu et al. 2012; Xu et al. 2014), less is known about whether TLR4 is involved in nicotine reward or withdrawal. Interestingly, a recent clinical study showed a potential association between *TLR4* polymorphism and lifetime smoking (Zerdazi et al. 2017). Based on the study from 514 bipolar disorder patients, El-Hadi and colleagues found that rs10759932 was significantly associated with tobacco smoking (Zerdazi et al. 2017). This finding suggests the involvement of TLR4 in smoking,

or further, nicotine addiction. However, studies also suggest that nicotine attenuates neuroinflammation induced by microglia activation in the brain (Park et al. 2007; Lutz et al. 2014), possibly through TLR4 signaling (Li et al. 2021). Nicotine and its metabolite cotinine targeted TLR4 co-receptor, MD-2, and inhibited LPS-induced production of TNF-α and nitric oxide, and further blocked microglia activation (Li et al. 2021). Moreover, this effect cannot be abolished by nicotinic acetylcholine receptor (nAChR) inhibitor or nAChRs siRNA (Li et al. 2021). These results seem inconsistent and add more complexity to the role of TLR4 in nicotine response.

2.3 Ethanol

Neuroinflammation contributes to the establishment of addiction of several substances, including alcohol. In vitro and in vivo studies have shown that ethanol produces neuroinflammation at least partially through TLR4 signaling pathway and leads to the activation of NFκB (Blanco et al. 2005; Fernandez-Lizarbe et al. 2009). For example, adolescent binge drinking increases the TLR4 expression in the adult prefrontal cortex, which is correlated with deficits in reversal learning and increased preservative behaviors (Vetreno and Crews 2012). Bing drinking also promoted the IL-1β mRNA expression in the basolateral amygdala (BLA). Consistently, intra-BLA infusions of IL-1 receptor antagonist (IL-1Ra) decreased the alcohol consumption without altering sucrose drinking and locomotion in mice (Marshall et al. 2016). Furthermore, studies utilized TLR4 transgenic animal models showed that TLR4 deficiency prevented ethanol-induced neuroinflammation along with synaptic changes and long-term behavioral and cognitive alterations (Fernandez-Lizarbe et al. 2009; Pascual et al. 2017; Montesinos et al. 2015; Montesinos et al. 2016; Montesinos et al. 2018; Shukla et al. 2018). Consistently, TLR4 antagonists like (+)-Naltrexone and Nalmefene prevented TLR4 activation and inhibited alcohol-induced upregulations of proinflammatory responses as well as alcohol intake and reward (Jacobsen et al. 2018a, b; Montesinos et al. 2017). However, a recent comprehensive study showed that TLR4 may not be essential to excessive alcohol drinking (Harris et al. 2017). Using different species, different tests of alcohol consumption, and different methods to inhibit TLR4 signaling, they found that TLR4 inhibition did not affect the drinking-in-the-dark or two-bottle choice chronic ethanol intake or ethanol self-administration (Harris et al. 2017). This study questioned the essentiality of TLR4 in alcohol reward. Nevertheless, they did agree on the effect of TLR4 modulation in alcohol-induced sedation and GABA receptor function (Harris et al. 2017).

Despite the complex results from preclinical studies, much efforts have been put on whether TLR4-related neuroimmune responses regulate alcohol intake in patients with alcohol use disorders (AUD). Studies showed that AUD patients had altered TLR4 methylation, which is correlated with alcohol consumption patterns (Karoly et al. 2017, 2018). Post-mortem human also showed upregulated TLR4-related immunoreactivity cells that correlated with lifetime alcohol consumption (Crews et al. 2013), although alcohol withdrawal may have differentiated effects

(Donnadieu-Rigole et al. 2016). In a randomized, placebo-controlled clinical study, however, ibudilast did not affect the subjective responses to alcohol. Meanwhile, it attenuated alcohol-induced stimulant and mood-altering effects in patients with more depressive symptoms (Ray et al. 2017), while other appetitive responses, like craving for high-fat/high-sugar diet, were not altered (Cummings et al. 2018). These results raised a question whether improvement of depressive symptomatology should be considered as a measurement for potential pharmacotherapies. Nevertheless, we are still at the very beginning to examine TLR4 as promising therapeutic target for the treatment of alcohol addiction, more comprehensive studies with larger sample size are warranted.

3 Possible Mechanisms Underlying the Role of TLR4 Signaling in Drug Addiction

Apart from the traditional neuronal mechanisms which involves dopaminergic, glutamatergic, and GABAergic system, drugs of abuse-induced glia activation are believed to contribute to the development of drug addiction. Opioid, psychostimulants, and alcohol all bind to the accessory receptor Myeloid Differentiation factor 2 (MD-2) and activate TLR4. This activation promotes the release of proinflammatory cytokines and chemokines, which subsequently alters the neuroadaptations and synaptic plasticity that is related to drug-induced aberrant behaviors. TLR4 is showed to play a role in NAc synaptic physiology and drug reward behavior (Kashima and Grueter 2017). TLR4-KO animals demonstrated a significantly decreased α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor/N-methyl-D-aspartate (NMDA) receptor ratio (A/N ratio) in the NAc core, suggesting a decrease in postsynaptic strength caused by a reduced AMPAR transmission or increased NMDAR transmission (Kashima and Grueter 2017). Meanwhile, TLR4-KO D1(−) MSNs showed significant slower NMDAR decay kinetics compared with WT, suggesting an altered NMDAR stoichiometry (Kashima and Grueter 2017). Because altered NMDARs in the NAc MSNs are related to behavioral adaptations affecting motivation and reward-associated learning, it is further shown that TLR4-KO mice exhibit deficits in long-term depression in the NAc core, paralleled with deficits in drug reward learning (Kashima and Grueter 2017). These results showed a direct association between TLR4 and drug-induced neuroadaptations.

The downstream effectors of TLR4 may also play a part in regulating drug addiction. Our recent study examined the role of IRAK4, a downstream molecule of TLR4 signaling, in opioid addiction. We found that IRAK4 antagonist PF06650833 reduced cue-induced reinstatement of morphine-seeking and fentanyl-seeking (Wu et al. 2021). Morphine self-administration induced activation of IRAK4 in the NAc, which was accompanied by increases in IKKα/β activity and expression level of soluble TNF-α (Wu et al. 2021). Furthermore, microinjection of RF06650833 into the NAc reduced cue-induced reinstatement of morphine-seeking (Wu et al. 2021). As IRAK4 is one of the keynotes of the TLR4 signaling cascade,

our results might suggest that TLR4 might participate in the cue-induced reinstatement of morphine-seeking via the IRAK4 signaling pathway.

