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Dakang Xu *Editor*

Regulation of Inflammatory Signaling in Health and Disease

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Regulation of Inflammatory Signaling in Health and Disease

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Preface

The innate immune system provides immediate defense against invading microbial pathogens and is found in all classes of living organisms, whereas the adaptive immune system is found only in vertebrates. This book covers recent research in innate immunity that has revealed a large number of receptors that sense the presence of microorganisms or cellular damage in tissues. In complex tissues, many of these sensing events occur simultaneously. Thus, downstream signaling pathways need to be integrated so that an appropriate inflammatory response can be initiated.

Current key concepts in innate immunity are discussed, including the formation of signalosomes, inflammasome formation and pyroptosis, methods of extrinsic cell communication, and examples of receptor cooperation. There is an overview of posttranslational modifications in different inflammatory signaling pathways and their essential roles in the regulation of inflammation. Emerging evidence suggests that epigenetic mechanisms play an important role in fine tuning the innate immune response. An understanding of epigenetic regulation of innate immune cell identity and function will enable elucidation of the relationship between gene-specific host defense and inflammatory diseases, as well as innate immune memory in health and disease. Inflammasomes determine the molecular and cellular processes of inflammation in response to microbial infection. Current data suggest that inflammasomes also have antimicrobial functions. Microbial factors are involved in regulating host inflammatory signaling pathways, the composition and load of the gut microbiota, the co-metabolism of the host and the microbiota, and the host immune system and physiology.

Novel aspects of functional genomics, epigenomics, transcriptomics, posttranslational modifications, the microbiome, and immunometabolism are reviewed in relation to inflammatory signaling and responses. Study of the inflammation that occurs in response to host-pathogen interactions is essential for the development of new therapies to improve human health. Here we review recent findings on the mechanisms underlying the regulation of inflammatory responses to pathogens, dysregulation of these responses in inflammatory disease, and the use of such mechanisms to boost or subdue the inflammatory response. Such as TLR agonists as

adjuvants for cancer vaccines, small-molecule HDAC inhibitors and other epigenetic regulators as drug targets in inflammatory diseases, and the potential role of miR-155 as a diagnostic, prognostic, and therapeutic target in the treatment of multiple sclerosis.

The cGAS–STING pathway detects the presence of cytosolic DNA and triggers the expression of inflammatory genes in response to DNA damage. It is also linked to the tumor microenvironment, where it is paramount to immune clearance of tumors. To date, little attention has been paid to the aging-associated alteration in intercellular communication known as "inflammaging," a proinflammatory phenotype in organs that occurs with aging, suggesting an alternative senescence-associated secretory phenotype. Telomere attrition induces not only epithelial stem cell senescence, but also low-grade inflammation in lungs.

We also review crosstalk between the innate and adaptive immune systems, in particular in relation to the newly emerged innate lymphoid cells (ILCs). Innate lymphoid cells are involved in both innate and adaptive immunity, and contribute to tissue homeostasis, as well as defense against pathogens and inflammatory disorders.

Finally, I thank all the authors for their valuable contributions. It is my hope that this book will stimulate scientists and clinicians to continue to explore this fascinating field. Bridging the gaps in understanding between the fields of human and mouse immunology will provide new insights into inflammatory-mediated disease and immune defense. These innovative perspectives in basic and clinical research will aid in the translation of knowledge to the clinic.

Shanghai, China Dakang Xu

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Chapter 1
Activation of the Innate Immune Receptors: Guardians of the Micro Galaxy

Activation and Functions of the Innate Immune Receptors

Dominic De Nardo

Abstract The families of innate immune receptors are the frontline responders to danger. These superheroes of the host immune systems populate innate immune cells, surveying the extracellular environment and the intracellular endolysosomal compartments and cytosol for exogenous and endogenous danger signals. As a collective the innate immune receptors recognise a wide array of stimuli, and in response they initiate specific signalling pathways leading to activation of transcriptional or proteolytic pathways and the production of inflammatory molecules to destroy foreign pathogens and/or resolve tissue injury. In this review, I will give an overview of the innate immune system and the activation and effector functions of the families of receptors it comprises. Current key concepts will be described throughout, including innate immune memory, formation of innate immune receptor signalosomes, inflammasome formation and pyroptosis, methods of extrinsic cell communication and examples of receptor cooperation. Finally, several open questions and future directions in the field of innate immunity will be presented and discussed.

Keywords Innate immunity • Innate immune receptors • Inflammasomes • Myddosome • PRRs • Innate immune memory • TLRs • NLRs • CLRs • RLRs

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1.1 Inflammation and the Innate Immune Response

Inflammation is a complex biological process critical for the host that mediates numerous pathological and physiological responses [1]. In response to microbial infection or the introduction of environmental irritants, the innate immune system acts to protect the host via the rapid detection and removal of foreign threats and the removal of dead/dying cells through inflammatory processes. Imbalances in homeostatic processes, such as during metabolic dysfunction or tissue injury, can also trigger sterile inflammatory responses. There are five classical signs used in the clinical description of inflammation: swelling, heat, pain, redness and loss of function [2]. On a cellular level, these symptoms reflect the actions of specialised tissue-resident innate immune cells (e.g. macrophages and dendritic cells) that send out signals (cytokines, chemokines and interferons) to mediate increased blood flow, allowing access to plasma proteins as well as infiltration of additional circulating white blood cells (e.g. neutrophils and monocytes/macrophages) to the tissue site of inflammation. Once the immune stimulus is removed, repair processes are initiated to restore tissue homeostasis. An effective inflammatory response is therefore acute and rapidly resolved following the removal of the stimuli. Low-level inflammation is also critical for physiological processes; for instance, the balance between the commensal microflora and the host immune system is necessary to maintain a healthy intestinal environment [3], as well as small systemic concentrations of type I IFNs under homeostatic conditions, which is important for priming immune cells for rapid responses to microbial insults [4, 5]. Controlled inflammation in the context of protection, repair and physiology is therefore beneficial for the host. In contrast, chronic inflammation can have detrimental outcomes for the host, often resulting from persistent infection or in response to sterile inflammation driven by metabolic abnormalities or the inability to remove environmental irritants [1]. Chronic inflammation is a hallmark of autoimmune and monogenic autoinflammatory diseases where, often, the proverbial innate immune receptor heroes are turned villains. Whilst the enduring inflammation in autoimmune disease results from continual activation of the immune system by endogenous stimuli, autoinflammatory diseases stem from specific gain-of-function genetic variations in single innate immune genes leading to unwarranted inflammation [6–8]. Some recent examples of autoinflammatory diseases include those stemming from genetic variations in genes encoding innate immune receptors, such as stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI) [9] and pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) [10]. Identification of such genetic variants has given molecular insights into the mechanisms of innate immune activation and signalling and may lead to better treatment options for affected patients.

The innate immune system is typically described as a non-specific form of host defence due to its rapid induction and apparent lack of any immunological memory. Recently, this conventional model was contested by findings that even in the absence of the adaptive immune response, myeloid cells (predominantly monocytes and

macrophages) can adopt long-term inflammatory phenotypes via epigenetic reprogramming [11]. This so-called innate immunological memory likely evolved prior to the adaptive immune system to confer protection upon secondary infections. Indeed, even in *Drosophila* phagocytosis of apoptotic debris was found to reprogramme macrophages, priming them to rapidly respond to future infections or tissue injury [12]. This system is also active in both murine and human myeloid cells. An emerging body of work shows priming myeloid cells, such as monocytes, with the potent bacterial agent lipopolysaccharide (LPS), and results in mature macrophages displaying a sustained immunosuppressive phenotype (known as 'tolerance'). In contrast, a primary infection with *Candida albicans* increases subsequent immune responses against secondary exposures with the same or different pathogens (termed 'trained immunity') [13]. These findings highlight an intriguing new capability to the innate immune response.

In order to better understand the inflammatory response, the mechanisms mediating the activation and functions of the innate immune system receptors central to the inflammatory process must be understood.

1.2 Triggering the Innate Immune System

All living organisms possess some form of physiological response to danger. Innate immune receptors, also known as pattern recognition receptors (PRRs), are highly conserved germ-line proteins, present throughout evolution to mediate host defence via the recognition of foreign and host-derived danger signals. This evolutionary conservation is best exemplified by the expression of numerous families of PRRs in sponges [14, 15], the most primitive of multicellular organisms. Collectively, PRRs recognise a wide variety of danger signals arising from both exogenous and endogenous sources (see Table 1.1). Exogenous danger signals are represented predominantly by highly conserved components of microbes, termed pathogen-associated molecular patterns (PAMPs). This includes structural elements found within bacterial and fungal cell walls, such as LPS (Gram-negative bacteria), peptidoglycans (Gram-positive bacteria) and ß-glucans (fungi), as well as forms of viral and bacterial nucleic acids. In addition, some PRRs respond uniquely to viability-associated PAMPs of the microbes encountered (e.g. prokaryotic mRNA), termed vita-PAMPs [16, 17]. In addition to pathogen-derived molecules, some exogenous environmental irritants (e.g. silica crystals) can also activate PRRs. Host-derived molecules that accumulate or become modified following tissue injury, metabolic dysfunction and uncontrolled cell death, and those that mediate sterile inflammatory responses via PRRs, represent danger-associated molecular patterns (DAMPs). A classic example is uric acid, which is soluble and innocuous in blood under physiological conditions; however, following drastic increases in levels of circulating uric acid (hyperuricaemia) results in a chemical-phase transition of soluble uric acid into immunoreactive monosodium urate crystals that deposit within joints and periarticular tissues driving the inflammation seen in gout [18]. Another DAMP that is increasingly implicated in mediating sterile inflammation is DNA leaked from

Table 1.1 Localisation of innate immune receptors and their stimuli

PRR	Localisation	PAMPs	DAMPs	Synthetic ligands
Toll-like receptors (TLRs)				
TLR1	Plasma membrane	Triacyl lipoproteins	Unknown	Pam3CSK4
TLR2	Plasma membrane	Lipoproteins, zymosan, mannan, fungal ß-glucans, peptidoglycan, lipoteichoic acid, type 1 fimbria	Versican	Pam2CSK4, Pam3CSK4
TLR3	Endolysosomal membrane	Viral dsRNA	mRNA	Endosomal poly(I:C), poly(A:U)
TLR4	Plasma‡endolysosomal membrane	Extracellular LPS[••], type 1 fimbria	Oxidised low-density lipoprotein, amyloid-ß	Lipid A derivatives, purified LPS
TLR5	Plasma membrane	Flagellin	Unknown	Recombinant flagellin
TLR6	Plasma membrane	Diacyl lipoproteins, lipoteichoic acid, zymosan	Oxidised low-density lipoprotein, amyloid-ß, versican	Macrophage-activating lipopeptide 2, synthetic diacylated lipoproteins, Pam2CSK4
TLR7	Endolysosomal membrane	Viral and bacterial ssRNA	Immune complexes, self-RNA	Thiazoquinoline and imidazoquinoline compounds (e.g. R848, imiquimod)
TLR8	Endolysosomal membrane	Viral and bacterial ssRNA	Immune complexes, self-RNA	Thiazoquinoline and imidazoquinoline compounds (e.g. R848, imiquimod)
TLR9	Endolysosomal membrane	Viral and bacterial CpG DNA, DNA:RNA hybrids	Chromatin IgG immune complexes	Class A, B and C CpG oligodeoxynucleotides
TLR10[^]	Plasma membrane	Unknown	Unknown	Unknown
TLR11[*]	Endolysosomal membrane	Profilin and flagellin	Unknown	Unknown
TLR12[*]	Endolysosomal membrane	Profilin	Unknown	Unknown
TLR13[*]	Endolysosomal membrane	Bacterial 23S ribosomal RNA (rRNA)	Unknown	23S rRNA-derived oligoribonucleotide

NOD-like receptors (NLRs)				
NOD1	Cytosolic	Peptidoglycan-containing iE-DAP motifs		Synthetic iE-DAP peptides
NOD2	Cytosolic	Muramyl dipeptide (MDP)		Mifamurtide
NLRP1◆	Cytosolic	*B. anthracis* lethal toxin[*], *T. gondii* effector proteins[*]	Unknown	Purified *B. anthracis* lethal toxin[*]
NLRP3◆[†]	Cytosolic	*S. hygroscopicus* pore-forming toxin (nigericin), influenza A virus PB1-FB peptide, influenza M2 protein	Cholesterol crystals, monosodium urate (MSU) crystals, calcium pyrophosphate dihydrate (CPPD) crystals, extracellular ATP, islet amyloid polypeptide (IAPP), amyloid-ß, extracellular ASC specks	Alum crystals, TiO_2 and SiO_2 nanoparticles, silica crystals
NLRP6	Cytosolic	Unknown	Unknown	Unknown
NLRP7	Cytosolic	Unknown	Unknown	Unknown
NLRP12	Cytosolic	Unknown	Unknown	Unknown
NAIP[^] NAIP1,2,5,6[*]	Cytosolic	Flagellin, components of the type 3 secretion system[*]	Unknown	Recombinant flagellin
NLRC4◆[†]	Cytosolic	Flagellin, components of the type 3 secretion system	Unknown	Recombinant flagellin

(continued)

Table 1.1 (continued)

PRR	Localisation	PAMPs	DAMPs	Synthetic ligands
PYRIN				
PYRIN◆[†]	Cytosolic	Rho GTPase modification: *C. difficile* toxin B (TcdB), *C. botulinum* C3 toxin, *V. parahaemolyticus* VopS, *H. somni* IbpA, *B. cenocepacia* type 6 secretion system components	Unknown	Purified TcdB
RIG-I-like receptors (RLRs)				
RIG-I	Cytosolic	Short viral dsRNA	Unknown	5′ppp-dsRNA, cytosolic poly(I:C)
MDA5	Cytosolic	Long viral dsRNA	Unknown	Cytosolic poly(I:C)
LGP2	Cytosolic	dsRNA?	Unknown	Unknown
C-type lectin-like receptors (CLRs)				
Dectin-1	Plasma membrane	Fungal ß-glucans, zymosan	Unknown	Unknown
Dectin-2	Plasma membrane	Fungal α-mannose, *M. furfur* Furfurman	Unknown	Unknown
Mincle (Clec4e)	Plasma membrane	Mycobacterial cord factor/ trehalose-6,6-dimycolate (TDM)	SAP130	Synthetic trehalose-6,6-dibehenate (TDB)
Cytosolic DNA receptors (CDRs)				
AIM2◆	Cytosolic/nucleus	Viral dsDNA, bacterial dsDNA	mtDNA Self-DNA Damaged nuclear DNA	Poly(dA:dT)

cGAS	Cytosolic	Viral dsDNA	mtDNA	Transfected dsDNA, poly(dA:dT), DNA-intercalating agents
		Bacterial dsDNA	Nuclear DNA	
		dsDNA provirus	Circulating tumour DNA	
		DNA:RNA hybrids	Damaged DNA	
		Y-DNA	Oxidised DNA	
			Self-DNA	
STING[†]	Cytosolic: ER‡ Golgi membrane	Bacterial cyclic dinucleotides (CDNs): 3′3-cGAMP, cyclic di-GMP, cyclic di-AMP	Endogenous second messenger CDN: 2′3′-cGAMP (produced via cGAS in response to dsDNA)	Synthetic CDNs, CMA*, DMXXA*

^human specific, *mouse specific, ‡ traffics to, ◆ forms an inflammasome, † receptor can be activated indirectly, ¨ intracellular LPS directly binds caspase-4, caspase-5 and caspase-11

membrane-compromised mitochondria, i.e. mitochondrial-derived DNA (mtDNA), which is linked to the activation of several PRRs [19]. Rather than responding to specific signals, some PRRs are activated more generally in response to perturbations in cellular homeostasis, recently coined homeostasis-altering molecular processes (HAMPs) [20]. Alterations to the normal cellular environment can be sensed following pathogen infection, for instance, modification to the actin cytoskeleton by bacterial toxins, as well as via induction of endogenous mechanisms, such as the alteration of intracellular ion levels.

1.3 Detecting Danger

The vertebrate innate immune system comprises families of PRRs expressed primarily on highly specialised immune cell such as monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells. PRRs are also expressed in other cell types that commonly encounter potential danger signals, including epithelial cells. The PRR families can be broadly classified according to their localisation on either the plasma and endolysosomal membranes or within the cytosolic compartment. Whilst Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) represent the membrane-bound receptors, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and several cytosolic DNA receptors (CDRs) represent those expressed in the cytosol. An overview of the major PRR families is presented in Fig. 1.1. Conceptually, the PRRs act as sensor proteins, which engage an adaptor(s) molecule, mediating the activity of downstream effectors, which results in production of inflammatory signals. Activation of most PRRs leads to precise intracellular signalling cascades culminating in specific transcriptional responses driven primarily through nuclear factor-kappa B (NF-κB) and the interferon regulatory factor (IRF) families of transcription factors, ultimately resulting in the production of inflammatory cytokines (e.g. TNF, IL-6), chemokines and type I interferons (IFNs). In contrast, rather than initiating transcription, some PRRs are able to form oligomeric protein structures, termed inflammasomes, that instigate proteolytic maturation of members of the IL-1 family of cytokines (i.e. IL-1ß and IL-18), as well as mediate the inflammatory form of programmed cell death termed pyroptosis. The eventual secretion of inflammatory mediators downstream of innate immune pathways mobilises recruitment of an army of host immune cells and facilitates acute inflammatory processes.

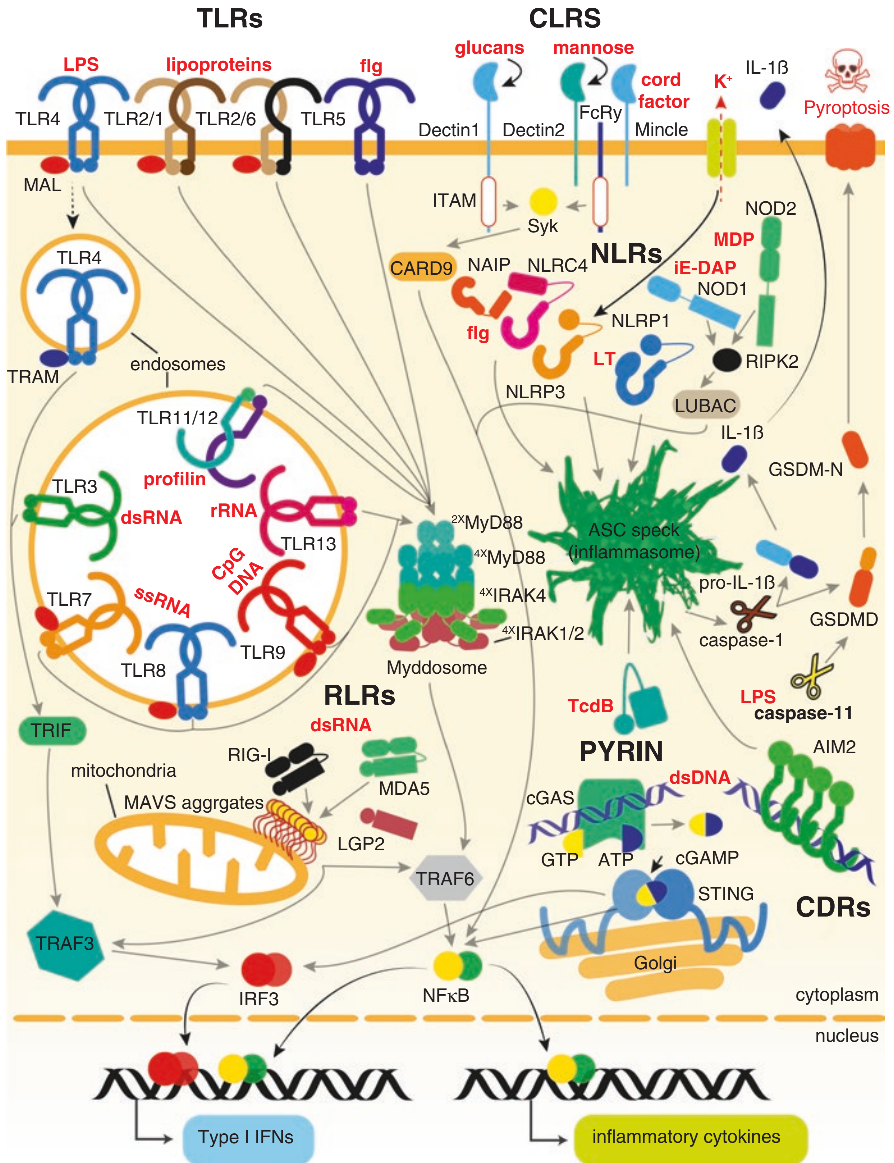

Fig. 1.1 Activation of the innate immune receptors and their signalling pathways (see text for details)

1.4 Innate Immune Signalosomes

An interesting concept emerging in innate immunity is the prerequisite for some PRRs to assemble into higher-order signalosomes in order to transduce their downstream signalling pathways [21, 22]. A feature common to these innate immune signalling platforms is the presence of molecules containing specialised protein–protein binding domains of the death domain (DD) superfamily comprising DDs, death effector domains (DEDs), pyrin domains (PYDs) and caspase recruitment domains (CARDs) [23]. These domains are found within the protein structures of PRR adaptor and effector molecules of several innate immune signalling complexes.