Immune factors like TNF-α and IL-β that are involved in the modulation of synaptic functions probably participate in drug reward as well. TNF-α is a key effector in the TLR4 signaling, and inhibition of TNF-α abolishes TLR4-mediated responses (Kawai and Akira 2010; Eidson et al. 2017). It is reported that TNF-α is involved in cocaine-induced plasticity (Lewitus et al. 2016). Drugs of abuse activate the glia cells in the NAc, which subsequently enhance the production of TNF-α. TNF-α is known to regulate the internalization of synaptic AMPA receptors (Lewitus et al. 2014). A recent study showed that cocaine activates striatal microglia and promotes TNF-α production, which suppresses the glutamatergic synaptic strength in the NAc core (Lewitus et al. 2016). Besides the AMPARs, TNF-α is also suggested to regulate the activity of presynaptic metabotropic glutamate receptors and $GABA_A$ receptors (Bezzi et al. 2001; Stellwagen et al. 2005; Pascual et al. 2012; Domercq et al. 2006). Like TNF-α, IL-β is also activated by TLR4 (Latz et al. 2013). IL-β is associated with long-term potentiation which underlies learning and memory, thus is implicated with drug-related aberrant memory (Rizzo et al. 2018). IL-β decreases glutamate supply through the inhibition of glial glutamate transporter activity, resulting in the attenuation of glutamate-glutamine cycle-dependent GABA synthesis. Moreover, IL-β also participates in the regulation of postsynaptic GABA receptor activity. These modulations are widely associated with synaptic plasticity which may contribute to TLR4 signaling-related neuroadaptations (Wang et al. 2000).

The activation of TLR4 by drugs of abuse produces neuroinflammation as well as neurodegeneration within key brain regions that are involved in drug addiction (Alfonso-Loeches et al. 2010; Pascual et al. 2011; Alfonso-Loeches et al. 2012). Conversely, inhibition of TLR4 abolishes the proinflammatory responses and blocks cell damage (Blanco et al. 2005). For example, neurodegenerations in the prefrontal cortex are associated with the loss of executive functions over behavioral inhibition or a lack of inhibitory control over mesolimbic areas, which may consequently promote the drug-taking behaviors (Crews et al. 2011, 2015). Generally, the loss of control over progression from initial recreational drug use to compulsive drug-taking may promote the development of drug addiction (Wu and Li 2020). Although much evidence has implicated the role of TLR4 and its signaling in drug addiction, the exact mechanisms and process remain unknown. Nevertheless, it should be kept in mind that drugs of abuse activation of TLR4 signaling may work in conjunction with the traditional well-established neuronal mechanisms, as the modulation of central immune system alone did not alter drug-related behaviors (Coller and Hutchinson 2012).

4 Future Directions

While extensive studies have suggested that TLR4 and its signaling play an important role in drug addiction, many questions remain to be answered before TLR4 modulators could be used as potential treatments for alleviating drug abuse-related symptoms. Firstly, conflicting results from preclinical studies suggest the complex effects of TLR4 in regulating drug addiction. Future comprehensive studies that examine the effect of TLR4 modulation in different drug class from different drug addiction stages (i.e. binge/intoxication, withdrawal and relapse) will help establish whether TLR4 is a promising and novel therapeutic target to treat drug addiction. Secondly, the mechanism underlying the effect of TLR4 modulations in drug addiction is no clear. More studies carefully investigate how TLR4-related activation contribute to the progression of drug addiction are urgently needed. More importantly, to answer how TLR4-related non-neuronal system communicate and synergize with the well-known neuronal system will help tremendously in understanding the mechanisms underlying drug addiction. Last but not least, increased recognition of TLR4 in regulating drug addiction leads to a growing interest in clinical investigations. However, we are still far away from reaching a solid conclusion from clinical settings that TLR4 modulators could be potential pharmacotherapies for drug addiction. Future randomized and placebo-controlled clinical studies with large sample size, which examine the long-term safety, tolerability, and efficacy of TLR4-based neuroimmune pharmacotherapies are warranted.

5 Conclusion

Drugs of abuse activate TLR4 and its signaling and enhance the production of proinflammatory cytokines and chemokines. Modulations of TLR4 and its signaling are shown to be involved in addiction to drugs from different class, including psychostimulants, opioids, and alcohol. Furthermore, increased evidence has suggested that TLR4 and related glial cell modulators could be potential treatments for addiction-related behaviors. This is a thriving topic that requires more comprehensive studies for both target validation and clinical efficacy verification to reshape the treatment for drug addiction.

Acknowledgements RW was partially supported by the National Natural Science Foundation of China (Grant 81701340) and Natural Science Foundation of Jiangsu Province (Grant BK 20170517). JXL was supported by National Institutes of Health National Institute on Drug Abuse (Grant R01DA047967).

Conflict of Interest The authors declare no conflicts of interest.

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Toll-Like Receptors as Drug Targets in the Intestinal Epithelium

Declan P. McKernan

Contents

Abstract

Toll-like receptors (TLRs) receptors are responsible for initiation of inflammatory responses by their recognition of molecular patterns present in invading microorganisms (such as bacteria, viruses or fungi) or in molecules released following tissue damage in disease states. Expressed in the intestinal epithelium, they initiate an intracellular signalling cascade in response to molecular patterns resulting in the activation of transcription factors and the release of cytokines, chemokines and vasoactive molecules. Intestinal epithelial cells are exposed to microorganisms on a daily basis and form part of the primary defence against pathogens by using TLRs. TLRs and their accessory molecules are subject to tight regulation in these cells so as to not overreact or react in unnecessary circumstances. TLRs have more recently been associated with chronic inflammatory diseases as a result of inappropriate regulation, this can be damaging and lead

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Handbook of Experimental Pharmacology, https://doi.org/10.1007/164_2021_563

to chronic inflammatory diseases such as inflammatory bowel disease (IBD). Targeting Toll-like receptors offers a potential therapeutic approach for IBD. In this review, the current knowledge on the TLRs is reviewed along with their association with intestinal diseases. Finally, compounds that target TLRs in animal models of IBD, clinic trials and their future merit as targets are discussed.

Keywords

Anti-inflammatory drugs · Inflammatory bowel disease · Intestinal epithelium · Toll-like receptors

1 Introduction

Inflammation can be initiated by infection, tissue injury or tissue stress and malfunction. The cardinal signs of inflammation are fever, redness, swelling and oedema, pain and loss of function (Taylor 1997). These hallmarks of inflammation are a means to an end, the end being the removal of the source of the noxious insult, the protection of the affected tissues, and ultimately the restoration of homeostasis. It generally involves the delivery of cellular and molecular components to the site of the noxious insult or infection via the circulatory system to complete these actions (Medzhitov 2008). Inflammatory mediators such as cytokines, chemokines and vasoactive peptides are produced by cells such as myeloid cells, lymphocytes, endothelial cells, epithelial cells and fibroblasts which are resident in the injured or infected tissue (Fullerton and Gilroy 2016). These mediators facilitate the entry of immune cells, such as neutrophils that are normally restricted to the vasculature, into the inflamed or infected tissue. Components of the mediators produced upon the initiation of the immune response allow the entry of these immune cells into the extravascular spaces (Friedl and Weigelin 2008).

Pattern recognition receptor (PRR) activity is a core component of the inflammatory response. These receptors play a key role in the recognition of and initiating an appropriate and regulated response to noxious insults. Many PRRs have been identified and can be grouped into four main families of receptors, the RIG-like receptors (RLRs), the NOD-like receptors (NLRs), the C-type lectin receptors and finally the Toll-like Receptors (TLRs). It has been reported in a number of disease states that PRR signalling can become dysregulated and therefore is a good potential candidate for therapy of such diseases (McKernan 2020). This review will discuss the current knowledge specifically on Toll-like receptors in the gastrointestinal epithelium, their potential as drug targets in chronic gastrointestinal diseases and also review molecules that target them that have recently been tested in animal models of disease and in clinical trials.

2 The Gastrointestinal Epithelium

While normally thought of for its role in digestion and absorption of nutrients, the gastrointestinal epithelium plays an essential part in the innate immune response. Resident cells in the epithelium produce antimicrobial molecules, mucus and also communicate with the trillions of resident microorganisms (including bacteria, fungi and viruses) known as microbiota and immune cells resident underneath the layer. Most of these microorganisms are not immediately harmful to the host with some intimately involved in host food and xenobiotic metabolism as well as vitamin production. Others produce molecules such as bacteriocins capable of destroying pathogenic bacteria (Gilbert et al. 2018). Many of these microorganisms are in constant communication with host cells directly or indirectly, sometimes releasing effector molecules, other times involving direct cell-to-cell interactions or phagocytosis which can facilitate tolerance (Belkaid and Harrison 2017; Donaldson et al. 2016).

The epithelium is only a single layer thick but maintains its own stem cell niche at the base of the crypts of Lieberkuhn with the layer replacing itself every 4–5 days (McKernan and Egan 2015; Gunther et al. 2013). This niche is responsible for generating both absorptive cells like enterocytes that transport digested food metabolites into the blood and secretory cells like Paneth cells, Goblet cells and enteroendocrine cells that produce mucus, antimicrobial peptides and hormones, respectively (Kurashima and Kiyono 2017). A wide variety of myeloid cells such as dendritic cells and macrophages are in direct contact with the basolateral (serosal) side of the epithelial cell layer (Farache et al. 2013b). Additionally, there are intraepithelial lymphocytes that protrude through intercellular gaps allowing them to sample luminal contents (Van Kaer and Olivares-Villagomez 2018) as well as lymphocytes located in the underlying lamina propria (Zeng et al. 2016; Rakoff-Nahoum et al. 2004) or in discrete follicles like Peyer's patches (Deshmukh et al. 2014). There are also activated leukocytes that are trafficked to the intestine where they can undergo antigen priming (Habtezion et al. 2016). Pattern recognition receptors like Toll-like receptors expressed in the intestinal epithelium are key to the communication between a variety of the cell types mentioned above (Abreu 2010; Allaire et al. 2018; Pott and Hornef 2012).

3 Toll-Like Receptors

Toll-like receptors (TLRs) recognise patterns present in structurally conserved molecules called pathogen associated molecular patterns (PAMPs) in bacteria and viruses as well as damage associated molecular patterns (DAMPs) from molecules released from damaged or dying cells (Fitzgerald and Kagan 2020). These receptors of which there are 10 in humans are located in intracellular and extracellular compartments of cells (Blasius and Beutler 2010). TLRs are widely expressed in many different tissues with basal expression levels varying between tissues (Nishimura and Naito 2005; Hennessy and McKernan 2016). Activation of these

receptors usually requires homo- or heterodimerisation of receptors and results in a signal transduction cascade that leads to the activation of transcription factors in the cytoplasm like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon regulatory factors (IRF) 3/5/7. These factors then translocate to the nucleus increasing the transcription of cytokines like interleukins (IL1, IL6, IL10) and type I interferons (IFNa) (Akira et al. 2006).

Toll-like receptors are compartmentalised in their location and function which affects the ligands they interact with. On the cell surface TLRs 1, 2, 4, 5 & 6 recognise bacterial and fungal PAMPs such as lipoproteins and intracellular TLRs 3, 7, 8 & 9 recognise viral or microbial PAMPs such as nucleic acids (Akira et al. 2006). Ligand recognition mostly occurs via the extracellular domain containing leucine rich repeats (LRRs). In some cases, accessory molecules like CD14 are required. Expression of accessory proteins in intestinal epithelial cells such as CD14 and MD2 and LPS binding protein in the periphery has a central role in determining TLR4 responses, in particular (Yu and Gao 2015). Intracellularly, the Toll-Interleukin-1 resistance (TIR) domain is responsible for initiating intracellular signalling via the recruitment of Myeloid differentiation primary response gene 88 (MyD88) or MyD88 adaptor-like (Mal) adaptors (Akira and Takeda 2004; O'Neill et al. 2003).

Cell surface TLRs mostly recognise bacterial and fungal lipopeptide and sugar moieties present on cell walls or external structures of the microorganism. TLR4 was the first family member discovered as it was identified as the receptor which responds to lipopolysaccharide (LPS) in Gram-negative bacteria known for inducing septic shock (Poltorak et al. 1998). Another cell surface TLR, Toll-like receptor 2 is activated by structurally conserved motifs in lipopeptides from bacteria, fungi and viruses (Akira et al. 2006). It forms heterodimers with either TLR1 or TLR6 that recognise different structures. For example, TLR1/2 heterodimers recognise triacylated lipopeptides from Gram-positive bacteria and mycoplasma while TLR1/TLR6 heterodimers recognise diacylated lipopeptides from Gram-negative bacteria and mycoplasma (Kang et al. 2009). Finally, TLR5 recognises the flagellin protein present in the flagella of motile bacteria via homodimer formation (Akira et al. 2006).