The first evidence for this phenomenon was presented in 2002 when the late Jürg Tschopp and colleagues found that in the cytosol of activated immune cells, the senor NLRP1, the adaptor ASC and the effector caspase-1 formed large protein complexes observed as significant shifts in the elution profiles of these proteins during size exclusion chromatography [24]. This sizeable inflammatory signalling complex was coined the 'inflammasome' and has subsequently been demonstrated to be a requirement downstream of a number of PRRs, which to date includes the NLR family members, NLRP1, NLRP3 and NLRC4, as well as the pyrin and HIN domain-containing protein (PYHIN) family member, the absence in melanoma 2 (AIM2) and the PYRIN receptor. Whether other PRRs, namely, NLRP6, NLRP7, NLRP12, RIG-I and IFN-γ-inducible protein 16 (IFI16), can also form genuine inflammasome complexes remains to be established. Upon activation, inflammasome-forming sensors oligomerise creating a seed for subsequent recruitment of the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), which forms large PYD-dependent filamentous structures [25]. This recruitment results in the majority of cytosolic ASC being relocalised to a single perinuclear polymer protein aggregate known as the ASC speck [26]. Formation of the ASC speck facilitates recruitment and autocatalytic activation of the effector inflammatory caspase, caspase-1 via homotypic CARD–CARD interactions to initiate cleavage and maturation (via cleavage) of IL-1ß and IL-18 and instigate pyroptosis. Although it was long recognised that pyroptotic cell death was entirely dependent upon caspase-1 activity, the exact molecular mechanisms leading to cellular rupture remained a mystery. The major breakthrough came when Gasdermin-D (GSDMD) was identified as the executioner protein in pyroptosis, by several groups in parallel, using independent screening approaches [27, 28]. These studies revealed that in addition to the pro-forms of IL-1ß and IL-18, GSDMD is also a caspase-1 substrate following inflammasome activation. Caspase-1 cleavage of GSDMD results in the release of an active amino (N)-terminal fragment termed Gasdermin-N that specifically binds to lipids (phosphoinositide and cardiolipin) of the inner leaflet of the plasma membrane. The binding of the Gasdermin-N fragment generates cytotoxic oligomeric pores that perforate the cellular membrane leading to cell swelling and lysis [29]. The specific lipid-binding preferences of Gasdermin-N to the inner leaflet of the plasma membrane ensure the intrinsic destruction of infected cells whilst

limiting damage to surrounding tissue and other host cells. Interestingly, Gasdermin-N reportedly displays some bactericidal activity, which is likely explained by the presence of cardiolipin as a component of bacterial outer membranes. It is conceivable that Gasdermin-N released following cellular lysis of infected cells could subsequently insert into extracellular bacterial membranes to cause cell lysis.

Several members of the RLR family also form higher-order signalosome platforms upon activation [30]. Following binding of ssRNA in the cytosol, monomers of RIG-I or melanoma differentiation-associated gene 5 (MDA-5) interact via tandem N-terminal CARD (2CARD) interactions forming tetrameric structures. This oligomerisation seeds the recruitment of the adaptor protein mitochondrial antiviral signalling (MAVS) [31], which subsequently forms CARD-dependent helical filament structures analogous to those of ASC formed upon inflammasome activation. Filamentous MAVS drives downstream signalling events leading to NF-κB and IRF3 transcriptional responses.

With the exception of TLR3, all the TLRs emit signals via engaging the adaptor molecule, myeloid differentiation primary response gene 88 (MyD88) upon activation. MyD88 contains two critical domains that fulfil its role as an adaptor: (1) a carboxyl (C)-terminal Toll/interleukin-1 (IL-1) receptor (TIR) domain that enables coupling to the intracellular region of TLRs or the IL-1R and (2) a DD at the N-terminus that facilitates protein–protein interactions with the four members of the IL-1 receptor-associated kinase (IRAK) family of serine/threonine effector kinases [32]. Although the key players and kinetics of TLR/IL-1R proximal events were identified some time ago, our understanding of the mechanisms controlling this process was greatly enhanced following the revelation that in solution the DDs of MyD88 form specific oligomeric complexes upon addition of IRAK4 DDs [33, 34]. This stable oligomeric complex was coined the Myddosome. The crystal structure of the complex revealed a tower-shaped oligomer of four layers: two upper layers comprising two and four MyD88 DDs, respectively, followed by a third layer comprising four IRAK4 DDs and a third and final layer of four IRAK2 DDs assembling as a left-handed helical structure [33]. Architecturally, the Myddosome is stabilised via specific type III DD–DD interactions, whilst the specificity of its formation is enhanced via complementary surface electrostatic charges and contour between adjacent layers. The finding that IRAK2 DDs only binds to stable MyD88–IRAK4 DD complexes rather than with the individual DDs of either MyD88 or IRAK4 suggests a sequential ordered assembly process of the Myddosome. Discovery of the Myddosome revealed new insights into mechanisms of IRAK activation, and more mechanistic intricacies of TLR signalling are sure to emerge [35–37].

1.5 The Families of Innate Immune Receptors

1.5.1 Toll-Like Receptors (TLRs)

TLRs were the first family of innate immune receptors described. To date ten TLRs have been identified in humans (TLRs1–10) with twelve expressed in mice (TLRs1–9 and TLRs11–13, with TLR10 a pseudogene) [32]. The TLRs are type I transmembrane proteins anchored into either the plasma membrane or endolysosomal membranes. Their cellular localisation is indicative of the ligands they bind, with cell surface TLRs recognising typically bacterial outer membrane components, whilst microbial nucleic acids are sensed from TLRs localised within acidified endolysosomal compartments (see Table 1.1). Most of the TLRs are well characterised, however the ligand and precise function of TLR10 remains in question with some reports suggesting that it signals as a heterodimer with TLR2 and upstream of MyD88 to induce signals in response to influenza infection, whilst others have shown that it plays an inhibitory role [38–42]. Structurally, the TLRs consist of three major domains: (1) a ligand recognition domain consisting of folded leucine-rich repeats (LRRs) at the amino (N)-terminus, (2) a central transmembrane region and (3) a cytoplasmic TIR domain at the carboxyl (C)-terminus [43]. Variations in the LRRs of the different TLRs are thought to give specificity to the ligands they sense. Ligand binding induces the dimerisation of most TLRs, whilst TLR7, TLR8 and TLR9 have been shown to exist as preformed homodimers. As well as forming homodimers, some TLRs can recognise additional ligands via the formation of heterodimers or in cooperation with co-receptors or accessory proteins. Whilst TLR2/TLR1 heterodimers specifically recognise triacylated lipoproteins, TLR2/TLR6 dimers sense diacylated lipoproteins [44–46]. TLR2 is also thought to act synergistically with Dectin-1 (see below) in response to fungal ß-glucans leading to robust production of pro-inflammatory cytokines [47, 48]. In mice TLR11/TLR12 dimers were shown to be responsible for engaging profilin from the outer membrane of *Toxoplasma gondii* [49]. Additionally, TLR4/TLR6 heterodimers in concert with the scavenger receptor, CD36 can form a trimeric complex able to recognise oxidised low-density lipoproteins and amyloid-ß to elicit sterile inflammatory responses [50].

In the presence of a ligand, TLR dimers undergo conformational changes that enable the intracellular TIR domains to engage specific adaptor molecules for initiating downstream signal transduction pathways. Four adaptors have been identified that induce positive signalling: MyD88, MAL (also known as TIRAP), TRIF and TRAM. Broadly, MyD88-depedent pathways predominantly trigger production of inflammatory cytokines, whilst TRIF-mediated signalling elicits secretion of type I IFNs. Whilst all TLRs, excluding TLR3, utilise MyD88 for signalling [51, 52], TLRs 2, 4, 7 and 9 have been shown to require the addition of MAL as a bridging adaptor [53–57]. MAL is thought to facilitate stronger binding between MyD88 and TIR domains that have incompatible electrostatic surface charges [58]. Formation of the Myddosome (see above) allows transient recruitment of the E3 ubiquitin (Ub)

ligase and TNFR-associated factor 6 (TRAF6) to the receptor complex through interactions with IRAK1/IRAK2 via C-terminal TRAF6-binding motifs [59]. TRAF6 is subsequently activated and released into the cytosol where it mediates activation of the NF-kappa B inhibitor (IκB) kinase (IKK) and mitogen-activated protein kinase (MAPK) signalling complexes leading to translocation of the major transcription factors, NF-κB, cyclic AMP-responsive element-binding protein (CREB), activator protein 1 (AP1) and IRF5 for induction of pro-inflammatory cytokines. In plasmacytoid dendritic cells (pDCs) specifically, MyD88 signalling can also lead to type I IFN production via IRAK1-dependent IRF7 activation [60–62].

A role for the TIR adaptor TRIF has been demonstrated downstream of both TLR3 and TLR4 [63–65]. Whilst TLR3 signals exclusively via TRIF, TLR4 requires the bridging adaptor TRAM to facilitate TRIF-dependent signalling [66]. Interestingly, activation of TLR4 induces signalling via MAL/MyD88 at the plasma membrane; however, the TRAM/TRIF pathway is only initiated following CD14-dependent endocytosis of the receptor complex [67, 68]. TRIF signals primarily through TRAF3 enabling TANK-binding kinase 1 (TBK-1) activity and successive IRF3-medaited transcription of type I IFNs [69]. In addition, TRAF6 is recruited, mediating a modest NF-κB response via receptor-interacting serine/threonine-protein kinase 1 (RIPK1) [70].

1.5.2 NOD-Like Receptors (NLRs)

The NLRs comprise the largest family of cytosolic receptors with a common central nucleotide oligomerisation domain (NOD), C-terminal LRRs of variable lengths and variable N-terminal protein-interacting domains that further classify the NLRs into four subfamilies [71]: (1) NLRAs have an acidic transactivating domain and contains class II major histocompatibility complex transactivator (CITTA); (2) NLRBs express a baculovirus inhibitor of apoptosis protein repeat (BIR) domain and is composed of NLR family apoptosis inhibitory proteins (NAIPs); (3) NLRCs harbour a CARD and include NOD1, NOD2, NLRC3, NLRC4 and NLRC5; and (iv) NLRPs with a PYD represent the biggest subgroup with NLRP1–14. Major structural differences within the NLRP family include NLRP10, which lacks the C-terminal LRRs and NLRP1 that contains an extended C-terminus with additional ‘function to find’ domain (FIIND) and CARD. Finally, NLRX1 is an orphan receptor that unlike the other cytosolic NLRs is anchored into the mitochondrial outer membrane via a unique N-terminal mitochondrial targeting sequence. The NLRs play various immune functions including response to infection, formation of inflammasomes (NLRP1, NLRP3, NLRC4) [72], regulation of antigen presentation (NLRC5, CIITA) [73, 74], regulation of microbiota homeostasis (NLRP6) [75–77] as well as modulatory roles in NF-κB (NLRP6, NLRP12, NLRC3), MAVS (NLRX1) [78] and STING (NLRC3, NLRX1) [79, 80] responses. Below, I will summarise the known roles for NLRs in activation of innate immune pathways.

NOD1 and NOD2 receptors sense specific motifs within the peptidoglycan (PGN) carbohydrate components of bacterial outer membranes within the cytosol. NOD1 recognises PGN containing the D-glutamyl-meso-diaminopimelic acid (iE-DAP) moiety most commonly present in Gram-negative bacteria [81, 82], whilst NOD2 detects the PGN muramyl dipeptide (MDP) structure found in almost all Gram-negative and Gram-positive bacteria [83]. Ligand binding to either NOD1 or NOD2 causes auto-oligomerisation of receptor molecules leading to CARD–CARD interactions with the kinase, RIPK2 that recruits the ubiquitin ligases, X-linked inhibitor of apoptosis protein (XIAP), baculoviral IAP repeat containing 2 and 3 (BIRC2, BIRC3) and the linear ubiquitin chain assembly complex (LUBAC) [84]. This induces subsequent activation of AP1- and NF-κB-dependent transcription downstream of the MAPK and IKK complexes, respectively, for the production of pro-inflammatory cytokines [84, 85].

NLRP1 is expressed as a single protein in humans, whilst mice express three paralogs due to multiple gene duplication events: NLRP1a, NLRP1b and NLRP1c [86]. Physiological activation has only been demonstrated for murine NLRP1b in response to anthrax lethal toxin from *Bacillus anthracis* and effector proteins from *Toxoplasma gondii* [87]. However, in mice deficiency of NLRP1 is associated with spontaneous obesity [88], whilst SNPs in human NLRP1 have led to implications in several autoimmune and autoinflammatory diseases, including vitiligo as well as inflammatory skin disorders relating to carcinoma [89–92]. A unique feature of the NLRP1 structure is the presence of both a FIIND and CARD at the C-terminus. In humans, the PYD is thought to structurally auto-inhibit NLRP1 via physically blocking accessibility to the CARD. However, following activation of NLRP1, the FIIND undergoes spontaneous proteolysis liberating a smaller C-terminal fragment containing the partial FIIND and the exposed CARD [92]. This cleavage fragment is therefore free to bind ASC via atypical CARD–CARD interactions to form a functional inflammasome.

NLRP3 is activated by a diverse array of stimuli (see Table 1.1), including endogenous and environmental crystals (e.g. cholesterol crystals), particulates (nanoparticles) and protein aggregates (amyloid-ß), as well as extracellular ATP, specific influenza viral components and the bacterial pore-forming toxin nigericin from *Streptomyces hygroscopicus* [93]. Hence, it is generally accepted that NLRP3 is activated downstream of a more generalised form of cellular stress. The suggested models to date include mitochondria dysfunction, intracellular ROS production, lysosomal rupture and changes to intracellular ion levels [94]. The most reproducible and consistent finding suggests that efflux of potassium leading to reduced intracellular levels is upstream of NLRP3 activation. This unified view is strengthened by the observations that addition of culture medium with reduced potassium alone is sufficient to activate immune cells and, conversely, high-level potassium medium can inhibit NLRP3 activation [95–97]. Additionally, potassium efflux appears to be associated with most NLRP3 activators [96]. Activation of the NLRP3 inflammasome is dependent upon a prerequisite priming step (e.g. via TLR activation) that enables both a transcriptional induction of NLRP3 expression and post-translational modifications that license its inflammasome activation [93].

NLRC4 is an indirect sensor of cytosolic bacterial flagellin, as well as the needle and rod subunits of the type 3 secretion system (T3SS). Direct binding of these DAMPs is to NAIPs that subsequently recruit and activate the NLRC4 inflammasome. In humans, only one NAIP exists that recognises *Salmonella* flagellin and the bacterial T3SS needle protein, whilst in mice multiple NAIPs are able to recognise flagellin (NAIP4 or NAIP5) plus the T3SS needle (NAIP1) and rod (NAIP2) components [98–100]. Upon recruitment to a single NAIP, NLRC4 undergoes significant conformational change leading to oligomerisation of 10–12 monomers of NLRC4 into a circular structure [101, 102]. NLRC4 is thought to require a specific serine phosphorylation event at residue 533 for its activation [103, 104]. Although the precise mechanistic details remain unclear, the NAIP/NLRC4 oligomeric complex then recruits ASC via CARD–CARD interactions and subsequently activates caspase-1 and its downstream effector functions.

Activation of NLRP7 and NLRP12 inflammasomes is implicated in bacterial infection. NLRP7 appears to be required for cytoplasmic detection of bacterial lipopeptides [105, 106], whilst NLRP12 may recognise T3SS components during *Yersinia pestis* infection [107]. However, more work is required to determine if these receptors play other roles in bacterial detection and indeed form a physiological inflammasomes upon activation.

1.5.3 PYRIN

Structurally, PYRIN is related to the tripartite motif (TRIM)-containing family of proteins due to the presence of two central B-box zinc finger domains and a coiled-coil domain, as well as a C-terminal B30.2 PRY-SPRY domain (absent from murine PYRIN). However, unlike other TRIM proteins, PYRIN expresses an N-terminal PYD domain enabling assembly of a functional ASC-dependent inflammasome [108]. The activation of the PYRIN inflammasome is triggered in response to specific bacterial toxins and effectors (see Table 1.1), such as toxin B from *Clostridium difficile* (TcdB) and *Vibrio parahaemolyticus* VopS [109]. Interestingly, rather than direct sensing of these bacterial proteins, PYRIN is triggered downstream of their ability to inactivate Rho GTPase, thereby inducing actin depolymerisation [109]. Mechanistically, PYRIN is held in the cytoplasm in an inactive state bound by 14-3-3 molecules to a specific motif containing a constitutively phosphorylated serine residue at position 242 [10, 110]. Serine phosphorylation is mediated via RhoA-dependent activation of the protein kinase C-family members, PKN1 and PKN2 [110]. Hence, RhoA inactivation results in loss of PKN kinase activity, dephosphorylation of serine 242, removal of 14-3-3 and activation of the PYRIN inflammasome.

1.5.4 RIG-I-Like Receptors (RLRs)

The family of RLRs consists of RIG-I, MDA5 and laboratory of genetics and physiology 2 (LGP2) that survey the cytosolic compartment for viral dsRNA [111–113]. RIG-I and MDA5 recognise specific RNA from different viral infections [114], and whilst RIG-I senses short dsRNA (up to ~1 kb), MDA5 binds larger dsRNA fragments (>2 kb) [115]. RIG-I sensing of dsRNA is enhanced by the presence of a 5′ triphosphate (PPP) moiety leading to increased type I IFN-inducing activity [116]. Both RIG-I and MDA5 are structurally similar, expressing two N-terminal CARD domains, a central helicase/ATP domain and a regulatory domain (RD) at the C-terminus. LGP2 also contains the helicase domain and the RD but lacks the 2CARD that is required to trigger downstream signalling. Hence, LGP2 was initially thought to act as a negative regulator by sequestering dsRNA and/or binding to RIG-I [117]; however, later studies suggested that LGP2 may act in concert with RIG-I and MDA5 to induce positive signalling [112]. RIG-I and MDA5 signal via the adaptor molecule MAVS (see above) [118–121], which recruits the E3 ubiquitin ligases TRAF3 and TRAF6, which elicit activation of TBK-1/IRF3 and NF-κB, respectively, culminating in a strong type I IFN antiviral response [122].

1.5.5 C-Type Lectin Receptors (CLRs)

CLRs comprise a large family of calcium-dependent carbohydrate-binding receptors. To date, only activation of the type II CLRs, namely, Dectin-1, Dectin-2 and Mincle (also known as Clec4e), is implicated in antifungal immune responses. These CLRs are transmembrane proteins expressed on the surface of immune cells containing a single N-terminal extracellular carbohydrate-recognition domain allowing detection of ß-glucans and α-mannose components of fungi by Dectin-1 and Dectin-2, respectively [123, 124] and, in the case of Mincle, an immunostimulatory factor from *Mycobacterium tuberculosis* [125] (see Table 1.1). Upon activation, these receptors engage spleen tyrosine kinase (Syk) via an immunoreceptor tyrosine-based activation motif (ITAM). This occurs directly in the case of Dectin-1 which contains this motif within its intracellular region, whilst Dectin-2 and Mincle must associate with the ITAM-containing adaptor FcRγ [126]. The recruitment and activation of Syk lead to signalling via the CARD9/Bcl-10/MALT-1 complex and downstream activity of NF-κB, resulting in production of pro-inflammatory cytokines [127, 128].

1.5.6 Cytosolic DNA Receptors (CDRs)

Two major cytoplasmic signalling pathways exist within the innate immune system downstream of dsDNA sensing: the cyclic GMP–AMP synthase (cGAS)/STING pathway and the AIM2 inflammasome pathway. Several other receptors have also been implicated in sensing intracellular DNA, such as IFI16- and DNA-dependent activator of IRFs (DAI); however, their precise functions remain unclear.