The intracellular TLRs detect intracellular non-self nucleic acids, and so act within the endosomal compartment so as to exclude host DNA (Blasius and Beutler 2010). TLR3 recognises double stranded RNA (dsRNA) from viruses (Alexopoulou et al. 2001). It induces the type I interferon production in addition to inflammatory cytokines. TLR7 recognises ssRNA from viruses such as vesicular stomatitis virus (VSV), influenza type A and human immunodeficiency virus (HIV) (Akira et al. 2006; Kawai and Akira 2006). TLR8 has been implicated in ssRNA sensing as well as in the development of many autoimmune conditions (Guiducci et al. 2013). TLR9 recognises the unmethylated CpG motifs that are present in bacterial and viral genomes not usually present in host cells. Their activation by these motifs results in activation of dendritic cells, B cells and also initiates a T_H1 response (Bafica et al. 2005). Specific details of individual TLR signalling pathways have been reviewed in depth elsewhere (Fitzgerald and Kagan 2020).

4 TLRs in the Gastrointestinal Epithelium

The role of TLRs within the intestinal epithelium is to detect pathogens and to convey suitable signals to neighbouring cells to mount an effective immune response. The cells of the layer have also been implicated in maintaining tolerance towards the resident commensal microorganisms of the lumen (O'Connell and McKernan 2017). In recent years, TLR signalling has also been linked with metabolism, proliferation, repair and cell death (Sommer et al. 2015). This is possible due to the release of different proteins after TLR signalling that are capable of interacting with multiple cell types. Such is their importance here that specific polymorphisms of TLRs (e.g. TLR4 D299G) have also been associated with gastrointestinal disease such as an increased risk of colon cancer (Eyking et al. 2011). It is worth remembering that the intestinal epithelium is a single cell layer that acts as a barrier to invading bacteria and viruses. Localisation of TLRs within epithelial cells is a key determinant of signalling. For intestinal epithelial cells, this is due to cell surfaces at both the apical (luminal) and basolateral (serosal) sides. Previous studies have demonstrated differential cytokine responses depending on whether receptor signalling occurs from the apical or basolateral side of the epithelium (Sabharwal et al. 2016; Stanifer et al. 2020). Intracellular TLR location can be determined by proteins such as UNC93B1 which act as chaperones for TLRs 3, 7, 8 & 9 (Yu and Gao 2015). For some receptors, such as TLR9 signalling pathways can diverge depending on whether stimuli are received from the apical or basolateral side resulting in different transcriptional responses (Lee et al. 2006). It is also important to highlight that there is not uniformity in response along the entire GI tract. This is due to regional differences in TLR responses between the duodenum, jejunum, ileum, caecum and the colon. This can result due to variations in the length of or complete absence of villi, presence or absence of certain cell types (e.g. Paneth cells), the varied density of myeloid/lymphoid cells along the tract, one versus two layers of mucus, the varying concentrations of specific nutrients/metabolites (e.g. vitamin A), the variation in microbial density and diversity (which increases towards the colon), production of microbial metabolites (e.g. SCFAs/indoles), presence of follicle associated epithelium (e.g. Peyer's patches/cryptopatches). Such variation has the possibility to create individualised responses to pathogens and commensals alike. It has been suggested that this variation may even influence individual susceptibility to chronic diseases such as inflammatory bowel diseases and allergy (Agace and McCoy 2017; Mowat and Agace 2014).

5 Mechanisms of TLR Defence

More generally, TLR signalling has been reported to be involved in the maintenance of gut homeostasis as well as repair, this has been demonstrated over the years by using knockout mice (Burgueno and Abreu 2020). For example, initially it was demonstrated that TLR-mediated recognition of commensals in the colon regulated the production of tissue protective factors and that TLR signalling protects from

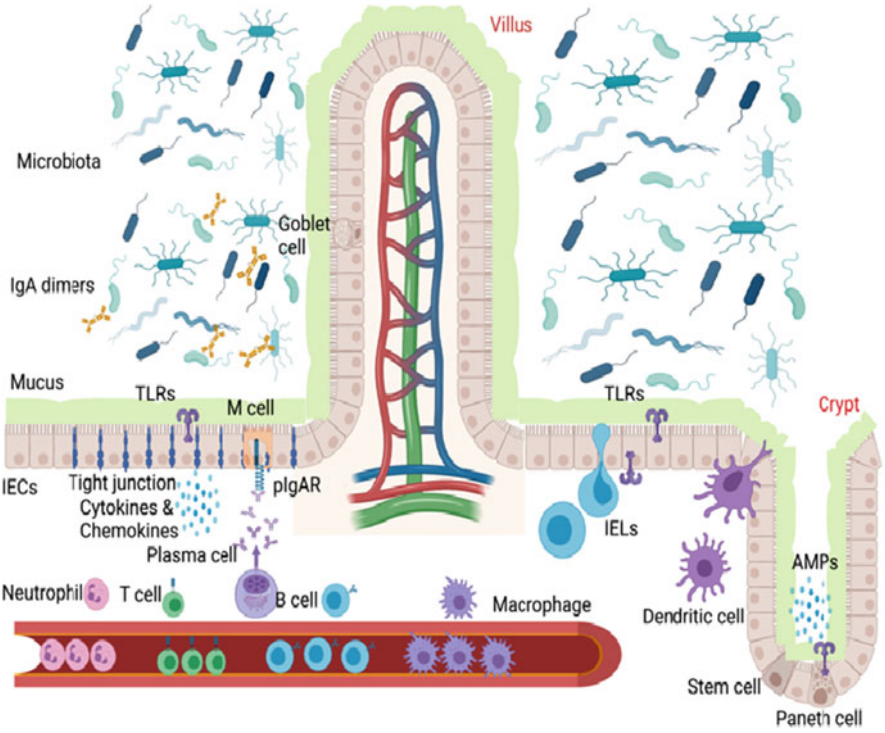

Fig. 1 TLR signalling in the intestinal epithelium. Shown in this figure are the various cell types of the intestinal epithelium and how TLRs affect their physiology. TLRs may induce expression of tight junction proteins between intestinal epithelial cells (IECs), the production of mucus from Goblet cells, the release of antimicrobial peptides (AMPs) from Paneth cells and facilitate the transport of antibodies via the expression of polymeric IgA antibody receptor (pIgAR). In addition, engagement of TLRs with microbes can induce the expression and release of cytokines and chemokines to recruit immune cells (indicated in the figure)

mortality caused by intestinal epithelial injury (Rakoff-Nahoum et al. 2004). More recently, a key role for TLR1 in the intestinal epithelium was also demonstrated. Genetic knockout of TLR1 was associated with an increase in mucosal-associated bacteria, gut permeability and a reduction in wound healing as well as systemic bacteria and an elevated innate immune response (Kamdar et al. 2018). Additionally, mice deficient in intestinal epithelial cell TLR5 developed low-grade inflammation, an altered microbiota and increased susceptibility to colitis (Chassaing et al. 2014). TLR9 signalling was demonstrated to be protective in cases of colitis by conferring intracellular tolerance to subsequent TLR challenges (Lee et al. 2006). TLR signalling has been linked with regulation in the microbiota composition in particular the numbers of mucus-associated and opportunistic bacteria (Frantz et al. 2012).