AIM2 is a member of the PYHIN family containing two major structural domains, a C-terminal HIN-200 DNA-binding domain and an N-terminal PYD to mediate protein–protein interactions with the adaptor molecule ASC. Several groups concurrently identified AIM2 as an inflammasome-forming sensor in response to cytosolic dsDNA [129–132], which occurs most prominently following bacterial infection [133]. Interestingly, AIM2 was recently shown to recognise radiation-induced DNA damage from within the nucleus [134]. Unlike the inflammasome-forming NLRs that can undergo self-oligomerisation by virtue of their NODs, AIM2 forms filaments along dsDNA via regular HIN domain binding, leading to assembly of PYDs from multiple AIM2 molecules and the polymerisation of ASC into filaments that eventually aggregate into ASC specks [135, 136]. This process facilitates the effector functions of caspase-1, maturation of IL-1β and IL-18 and pyroptotic cell death.

cGAS is a cytosolic sensor of dsDNA most commonly in the context of viral infection [137]. However, rather than directly inducing a downstream signalling cascade in response to DNA, the enzymatic activity of cGAS produces an endogenous second messenger that engages STING to elicit a strong antiviral immune response [138, 139]. Upon binding cytosolic DNA, cGAS forms dimers and undergoes structural changes facilitating the recruitment and catalysis of GTP and ATP into the cyclic dinucleotide (CDN) [140, 141], cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP–AMP or 2′3′-cGAMP) [142–146]. Interestingly, unlike other PRRs cGAS is not able to discriminate between the origins of dsDNA it encounters. Hence, cGAS can also produce cGAMP in response to host-derived DNA, such as from the mitochondria, nucleus or oxidised DNA [147, 148]. Not surprisingly, the cGAS/STING pathway is implicated in a number of autoimmune disorders including Aicardi–Goutières syndrome and systemic lupus erythematosus [149–152]. In addition to microbial and host dsDNA, cGAS activity has been reported in response to other forms of DNA, such as RNA/DNA hybrids and specific HIV-1-derived Y-DNA [153, 154] (see Table 1.1).

Preformed STING dimers reside within the endoplasmic reticulum (ER) membrane by virtue of four transmembrane domains at the N-terminus of each STING monomer [138]. STING dimers contain a V-shaped pocket that enables the binding of CDNs [155, 156]. In addition to endogenous 2′3′-cGAMP, STING was originally shown to bind CDNs of bacterial origin, such as 3′5′-cGAMP produced upon *Listeria monocytogenes* infection [157]. The binding of CDNs to STING leads to recruitment of TANK-binding kinase-1 (TBK-1) and trafficking of the dimeric STING/TBK-1 complex to perinuclear Golgi compartments. TBK-1 then phosphor-

ylates the transcription factor IRF3, leading to nuclear translocation and strong induction of type I IFN genes [158]. In addition, STING activation also results in downstream NF-κB activation to further promote maximal transcription of IFNβ and for the production of pro-inflammatory cytokines.

1.6 Spreading Inflammatory Signals

An effective innate immune response is dependent upon the transfer of inflammatory signals between an activated host cell and neighbouring immune cells. This occurs most notably via production of cytokines, chemokines and type I IFNs upon their transcription and subsequent secretion into the extracellular environment. These inflammatory signals can act on the host cell themselves (autocrine), on proximal cells (paracrine) and occasionally even on distal cells (endocrine). As their name suggests, chemokines act to induce directed chemotaxis of neighbouring immune cells to sites of inflammation. Whilst pro- and anti-inflammatory cytokines orchestrate the magnitude and specificity of general responses to microbial infections, type I IFNs, such as IFNα and IFNβ, are particularly important for mounting an immediate and effective antiviral response. Type I IFNs mediate their effects by binding to the IFNα/β receptor (IFNAR), which is comprised of an IFNAR1/IFNAR2 heterodimer and results in the transcription of numerous IFN-stimulated genes (ISGs) encoding proteins with diverse antiviral functions [159, 160]. Although effective, this form of cell-to-cell communication is often dependent upon transcription, translation and secretion of new proteins, which takes time. In addition, initiation of innate immune pathways in infected cells is commonly targeted by the invading pathogens that have evolved immune evasion strategies to escape detection. Hence, more rapid methods of host cell-to-cell communication have evolved to initiate and amplify inflammatory signals in uninfected cells. For instance, the transfer of messages packaged within exosomes can be an effective means of communication between cells. However, perhaps the most rapid form of intercellular communication is the transfer of ions and signalling molecules through gap junctions, which are small channels directly connecting the cytoplasm of two adjacent cells. Such a mechanism has been noted to propagate both NF-κB- and IFN-dependent responses [161, 162]. Recently, it was demonstrated that the endogenous cGAS-derived second messenger, 2′3′-cGAMP, is efficiently trafficked through gap junctions from activated immune cells to neighbouring cells to directly engage and activate STING, thereby propagating the antiviral response [163]. A similar mechanism exists whereby transfer of cGAMP from HIV-infected host cells to recipient cells via HIV envelope membrane fusion sites results in an antiviral response in target cells and protection from HIV infection [164]. Interestingly, in other studies, infected immune cells were also shown to transmit an antiviral state to bystander cells through packaging of cGAMP into progeny virus particles that subsequently go on to infect surrounding cells and elicit STING-dependent responses [165, 166]. Mechanisms involving the transfer of cGAMP may be of particular relevance for

cells that express no or low amount of cGAS but have sufficient STING levels, such as in certain epithelial cell populations.

Following inflammasome activation, plasma membrane integrity is severely compromised via the pore-forming activity of GSDMD, which ultimately results in cell lysis and leakage of its contents into the peripheral environment. This includes the release of preformed ASC specks, which were recently demonstrated to retain their activity in the extracellular space leading to further activation of caspase-1 and IL-1ß, thus promoting an inflammatory environment [167, 168]. Furthermore, in a similar manner to other large protein aggregates, extracellular ASC specks were shown to be taken up by macrophages acting as DAMPs to trigger subsequent activation of the NLRP3 inflammasome via lysosomal rupture [168]. Of note, ASC specks were observed in the bronchial lavage fluid of chronic obstructive pulmonary disease patients and the serum of autoinflammatory disease patients with cryopyrin-associated periodic syndromes (CAPS) [167, 168]. These findings suggest that whilst release of ASC specks represents a rapid way for cells to signal danger to their local microenvironment, in cases of chronic inflammation, ASC specks may contribute to a sustained inflammatory response.

1.7 Receptor Cooperation

Innate immune responses to pathogenic infection must be finely orchestrated in order to provoke a suitable reaction. This can include the cooperation between multiple PRRs to distinct stimuli and multiple PRRs to the same stimuli from distinct locations (Fig. 1.2). Through in vitro cell stimulation assays with synthetic or purified PRR ligands, we have learnt a great deal about the mechanisms and function of

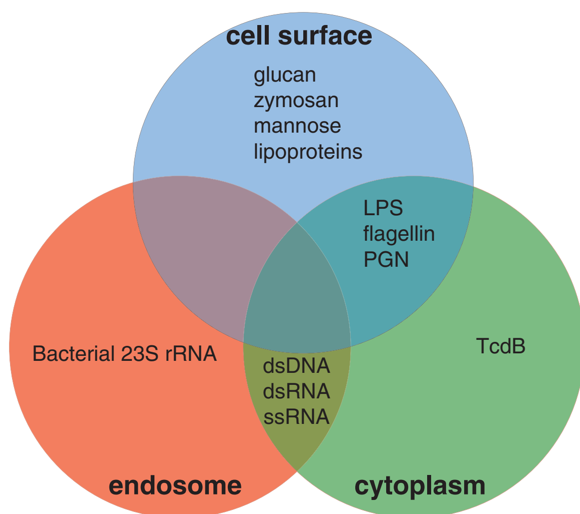

Fig. 1.2 Localisation of DAMP sensing

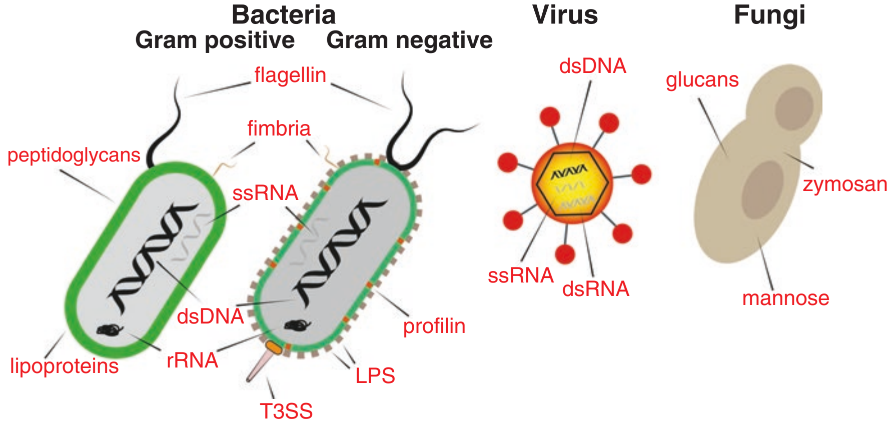

Fig. 1.3 Multiple DAMPs expressed by pathogens

the innate immune system. These assays are often performed using singular ligands in order to reduce the influence of other factors and reveal specific mechanisms of regulation and activation. However, this often leads us to think of the activation of PRRs in isolation, when in fact pathogens invariably carry numerous ligands that can potentially activate multiple PRRs simultaneously and/or sequentially during infection (Fig. 1.3 and Table 1.1). For instance, Gram-negative bacteria can harbour multiple ligands, including LPS on the outer cell membrane detected by TLR4, flagella recognised by TLR5, dsDNA sensed in endolysosomes via TLR9 or in the cytosol via cGAS as well as prokaryotic CDNs (e.g. cyclic di-GMP), directly activating STING. Hence, coordinating multiple PRR responses must be tightly regulated in order to induce an effective and specific immune response. For instance, it is well documented that a prerequisite step for maturation of IL-1ß by the inflammasome is an NF-κB transcriptional response to induce the expression of the pro-form of IL-1ß. This is likely mediated via initial detection of pathogens by TLRs at the cell surface or in endosomal compartments prior to secondary activation of inflammasome-forming receptors in the cytosol. One example is the Gram-positive bacterium, *Listeria monocytogenes*, which triggers TLR2 activation at the cell surface and subsequently NLRP3, AIM2 and NLRC4 responses within the cytosol [169–172].

In addition to multiple danger signals provoking activation of several PRRs upon infection, some singular ligands can also engage multiple PRRs from differing cellular locations in order to effectively destroy the foreign threat. An excellent example of this is during the detection of the potent immunogenic agent, LPS (also known as endotoxin) from multiple locations. LPS accounts for the majority of the structure of outer cell walls of Gram-negative bacteria and as such is detected in the extracellular environment via TLR4 in concert with the co-receptor CD14 and MD2 during infection. This recognition triggers a strong inflammatory response via activation of both NF-κB and type I IFN responses. Interestingly, LPS leakage into the

host cytosol also elicits a more drastic all or nothing inflammasome response via activation of the so-called 'noncanonical' inflammasome pathway following direct binding of LPS to caspase-11 (caspase-4 and caspase-5 in humans) [173–180]. This process was shown to be dependent upon lysis of intracellular vacuoles by the IFN-inducible GTPases, guanylate-binding proteins (GBPs), thus exposing bacteria to the cytosol for activation of caspase-11 [181]. Activation of caspase-11 or caspase-4/caspase-5 mediates GSDMD cleavage and pore formation driving two separate intracellular pathways within the host cell: (1) pyroptosis and (2) activation of the NLRP3 inflammasome driving caspase-1-depedent IL-1ß release. The activation of NLRP3 downstream of caspase-11 or caspase-4/caspase-5 appears to be mediated via the efflux of intracellular potassium through GSDMD-dependent pore formation as was recently demonstrated for NLRP3 activation downstream of the activity of plasma membrane pore-forming MLKL [95]. Another fantastic example of effective PRR crosstalk is in response to infection by the Gram-positive bacterium, *Francisella novicida*. Upon entry into the cytosolic compartment, *F. novicida* activates a type I IFN response via cGAS/STING (potentially via low levels of dsDNA release), inducing IRF1-dependent expression of the IFN-inducible GBPs and IRGB10, which target the bacterial cell membrane to allow significant levels of dsDNA access into the cytosol [182–184]. Cytosolic *F. novicida* DNA then binds and activates the AIM2 inflammasome. Interestingly, the bacteriolysis activity of GBPs and IRGB10 also facilitates LPS-induced caspase-11 activation and subsequent NLRP3 inflammasome formation [183].

1.8 Open Questions and Future Directions

In this review, I have presented an overview of the activation of the PRRs in the context of innate immune function. The innate immune system is infinitely complex, and this review merely scratches the surface of the precise mechanisms controlling these processes and the exciting and emerging concepts in this fundamental and rapidly developing field of research. Below, I will discuss some of the pertinent open questions relating to this review.

Innate immunological memory is a recent and interesting concept shown to provide important protection against bacterial and fungal infection. Importantly, innate immune memory has also been linked to atherosclerosis progression [185]. Hence, understanding the exact mechanisms of innate immune memory in specific contexts is of therapeutic interest and begs the question: can innate immune memory be exploited for clinical benefits? Recently, innate immune memory was found to form the molecular basis for protection provided by various vaccines, suggesting that manipulating this process could provide benefit in other therapeutic settings [186]. In atherosclerosis, oxidised low-density lipoprotein particles, which drive damaging local inflammation, have been shown to induce a long-term pro-inflammatory phenotype in human monocyte-derived macrophages in vitro [185]. Meanwhile, high-density lipoproteins (HDLs) are well characterised to be anti-inflammatory in

human and murine myeloid cells via instigating epigenetic down-modulation of the pro-inflammatory transcriptional landscape [187, 188]. Hence, understanding if HDL can instal counteractive anti-inflammatory innate immune memory in monocyte-derived macrophages of the heart would give great therapeutic power to HDL-based therapies and potential modulators of this process.

Another concept that has recently regained significance is the relationship between inflammasome-mediated cytokine release and cell death: is the release of IL-1 cytokines downstream of inflammasome activation merely cellular cargo passively lost from activated cells undergoing GSDMD-mediated pyroptosis? Or is this cytokine release an active cellular process? IL-1ß lacks a conventional secretory signal sequence, and, indeed, in the majority of studies on inflammasome activation in innate immune cells, it appears these events are intrinsically linked. However, it was recently shown that neutrophils are able to secrete IL-1ß upon activation of NLRP3 or NLRC4 without undergoing pyroptosis [189]. Additionally, in response to LPS stimulation, human monocytes can secrete IL-1ß in the absence of pyroptosis via an alternative caspase-8-induced NLRP3 inflammasome [190]. These findings are supported by recent work in which a direct caspase-1 dimerisation system (inflammasome independent), in combination with single-cell cytokine and viability measurements, was used to demonstrate that IL-1ß is released from live cells following caspase-1 activation, in the absence of cell death [191]. Further work is required to understand if these processes are indeed independent events or if this uncoupling is merely cell type and/or context specific.

The requirement for some PRRs to form large oligomeric signalosomes to induce downstream signalling is an interesting theme. ASC- and MAV-dependent protein aggregation is well established and readily reported upon physiological activation of either the inflammasome or RIG-I/MDA5, respectively. However, formation of the Myddosome structure has only been resolved during crystallisation studies of the MyD88, IRAK4 and IRAK2 DDs in vitro and as such raises the question of whether or not the Myddosome holds any physiological relevance for TLR activation. To date, the only evidence to suggest the biological existence of the Myddosome is the occurrence of natural MyD88 variants expressing amino acid changes within the DD that would disrupt the specific interaction required for Myddosome assembly. Endogenous interactions between Myddosome components are commonly reported using standard biochemical techniques [57, 192]; however, direct visual evidence of Myddosome formation in living cells remains to be achieved. This may be due to the potentially small size of the Myddosome complex, whilst ASC and MAVS aggregates form to sizes of up to ~0.4–1 μm and are readily visible by current confocal imaging techniques. Visual confirmation of physiological Myddosome assembly may be more feasible in the near future with the rapid advances being made in high-resolution imaging techniques, such as lattice light sheet technology.

An open question in the field remains: what is the precise mechanism and interactor(s) that mediate activation of the NLRP3 inflammasome? This seemly simple question has been a persistent thorn in the side of inflammasome researchers, despite a plethora of published examinations on the topic. Although activators of NLRP3 seem to converge upstream of potassium efflux, what directly binds or con-

trols activation of NLRP3 in response to potassium efflux remains a mystery. Several post-translational modifications have been proposed in NLRP3 activation, suggesting that this could be indirectly mediated by potassium efflux. To date, fully understanding what the genuine mechanism of NLRP3 activation entails is still being significantly pursued by researchers.

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Chapter 2
Posttranslational Modification Control of Inflammatory Signaling

Yibo Si, Yunlu Zhang, Zijuan Chen, Ruixue Zhou, Yihua Zhang, Doudou Hao, and Dapeng Yan

Abstract Inflammation is usually the defensive reaction of the immune system to the invasion of pathogen and the exogenous objects. The activation of inflammation helps our body to eliminate pathogenic microbe, virus, and parasite harming our health, while under many circumstances inflammation is the direct cause of the pathological damage in tissues and dysfunction of organs. The posttranslational modification (PTM) of the inflammatory pathways, such as TLR pathways, RLR pathways, NLR pathway, intracellular DNA sensors, intracellular RNA sensors, and inflammasomes, is crucial in the regulation of these signaling trails. Ubiquitination, phosphorylation, polyubiquitination, methylation, and acetylation are the main forms of the PTM, and they respectively play different roles in signaling regulation. The effects of the PTM range from the production of pro-inflammatory factors and the interaction between adaptors and receptors to cell translocation in response to the infectious or other dangerous factors. In this chapter, we will have an overview of the different ways of the posttranslational modifications in different inflammatory signaling pathways and their essential roles in regulation of inflammation.

Keywords Posttranslational modification • TLR pathways • RLR pathways • NLR pathway • Intracellular DNA sensors • Intracellular RNA sensors • Inflammasomes

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2.1 Introduction

The definition of inflammatory signaling pathways is the mechanism of how inflammatory signals transduce and activate relative cells and thus induce inflammation. The innate immune system contributes a broad range of inflammatory signaling pathways with its pattern recognition receptors (PRRs). The PRRs represent many receptors that are able to recognize pathogen-associated molecular patterns (PAMPs) encoded by the pathogenic microbe, virus, and parasites living in the nature. The formation of the pairing PAMPs and PRRs is the result of the fight between our bodies and pathogens through the evolution. Besides PAMPs, the PRRs can also recognize the danger-associated molecular patterns (DAMPs) released from damaged cells in our bodies, the most important mechanism of pathological injury in tissues and organs when diseases happen. When PAMPs or DAMPs are derived and detected by PRRs, downstream molecular interaction occurs and eventually activates the inflammatory pathways. The best-known PRRs are Toll-like receptors, which are well studied these years. The classic TLR pathways include the interaction between myeloid differentiation primary response protein 88 (MyD88) adaptor-like (Mal) and MyD88, thus activating TNF receptor-associated factor 6 (TRAF6) and eventually activating the translocation of nuclear factor-κB (NF-κB), while there is an exception when it comes to TLR3. Other PRRs, such as retinoic acid-inducible gene I-like receptors (RIG-I-like receptors, RLRs) and nucleotide-binding oligomerization domain-containing protein I-like receptors (NLRs), also play essential roles in the recognition and elimination of pathogens attacking our body. RLR pathways are likely to recruit melanoma differentiation-associated proten-5 (MAV5) and caspase activation and recruitment domain-containing protein 9 (CARD9) that eventually activates the IRF3 and NF-κB, respectively; NLR pathways function as inflammatory signaling pathways using NOD-1/NOD-2 and receptor-interacting protein 2 (RIP2) to initiate NF-κB and cause inflammation. The receptors mentioned above are the initiators of their own inflammatory signaling pathways, and once they have detected the PAMPs or DAMPs that can be recognized and bind to them, they can recruit and activate downstream molecules (peptide, protein, or enzyme) which are responsible for the inflammation formation. Recently, more attentions are paid to other PRRs. Cytoplasmic sensors including cyclic GMP-AMP synthase (cGAS), DNA-dependent activator of IRFs(DAI), and IFN-inducible protein-16 (IFI16) are a series of molecules that are able to detect ds-DNA produced by pathogens, and all downstream molecular of them is stimulator of interferon genes (STING) which eventually trigger interferon production; cytoplasmic RNA sensors including RIG-I and MAVS are molecules monitoring abnormal cytoplasmic RNA and consequently activating IRF3. Also, inflammasome signaling via NLR family pyrin domain-containing protein 3 (NLRP3), NLR family CARD domain-containing protein 4 (NLRC4), AIM2, and so on can activate the assembly of inflammasomes, which eventually activate caspase-1 and accordingly produce inflammatory factors.

Posttranslational modification (PTM) is a process that is operating (adding or cutting) proteins, enzymes, or peptides with different functional chemical groups with the help of specific enzymes, which happens after the translation of these proteins. There are diverse PTMs, among which phosphorylation and ubiquitination are the two most prevalent modification forms involved in many signaling pathway regulations. Besides phosphorylation and ubiquitination, polyubiquitination, methylation, acetylation, sumoylation, and succinylation also participate in some protein modifications. PTM exerts profound influence on the fate of proteins. Different PTM types have different roles to different proteins, such as translocation, secretion, function, and elimination. These effects control a group of cells whose proteins are modified by PTM mechanism, and the response of these cells ranges from division, differentiation, and migration to apoptosis.