There have been a number of discrete mechanisms employed following epithelial TLR signalling to prevent pathogen translocation (summarised in Fig. 1). These mechanisms are specifically related to the cells of the epithelium itself and include

maintenance of tight junctions, mucus production, antimicrobial peptide release and facilitative antibody transport (McKernan 2019). Although it is only one cell thick, this barrier manages to exclude food and microorganism through the use of tight junction proteins that bind neighbouring cells together very tightly thus preventing leakage of luminal contents. Tight junction proteins expressed here include zonula occludens 1 (ZO-1), ZO-2 and claudins. The mechanisms of their regulation during homeostasis and infection are only now being elucidated. It is now known that TLR (TLR2 & TLR4) activation by commensal bacteria has a role in this. TLR signalling activates protein kinase C resulting in a reorganisation of tight junction proteins, leading to an increase in transepithelial resistance and an increase in IEC survival, thus strengthening the barrier (Cario 2008; Oppong et al. 2013; Sommer et al. 2015). Intercellular gap junctional proteins such as connexin 43 have been implicated in a number of barrier diseases including enterocolitis and cancer. TLR2 signalling prevents the occurrence of spontaneous colonic inflammation as it increases the expression of connexin 43 (Ey et al. 2009). It has also been suggested that the integrity of the underlying enteric nervous system as well as in neurochemical coding can be influenced by TLR2. As a consequence, this seems to lead to an alteration in intestinal motility and thus an alteration in the transit of bacteria through the gut which can then have an effect on inflammation in the intestine (Brun et al. 2013). In the case of infection, it seems that TLR4 is also implicated but this time in increasing the epithelial permeability in response to lipopolysaccharide and may be species dependent (Nighot et al. 2017).

Mucus production by Goblet cells in the epithelium is a second means to prevent infection. Mucus is used to trap pathogens and is composed of glycoproteins and trefoil factor 3 (TFF3). Stimulation of TLR2 by commensals and signalling via a PI3K/Akt was shown to regulate TTF3 expression and can lead to colitis in its absence (Lin et al. 2013; Podolsky et al. 2009). In addition, mucin 2 production by Goblet cells is also regulated by TLR ligands and commensal bacteria (Birchenough et al. 2016; Johansson et al. 2008). As well as tight junction proteins and mucus production, TLR signalling also regulates the production of antimicrobial peptides and enzymes by Paneth cells. Their production is dependent on TLR signalling and induction of degranulation. Such peptides include regenerating islet-derived protein IIIγ (RegIIIγ) which is a C-type lectin that binds bacterial peptidoglycan. Resistin-like molecule β (RELMβ) is also regulated by TLRs and promotes the secretion of mucin 2a as well as being a modulator of macrophage and T cell responses. Finally, CRP-ductin activity is TLR-dependent and it agglutinates Gram-positive and Gram-negative bacteria, cathelicidin and β-defensin (Vaishnava et al. 2008; Vora et al. 2004; Kinnebrew et al. 2012; Ta et al. 2017). Their effects or lack of are most commonly seen during gastrointestinal infection. For example, *Salmonella typhimurium* infection induces the production of Muc2 and TFF3 from Goblet cells as well as the antimicrobial proteins RegIIIγ and RELMβ via the MyD88 adaptor. This was illustrated by infection of MyD88 knockout mice which had enhanced tissue damage and colitis in the absence of TLR signalling (Bhinder et al. 2014). Also, in a *Citrobacter* infection model, host resistance to infection

was mediated by intestinal epithelial cell MyD88 signalling as it led to the induction of RegIIIγ and promoted barrier function (Friedrich et al. 2017).

As well as innate immune mechanisms, TLR signalling provides a link to adaptive immunity in particular antibody transport. It has been demonstrated that dendritic cells that have come in contact with commensal bacteria can activate antibody producing plasma cells in the underlying lamina propria (LP) via TLR signalling (Zeng et al. 2016) and that these cells then produce soluble IgA (sIgA) molecules that are polyreactive, meaning they can bind many components of different microbial species in the lumen (Macpherson and Uhr 2004). Binding with sIgA then prevents these bacteria from interacting with the epithelium and as a result the composition and metabolic function of gut microbiota can be determined by sIgA interactions and aids in the maintenance of homeostasis (Nakajima et al. 2018). The polymeric immunoglobulin receptor (pIgR) is present on the basolateral surface of intestinal epithelial cells and its expression is regulated by TLR3 and TLR4 stimulation. It is required for the transport of dimeric sIgA from the LP into the lumen. Following transport into the lumen it can interact with both commensals and pathogens (Schneeman et al. 2005; Bruno et al. 2011).

Apart from the enterocytes, Paneth cells, Goblet cells and hormone secreting enteroendocrine cells also express functional TLRs (Bogunovic et al. 2007). Signalling via TLRs may induce muscular contraction in the intestine by inducing these cells to secrete hormones (Palazzo et al. 2007) as well as chemokines like CXCL1 (Selleri et al. 2008). Hormones involved in the control of food intake and gut motility like peptide YY (PYY) can increase in expression in response to a number of TLR ligands and further increase in the presence of commensal metabolites such as butyrate (Larraufie et al. 2017). This limits the potential contact time between these bacteria and the epithelium.

6 Myeloid Cells

As well as defence mechanism pertaining to the cells of the epithelium, TLR signalling in these cells is also directly involved in alerting underlying and distant immune cells to potential dangers by recruiting phagocytes, facilitating antigen uptake, inducing the expression of integrins and other adhesion molecules, tolerising antigen presenting cells and switching phenotypes of lymphocytes (Wittkopf et al. 2014). Cell types affected by TLR signalling in the epithelium come from both myeloid and lymphocyte lineages and include neutrophils, macrophages, dendritic cells and B and T lymphocytes. TLR signalling enhances recruitment of such cells, as well as their extravasation and maturation depending on the nature of the interaction, for example whether it engages with commensals or pathogens. Often following such interactions, epithelial cells release large amount of the chemokine IL8 known to induce neutrophil infiltration into the mucosa (Kucharzik et al. 2005; Schuerer-Maly et al. 1994; Fukata et al. 2005). In addition, TLR-induced cytokine release facilitates extravasation from the blood by increasing the expression of adhesion molecules intercellular adhesion molecule (ICAM1) and vascular cell

adhesion molecule (VCAM1) on endothelial cell walls (Maaser et al. 2001) and ICAM1 on epithelial cells which then allows for neutrophil adhesion to the layer (Huang et al. 1996).

Priming and activation of recruited cells is also a significant consequence of TLR signalling in the epithelium and is an integral part of the overall immune response. TLR signalling (specifically TLR2 and TLR4) can mediate phagocytosis and translocation of bacteria across the epithelium which allows priming of immune cells in the lamina propria via antigen presentation (Neal et al. 2006; Oppong et al. 2013). However, if not controlled can lead to spread of pathogens. For example, knockout of the TLR4 gene in mice led to increased bacterial translocation to the mesenteric lymph nodes compared to their wild-type mice (Fukata et al. 2005). Epithelial TLR detection of microorganisms can influence the cytokines released of nearby monocytes and macrophages including skewing the Th profile. This was shown with the binding of TLR4 and TLR9 in a co-culture system and the increased release of TNFα, IFNγ, IL12 and IL6 that can then modulate the phenotype of neighbouring T cells and monocytes (de Kivit et al. 2011). Crosstalk between epithelial cells and macrophages via TLR4 signalling led to increased expression of anti-inflammatory IL10 in intestinal epithelial cells and was shown to be important in maintaining intestinal homeostasis (Hyun et al. 2015). Antiviral responses can result from priming of dendritic cells, monocytes and T cells via response to TLR8 in the epithelium (Angelini et al. 2017).

Professional antigen presenting cells like dendritic cells are in close proximity to the epithelium (Jin et al. 2012). Dendritic cells extend projections into the lumen to sample intestinal contents. This extension is thought to be influenced by TLR signalling in the epithelium particularly after interaction with species of *Salmonella* (Chieppa et al. 2006; Farache et al. 2013a). TLR-mediated intestinal epithelial release of transforming growth factor (TGFβ) and thymic stromal lymphopoietin (TLSP) from epithelial cells primes these cells when engaging with commensal bacteria diverting dendritic cells away from Th1 signalling and towards a more tolerogenic phenotype (Iliev et al. 2009; Rimoldi et al. 2005; Zeuthen et al. 2008). Additional anti-inflammatory properties imprinted on dendritic cells include production of Muc2 derived from intestinal epithelial cells which contributes to tolerance of commensal microbes (Shan et al. 2013). Other notable contributors include retinoic acid in dendritic cells, which requires TLR signalling (de Kivit et al. 2017; Villablanca et al. 2011; Wang et al. 2011). It has been reported that there can be different levels of TLR expression in dendritic cells subsets and so may respond differently to various molecular patterns interacting with these receptors (Dillon et al. 2010; Monteleone et al. 2008).

7 Lymphocytes

In addition to innate responses, epithelial TLR signalling has been reported to also influence adaptive responses by activation of lymphocytes but also by helping to maintain tolerance and prevent excessive adaptive response when needed. For

example, recognition of Gram-negative commensal bacteria by TLR4 facilitates crosstalk between intestinal epithelial cells and intestinal intra-epithelial lymphocytes leading to the release of the T cell growth factor IL15 and migration of T cells to the epithelium (Kaneko et al. 2004; Yu et al. 2006). Intra-epithelial lymphocytes can use occludin to project dendrites between epithelial cells into the luminal space (Edelblum et al. 2012), thereby limiting the ability of pathogens to cross the epithelium and cause disease (Edelblum et al. 2015). It has also been demonstrated that TLR signalling in the epithelium in response to the microbiota in the small intestine can communicate with intra-epithelial lymphocytes that can then lead to an antimicrobial response via release of the C-type lectin RegIIIg (Ismail et al. 2011).

Regulatory T cells (Tregs) are a subpopulation of T cells (that express CD4, Foxp3 and CD25) that play a key role in regulating immune responses to infection and in autoimmunity. *Fusobacterium nucleatum* has been detected in patients with inflammatory bowel diseases (IBD) and shown to induce Tregs following TLR2/4 signalling in intestinal epithelial cells (Jia et al. 2017). Commensal *Clostridium* species have been shown to induce the release of TGFβ from intestinal epithelial cells which can then activate Foxp3+ Tregs (that release IL10) in the lamina propria of the colon reducing colitis in mice (Atarashi et al. 2011). Communication between both cell types is critical to maintaining gut homeostasis. This has been highlighted in studies using Foxp3 knockout mice where there is a restraining of tonic microbial-dependent proinflammatory signals in intestinal epithelial cells (Rivas et al. 2012). Th17 T cells in the lamina propria can be induced by colonisation of the small intestine by segmented filamentous bacteria by serum amyloid A and protects against infection by *Citrobacter* mice (Ivanov et al. 2009). Following *Salmonella* infection, TLR-dependent release of IL23 from intestinal epithelial cells can stimulate IL22 release from intra-epithelial lymphocytes, which then stimulates Paneth cells to release angiogenin 4 a bactericidal protein into the lumen (Walker et al. 2013). These studies highlight how intestinal epithelial cell and lymphocyte communication can vary their responses.

Finally, B cells in the vicinity of the epithelium are also under the influence of TLR signalling in epithelial cells. It has been shown that in response to viral RNA, epithelial cells can release TLSP which then causes the release of B-cell activating factor (BAFF) from dendritic cells which then induces the expression of cytidine deaminase causing class switch recombination (CSR) in B cells (Xu et al. 2007). Interaction of commensal bacteria with TLRs on intestinal epithelial cells mediated the release of TLSP, the recruitment of B cells and class switch recombination of B-cell IgA2 by inducing dendritic cells to release a proliferation inducing ligand (APRIL) without the use of T cells (He et al. 2007; Shang et al. 2008). These events illustrate the influence of TLR signalling in the epithelium on the specificity of the adaptive immune response.

8 Pharmacological Targeting of TLRs in the Intestinal Epithelium

Pharmacologically, TLR signalling could be targeted at a number of different points in the pathway. These include the receptors themselves, which as described above can be located at the cell surface or intracellularly in endosomal compartments. Molecules have also been designed to interfere with many of the signalling molecules (often kinases); however, these are very often shared between the PRR pathways as well as some other signalling pathways (Bryant et al. 2015). This review will focus specifically on the receptors as targets rather than discussing molecules targeting proteins further downstream. To date both agonists and antagonists (including antagonistic antibodies) have been designed for TLRs in addition to probiotic which have subsequently been shown to affect TLR expression and activity. These molecules and preparations have been tested in animal models as well as in clinical trials for inflammatory diseases of the intestine.