2.2 The PTM in TLR Pathways

The Toll-like receptor (TLR) pathways constitute an important mechanism in the activation of innate immune cells including monocytes, macrophages, and dendritic cells, and the activation of these cells can result in the formation of inflammation. PTMs are broadly and essentially involved in signal regulating of TLR pathways.

2.2.1 The Overview of TLR Pathways

Toll-like receptors (TLRs) are type I transmembrane glycoproteins which play an important role in recognizing infectious factors as well as the DAMPs released by the apoptotic cells and damaged cells. Ten TLRs have been found up to date termed as TLR1–10. TLRs 1, 2, 4, 5, 6, and 10 are located in the cell surface, while TLRs 3, 7, 8, and 9 have an endosomal localization [1]. The signaling pathways initialized by Mal (MyD88 adaptor-like) are called MyD88-dependent pathways which trigger TLR2/4 receptor-related pathways. While TLR3 and endosomal TLR4 receptor-related TLR pathways are resulted from TRAM/TRIF complex, so-called TRIF pathways. In MyD88-dependent pathways, after TLR2/4 activated by PAMP/DAMP, Mal is recruited to the endosomal parts of TLRs and sequentially recruits the TNF-receptor-associated factor 6 (TRAF6). TRAF6 then dissociates from the receptor and forms a complex with TAK1 (transforming growth factor β-activating kinase), TAB1 (TAK1-binding protein 1), and TAB2 (TAK1-binding protein) at the plasma membrane, which induces the phosphorylation of TAB2 and TAK1 [2]. After that, the complex TRAF6/TAK1/TAB1/TAB2 translocates to the cytosol from cell membrane leading to the ubiquitination of TRAF6 and activation of TAK1. Activated TAK1 mediates the phosphorylation of IκB kinase (IKK) composed of IKKα, IKKβ, and the modulating part IKKγ/NEMO. Phosphorylated IKK complex is activated and thus phosphorylates the IκBs, which triggers the polyubiquitination of IκBs

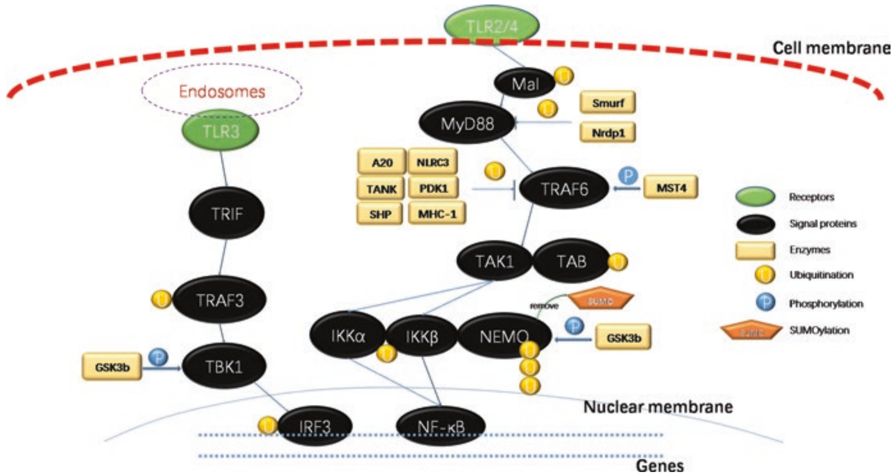

Fig. 2.1 Inflammatory signaling transduction and PTMs of TLR pathways

causing its degradation. Eventually, degraded IκBs result in the activation of NF-κB which activates the pro-inflammatory genes and causes the production of inflammatory factors such as IL-6. TAK1 also activates the MAP kinase kinase 3 MKK3/6-p38 signaling cascade, leading to the activation of cAMP response element binding (CREB) nuclear transcription factor and the MKK4/7-Jun N-terminal kinase (JNK) mediating the activation of the transcription factor activator protein-1 (AP-1) [2] which triggers the production of pro-inflammatory factors cooperatively with NF-κB. In TRIF pathways lacking the existence of MyD88, activated TLR3/endosomal TLR4 recruits TRAM and TRIF to their TIR domain to form a complex. Then the complex continuously recruits TRAF3 and TBK-1 (TANK-binding kinase 1). Activated TBK-1 can phosphorylate IRF3 to initiate its translocation to the nucleus and thus triggers the production of interferon, which plays a crucial role in antiviral response and local inflammation.

2.2.2 *The PTMs in TLR Pathways*

In the two main signaling pathways of TLRs, PTMs take the responsibility as the molecular mechanism in cascading signal transduction in the cells (Fig. 2.1). The phosphorylation of NF-κB inhibitor (IκB) kinase a (IKKa), IKKb, IκBa, and IRF3 is indispensable to their normal function. Additionally, phosphorylation occurring at a conserved pLxIS motif of TRIF is necessary for the translocation of IRF3 as well as IFN production [3]. Autophosphorylation of TBK1 modified by glycogen synthase kinase 3b (GSK3b) is essential of IRF3 activation [4]. Numbers of proteins are demonstrated that they act as the enzymes involved in phosphorylation-modification,

such as Src homology-containing protein tyrosine phosphatase-1(SHP1) [5] and mammalian STE20-like protein kinase 4(MST4) [6]. For example, the kinase MST4 can phosphorylate the TRAF6 molecules that plays an important role in TLR-MyD88-dependent pathways causing its dysfunction in oligomerization and autoubiquitination, thus causing the repression of the TLR pathway. MST4 knockdown mice show severe inflammation and death in septic shock, which can be restored by heterozygous deletion of *Traf6* [6]. Moreover, in the NF-κB signaling pathway, NEMO, an adaptor protein involved in the activation of the IKK, is phosphorylated by GSK-3β (glycogen synthase kinase-3β) at S (8, 17, 31, 43), which facilitates the NF-κB activity [7]. The ubiquitination also participates in the regulation of TLR pathways. The form of lysine 63(K63)-linked ubiquitination/polyubiquitination is related to the signal transduction, while it is known that K48-linked ubiquitination is responsible for proteasomal degradation [8]. In the TLR pathways, K63-linked polyubiquitination of TRAF6, TAB2/3, NEMO, and TRAF3 is required for NF-κB and IRF3 activation [9, 10]. K48-linked ubiquitination of IκB leads to the proteasomal degradation of IκB and thus activates the NF-κB. Linear ubiquitination of NEMO (NF-κB essential modulator) is involved in the activation of NF-κB. The LUBAC ligase composed of the two RING finger proteins HOIL-1 L and HOIP activates the canonical NF-κB pathway by binding to NEMO and conjugates linear polyubiquitin chains onto specific Lys residues in the CC2-LZ domain of NEMO. In *HOIL-1* knockout mice and cells derived from these mice, NF-κB signaling induced by proinflammatory cytokines such as TNF-α and IL-1β was suppressed [11]. NEMO is able to bind many types of ubiquitin chains including K63, K48, K27, and linear-linked ubiquitin chains [8]. Additionally, TLR pathways can be inhibited via ubiquitination modification: Mal can be inhibited by suppressor of cytokine signaling 1 (SOCS1) [12]. MyD88 can be inhibited by neuroregulin receptor degradation protein 1 (Nrdp1) [13] and smad ubiquitin regulator factor proteins (Smurf). Another example for the suppression effect of ubiquitination in TLR pathways is that PDK1 inhibits tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) ubiquitination by interrupting the complex between transforming growth factor beta-activated kinase 1 (TAK1) and TAK1-binding protein 2 (TAB2), which negatively regulates TAK1 activity [14]. TRAF6 can be inhibited by A20 [15], TRAF family member-associated NF-κB activator (TANK) [16], β-arrestin [17], SHP [18], MHC-1, and NLRC3 [19]. To some other special PTM forms, SUMO-specific protease 6 (SENP6) inhibits TLR inflammatory responses by catalyzing the de-SUMOylation of IKKγ/NEMO [20].

2.3 The PTMs in RLR Pathways

The RLR pathways (retinoic acid-inducible gene I-like receptors) are PRRs that detect RNA and DNA produced by intruding virus locating in cytoplasm. Basically, the triggered cellular RLRs include two members termed as RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated protein 5).

While the regulation of RLR pathways is dominated by a load of factors, PTM is indispensable.

2.3.1 The Overview of RLR Pathways

The sensors belonging to the RLR family are intracellular localized. Within the RLR pathways, RIG-I recognizes RNA produced by virus with short double-stranded RNA (dsRNA) stretches and a 5′-triphosphate or 5′-diphosphate moiety, while MDA5 detects longer dsRNA or viral RNA. RIG-I and MDA5 are DExD/H--box-containing RNA helicases with similar structures: both possess two N-terminal caspase activation and recruitment domains (CARDs) for initiating downstream signaling and a central helicase domain and carboxyl-terminal domain (CTD), which are both required for RNA binding [21]. Binding with RNA, RIG-I/MDA5 is activated and thus binds to the mitochondrial antiviral-signaling protein (MAVS). MAVS then recruits the TBK1 (TANK-binding kinase 1) and IKKε which respectively phosphorylates the IRF3/7 and aggregates IKKα-IKKβ-IKKγ complex. Sequentially, phosphorylated IRF and NF-κB are translocated into the nucleus, triggering the transcription of antiviral genes and inflammatory genes. Besides RLR pathways, proteins of RLRs play their roles in many other signal transduction trails, such as RNA sensor-mediated inflammation formation. Among them, reversible Ser/Thr phosphorylation and K63- and K48-linked polyubiquitination are critical signals to control pro-inflammatory cytokine induction triggered by RLRs [21].

2.3.2 The PTM Involved in RLR Pathways

2.3.2.1 Phosphorylation

Phosphorylation of the relevant proteins in RIG-1 signaling pathway plays a critical role not only in recruiting and activating downstream proteins but also in activation of other PTM like ubiquitination. Siglec-G, a member of the immunoglobulin-like lectin family, binds to activated RIG-I and recruits tyrosine phosphatase SHP2 (phosphatase Src homology 2) which in turn phosphorylates Siglec-G leading to the promotion of interaction between Siglec-G and RIG-1. Then Siglec-G/SHP2/RIG-I complex recruits and phosphorylates c-Cbl (Casitas B-lineage lymphoma, a E3 ligase), which induces the K48-linked ubiquitination and degradation of RIG-1 at K813 and IRF3 in a dose-dependent manner [22] (Fig. 2.2). Lyn, a member of STKs (Src-family-tyrosine kinases) family, interacts with the helicase domain of RIG-I by its SH2/SH3 domain forming Lyn-RIG-I-MAVS trimolecular complex, which activates the signaling pathway by phosphorylation. Lyn itself is also activated by phosphorylation at T396 [23]. In addition, the Thr-170 phosphorylation, a phosphorylation closely located in proximity to Lys-172 which regulates the RLRs pathways by

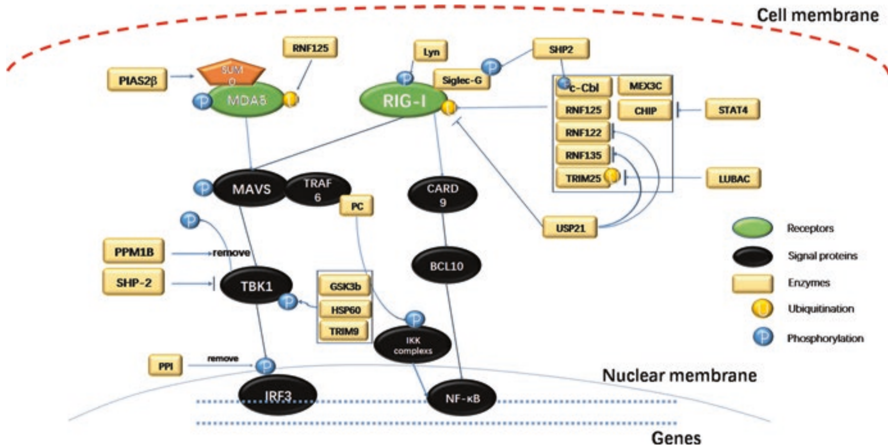

Fig. 2.2 Inflammatory signaling transduction and PTMs of RLR pathways

promoting the ubiquitination of RIG-I, antagonizes the Lys-172 ubiquitination and leads to the suppression of RLR activation [24]. Phosphorylation of Ser-8 of RIG-I is displayed in uninfected cells and decreases in virus-infected cells, serving as an inhibitory modification of RLRs to prevent cells from unwanted inflammatory response via RLRs [25].

In expect of RIG-I itself, the phosphorylation of other molecules in the RIG-I signaling pathway also effectively regulates the on-off of the pathway.

MAVS MAVS is located in the outer mitochondrial membrane. The interaction with it is indispensable for the phosphorylation and activation of IRF3 and TBK1, while both MAVS polymerization and phosphorylation are required for MAVS-IRF3 interaction. Phosphorylation of MAVS at S442 depending on its polymerization and TRAF-mediated polyubiquitin synthesis is critical for IRF3 activation but dispensable for TBK1 and IKK activation [3]. PC, a member of biotin-containing enzyme family, interacts with MAVS/TRAF6, which promotes phosphorylation of IKK (IκB kinase complex) and IκBα and induces the redistribution in nucleus of NF-κB [26].

TBK1 As a key kinase in both RNA and DNA sensing pathway, TBK1 is attenuated by diverse molecules with different mechanisms. Negative regulation of the activity of TBK1 is critical to avoid excessive generation of IFH-β and other inflammatory cytokines. PPM1B (protein phosphatase Mg^{2+}-/Mn^{2+}-dependent 1B) dephosphorylates TBK1 at S172 and decreases the IRF3 activation [27]. Moreover, SHP-2 interacts with TBK1 kinase domain to block its activity as a kinase [28]. In zebra fish, STAT6 also negatively regulates the phosphorylation of TBK1, which was not found in mammalian cells [29]. TRIM9s (TRIM9 short) interacts with TBK1 to facilitate phosphorylation of TBK1 via the RING domain of TRIM9s. In addition, TRIM9s, bridging GSK3β and TBK1, also initiates TBK1 oligomerization which is critical to its activation [30].

IRF3 MAVS also recruits TBK1 to phosphorylate IRF3 typically at 5T/S cluster (396–405) and S385–386. Then phosphorylated mono-IRF3s dissociate from MAVS to form dimers with a C-terminus-C-terminus interaction [22]and translocate to the nucleus. PPI, one of the most abundant phosphatases in eukaryotic, regulates the RIG-I signaling pathway by dephosphorylating IRF3 [31]. HSP60 (heat shock protein 60, also known as HSPD1) interacting with IRF3 by directly phosphorylating IRF3 that triggers its dimerization [32].

2.3.2.2 Ubiquitination

Modification of RIG-I 2CARD with K63-linked ubiquitination is important for signaling activity. The tetrameric architecture of 2CARD mentioned above binds to six Ub molecules in the asymmetric unit. Both the activation and degradation of RIG-I are attenuated by several host molecules.

RNF Family RNF125, a RING-type E3 ubiquitin ligase, polyubiquitylates RIG-I at K181 and leads to K48-linked proteasome-dependent degradation, which impedes the RIG-I signaling pathway as a negative feedback regulation [33]. Similarly, the TM domain of RNF122 directly binds to the 2CARD domain of RIG-I which mediates K48-linked ubiquitination at K115/K146 of 2CARD domain of RIG-I. Located on ER membrane, RNF122 can also mediate self-ubiquitin degradation [34]. Conversely, RNF135, also called REUL, positively regulates the RIG-I pathway via facilitating the K63-linked polyubiquitination of RIG-I [35]. In addition, RNf121 facilitates the proteasomal degradation of IκBα, whose degradation is critical for NF-κB to translocate into the nucleus and trigger IFN-β expression [36]. Moreover, RNF5 promotes the K48-linked polyubiquitination that degrades MAVS and STING on L150 [37].

TRIM Family RLR-MAVS-dependent signal pathways are under strict control of PTM including polyubiquitination and phosphorylation. The ubiquitin E3 ligase TRIM25 modifies RIG-I with K63-linked polyubiquitination [38]. TRIM25 (tripartite motif 25) composed of a RING domain at N-terminal, one or two B-box domains, a coiled-coil dimerization domain, and a C-terminal SPRY domain binding to 2CARD domain, catalyzes RIG-I at K172 via K63-linked polyubiquitination. Furthermore, the coiled-coil domain of TRIM25 is a stable, antiparallel dimer possessing two catalytic RING domains on opposite ends of an elongated rod. RING dimerization is required for catalysis, TRIM25-mediated RIG-I ubiquitination, interferon induction, and antiviral activity [39]. The interaction between TRIM25 and RIG-I delivering the Lys 63-linked ubiquitin moiety to the N-terminal CARDs of RIG-I results in an increase in RIG-I downstream signaling activity. The ubiquitination of RIG-I enables its oligomerization and thus induces RIG-I interacting with MAVS. Mutations of conserved residues of RIG-I that disrupt its ubiquitin binding also abrogate its ability to activate IRF3 demonstrating that CARD domain of RIG-I is the structural basis for the IRF-inducing ability of RLRs [40]. NS1 (nonstructural protein 1), a virulence factor of *influenza A virus*, not only binds to

TRIM25 directly impeding the K63-linked ubiquitination of RIG-I but also prevents RIG-I from sensing the viral dsRNAs [41]. In addition, TRIM56 and TRIM32, both positive modulators in STING-dependent IRF3 activation, facilitate the K63-linked ubiquitination of STING required for recruitment and activation of TBK 1 [42, 43]. Conversely, TRIM23 interacts with NEMO and catalyzes the conjugation of K27-linked polyubiquitin chain onto NEMO, leading to its proteasome-dependent degradation [44]. TRIM21, bound to K63 E2 conjugating enzyme UBC13, facilitates TAK1-TAB1-TAB2, IKKα-IKKβ-NEMO, and IRF3/5/7 complex activation via K63-linked ubiquitination [45]. Additionally, TRIM4 is another E3 ligases identified as enzymes modifying RIG-I with K63-polyubiquitin chains [46].

Other Protein Families MEX3C interacts with RIG-I directly via its 382–599 domain, while its RING domain ubiquitylates RIG-I at K99/K169 causing the activation of RIG-I [47]. STAT4, in spite of a classical kinase, blocks the degradation of RIG-I via interacting with CHIP and disrupts the association between RIG-I and CHIP, which induces K48-linked ubiquitination and degradation of RIG-I [48]. LUBAC (linear ubiquitin chain assembly complex) natively regulates RIG-I signaling pathway in two ways. One is that LUBAC, directly bound to TRIM25 which facilitates the K63 ubiquitination of RIG-I (mentioned above), catalyzes the K48-linked polyubiquitination of TRIM25 resulting in the degradation of TRIM25 [49]. The other is that LUBAC catalyzes the linear ubiquitination of NEMO which plays a critical role in the activation of NF-κB signaling pathway, while the linear-ubiquitinated NEMO impedes the interaction between MAVS and TRAF3 [50]. C-Cbl, an E3 ligase, promotes the K48-linked polyubiquitination and proteasomal degradation of RIG-I [51], which is phosphorylated by Siglec-G. USP21 functions in few ways. It blocks the K63-linked ubiquitination of RIG-I, promotes the deubiquitination of RIG-I and MAVS, and also inhibits TRIM25- and RNF135-mediated RIG-I polyubiquitination and activation [52]. USP15 interacts with the CARD domain and C-terminal of RIG-I by its UCH domain while deubiquitinates the USP15 by its active sites, His862 [53]. P97, forming Np14-Ufd1-p97 complex, promotes the K48-linked ubiquitination of RIG-I by facilitating RNF125. Though Np14 binds to RIG-I at CARD of non-ubiquitination RIG-I to promote the K63-linked ubiquitination of RIG-I at K172/K170, then the activation of K63-linked progress triggers the interaction among Np14, Ufd1, and p97, which functions as a negative feedback regulation [54]. Smurf 2(Smad ubiquitin regulatory factor 2), another E3 ubiquitin ligase, physically interacts with MAVS and triggers the K48-linked ubiquitination and degradation of MAVS [55]. Similarly, PCBP1 or PCBP2 recruits AIP4, a HECT domain-containing E3 ligase, which induces the K48-linked polyubiquitylation and degradation of MAVS [56]. RAUL, a HECT domain E3 ubiquitin ligase, induces the K48-linked polyubiquitination of IRF3/7 that blocks the RIG-I signaling pathway [57].