Inflammatory bowel diseases (IBD) are a group of disorders known to cause chronic inflammation within varying segments of the digestive tract. These disorders are thought to be caused by excessive activation of the innate immune response in the mucosal cell lining (Maloy and Powrie 2011). The primary subtypes of IBD, Crohn's disease (CD) and ulcerative colitis (UC) share similarities in pathologies but differ in the location within the gastrointestinal tract in which they manifest. Ulcerative colitis is characterised by chronic inflammation localised to the colon and rectum (Maloy and Powrie 2011). Crohn's disease can manifest across any region of the gastrointestinal tract and can be further classified according to the region affected and its clinical presentation (Maglinte et al. 2003). TLR dysregulation and their cytokine products have been implicated in the development of these conditions (Friedrich et al. 2019; Lu et al. 2018). Following their discovery, expression levels of TLRs in the mucosa were measured from biopsy samples. TLR4 levels were upregulated in both CD and UC. In contrast, TLR3 was significantly downregulated in intestinal epithelial cells (IECs) in CD but not in UC (Cario and Podolsky 2000; Cario 2010). TLR5 expression was also upregulated in those with active UC and downregulated in those in a quiescent phase (Sanchez-Munoz et al. 2011). Similarly, TLR9 has also been shown to be dysregulated in IBD, with peripheral B cells taken from patients with IBD patients shown to have significantly higher expression of TLR9. The study also showed a positive correlation between TLR9 expression and IBD severity (Berkowitz et al. 2013).

As highlighted above, TLR signalling in the epithelium plays a key role in maintaining homeostasis. They are central to the innate response and also inform the adaptive immune response. If TLR expression and signalling in the epithelium is altered in IBD, it could be an important drug target. This could be achieved either by designing small molecules/antibodies for the receptors or using dietary supplements or probiotics that affect their expression and activity. Animal models such as the DSS and TNBS colitis models in rodents have been used to evaluate efficacy and to suggest specific mechanisms of action of such molecules. TLRs (particularly TLRs 2, 4, 5 & 9) have been reported to play a central role in the development of

inflammation and injury in these animal models making them useful in such evaluations (Cario et al. 2007; Ivison et al. 2010; Obermeier et al. 2005; Shi et al. 2019).

Many molecules targeting TLRs have been tested over the past decade, some examples will now be discussed. A TLR2 inhibitory peptide reduced the expression of proinflammatory cytokines in the colonic mucosa and ameliorated DSS-induced colitis in mice as assessed by colonoscopy score and histology (Shmuel-Galia et al. 2016). The TLR3 agonist poly I:C also protected against DSS-induced colitis in mice by increasing the intestinal expression of zona occludens 1, occludin and claudin 1 (Zhao et al. 2017). Others have since shown that these increases in tight junction proteins can be further increased in the presence of probiotics strains of bacteria (Kanmani and Kim 2019). The TLR4 antagonist, FP7 ameliorated DSS-induced colitis in mice and reduced mucosal release of proinflammatory cytokines (Facchini et al. 2020). Similarly, blockade of TLR4 using an antagonistic antibody also protected in this model and reduced proinflammatory cytokine release in mice as well as infiltration of immune cells to the lamina propria (Ungaro et al. 2009). The TLR4 antagonist TAK-242 was proposed to improve colitis by altering specific phyla of microbes in the gut (Wang et al. 2020). Additionally, TLR4 antagonist C34 was shown to attenuate inflammation in a mouse model of necrotising enterocolitis as well as in an ex vivo human model (Neal et al. 2013). The TLR7 agonist imiquimod ameliorated DSS-induced colitis in mice via oral administration by inducing type I interferons and antimicrobial peptides. An improvement was also seen when the agonist was administered topically (via enema) (Sainathan et al. 2012). Finally, a novel immunomodulatory microparticle called MIS416 that consists of muramyl dipeptide and bacterial DNA was shown to activate TLR9/NOD2 signalling. It was also shown to improve the therapeutic effects of mesenchymal stem cells in a DSS-induced colitis model in mice (Lee et al. 2018).

Probiotics have in recent years shown to be efficacious in many animal models of disease. Specific strains have shown to be of benefit in preventing infection, improving diet, altering gut transit, targeting visceral pain, ameliorating allergy and resolving inflammation (Sanders et al. 2019). These effects can be mediated by direct interaction with pattern recognition receptors on the epithelium or from the release of metabolites such as short chain fatty acids (e.g. butyrate) or neurotransmitters (e.g. GABA). Many of these mechanisms present new therapeutic opportunities in the treatment of a wide variety of conditions (Long-Smith et al. 2020; Plaza-Diaz et al. 2017). There have been a number of in vitro studies in recent years that suggest that specific strains of probiotics may have benefit in resolving gut inflammation by enhancing and/or engaging with TLR receptors or interfering with their signalling pathways (Plaza-Diaz et al. 2017). This has also been extended to ex vivo and animal models of intestinal inflammation. It has been shown that feeding microbiota to Germ-free mice can augment TLR2 expression in the epithelium as well as affecting epithelial signalling and proliferation in the mucosa (Hormann et al. 2014). On the contrary, treatment with antibiotics increased the expression of TLR4, TLR5 and TLR9 in the ileum and TLR3, TLR4, TLR6, TLR7 and TLR8 in the colon, and it

reduced the expression of TLR2, TLR3 and TLR6 in the ileum and TLR2 and TLR9 in the colon (Grasa et al. 2015).

Most probiotics that have effects on TLR expression and activity have come from the Bifidobacterium or Lactobacillus species. For example, *Bifidobacterium longum* by engaging with TLR2 and NOD2 was shown to promote epithelial barrier function (by increasing expression of zonulin & occludin), downregulating proinflammatory cytokines and ameliorating cell damage caused in a DSS colitis mouse model (Srutkova et al. 2015). A similar mechanism was shown in vitro with *Lactobacillus plantarum* that acted via TLR2 and upregulated expression of zona occludens 1 and occludin (Karczewski et al. 2010). *Lactobacillus casei* is thought to protect from TNBS induced colitis in mice by inducing a T regulatory cell response via TLR2 and increased production of anti-inflammatory IL10 and TGFB (Thakur et al. 2016). TLR2 is also central in the protective effects of *Lactobacillus reuteri* against necrotising enterocolitis in mice (Hoang et al. 2018). *Lactobacillus rhamnosus* was shown to protect against an infection induced colitis model in mice also via TLR2 (Ryu et al. 2016) This same effect was also demonstrated in human blood taken from individuals fed *Bifidobacterium infantis* (Konieczna et al. 2012). In humans, *L. casei* supplementation in UC patients was shown to decrease expression of both TLR2 and TLR4 measured in colonic biopsies. This was also accompanied with increased levels of anti-inflammatory IL10 (D'Inca et al. 2011).