2.3.2.3 Others

Besides phosphorylation and ubiquitination, there are other special PTMs required for the regulation of IRG-I signaling pathway. FAT10 negatively regulates the pathway by preventing RIG-I from ubiquitin-dependent activation and interaction with IRF3. Also excessive FAT10, bound to the CARD domain of RIG-I directly, sequesters the active form of RIG-I into insoluble precipitate [58]. What's more, several DUBs removing K63-linked polyubiquitin from RIG-I to balance pro-inflammatory responses are identified, including USP3 (ubiquitin-specific protease 3) [59], CYLD [60], and USP21 [52]. In addition, it is reported that ISG15-modified RIG-I displays lower activity to trigger the downstream response [61]. Similar to ubiquitylation, SUMOylation is a multistep reaction that covalently conjugates a 12-kDa small ubiquitin-like modifier (SUMO) to target proteins by a single E1-activating enzyme (Aos1/Uba2), a unique E2-conjugating enzyme (Ubc9), and an array of different E3 ligases [62]. The direct interaction between UBC9 and target proteins is required for SUMOylation to transfer SUMO from E1. Therefore, when UBC9 binds to RIG-I directly, activated SUMOylation of RIG-I alters the protein folding to assist RIG-I ubiquitylation and enhances the interaction with MAVS [63]. IRTKS (insulin receptor tyrosine kinase substrate) can also recruit Ubc9 to SUMOylate PCBP2 (a negative regulator of MAVS) by SUMO2 at K37 of PCBP2, which is required for cytoplasmic translocation of PCBP2 then leading to MAVS degradation [64]. Similarly, Ndfip1 promotes the degradation of MAVS by triggering the autoubiquitination of Smurf1, an E3 ubiquitin ligase with multiple HECT domains, which then interacts with and finally degrades MAVS [65]. When it comes to IRF family, α-LA (LA, lipoic acid, 1,2-dithiolano-3-pentanoic acid; $C_8H_{14}O_2S_2$) promotes SUMOylation of IRF-1 by SUMO1 which negatively regulates its transcription activity [66]. Both of IRF-3 and IRF-7 can be SUMOylated by SUMO 1/2/3 at K152 of IRF3 and K406 of IRF7, which induces a lower level of IFN mRNA [67]. In addition, amidation of RIG-I inhibits IFN induction, so the deamidation catalyzed by phosphoribosylformyglycinamide synthase (PFAS) of RIG-I at Q10, N245, and N445 residues converts the suppression. Upon viral RNA stimuli, the CARD domain of RIG-I interacts with the GAT domain of vGAT (glutamine aminotransferase); the latter was a pseudo enzyme recruiting PFAS to RIG-I which triggers the downstream pathway [68]. Moreover, as in uninfected cells, RIG-I is acetylated which restricts not only RIG-I translocation to intracellular membrane for interaction with MAVS but also the oligomerization of RIG-I, in infected cells, HDAC6 (histone deacetylase 6) deacetylates RIG-I to activate RIG-I depending IRF3 activation [69, 70].

2.4 The PTMs of NLR Pathways

NLR pathways refer to nucleotide-binding oligomerization domain containing protein 1-like receptor-related signaling pathways. NLR proteins are cytosolically located detecting PAMPs invading into the host cells. Up to now, more than 20 genes are identified in human genome [71], and certain roles of several NLR proteins including NOD1, NOD2, NLRP3, and NLRC4 are studied. Among different signaling transduction ways, PTM displays its roles in them.

2.4.1 The Overview of NLR

NLR proteins include NOD1/2, Nalps, Ipaf, and Naip. The essential N-terminal domains of NLR can be identified as characters of different proteins, NOD1/2 and Ipaf with CARD, Nalps with PYD (pyrin domain), and Naip with BIR (baculovirus inhibitor of apoptosis protein repeat domain) [72]. NOD1/2 are responsible for detecting microbe-derived peptidoglycan [73], and once activated, they can recruit a serine-threonine kinase called RIP2 (receptor-interacting protein 2) via CRAD-CARD interactions [74].Then, RIP2 directly interacts with the IKKγ (NEMO) and promotes its ubiquitylation; thus IκBα is degraded, and NF-κB is able to translocate into nuclear to promote pro-inflammatory chemokine production.

2.4.2 The PTMs Involved in NLR Pathways

Since RIP2 is the key protein linking NOD and NF-κB activation, RIP2 itself arises a lot of attentions for its capacity in regulating NLR pathways (Fig. 2.3). It has been proved that RIP2 undergoes autophosphorylation at Tyr 474 via tyrosine kinase activity in RIP2 itself, and this phosphorylation is necessary for effective NOD-related NLR signaling transduction [75]. Other phosphorylation sites identified include S168/176 in RIP2 kinase domain [76, 77], S363/393 in interdomain region of RIP2 [77], and S527/529/531/539 in the flexible C-terminal region of RIP2 [78, 79]. Additionally, RIP2 is also related to the ubiquitination of NEMO (IKKγ), important for sequent IκBα degradation and NF-κB activation [80]. Pellino3-mediated K63-linked polyubiquitination of RIP2 plays a positive role in NOD2-RIP2-NF-κB pathway [81]. Ubiquitination of RIP2 mediated by clAPs (cellular inhibitors of apoptosis) is a promotive factor of NLR-related NF-κB and JNK pathways [82]. Similarly, XIAP mediating the ubiquitination of RIP2 also increases the activation of RIP2-mediated NF-κB [83].

While some PTMs are responsible for enabling and increasing NLR pathway activation, some others negatively regulate them. RIP2 is polyubiquitinated by ITCH, an E3 ubiquitin ligase, which inhibits the NF-κB activation and JNK

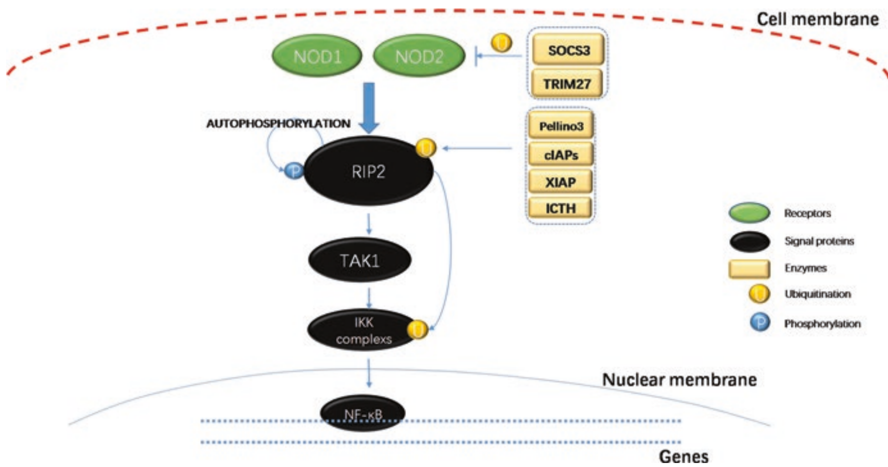

Fig. 2.3 Inflammatory signaling transduction and PTMs of NLR pathways

activation via phosphorylation of JNK and MAPK [84]. For NODs, SOCS3 and TRIM27 induce polyubiquitination and proteasomal degradation of NOD2 [85, 86] and thus attenuate the whole signal transduction mediated by NOD2.

2.5 Intracellular DNA Sensors

2.5.1 Overview of Cytosol DNA Sensor

Intracellular DNA sensors recognize the dsDNA and ssDNA of virus that invade into the cytoplasm, which then mediate a type I IFN response and other inflammatory cytokines such as TNF-α and IL-6 through recruitment and activation of their adaptor proteins, like cGAMP, STING, TBK1, and so on. One of critical DNA sensors that we have investigated most is cGAS (cyclic GMP-AMP synthase). In addition, many other DNA sensors in the cytoplasm have been identified, including IFI16 (interferon-inducible protein 16), AIM2 (absent in melanoma 2) inflammasomes, and so on.

2.5.2 PTM of cGAS Signaling Pathways

Triggered by viral dsDNA, the mammalian cGAS enzyme with a single active site converts GTP and ATP into a mixed dinucleotide species G[2′-5′]pA[3′-5′]p (cGAMP) containing a 2′-5′ phosphodiester bond [87]. cGAMP then binds to STING resulting in the translocation of the adaptor protein from the endoplasmic

reticulum to the Golgi and perinuclear sites, where activated STING dimerizes and binds to TBK1 and IRF3 and the STING-TBK1-IRF3 complex that finally triggers the IFN and ISG gene expression [21]. Here we introduce the posttranslational modification (PTM) of the relevant molecules in this pathway (Fig. 2.4).

2.5.2.1 Phosphorylation

Phosphorylation is one of the most common modifications to regulate the target protein functions by inducing their conformational changes or recruiting of other proteins. Akt kinase, one of the most critical and versatile protein kinases in higher eukaryotes, negatively regulates cGAS by phosphorylating cGAS at S291 of mouse and S305 of human [21]. Phosphorylation is also critical for activation and translocation of STING. ULK1/ATG (UCN-51-like kinase) can phosphorylate STING at S366 that suppresses the IRF3 function, while ULK1/ATG itself is activated via dissociation from unphosphorylated AMPK which is typically elicited by cGAMP [88]. In addition, viral interferon regulatory factor (vIRF1) of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) inhibits the phosphorylation and activation of STING by inhibiting its interaction with TBK1 [89].

2.5.2.2 Ubiquitination

Protein ubiquitination is inversely regulated by E1, E2, and E3 enzymes or DUB which regulates degradation and activation of a wide variety of molecules in different immunological processes. TRIM (tripartite motif) is one of the most dominant E3 ligases families. TRIM14 not only recruits USP14 (ubiquitin-specific protease

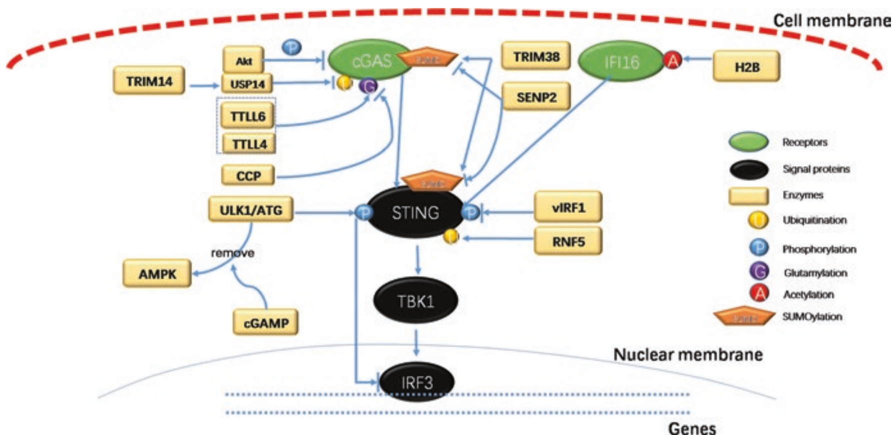

Fig. 2.4 Inflammatory signaling transduction and PTMs of intracellular DNA sensor pathways

14) that cleaves K48-linked ubiquitin chains of cGAS at K414 to prevent it from degradation but also blocks p62-mediated selective autophagic degradation of cGAS [43]. Moreover, STING is also a target for ubiquitination, of which K48-linked ubiquitination leading to degradation while K11-linked and K63-linked ubiquitination facilitating activation [43].

2.5.2.3 Others

Besides phosphorylation and ubiquitination, there are also other essential modifications that regulate the cGAS signaling pathway. Glutamylation of the cGAS suppresses its activation via two ways, in one is polyglutamylated at Glu272 by TTLL6 (tubulin tyrosine ligase-like glutamylases 6) and the other one is monoglutamylated at Glu302 by TTLL4. Conversely, CCP inverses this progress by CCP6 (cytosolic carboxypeptidases 6) via removing the polyglutamylation of cGAS whereas CCP5 hydrolyzing the monoglutamylation of cGAS [90]. What's more, sumoylation of cGAS at K217 and K464 and of STING at K337 by TRIM38 promotes their protein stability and prevents their ubiquitination and degradation, while in the late phase of viral infection, desumoylation of both cGAS and STING mediated by SENP2 avoids the sustained inflammatory response, and SENP2 is recruited by phosphorylated STING at S365 [91].

2.5.3 PTM of IFI16 Signaling Pathways

Interferon-inducible protein 16 (IFI16) is a critical DNA sensor binding to ssDNA, albeit more weakly than to dsDNA in the cytoplasm [92]. After triggered by viral DNA, IFI16 interacts with other adaptor proteins to form BRCAI-IFI16-ASC-procaspase-1 complex, which induces IL-1β and IFN-β production and STING activation. Acetylation of IFI16 complex by H2B, a histone acetyltransferase, enhances its interaction with STING that induces the redistribution of STING from cytoplasm to nucleus and the interaction with cGAS promoting cGAMP production and generation of IL-1β and IFN-β [93].

2.5.4 PTM of AIM2 Signaling Pathway

Located in the cytoplasm, absent in melanoma 2 (AIM2) inflammasome directly interacts with dsDNA driving activation of caspase 1, which promotes pyroptosis and the release of the pro-inflammatory cytokines. This progress requires the type I IFN-potentiated expression of GBPs (guanylate-binding proteins) via the transcriptional factor IRF1 [94]. AIM2 indirectly inhibits the phosphorylation of STAT1 (the signal transducers and activators of transcription 1) which impedes the

phosphorylation of NF-κB p65 at S536 and acetylation of it at L310, the modification facilitating NF-κB translocation and activation, while deacetylation of it by SIRT1/HDAC3 blocks the activation pathway [95].

2.6 Intracellular RNA Sensors

RNA sensors sense atypical RNAs associated with virus infection, recognizing the 555p' RNA and 55p' RNA not protected by a 5′-cap. The typical RNA sensors RIG-1 and MDA5 share the similar functional domains including CARD domain of N-terminal that not only recruits downstream proteins like MAVS, IRF3, and TBK1 but also functions in polyubiquitination and C-terminal domain (CTD) binding to exogenous RNA, consistent with their similarity in immune response. PTM plays an important role in regulation of RNA sensor signaling pathway (Fig. 2.2).

2.6.1 PTM of RIG-I Signaling Pathways

As mentioned in Sect. **2.3**

2.6.2 PTM of MDA5 Signaling Pathways

MDA5 with 2CARD domains in high degree of heterogeneity can self-assemble into a defined oligomeric arrangement, while CARD domains form discrete patches of oligomers along MDA5 filament about 11 monomers per patch [96]. The phosphorylation of MDA5 at S88 in the first CARD and S828 in the CTD dampen its ability to interact with MAVS in uninfected cells [97, 98]. TRIM13, one member of the TRIM family, an E3 ligase family, inhibits the activation of MDA5 and the subsequent MDA5-mediated production of IFN-β but regulates RIG-I pathway in a positive way, whose role in the RNA-sensing signaling network needs to be further investigated [99]. In addition, RNF125 also facilitates the K48-linked polyubiquitination as the proteasome-dependent degradation of MDA5. What's more, PIAS2β, an E3 ligase of SUMOylation, SUMOylates at the C-terminal region of MDA5, which promotes the MDA5-mediated IFN induction, while the SUMOylation has no influence on the K48-linked ubiquitination of MDA5 [100].

2.7 Inflammasomes

Inflammasomes are protein complexes assemble on recognition of exogenous and endogenous danger signals and serve as platforms for activation of canonical caspase 1 or noncanonical caspase11 and secretion of pro-inflammatory cytokines: such as IL-1β, IL-18, and HMGB1 (high-mobility group box 1). Different types of inflammasomes vary in types of activation and downstream molecules. The most widely studied inflammasome up to now is the NLRP3 inflammasome, while NLRC4, AIM2, NLRP1, and other inflammasomes require more experimental investigations. As their effective influence on immune response, the activity of inflammasomes is tightly regulated to avoid from the generation of excessive inflammatory functions. Generally speaking, there are mainly two aspects of regulation in host cells. (A) The expression of inflammasome-relevant proteins is limited at a low level. For example, the level of NLRP3 in many cell types is particularly low with a priming signal to be triggered [101]. (B) The variants of inflammatory components alter in distinct, even controversial functions. Splice variants of ASC have been identified with different activities, with one variant even inhibiting the signaling pathway but not facilitating it as usual [102].

Adaptor protein ASC bridges the sensor protein (NLRP3 and AIM2) and caspase 1 to form ternary inflammasome complexes by its PYD and CARD domains. Then NLRP3 or NLRC4 inflammasomes recruit ASC through their PYD domains, while ASC recruits caspase 1 by the CARD/CARD interaction. Activated ASC trends to form filament, of which the PYD domain is nuclear for ASC^{PYD} forming filament, required for ASC^{PYD} removing His-MBP from the fusion protein [103].

2.7.1 PTM of NRLP3 Signaling Pathway

NLRP3 inflammasome senses a wide range of pathogens including bacteria, virus, fungi, and parasites through both DAMP and PAMP pathways. In addition, NLRP3 can be also activated by the reactive oxygen species (ROS) and lysosome destabilization [104]. In majority of cases, NLRP3 does not interact with pathogens directly. Excessive ROS are sensed by a complex of thioredoxin and thioredoxin-interacting protein (TXNIP), which leads to the dissociation of the complex. Then subsequent binding of TXNIP to NLRP3 triggers the recruitment of ASC and pro-caspase-1, together forming the active inflammasome complex. In addition, NLRP1, AIM2, NAIP, and NLRP4 that can bind to pathogens directly and proteases caused by lysosomal destruction facilitate the interaction between ASC, pro-caspase 1, and NLRP3 [102]. PTM plays an important role in regulating the whole progress (Fig. 2.5).

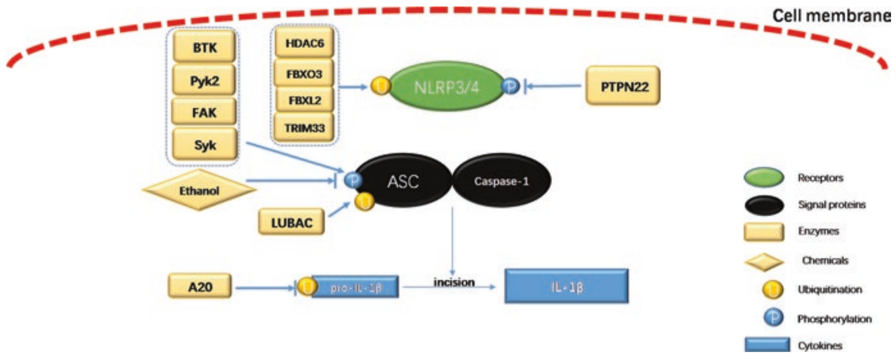

Fig. 2.5 Inflammatory signaling transduction and PTMs of inflammasome pathways

2.7.1.1 Phosphorylation

PKR (double-stranded RNA-dependent protein kinase) physically binds to NLRP3 as a PKR-NLRP3 complex and promotes the inflammasome formation. PKR also plays a critical role in activation of the NLRP1, AIM2, and NLRC4 [105]. Interacting with NLRP3 directly, PTPN22 interferes the tyrosine phosphorylation at Tyr86 of NLRP3, while phosphorylation of NLRP3 negatively regulates the activation of the complex. ASC is necessary for NLRP3 activation [106]. Ethanol inhibits NLRP3 inflammasome by impeding the phosphorylation of ASC at Y146 of human [107]. Syk (spleen tyrosine kinase) interacts with ASC and NLRP3 directly by its kinase domain but without procaspase-1, which phosphorylates ASC at Y146 and Y187 to promote the NLRP3-dependent caspase-1 activation [108]. As downstream proteins of Syk, Pyk2 (proline-rich tyrosine kinase) and FAK (focal adhesion kinase) also phosphorylate ASC at Y146 required for ASC oligomerization, caspase-1 activation, and IL-1β secretion. And only the depletion of FAK leads to the significant inhibition of AIM2-mediated IL-1β secretion [109]. In addition, BTK (Bruton's tyrosine kinase) phosphorylates ASC facilitating its oligomerization and distribution in macrophages required for NLRP3 activation, which also phosphorylates PLCγ to enhance Ca^{2+} influx as an assistant factor to NLRP3 inflammasome formation [110]. Conversely, AMPK (adenosine monophosphate-activated protein kinase) phosphorylation negatively regulates the activation of NLRP3 inflammasome [111]. Mechanism different from the kinases mentioned above, though Nek-7 interacts with NLRP3 by its NOD, LRRs and N-terminal (amino acid residues 34–212) directly, it does not function in phosphorylation but improves the K^+ efflux to activate NLRP3 inflammasome [112].

2.7.1.2 Ubiquitination

LUBAC plays a critical role in ASC foci formation and NLRP3-ASC inflammatory assembly required for linear ubiquitination of ASC and ASC-dependent NLRP3 inflammasome activation [113]. Two members of F-box protein as ubiquitin E3 ligases with a RING domain, FBXO3 (f-box O3) and FBXL2 (f-box L2), function in regulating the ubiquitination of NLRP3. FBXL2 mediates directly degradation of NLRP3 at K689 which impedes the activation of the inflammasome, while FBXO3 targeting on FBXL2 facilitates the ubiquitin-dependent degradation of FBXL2 promoted by the increased level of intracellular LPS [114]. TRIM33 directly interacts with NLRP3 at PYD domain of NLRP3 by its second C-terminal coiled-coil domain to facilitate the K48-linked proteasomal degradation of formation and activation via binding to the HA2-DUF region of DHX33 which induces the K63-linked ubiquitination at K218 [115]. DUBs (deubiquitinases) like OTUB1, UCH-L1, USP7, or CYLD have been shown to play a crucial role in ubiquitin-mediated cellular processes. It is also required for inflammasome-dependent IL-1β processing via promoting oligomerization of ASC to improve the activation of AIM2/NLRP3 inflammasomes; activation of the latter is more evident [116]. For example, UCH-L5 (ubiquitin C-terminal hydrolase 37, UCH37) knockdown leads to decrease in inflammasome-dependent IL-1β release [117]. In addition, upon LPS stimulation, A20, a ubiquitin-modifying enzyme, binds to pro-IL-1β to restrict the K63-linked ubiquitination of pro-IL-1β at K133 that promotes its proteolytic processing [118]. Interestingly, a deacetylase mentioned before, HDAC6 negatively regulates the NLRP3 pathway independent of deacetylation but by facilitating the ubiquitin-dependent degradation of NLRP3 with the Buz (binder of ubiquitin zinc finger) domain of HDAC6 [119].

2.7.1.3 Others

JAK/STAT1 is required for LPS- and IFN-β-induced HMGB1 acetylation catalyzed by the two NLS (nuclear localization sequence) domains of the acetylase, key to efficient HMGB1 release [120]. In addition, UBC9, a sole SUMO-conjugating enzyme, mediates the SUMO-1 SUMOylation to prevent IkBα from degradation, which inhibits the activation of NF-κB. Upon LPS stimuli, CDK1 phosphorylates UBC9 that promotes this progress [121].

2.7.2 PTM of Other Inflammasome Signaling Pathway

NLRC4 Different from NLRP3, NLRC4 is only activated by specific bacterial proteins but not binding directly [122]. NAIP proteins sense bacterial proteins like *S. typhimurium flagellin* causing the recruitment of NLRC4 and assembly of the NLRC4 inflammasome [104]. PKCζ and PAK2 phosphorylate NLRC4 at S553, but

the depletion of PKCζ only reduces the phosphorylation of NLRC4 resulting in ASC focus formation, caspase-1 activation, and pyroptosis. Upon certain stimuli, PKCζ itself is activated via its phosphorylation at Y311 by PMA [123].

Pyrin Encoded by MEFV gene, pyrin recognizes the Rho GTPases-modified bacterial toxins like *C. difficile* cytotoxin TcdA/B, rather than microbial products like other inflammasomes. After phosphorylated at both S205 and S241, pi-pyrin recruits 14-3-3 protein which assists with the activation of pyrin upon specific Rho-modifying toxin stimuli, which triggers the dephosphorylation, ASC recruitment, and assembly of pyrin inflammasome [124].

2.8 PTM-Related Diseases and Immune Disorders

Since PTMs exert a wide range of influence on our immune system, any incapacity in regulations of posttranslational modifications can be enervating factors to immune homeostasis, which is responsible for a series of disease directly and indirectly. Up to now, several disorders in modulation of PTMs are identified and proved to be relative with certain diseases, such as Crohn's disease, a disease caused mainly by immune disorders, as well as exogenous factors that are able to effect the PTM patterns and lead to acquired disorders in immune systems, which cause the upregulation or repression in inflammation. A clear study of the factors, whatever endogenous or exogenous, is helpful for a precise remedy against the diseases derived from them.

Several notorious diseases are actually relative with the profound influence caused by PTM disorders. The Crohn's disease, marked by symptoms including severe stomachache, diarrhea, fever, and chronic anemia, is demonstrated related to the PTM disorders in the immune system. It is proved that NOD-2-involved inflammatory signal pathways are responsible for the onset of Crohn's disease. The mutation of NOD-2 including *L1007fsinsC* which removes the last 33 amino acids of its polypeptide is detected common in Crohn's patients [125, 126], and this specific mutation results in the defect in inducing RIP2 tyrosine autophosphorylation, which is discussed above in NLR pathways that are crucial for the activation of NOD-2 signaling pathways [75].With this certain deficiency, the immune systems of patients with Crohn's diseases are failed to eliminate the infective microbe colonized in our intestines leading to the overbudden of bacteria in gut cells. Also, the ITCH can recognize the phosphorylated RIP2 and thus deactivates it for a moderate NF-κB activation, which serves as an inhibitory role of NOD-2 pathway. As a result, the antagonists, gefitinib and erlotinib, against the autophosphorylation of RIP2 are able to repress the overreaction of NOD-2 pathways downregulating the inflammatory conditions [75].

Some pathogens are capable to induce enzymes responsible for PTMs that negatively regulate our immune response in order to escape the elimination executed by inflammatory conditions. RIG-1, serving both as sensors in RLR pathways and

RNA sensors, is essential for RNA virus defense. However, it is reported that some RNA virus are able to recruit lectin family number Siglec-G which induces and E3 ubiquitin ligase c-Cbl, and this enzyme is responsible for the degradation of RIG-I via K48-linked ubiquitination at Lys813. Sequentially, the degradation of RIG-I dampen the RNA sensor pathway activations as expected, and thus RNA virus makes their own escape [22]. Actually, this feedback-like relationship between virus and our immune systems represents a widespread rivalry.

Not all of PTMs that suppress the inflammatory signaling pathways are unwelcomed. In fact, under most circumstances, our immune system remains inactivated state to avoid unexpected inflammation, which is harmful to tissues. In RLR pathways, K48-linked ubiquitination displays dual roles in regulation. While the K48-linked ubiquitination modified by RNF135 is a positive regulation, those modified by RNF122/125 are responsible for degradation of RIG-I, MDA5, and MAVS, which abrogate the activation of RLR pathways [33, 34, 54]. Also, the K48-linked ubiquitination of TRIM25, a crucial modifier of RLR pathways, displays similar role of inducing degradation of TRIM25 [49]. For MAVS, Smurf1/2, RNF5, AIP4, and MARCH5 are responsible for its K48-linked ubiquitination. In NLR pathways, the ubiquitination modified by ITCH of the RIP2 is an inhibitory factor for activation, which serves as a negative feedback mechanism to limit the inflammation. Similarly, every negative modification mentioned in this article is set to protect cells from dysbiosis brought by improper or overrated inflammation.

To clinical aspect, PTMs that are involved in disease generating are new molecular sites for pharmaceutical design. Although the complete acknowledge of the PTMs is required for a more precise and safer remedy targeting specific disease, well-designed studies of such drugs are promising.

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Chapter 3
Emerging Roles for Epigenetic Programming in the Control of Inflammatory Signaling Integration in Heath and Disease

Yiqun Hu, Feng Yan, Le Ying, and Dakang Xu

Abstract Macrophages and dendritic cells initiate the innate immune response to infection and injury and contribute to inflammatory signaling to maintain the homeostasis of various tissues, which includes resident macrophages for the elimination of invading microorganisms and tissue damage. Inappropriate inflammatory signaling can lead to persistent inflammation and further develop into autoimmune and inflammation-associated diseases. Inflammatory signaling pathways have been well characterized, but how these signaling pathways are converted into sustained and diverse patterns of expression of cytokines, chemokines, and other genes in response to environmental challenges is unclear. Emerging evidence suggests the important role of epigenetic mechanisms in finely tuning the outcome of the host innate immune response. An understanding of epigenetic regulation of innate immune cell identity and function will enable the identification of the mechanism

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between gene-specific host defenses and inflammatory disease and will also allow for exploration of the program of innate immune memory in health and disease. This information could be used to develop therapeutic agents to enhance the host response, preventing chronic inflammation through preserving tissues and signaling integrity.

Keywords Epigenetic • Inflammatory signaling • Innate immune memory • Inflammatory diseases

3.1 Introduction

The immune system is composed of an innate (non-specific) system with the same protective response regardless of the initiating infection and an adaptive (specific) response with specificity that can generate immunological memory [1]. Innate immunity, which is constitutively present, is the first line of defense and is immediately mobilized following infection within a few minutes to hours in contrast to the adaptive immune system, which is slower and requires a few days to weeks to mount a response [2]. The primary component of innate immunity is inflammation [3]. Inflammation is part of the complex biological response of body tissues to harmful stimuli from pathogens and damaged cells that provide a protective process. The function of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged by the original insult during the inflammatory process, and initiate tissue repair [4]. Injured cells release pro- and anti-inflammatory cytokines and other factors to limit the spread of infection and promote healing. At the same time, the innate response induces phagocytes and neutrophils that subsequently attract leukocytes and lymphocytes through these proinflammatory mediators [5]. Activation of the accompanying cascade enhances the innate response, which is an important consequence of complement cascade activation through inflammatory signaling, including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). Through pattern recognition receptors (PRRs), these pathogen-associated molecular patterns (PAMPs) play a critical role in innate immune responses, and PAMP signaling prompts the induction of cytokines and other subsets of genes, the ingestion of complement-tagged pathogens, and the destruction of microorganisms by phagocytes [6]. The innate response involves various cell types, including neutrophils, macrophages, and dendritic cells. TLRs recognize PAMPs in the extracellular milieu and endosomes, while NLRs patrol the cytoplasm. A set of Nod-like receptors that include NLRP1b, NLRP3, and NLRC4 assemble multi-protein complexes termed inflammasomes. Inflammasome assembly is critical for the activation of caspase-1, which ultimately cleaves

pro-IL-1β and pro-IL-18 into their mature bioactive forms, IL-1β and IL-18. Current studies have demonstrated inflammasome involvement during infection, autoimmune disease, and injury [7–15]. A comprehensive review of the inflammasome can be found in this book by Guangxun Meng. Another important function of the innate immune system is to stimulate the adaptive immune response via cross-presentation [16]. TLRs are one of the largest and best studied families of PRRs and are activated following recognition of specific, conserved PAMPs present in microbial proteins, bacteria, and nucleic acid variants that are normally associated with viruses, lipids, or carbohydrates. Recognition of these molecules by TLRs triggers signal transduction cascades that activate the transcription factors nuclear factor (NF-κB) and interferon-regulatory factors (IRFs), which induce the expression of proinflammatory cytokines and interferons or interferon-stimulated gene (ISG) and dictate the outcome of innate immune responses [17, 18]. Activation of these pathways is controlled by several molecular events, the basis of which depends on the interplay of a variety of elements, including transcription factors, epigenetic regulation, and post-transcriptional control mechanisms [18]. A growing number of results have documented the control of signaling pathways by transcription factors and epigenetic factors/chromatin-based mechanisms, which have provided critical evidence for context-specific gene expression in diverse innate immune cell types and chronic inflammatory autoimmune disease [19–22]. Epigenetic regulation comprises the posttranslational modification (PTM) of histones (histone methylation and histone acetylation and deacetylation), DNA methylation, and noncoding RNA. Epigenetic regulation is not only coupled with transcription factor-mediated regulation but is also linked with upstream signaling pathways that connect external signals and gene function to shape the identity and function of innate immune cells. In light of the recent data from high-throughput epigenomic techniques and human macrophage biology [23, 24], we are able to build an understanding of these regulatory mechanisms from detailed information to connect how innate immune cells develop functions in association with epigenetic changes.

In this chapter, we will discuss the role of epigenetic regulation in innate immune cell functional responses to insulting stimuli, the inflammatory process, and disease. In particular, we focus on the prominent role of DNA demethylation, histone modifications, promoter–enhancer interactions, and noncoding RNA-mediated regulation. Finally, we will describe newly emerging data to emphasize the important role of epigenetic mechanisms in the control of memory-type responses in innate immune cells. We will also summarize the future of innate immunity in epigenetics and medical translation.

3.2 Epigenetic Control of Gene Expression

Epigenetic changes in gene activity or expression occur without alteration of the underlying DNA sequence, which results in a change in phenotype without a change in genotype [25]. Genomic DNA is packaged by histones to form protein/DNA complexes termed chromatin in eukaryotic cells; chromatin alterations include DNA methylation, histone modifications, promoter–enhancer interactions, and non-coding RNA-mediated regulation. Epigenetic modulations are determined by the influence of chromatin organization/compaction and function and the critical regulation of gene expression mechanisms at the molecular level through cellular, tissue, and organ levels [26, 27]. The coordinated actions of these multifaceted epigenetic modulations determine cell fate, cell cycle regulation, and development and ultimately control responses in health and disease [28, 29].

In the genome, negatively charged, linear DNA is highly compacted and organized into three-dimensional (3D) chromosomes. DNA is coiled around histones (the main proteins of chromosomes) to form nucleosomes, the basic structural units of chromosomes. Histones are positively charged in the N-terminus with abundant lysine and arginine residues and can thus bind tightly to DNA to constrain its accessibility [30]. Each nucleosome contains a histone octamer that consists of two of each histone monomer (H2A, H2B, H3, and H4) [31]. These highly regulated organization mechanisms not only compact linear DNA into chromosomes but also allow the selective accessibility of transcription machinery [e.g., transcription factors (TFs) and cofactors] to specific genomic elements, including enhancers (facilitate transcription), promoters (initiate transcription), gene body/open reading frames (transcribe and translate into proteins), silencers (suppress transcription), and dielectric (block promoter–enhancer interactions). Epigenetic mechanisms chemically or structurally alter chromatin through modifications of DNA and histones or through chromatin remodeling and inter-/intrachromosomal DNA–DNA interaction, respectively [32] (Fig. 3.1). In DNA methylation, a methyl (CH3) group is enzymatically added onto the cytosine rings of DNA with temporal and spatial precision. In the human genome, DNA methylation occurs in most CpG dinucleotides [33], mainly to form 5-methylcytosine (5-mC). Methylation was once thought to be a stable heritable genetic trait, but recent studies have indicated that methyl groups can be dynamically added or erased [34]. DNA methylation occurs in promoter regions, such as transcription start sites (TSSs), and usually suppresses the expression of downstream genes through the recruitment of DNA-binding proteins and histone modifiers that repress transcription [35, 36]. Methylation of other genomic regions, such as enhancers and dielectric, has also been identified, but its functional importance remains to be investigated.

DNA methylation is a dynamic process in which methylation patterns with methylation marks in genomic regions can be synthesized de novo, maintained, or removed. DNA methylation is processed by an intricate balance between DNA methyltransferases (DNMTs) and DNA demethylases. The genome encodes three DNMTs (DNMT1, DNMT3A, and DNMT3B) to catalyze DNA methylation.

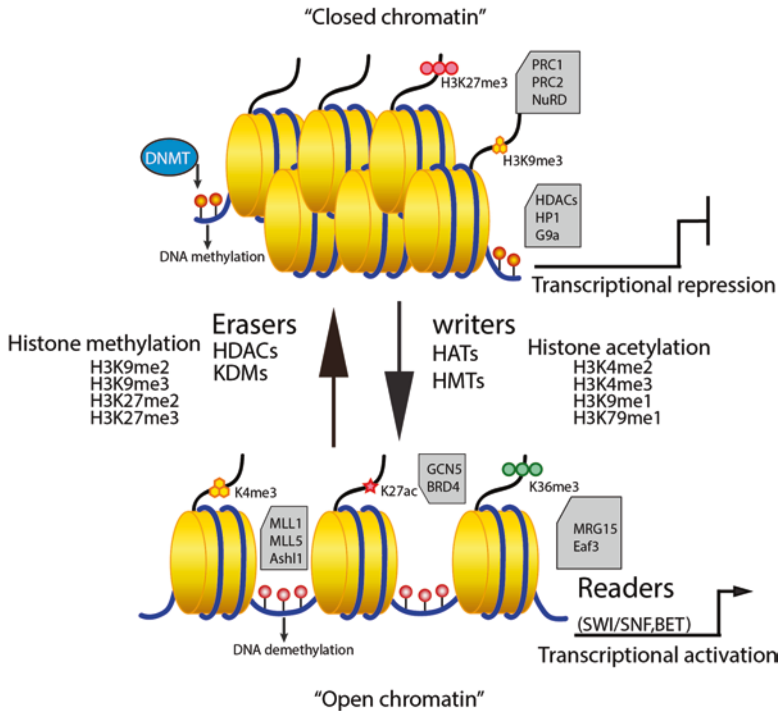

Fig. 3.1 Chromatins can exist as euchromatin (open) or as heterochromatin (compact). The chromatin state is marked (open) by promoter DNA hypomethylation and various posttranslational histone (H) modifications, especially H3, including H3K4me3, H3K27ac, and H3K36me3. In this state, DNA is loosely wrapped around nucleosomes and enables active (on) transcription. The heterochromatin state is marked (compact) by promoter DNA hypermethylation, H3K27me3, and H3K9me3, where compact DNA nucleosomes repress (off) transcription
The on and off states of transcription can be switched by the epigenetic markers writers, readers, and erasers, for example, "writers" by DNA methyltransferases [(DNMTs), histone acetyltransferases (HATs), and histone methyltransferases (HMTs)] and epigenetic "erasers" by DNA demethylases [(TETs), histone deacetylases (HDACs), and histone-lysine demethylases (KDMs)]. "Readers" by epigenetic marks on DNA or histones appear in *gray boxes*

DNMT3A and DNMT3B play major roles in de novo DNA methylation [37]. The major pathway mediating DNA demethylation is catalyzed by 11 translocation methylcytosine dioxygenase (TET) family proteins, with the methyl group of 5-mC oxidized to yield 5-hydroxymethyl cytosine (5-hmC), which can be further oxidized to 5-formylcytosine and 5-carboxylcytosine [38]. These derivatives of 5-mC are novel epigenetic marks that are linked to further biological functions.

Histone modification is subjected to posttranslational modifications (PTMs), including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination [39] (Fig. 3.1). Histone PTMs are widely distributed throughout the whole genome to form the histone code, which can control the accessibility of DNA and recruit transcription factors and coactivators/cosuppressors to result in active,

poised, or silenced transcription [40–42]. In conjunction with DNA methylation, the histone code affects the transcriptional states of genomic regions of promoters, enhancers, and gene bodies, which can determine transcriptional programs. Distinct histone modifications can lead to activation or inactivation of adjacent genes by recruiting chromatin remodeling complexes, TFs, and transcriptional coactivators/cosuppressors. In particular, H3K27ac [acetyl-histone H3 (Lys-27)], H3K4me3 [trimethyl-histone H3 (Lys-4)], H3K4me1 [methyl-histone H3 (Lys-4)], and H3K36me3 [tri-methyl-histone H3 (Lys-36)] are associated with the active transcription region. In contrast, H3K27me3 [tri-methyl-histone (Lys-27)] and H3K9me3 [tri-methyl-histone H3 (Lys-9)] are mainly distributed in the inactive gene locus [43, 44]. These histone PTMs are illustrated in Fig. 3.1. The three main classes of regulatory models are presented as follows: (1) "writers" that create covalent modifications for DNA methylation and histone methyltransferases and acetyltransferases, (2)"erasers" that eliminate modifications for demethylases and histone deacetylases, and (3)"readers" that recognize modification through specific protein domains for bromodomain-containing proteins and mating-type switching (SWI) and sucrose fermentation (sucrose nonfermenting – SNF) SWI/SNF nucleosome remodelers, which are the major players that govern dynamic changes in chromatin structure for development or response to environmental stimuli [45]. Histone modifications can affect transcriptional activities by the two following major mechanisms: (1) histone PTMs can alter chromatin structure and conformation. For example, H3K27 acetylation inhibits the positive charge of histones, thus decreasing their binding to DNA and increasing their accessibility for positive regulation of gene expression [46]. (2) Histone PTMs can provide signals for the "reader" enzymes to further recruit transcriptional activators/suppressors. For instance, H3K27 trimethylation is recognized by the polycomb repressive complex, and it can ubiquitinate histone H2A, leading to transcriptional repression [47]. For each histone PTM, there are specific enzymes that catalyze the dynamic "writing," "reading," or "erasing" of these modifications (Fig. 3.1). The "writers" include histone acetyltransferase (HAT), histone methyltransferase (HMT), and protein arginine methyltransferase, whereas the "erasers" are histone deacetylase (HDAC) and lysine demethylase (KDM). The "reader" proteins containing bromodomain-containing proteins or SWI/SNF nucleosome remodelers can recognize differentially modified histones. These enzymes are also subjected to PTMs elicited by upstream signal transduction. A study has shown that sumoylation of HDAC1 increases its transcriptional repression activity [48] and that phosphorylation of HDAC1/2 renders them enzymatically active [49].

Noncoding RNAs (ncRNAs) are RNAs that are not translated into proteins. Although 30% of the human genome can be transcribed, only a small portion of genes are translated into proteins [50]. ncRNAs can be classified on the basis of their size into small ncRNAs [small interfering RNAs (siRNAs), microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), transfer RNAs (tRNAs), and small nucleolar RNAs (snRNAs)] and long ncRNAs (lncRNAs)]. According to their modes of function, lncRNAs have been divided into four major types (signals, decoys, guides, and scaffolds) [51]. lncRNAs with functions related to signals or decoys contribute

to gene activation or suppression, respectively. lncRNAs that act as guides recruit chromatin-modifying enzymes to regulate gene expression in *cis* or *trans*. lncRNAs act as scaffolds and recruit multiple proteins to assemble ribonucleoprotein complexes, which usually function on chromatin and/or modulate histone marks.