Other species such as *Bifidobacterium longum sp infantis* are thought to have anti-inflammatory effects in the intestine via TLR4 as beneficial effects were lost in TLR4 knockout intestinal organ cultures (Meng et al. 2016). *Lactobacillus rhamnosus* was shown to increase TLR3 mRNA expression in both human intestinal organoids and in mouse intestine after 7 days and led to a greater response as indicated by IFNa and CXCL1 expression following Poly I:C stimulation in the probiotic group (Aoki-Yoshida et al. 2016). Other strains such as *Lactobacillus plantarum* reduce proinflammatory cytokine expression in intestinal epithelial cells as well as cell death and blood cell counts in response to the TLR3 agonist Poly I:C in mice (Mizuno et al. 2020). Given the genetic link between IBD and TLR9 (Torok et al. 2004), it is not surprising that some probiotics mediate their effects via this receptor in DSS colitis models (Rachmilewitz et al. 2004). Finally, some *Lactobacilli* strains are proposed to reduce inflammation by targeting negative regulators of TLR signalling such as A20, Tollip, SIGGIR and IRAKM (Kanmani and Kim 2020). Recently, it has been suggested that some of the innate signalling pathways may not be as dependent on strains of microorganisms themselves as once thought but more on the spatial expression of pattern recognition receptors like TLR4 in the epithelium which itself is intrinsically programmed (Kayisoglu et al. 2020).

Clinically there is still a large dependence on a small number of molecules including anti-inflammatories, immunosuppressives and biologics in the treatment of IBD. Unfortunately, some of these drugs have a long list of side effects. Also, many of these molecules are not successful in subgroups of patients so there is a strong need to develop a variety of molecules to deal with the diverse profile of those suffering from IBD (Neurath 2017). While some probiotic strains have also shown

some efficacy in the clinic in small scale trials improving clinical symptoms in UC and CD, the role of any of TLRs in these improvements has not been fully elucidated in humans (Groeger et al. 2013; Krag et al. 2013; Tamaki et al. 2016; Yoshimatsu et al. 2015). To date, TLR agonists have mostly been trialled in oncology including in colon cancer and TLR3, TLR7, TLR8 and TLR9 agonists have all been tested in patients as adjuvants or therapies but this is discussed in more detail elsewhere (Frega et al. 2020; Le Naour et al. 2020; Bourquin et al. 2019; Karapetyan et al. 2020). Surprisingly, there have not been a large number of TLR based compounds developed or even trialled for intestinal inflammation. Recently, a trial for the TLR2 agonist VB-201 in ulcerative colitis patients (NCT01839214) was completed but there have been no results published as of writing. Humanised antibodies against TLR2 (e.g. OPN-305) have been developed and tested in phase I and seem to be well tolerated and produce full blockade of the receptor (Reilly et al. 2013). This was also the case for the anti-TLR4 antibody (NI-0101) used in phase I where it was tolerated and also prevented cytokine release when volunteers were challenged with LPS (Monnet et al. 2017). Neither of these has been tested in IBD patients yet however.

The only target that has had any success to date have been TLR9 agonists. DIMS015 (cobitolimod, Kappaproct), which is a topically (by rectal enema) administered modified single stranded DNA-based oligonucleotide. It was initially used as an add-on therapy to glucocorticoids and showed efficacy in a small number of patients by restoring steroid sensitivity and significantly reducing the need for colectomy (Musch et al. 2013). It was then tested in a randomised, dose optimisation phase IIb trial (NCT03178669) to evaluate safety and efficacy in patients ($n = 213$) with moderate to severe ulcerative colitis prompting further evaluation in phase III trials. In this placebo-controlled double-blind randomised phase III trial (NCT01493960) cobitolimod was assessed as an add-on therapy in chronic treatment refractory ulcerative colitis patients ($n = 131$). It was reported that certain biomarkers (CD163, TSP1 and IL1RII) may be able to predict responders to such therapy (Kuznetsov et al. 2014). Results from phase IIa and IIb showed that the drug was well tolerated and that more patients on cobitolimod had mucosal healing and histological improvement as well as a higher proportion achieving symptomatic remission (Atreya et al. 2016, 2018, 2020). A second TLR9 agonist, BL-7040 which is administered orally was evaluated for safety and efficacy in an open label phase II study with moderatively active ulcerative patients (NCT01506362) and shown to be efficacious (with a clinical response in half of those treated) showing reduced mucosal neutrophils and inflammatory cytokines and also well tolerated (Dotan et al. 2016).

9 Conclusions

Individuals with chronic inflammatory disorders like IBD show an over activation of their innate and immune responses in the intestine. Many of these individuals have genetic predisposition to the disease thought to be linked to specific TLRs. The importance of TLRs in the epithelium has been discussed here. These receptors play

a prominent role in the innate and adaptive responses to pathogens as well as developing tolerance to commensals. This has been shown to be dysregulated in some individuals and predisposes them to chronic disease. Therefore, it is sensible to investigate these molecules as targets which would potentially reduce specific TLR activity but still allow other TLRs to respond to infection. There are a number of approaches that could be taken such as targeting specific domains in the PRR structure such as the LRR domain involved in ligand recognition, targeting the TIR domain or recruitment of adaptors like Mal, targeting kinase activity or targeting some of the regulatory proteins involved in signalling.

This review has focused specifically on those molecules that act as agonist or antagonists at the receptor itself. The compounds reviewed here have already made it to clinical trials and are predominantly targeting TLR9 at present. In the future, it would be prudent to investigate targeting TLRs expressed on other cell types in the gut as these may also be viable targets. For example, TLR4 on enteric glial cells may play a role in mediating pain and inflammation in ulcerative colitis and has been shown to be modulated by palmitoylethanolamide (PEA) (Esposito et al. 2014; McKernan and Finn 2014). There is still a need to identify more specific endogenous ligands, further elucidate signalling and to investigate their role in sterile inflammation. There have been very few successes in developing molecules that target TLRs into compounds in the clinic despite TLRs being discovered over two decades ago. This may be due to similarities in signalling pathways with other pattern recognition receptors or, which is why unique features of each pathway need to be identified. Overall, these receptors still hold a lot of promise as targets in inflammatory and infectious diseases.

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