Understanding the dynamic changes in the 3D conformation of chromatin is very challenging; thus, precise mapping of the epigenome, such as genome-wide epigenetic modifications that require high-throughput profiling technologies at nucleotide resolution, has been carried out. With the achievement of the Human Genome Project and the development of next-generation sequencing (NGS) at high-throughput levels, a large number of RNA-seq profiling studies have been performed in the past few years. FANTOM5 provides novel insights from a large network into human macrophage biology [52, 53], and with more advanced bioinformatics tools, we could build the foundation and principles to allow for investigations of epigenetics by different disciplines. Histone codes are comprehensibly presented in the Encyclopedia of DNA Elements (ENCODE), which was developed to identify all functional elements of the human genome [54, 55]. ENCODE has collected a large amount of data on epigenetic regulation from various cell types and tissues and made this information publicly accessible for us. The archived large-scale data sets, including functional DNA elements, transcriptional machinery, and histone modifications, can be easily uploaded to the human genome browser, which allows us to perform further integrative analyses.

3.3 DNA Methylation and Innate Immune Cell Responses

A growing number of data sets have shown that epigenetic modification mechanisms, including DNA methylation, histone modifications, and noncoding RNAs, contribute to the regulation of inflammatory signaling for innate immune responses [18, 56] (Fig. 3.1). DNA methylation occurs in several regions, including intragenic, intergenic, and promoters with CpG islands in mammals [57]. Methylation of promoters leads to gene expression silencing, while methylation of intragenic regions can result in alternative transcripts and expression, which may lead to tissue- and cell-specific changes [57]. DNA methylation of CpG islands impacts negative regulation of gene expression. Previous research has reported that the switching of M1 and M2 phenotypes occurs by inactivating state-specific signature genes through DNA methylation of CpG islands in gene promoters. The influence of global methylation on gene expression in macrophages is evidenced by numerous hypomethylated regions in intragenic and intergenic regions in genes [58]. DNA methylation is a heritable DNA modification, which refers to the addition of a methyl group to 5-cytosine (C) to DNA catalyzed by DNA methyltransferase, such as DNMT3a and DNMT3b, to form 5-methylcytosine (5mC). DNA methylation, which is a transcriptional repression mark, plays an important role in many biological processes, such as development, tumorigenesis, and immune responses [34]. In Dnmt1 and Dnmt3a double knockout (DKO) neurons, upregulation of MHC class I gene and

transcription factor signal transducer and activator of transcription 1 (STAT1) was found to subsequently lead to the expression of ISGs that are known to contribute to synaptic plasticity, and this regulation was associated with a significant decrease in DNA methylation, indicating that ISGs are potential targets of DNMTs [59]. Hypermethylation of the suppressor of cytokine signaling 1 (SOSC1) promoter under DNMT1 silencing causes overproduction of the proinflammatory cytokines TNF- and IL-6 during lipopolysaccharide (LPS)-induced activation of macrophages [60]. DNMT3b expression was also significantly elevated in isolated tissue resident macrophages, specifically adipose tissue macrophages from *ob*/*ob* mice. In obesity, elevated saturated fatty acids enhance DNMT3b expression, leading to DNA methylation of CpG sites on the peroxisome proliferator-activated receptor γ1(PPARγ1) promoter, a key transcriptional factor that regulates adipose tissue macrophage polarization, inflammation, and insulin resistance [61]. Genome-wide DNA methylation sequencing with isolated tissue macrophages and endothelial cells from ischemic muscle of hyperlipidemic and type 2 diabetes mellitus mice demonstrated significant promoter hypomethylation of genes typical of active proinflammatory M1 macrophage gene promoters, including Cfb, Serping1, and Tnfsf15, and simultaneous hypermethylation of anti-inflammatory, alternatively activated M2-Mϕ gene promoters, including Nrp1, Cxcr4, Plxnd1, Arg1, Cdk18, and Fes. This alternative activation skewed macrophage phenotypes toward M1 lineage [62].

Epigenetic modifications triggered by environmental factors are an important mechanism that leads to altered gene expression. DNA methylation has an important role in the regulation of inflammatory signaling, while aberrations in DNA methylation are involved in autoimmune and inflammatory diseases. Periodontitis is a common chronic inflammatory disease that poses significant global health and financial burdens; the initiation and maintenance of chronic inflammatory periodontitis occurs through a complex microbial biofilm in which the predominant pathogen is *Porphyromonas gingivalis* [63]. TLRs play an important role in periodontitis by recognizing *Porphyromonas gingivalis* pathogens and maintaining tissue homeostasis [64]. In an analysis of the TLR2 promoter CpG island, increased DNA methylation was observed in the gingiva of mice infected with *P. gingivalis* in a periodontitis oral gavage model. Infection was correlated with higher CpG methylation in dysregulated gingiva. Furthermore, tissues obtained from periodontitis patients also exhibited differential TLR2 promoter methylation through bisulfite DNA sequencing analysis. TLR2 promoter DNA methylation creates innate immune dysbiosis [65, 66]. DNMTs may also affect innate immune signaling.

3.4 Histone Methylation and Innate Immune Cell Responses

Increasingly, studies have indicated that histone methylation has an important role in the regulation of inflammatory triggered immune responses by controlling the transcriptional regulation of target-related genes [67]. Several histone

methyltransferases (HMTs) and demethylases have been listed as key regulators that control the innate immune response genes, which include transcription factors, cytokines, chemokine, IFNs, and encoding enzymes [68]. H3 histone-lysine 4 trimethylation (H3K4me3), a modification localized to the transcription start site of active genes, is controlled by mixed lineage leukemia (MLL) enzymes. MLL contains a central zinc finger domain homologous to that of the *Drosophila* protein trithorax (Trx) with transactivation activity and a carboxyl terminal SET domain homologous to regions of the suppression of variegation 3–9 [Su(var)3–9], enhancer of zeste [E(z)], and trithorax *Drosophila* proteins. Trx and E(z) are encoded by members of the *trx* and *Polycomb* groups (*trx-G* and *Pc-G*) of developmental regulatory genes, respectively [69–71]. SET and MLL proteins have been associated with a wide variety of diseases [72–75]. MLL4 (the mouse ortholog is Wbp7) has a key role in the innate immune response. Wbp7 is required for robust activation of pathogen-mediated gene responses. Severe response defects were observed in Wbp7-deficient macrophages after LPS challenge, resulting in dysregulation of a very specific subset of genes, such as *Pigp*. The H3K4me3 levels of phosphatidylinositol-glycan biosynthesis class P protein (PIGP) were directly downregulated by Wbp7 depletion. PIGP regulates the glycosylphosphatidylinositol (GPI)-anchor synthesis pathway, which directs CD14 to the extracellular membrane. Membrane-tethered CD14 serves as an important accessory receptor for TLR4 and is critical for innate immune responses [76]. Nuclear translocation of another H3K4 methyltransferase, MLL1 (also known as KMT2A), to chromatin was found to function in innate immunity by selectively regulating the activation of genes downstream of NF-κB-mediated genes, such as interleukin-6 (IL-6) by tumor necrosis factor-α (TNF-α) and LPS. [77]. Ash1l with a SET (Su[var]3–9, E[z] and trithorax) domain, a H3K4 methyltransferase, suppressed IL-6 and TNF-α production in TLR-triggered macrophages, protecting mice from sepsis. The mechanism involved Ash1l promoting A20 (Tnfaip3) expression by increasing H3K4 methylation at the Tnfaip3 promoter that in turn facilitates A20-mediated deubiquitination of NF-κB signaling pathways and the production of proinflammatory cytokines. Ash1l-deficient mice were more susceptible to sepsis with *Escherichia coli* challenge. Ash11-deficient mice also had increased susceptibility to collagen-II-induced arthritis, and compared with wild-type mice, more Ash11-deficient mice suffered from spontaneous systemic autoimmune disease [78]. In addition, Polycomb group (PcG) proteins mediate gene silencing and repress transdifferentiation in a manner dependent on histone H3 lysine 27 trimethylation (H3K27me3). JmjC-domain protein Jmjd3 (also known as KDM6B) is a H3K27me3 demethylase expressed in macrophages; Jmjd3 binds PcG target genes and regulates their H3K27me3 levels and transcriptional activity. Research has shown that macrophage terminal differentiation is associated with a sharp drop in Jmjd3 occupancy of both *HoxA7* and *HoxA11*, which correlated with an increase in H3K27me3 levels and gene inactivation. As an inducible enzyme, Jmjd3 erases a histone mark that controls differentiation and cell identity, which provides a link between inflammation and reprogramming of the epigenome [79]. In previous experiments, interleukin-4 (IL-4) treatment led to upregulated Jmjd3 expression and decreased H3K27me3 at the promoter of M2

marker genes, such as Ym1 (*Chi3l3*), FIZZ1 (*Retnla*), and Arginase 1 (*Arg1*), and a concomitant increase in Jmjd3 expression. IL-4 stimulation leads to increased activated transcription factor STAT6 and binds to consensus sites at the Jmjd3 promoter. Increased Jmjd3 contributes to the decrease in H3K27 dimethylation and trimethylation (H3K27me2/3) marks, as well as the transcriptional activation of specific M2 marker genes [80]. A later study also identified interferon-regulatory factor 4 (IRF4) as a key transcription factor that controls M2 macrophage polarization. Jmjd3-mediated H3K27 demethylation is crucial for regulating M2 macrophage polarization, and TLRs mediated innate immune responses against helminth infection by regulating the expression of *Irf4* [79, 81]. In this study, Jmjd3 is recruited to transcription start sites with RNA polymerase II complex to bind Jmjd3 to target genes by the presence of the activation marker H3K4me3; this action is a H3K27 demethylation-independent mechanism. This finding indicates that histone methylation state exchange in H3K4 and H3K27 methylation can be an important epigenetic process for the control of innate immune response genes [81]. The histone-lysine N-methyltransferase, enhancer of zeste homologue 1 (EZH1), is another H3K27 methyltransferase. Ezh1 was the most upregulated HMT during DC maturation, and silencing of Ezh1 significantly reduced TLR-triggered production of cytokines, including IL-6, TNF-α, and IFN-β, in DCs and macrophages by suppressing the TLR negative regulator Toll-interacting protein (TOLLIP); the regulation mechanism directly targeted the proximal promoter of tollip and maintained the high level of H3K27 methylation for repression [82]. Furthermore, the H3K9 methyltransferase G9A (also known as EHMT2) with SET domain-containing histone-lysine methyltransferase (HKMT), is crucial for the negative regulation of IFN and ISG for antiviral responses, genetic ablation, or pharmacological inactivation of lysine methyltransferase G9a, which is essential for the generation of H3K9me2, which resulted in highly potent IFN-producing cells and rendered these cells resistant to pathogenic RNA viruses [83]. Interestingly, recent data show that methyltransferase Setdb2 was the only protein lysine methyltransferase induced during infection with influenza virus and repressed the expression of CXC-chemokine ligand 1 (*Cxcl1*), coinciding with occupancy by Setdb2 at the Cxcl1 promoter, which displayed diminished trimethylation of histone H3 Lys9 (H3K9me3) in setdb2 genetrap mice. Mice with a hypomorphic gene trap construct of Setdb2 exhibited increased infiltration of neutrophils during sterile lung inflammation and were less sensitive to bacterial superinfection after infection with influenza virus. This finding indicated cross talk between virus-induced susceptibility and bacterial superinfections [84]. TET2, an enzyme that modifies the DNA base methylcytosine to 5-hydroxymethylcytosine (5HMC), is upregulated in macrophages after LPS stimulation, and TET2 and TET2-induced 5HMC have a feedback loop to prevent persistently high transcription of IL-6 during an innate immune response. In a previous study, the authors found that IkBζ targets TET2 to the IL-6 promoter to indirectly recruit HDAC2, which deacetylates H3 and H4 histones and suppresses transcription [85]. Another recent study provides a unique glimpse of yet another aspect of coordinated DNA methylation and protein acetylation in the host response to pathogenic stimuli. Dnmt3a selectively impaired the production of type I

interferons triggered by PRRs through an epigenetic mechanism by maintaining high expression of HDAC9. HDAC9 directly maintained the deacetylation status of the key PRR signaling molecule TBK1 and enhanced its kinase activity. Understanding how the complex coordination between methyltransferases and histones impacts signaling intermediates and transcriptional regulators at different levels of gene expression is necessary [86]. Overall, these findings indicated that epigenetic modification enzymes are recruited by multi-protein transcriptional complexes or corepressors with histone markers to target gene promoters, where they either directly or indirectly coordinate with DNA or histone modification for transcriptional regulation control.

3.5 Histone Acetylation, Deacetylation, and Innate Immune Cell Responses

The regulation of histone acetylation and deacetylation, which are essential processes for gene regulation, is controlled by HDACs and HATs [87]. HATs are enzymes that transfer acetyl groups to core histones, which have subsequent effects on chromatin remodeling and gene expression. HATs have the two following families [88]: (1) p300/CBP, Taf1, and nuclear receptor coactivators that also possess catalytic acetyltransferase activities and (2) Gcn5 N-acetyltransferases (GNATs) and Morf, Ybf2, Sas2, and Tip60 HATs. HATs are involved in initiating gene expression in macrophages during inflammation [89, 90]. However, we only understand global histone acetylation and its role in regulating gene expression. HATs may also interact with the opposing HDAC enzymes to enhance acetylation and eventually activation of antiviral gene promoters. HATs p300/CBP were recruited to the inactive IFN promoter upon transcription factor interferon regulator factor 5 (IRF5) phosphorylation and displaced the SMRT/Sin3a repressive complexes. IRF5 is subsequently acetylated by p300/CBP, facilitating H3 histone acetylation of target genes, histone phosphorylation by MAPK cascades, and recruitment of HATs p300/CBP by signaling transcription factors such as NF-κB and STATs, but little is known about how TLR-induced signals are propagated to chromatin and histones, including TNF-α and IL-6 [54]. A limited number of reports have shown that HATs catalyze GNATs and Morf, Ybf2, Sas2, and Tip60 HATs in the regulation of the expression of specific M1- or M2-associated genes.

The enzymes that oppose HAT functions are HDACs. Histone deacetylation is a dynamic process and may be the result of other posttranslational modifications. HDAC functions may induce further epigenetic changes and alternative gene expression. Eighteen mammalian HDACs have been identified, which are classified into the five following groups: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), and class III (consists of the NAD + −dependent HDACs) and HDAC11, which constitutes a class of its own [118]. The effects of HDACs during macro-

phage activation include HDAC sirtuin 6 repressing the transcription of NF-κB target genes through binding to NF-κB p65 and deacetylating H3K9 at the regulated gene promoters [91]. In addition, promyelocytic leukemia zinc finger (PLZF) protein, also known as Zbtb16 or Zfp145, suppressed the expression levels of inflammatory cytokines IL-6 and IL-12p40 by forming a corepressor complex that has HDAC activity to modify chromatin in a subset of transcription factor NF-κB regulatory elements [40, 92]. Using small-molecule inhibitors of HDAC classes I and II in several macrophage populations results in reduced levels of TLR receptors, cytokines, and chemokines. These inhibitors also impact cellular processes, such as chemotaxis, phagocytosis, apoptosis, and cellular metabolism [30, 55–57]. Inhibition of HDACs may also be beneficial in a complex inflammatory environment due to interactions between macrophage populations and other resident cells that occur for host defense. For example, HDAC inhibition rescued oligodendrocytes during traumatic brain injury via induction of the M2 phenotype in resident microglia [59]. There are many probable explanations for the HDAC class I and II effects on macrophage activation status. It has been reported that HDAC inhibition increase the recruitment of the repressive complex to the promoters of M1 activation state genes like IL-6 [55]. Another possibility is that these effects are a result of the decline in PU.1 levels in macrophages treated with TSA; PU.1 transcription factor is known to be important for the transcriptional control of macrophage development and function [60, 61]. Interestingly, Serrat et al. proposed that TSA induces acetylation-mediated repression of C/EBP, which binds with lower efficiency to the Arg1 promoter in macrophages [62]. A more comprehensive review of HDACs can be found in this book by Bandar Suliman.

3.6 Noncoding RNA and Innate Immune Responses

3.6.1 miRNAs

miRNAs are small noncoding RNAs of ~22 nucleotides in length that are produced by two RNases – Drosha and Dicer. The main function of miRNAs is RNA silencing by targeting protein coding transcripts. miRNAs regulate TLR signaling pathways at several levels, including regulation of TLR expression, TLR-associated signaling proteins and regulatory molecules, and TLR-induced transcription factors and functional cytokines [93, 94]. Therefore, miRNAs greatly contribute to inflammatory signaling and have been extensively studied. A comprehensive review of miRNAs can be found in this book by Claire McCoy.

3.6.2 lncRNAs

Thousands of lncRNAs have been discovered by high-throughput transcriptome sequencing [95, 96]. lncRNAs interact with small RNAs and RNA-binding protein and play important roles in controlling chromatin structure, gene expression, and mRNA translation via posttranscriptional mechanisms targeting splicing and stability [97]. Hundreds of lncRNAs are induced by innate immune activation through TLR ligation stimulation experiments. lnc-DC was expressed exclusively in DCs, which has been recently identified as a requirement of LPS-induced functional maturation and activation of DCs [98]. lnc-DC directly bound to STAT3 in the cytoplasm, which promoted STAT3 phosphorylation on tyrosine-705 by interfering with STAT3 binding and dephosphorylation by SHP1, which regulates DC differentiation. RNA-seq analysis revealed that 72 lincRNAs were significantly upregulated in mouse bone marrow-derived macrophages after treatment with bacterial lipoprotein Pam3CSK4 (TLR2) [99]. One of these lincRNAs, (lincRNA)-*Cox2*, was shown to act as a regulator of a MYD88- and NF-κB-mediated program in macrophages, and both activate and repress distinct subsets of proinflammatory genes. A later study showed that lincRNA-Cox2 is assembled into the SWI/SNF complex after TLR treatment in macrophages. The lincRNA-Cox2/SWI/SNF complex can modulate the assembly of transcription factor NF-κB subunits p65/p50 to the SWI/SNF complex, leading to chromatin remodeling and transactivation of the late primary inflammatory response genes in response to microbial challenge [100]. lincRNA-Cox2 is highly induced by multiple inflammatory triggers, such as TLR ligands (LPS) and Pam3CSK4 and microbial pathogens (*Sendai virus* and *Listeria monocytogenes*) [101]. Lethe, a functional pseudogene (Rps15a-ps4) lncRNA, is also highly inducible by TNF-α and IL-1β treatment. In addition, after treatment with an anti-inflammatory glucocorticoid receptor agonist, dexamethasone, Lethe expression is induced and impacts IL6, IL-8, and superoxide dismutase 2 (SOD2) expression levels through inhibiting transcription factor p65 (RelA) occupancy at its target gene promoter [102]. Furthermore, THRIL is another immunoregulatory lincRNA (TNF- and HNRNPL-related immunoregulatory lincRNA) that is induced by TNF stimulation in the human monocyte-like THP-1 cell line. THRIL was also differentially expressed in response to Pam3CSK4 (TLR2). Knockdown of nine lincRNAs, including THRIL, led to impairment of IL-6 and/or TNF-α induction [103]. lincRNA-Tnfaip3 is also required for the transactivation of NF-κB-regulated inflammatory response genes after LPS treatment. lincRNA-Tnfaip3 physically interacts with the high-mobility group box 1 (Hmgb1), assembling a NF-κB/Hmgb1/lincRNA-Tnfaip3 complex after LPS treatment. The resulting NF-κB/Hmgb1/lincRNA-Tnfaip3 complex can modulate Hmgb1-associated histone modification [104]. More recently, lincRNA-EPS was found to be regulated in macrophages and impacted control of the expression of immune response genes from transcriptome profiles of macrophages in lincRNA-EPS-deficient mice. Additionally, a gain-of-function study and rescue experiments indicated that lincRNA-EPS can functionally restrain inflammatory gene expression, which occurs through lincRNA-EPS

interacting with heterogeneous nuclear ribonucleoprotein L through a CANACA motif located in its 3′ end [105]. Overall, lncRNA was highlighted as an emerging and important regulator that controls gene expression in the innate immune cell response.

3.7 Epigenetic and Innate Immune Cell Memory

The innate immune defense through macrophages is tightly controlled by timing and space. Macrophages have the ability to respond readily and balance inflammatory signaling for the host defense against pathogens through the process of resistance and tolerance [106]. Resistance will reduce pathogens, whereas tolerance will limit tissue damage. These processes achieve effective host protection [107]. More recent evidence has shown that macrophages display long-term changes in their functional programs after infection or vaccination, and those changes affect its responsiveness to cytokines and inflammatory mediators to eliminate infection upon secondary pathogen stimulation [108]. This finding revealed the existence of innate immune cell memory in addition to classical adaptive immune system memory. Emerging studies have shown that chromatin structure and epigenetic modifications play an essential role in the behavior of trained immunity and endotoxin tolerance for the macrophage memory phenotype [109].

Priming macrophages with an initial infection or vaccine challenge results in enhanced macrophage responsiveness to a secondary challenge [110, 111]. Mice lacking functional T and B lymphocytes are protected against reinfection with nonlethal infection with *Candida albicans* in a monocyte-dependent manner, which lead to enhanced cytokine production both in vivo and in vitro. *C. albicans* and fungal cell wall β-glucans can induce functional reprogramming of monocytes [112]. This finding also applies to human monocytes treated with *C. albicans*, which increased the inflammatory response following subsequent stimulation. Monocyte training by β-glucans was associated with H3K4me3, which suggests the involvement of epigenetic mechanisms in this phenomenon [112]. H3K4 methylation (H3K4me3) and H3K27 acetylation (H3K27ac) occupied the regulatory element, which is in the promoter during the first infection stimulus in some cases. Recent studies have termed regulatory elements latent enhancers; after stimulation, transcription is terminated, and H3K4 methylation and H3K27 acetylation also disappear, but H3K4 methylation persists at latent enhancers and constitutes the basal level occupying the region for a faster and enhanced response until restimulation [113, 114] (Fig. 3.2). In β-glucan-trained macrophage cells, we can see the alteration of glycolysis enzymes, which form a link between metabolism and inflammation, and more detail has shown that increased glycolysis and suppressed oxidative phosphorylation under sufficient oxygen supply occur through the Akt-mTOR-HIF-1α pathway [115]. After training with *C. albicans*-derived β-glucan, macrophages regulated TNF-α, IL-6, and IL-18 under stable changes in H3K4me3, showing that both histone methylation and acetylation patterns change with envi-

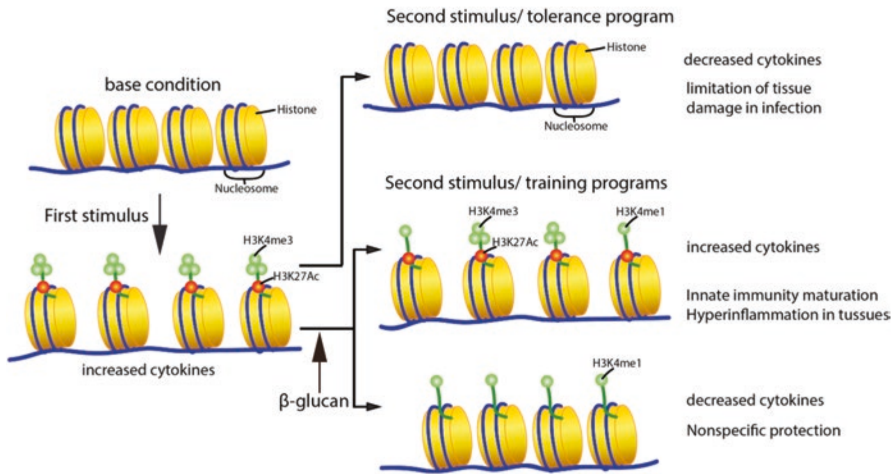

Fig. 3.2 The figure represents innate immune memory with endotoxin tolerance and trained immunity after pathogen-associated molecular patterns (PAMPs) challenge; innate immune cell recognition of pattern recognition receptors (PRRs) leads to functional reprogramming of the cell. The model of trained immunity with β-glucan stimuli from *Candida albicans* leads to metabolic changes with increased glycolysis and suppressed oxidative phosphorylation that links epigenetic reprogramming to the innate immune response through histone modifications (methylation and acetylation). Subsequent PRR stimulation may cause decreased or increased responsiveness of these cells, which depends on the initial type of stimulus. Initial PRR stimulation leads to H3K4me3 and H3K27Ac on the promoters of proinflammatory genes. In the endotoxin tolerance model, the removal of the stimulus causes the disappearance of H3K4me, and gene expression returns to basal levels. Following subsequent stimulus, some tolerized genes will lose H3K4me3 or H3K27Ac and will remain silent in response to a second stimulation (*top figure*). In the trained immunity model, proinflammatory genes will maintain enhancers marked with H3K4me1. Subsequent stimulus will lead transcription factors to bind to both the enhancers and the promoters of innate immune response genes and recruit a chromatin modifier complex, including histone acetyltransferase p300 and elevated H3K4me3, for enhancing the expression of a subset trained genes (*middle figure*). On the other hand, some genes will prevent H3K4me3 and have less gene expression induction (*bottom figure*)

ronmental challenges, which is most likely the metabolic switch for macrophage training and activation [109, 115].

Endotoxin tolerance is another form of memory that contrasts with trained macrophages; the underlying mechanism first involves stimulation with TLR4 ligands that causes the cell refractory state, which leads to tolerization from gene expression after restimulation [116]. Tolerized genes will be diminished, and non-tolerized genes will be increased or unchanged. This pattern reflects clinical situations such as sepsis in which patients enter a suppressed response state [117] (Fig. 3.2). The transcription active mark H3K4 methylation disappears after first stimulation, which is associated with the re-induction of tolerized inflammatory cytokines [19, 118]. The contribution of DNA methylation changes to macrophage memory has also shown that high levels of TET2 in cells have the ability to demethylate DNA; we would like to explore the regulatory mechanism of macrophage functional plas-

ticity that depends on stimuli and restimuli. DNMT3B affects the control of inflammatory signaling, which impacts macrophage polarized phenotypes for M1-like and M2-like states. Therefore, DNA methylation is another factor that contributes to macrophage memory [61, 119]. A recent study has indicated that environmental factors, such as circulating glucose levels and gut microflora, may alter innate immune responsiveness through modification of the epigenetic states of peripheral blood monocytes and resident tissue macrophages. Increasing expression levels of TLR1, TLR2, and TLR4 by high glucose through circulating glucose and insulin levels have acute effects on peripheral blood mononuclear cell (PBMC) receptor levels, indicating a direct impact on innate immune receptor expression [120]. High glucose drives epigenetic changes in monocytes that result in increased expression of proinflammatory stressors, promoting epigenetic changes and facilitated binding of transcription factor NF-κB subunits in monocytes that result in increased expression of the inflammatory cytokines IL-6 and TNF-α [121]. Under high-glucose conditions, enhanced binding of NF-κB to its target genes occurs through epigenetic changes with reduced HDAC2 and increased HAT activity [122].

Tissue macrophages are also regulated by environmental cues, and this relationship is evidenced by disease phenotypes, such as colitis models. There are correlations between gut microbiota and the immune responsiveness of resident macrophages; in humans, alteration of commensal bacteria populations is associated with inflammatory bowel disease (IBD), and in mice, enhanced cellular responses to dextran sodium sulfate (DSS)-induced colitis are observed in germ-free mice [123]. Regarding the deletion of TLR2 in multidrug resistance gene (MDR1/ABCB1) deficiency, TLR2/MDR1A double-knockout mice exhibited a fulminant pancolitis phenotype with early expansion of CD11b(+) myeloid cells and rapid changes in Th1 immune responses in the lamina propria during colitis models [124]. The double-knockout CD11b(+) myeloid cells expressed MD-2/TLR4 and hyperresponded to LPS or nonpathogenic *Escherichia coli* with reactive oxygen species and caspase-1 activation and release of proinflammatory IL-1β, leading to pyroptosis. Compared with active ulcerative colitis (US) with no polymorphisms, active ulcerative colitis (UC) with TLR2-R753Q and MDR1-C3435T polymorphisms was associated with increased expression of caspase-1 protein and cell death in acutely inflamed tissues. The results demonstrate that the restricted TLR signaling network is important in maintaining healthy intestinal homeostasis [125]. Interestingly, "training" mouse or human hematopoietic progenitor cells by prior exposure of differentiated macrophages to a TLR2 agonist ("tolerance") suppress inflammatory cytokine production but elevate reactive oxygen species. Soluble factors produced following the exposure of progenitor cells to a TLR2 agonist can also act in a paracrine manner to influence the function of macrophages [126].

3.8 Epigenetics in Host Defense and Inflammatory Disease

The majority of disease-associated single nucleotide polymorphisms (SNPs) that were identified by genome-wide association studies (GWAS) are localized to regulatory regions of the genome, and less than 10% of SNPs are located in protein coding genes [127]. Regulatory regions of the genome, including regulatory elements of promoters, enhancers, and intergenic regions of the genome, are rich in miRNAs and lncRNAs [128]. Those genetic variations could affect the expression and/or function of RNAs and impact human diseases. Dysregulated expression of enhancers, miRNAs, and lncRNAs could lead to several immune-related diseases, such as diabetes, multiple sclerosis, and IBD [129]. The important, highly specific miR-150 was found in leukocytes and was upregulated in mucosal tissue of patients with IBD for both ulcerative colitis and Crohn's disease and murine models of experimental colitis; this upregulation may represent a colitis-associated increase in mucosal leukocyte infiltration as opposed to representing pathophysiological relevance. Investigations into miRNA function in IBD will continue to be challenging given the high rate of inflammatory cell infiltration related to fibrosis and turnover of epithelial cells [130]. An atherosclerosis-associated SNP located in the intron of lncRNA LINC00305 was recently identified by a search of the GWAS database. LINC00305 expression is enriched in atherosclerotic plaques and monocytes. Overexpression of LINC00305 promoted the expression of inflammation-associated genes in THP-1 cells and reduced the expression of contractile markers in co-cultured human aortic smooth muscle cells [131]. Higher mRNA expression levels of DNMT1 and MBD2 were found in PBMCs from rheumatoid arthritis (RA) patients who had lower levels of global DNA methylation, which indicated that DNA hypomethylation results in overexpression of autoimmune-related genes that contribute to RA [132]. The current challenge is to define the functional role of these regulators (miRNA, IncRNA, and SNPs in regulatory elements in genomic DNA) in the context of disease pathophysiology. Recent use of promoter capture high-throughput chromosome conformation capture (Hi-C) will enable identification of novel candidate genes and complex long-range interactions with related autoimmune risk loci. Long-range interactions between regulatory elements and gene promoters are uncharted and will provide us with a complete picture of the link to understand genome control [133]. A study has identified interacting regions of 31,253 promoters in 17 human primary immune cell types. The data show that promoter interactions are highly cell type specific and enriched for links between epigenetically marked enhancers and active promoters [134]. The power of primary cell promoter interactomes to reveal insights into genomic regulatory mechanisms and rich resources to connect noncoding disease variants with putative target promoters can help us understand disease pathways and screen candidate genes. Genetic variants that affect gene expression can be identified using expression quantitative trait loci (eQTLs), which is a novel approach to analyzing regions of the genome containing DNA sequence variants that influence the expression level of one or more genes. This approach is crucial to addressing genes examined in the

context of human inflammatory diseases. Performing eQTL mapping and parallel analysis in multiple leukocyte subsets from patients with active disease provides new insights into the genetic basis of immune-mediated diseases [135]. Overall, the aberrant expression of miRNA, IncRNA, and SNPs in promoter or enhancers may play a causal role or could be the consequence of disease pathology, which requires further investigation.

3.9 Respective of Innate Immunity in Epigenetics

NGS technology offers unique ways to profile genome-wide epigenetic changes, including genome methylation and histone modifications. Three-dimensional genome architecture reveals chromatin topology, including ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag Sequencing), which was designed to detect genome-wide chromatin interactions mediated by specific protein factors [136], whereas Hi-C was developed to capture all chromatin contacts [137]. Hi-C has been proven effective for mapping large-scale structures, such as topologically associated domains [138, 139], based on the methods described in the previous sections [24]. Bioinformatics tools have been developed for the analysis of these NGS data to infer precise methylation changes, TF binding, and topological interactions. Publicly accessible databases have been established to enable researchers. We can use various methods to conduct profiling and analysis but can still limit the investigations to conduct epigenetic analyses with bioinformatics data and tools [140–142]. Understanding epigenetics requires a systems biology approach with concern regarding the kinetics of various processes, such as methylation, chromatin modifications, topological reorganization, TF activation, gene transcription, translation, and cellular processes, that span a range of timescales from milliseconds to hours. To understand how to uncover causal information, we have to analyze the sequence of time points, which provides a dynamic perspective of epigenetic changes. Therefore, we can ultimately build directed networks that regulate homeostasis and disease stage. There have been several recent reports of integrative analyses involving epigenetics, transcription, and, in some cases, cellular signaling. For example, we used TLR-induced macrophages as a model and combined time series transcriptomic profiling, cluster analysis, ChIP-seq, and network modeling to identify a prominent role of activating transcription factor 3(ATF3) in high-density lipoprotein-mediated reprogramming of macrophages in anti-inflammatory signaling on TLR responses [143].

Systems models provide a powerful way to guide designs for perturbation experiments, as well as therapeutic interventions. Existing methods used in epigenetics research, including NGS, are largely bulk assays, which provide global information averaged from a large number of cells. However, current approaches are moving to studies at the single-cell level. For example, by using the methyl-CpG-binding domain of methyl-CpG-binding domain protein 1 tagged with enhanced green fluorescent protein, we can achieve single-cell visualization of methylated DNA [144],

and FRET-based sensors have been used for single-cell detection of histone acetylation states [145]. Chromatin is a recently developed technique, which is a quantitative high-resolution imaging approach combining fluorescence in situ hybridization, immunostaining, and array tomography imaging that enables us to investigate chromatin organization in tissue specimens at the cellular level [146]. This excellent novel real-time imaging technology can be used in living cells with high sensitivity and specificity at locus-level resolution. The CRISPR (clustered regularly interspaced short palindromic repeats) bacterial immune system and derived Cas9 protein have provided unprecedented opportunities for genomic editing by introducing sequence-specific gene mutations [146]. Although CRISPR/Cas9 represents a valuable way to manipulate both the *cis* and *trans* elements involved in epigenetics both in vitro and in vivo, researchers are still improving undesirable off-target mutations, the lack of tissue specificity, and the efficacy of in vivo delivery, which will be expected in the future.

A growing understanding of how epigenetic mechanisms control gene expression patterns has highlighted the potential for targeting epigenetic mechanisms for the treatment of inflammation and related diseases [147]. Numerous HDAC inhibitors have been investigated for their effects on innate immune responses to PAMP stimulation. For example, vorinostat has been shown to reduce inflammatory cytokine production elicited both by LPS stimulation of human PBMCs and after LPS injection into mice [148, 149]; HDAC inhibitors have also been tested for their effects on human disease tissues, with studies primarily focusing on RA. In this context, TSA, vorinostat, and sodium phenylbutyrate were all shown to inhibit cytokine production by macrophages derived from the inflamed joints of patients with RA and cytokine production from RA synovial explants [150]. In addition, TSA and an HDAC3 inhibitor (MI192) were reported to inhibit IL-6 production from RA PBMCs stimulated with LPS [151]. Bromodomain and extraterminal (BET) proteins recognized acetylated lysine residues in the histone tail, which can also contribute to the transcription of inflammatory cytokine gene expression by forming a complex with positive transcription elongation factor-b and RNA polymerase II at the transcription start site [152, 153]. BRD-containing proteins bound to chromatin loci such as enhancers are often deregulated in disease, leading to aberrant expression of proinflammatory cytokines and growth-promoting genes. Recent developments targeting the BET subset of BRD proteins demonstrated remarkable efficacy in murine models, providing a compelling rationale for drug development and translation to the clinic [154]. Using I-BET (a synthetic compound) to inhibit inflammatory cytokine genes in macrophages after treatment LPS, the mechanism of this drug appears to comprise interfering with the binding of BET to acetylated histones, which can prevent inflammatory disorders [155, 156]. Jmjd3 could be another therapeutic target for inflammatory diseases. Indeed, a selective jumonji H3K27 demethylase (catalytic site inhibitor) [157], GSK-J4 (GlaxoSmithKline corporate compound collection), was recently reported to inhibit LPS-induced production of inflammatory cytokines in human macrophages from healthy volunteers.

Epigenetic research has had a profound impact on personalized medicine. Advances in NGS technology have enabled the sequencing of the human genome

within days with reduced cost. Collectively, DNA-, RNA-, and ChIP-seq data are anticipated to provide genetic and epigenetic information regarding inflammatory signaling at the homeostasis stage or inflammatory disease stage, which will allow us to understand how epigenetic mechanisms finely tune the outcome of host innate immune response and will also provide valuable information or guidance on human inflammatory disease etiology, diagnosis, treatment, and prognosis. The integration of NGS technology and data analysis by bioinformaticians and immunologists, using clinical information from physicians, will revolutionize translational medicine.

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Chapter 4
Roles of HDACs in the Responses of Innate Immune Cells and as Targets in Inflammatory Diseases

Yiqun Hu and Bandar Ali Suliman

Abstract Histone deacetylases (HDACs) are an emerging class of molecules involved in the epigenetic regulation of innate immune responses through Toll-like receptor (TLR) and interferon (IFN) signaling pathways. HDACs are also key drivers of inflammatory diseases via epigenetic regulation through chromatin DNA and histone modification by methylation and acetylation, among other mechanisms, which control innate immune cell gene expression. Importantly, these epigenetic changes are reversible, and HDACs may also be targeted by small-molecule HDAC inhibitors, which have been used in clinical settings for cancer therapy. Here, we highlight HDACs as strong therapeutic molecules and explore HDAC-induced mechanisms regulating innate immune responses and inflammatory cytokine control, with the goal of developing personalized medicine for the treatment of human diseases, including inflammatory diseases and immune disorders. Currently, this novel class of immunomodulatory therapeutics is being evaluated in the laboratory, in preclinical models, and in the clinic.

Keywords Histone deacetylases (HDACs) • HDAC inhibitors • Inflammatory signaling • Inflammatory diseases

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4.1 Introduction

4.1.1 The Innate Immune System

The innate immune system is a unique collection of immune cells and tissues that share a common goal: the nonspecific identification of pathogens [1]. Most innate immune cells are present at birth and harbor genetically conserved molecular receptors that are capable of identifying self from nonself molecules. These *innate immune receptors* are called *pattern recognition receptors* (*PRRs*) and are used by the immune system to recognize genetically conserved molecular structures, called *pathogen-associated molecular patterns* (*PAMPs*), which are shared by many microbial species. PAMP structures consist of combinations of lipid, sugar, protein, and nucleic acid sequences and constitute the outward-facing biological components of viruses, bacteria, fungi, and parasites. One of the hallmarks of the innate immune response is *speed*. The innate immune response is initiated by the interaction of PRRs with their ligands (i.e., PAMPs). Despite the molecular diversity of PAMPs in many microbial species, the immune responses initiated by innate immune cells are rapid and occur within seconds of successful recognition [2]. Receptor-ligand interactions are functionally translated into two distinct pathways: phagocytosis and the inflammatory response. Phagocytosis is the biological process by which professional innate immune cells, such as neutrophils, macrophages, and dendritic cells, engulf and destroy pathogenic molecules to extract specific protein structures called epitopes, which activate the adaptive arm of the immune system [3]. The inflammatory response, in contrast, consists of the production and subsequent release of regulatory chemical molecules aimed at orchestrating the functions of other immune cells [4].

This dependency of the adaptive immune response on the innate arm of the immune system demonstrates the crucial and important role of innate immune cells [5]. The human body requires this general and nonspecific immune response to achieve the full spectrum of adaptive immune recognition, either by supplying specific epitopes that are targeted by the adaptive immune system or by providing the necessary chemical modulators required for successful immune signaling.

4.2 Inflammatory Response Signaling

The immune system represents one of the largest signaling networks in the human body. Intertwined signaling cascades provide protection via millions of cells that circulate in the human body and defend it not only against external microorganisms but also internal defective and cancerous cells. There is an immaculate harmony between the two arms of the immune system. The innate immune cells, which are responsible for initiating the adaptive immune response, control the development and differentiation of adaptive immune cells. They also participate in the positive

and negative regulation of the inflammatory response to fine-tune the involvement of different immune cells in the overall immunological reaction [6].

Many studies have investigated the inflammatory response and how each and every molecule plays a part in its execution. Innate immune cells such as macrophages, fibroblasts, and dendritic cells recognize pathogens and pathogenic epitopes and subsequently initiate complex immune signaling cascades by which they communicate their findings, the scope of the inflammatory reaction, and the exact level of the adaptive immune system response required to eliminate the threat [7]. To achieve such a delicate outcome, stimulated innate immune cells control both the spectrum and scope of the inflammatory reaction by controlling intermediary lipid molecules and enzymes that work together at the transcriptional level for each specific cell. Although many pathogenic molecules are recognized by the antigen-presenting cells of the innate immune system, genetic signatures at the level of transcriptional regulation are commonly shared among those cells.

Cytokine production is the hallmark and the ultimate outcome of the innate immune response [8]. When a macrophage encounters a virus, bacterium, or other microorganism, it produces cytokines known as interleukins that instruct other immune cells. The responses of these stimulated cells are not only directed toward other immune cells but also toward many other cells and tissues with essential roles in the immune system response to combat the infection. Some of the chemical mediators produced by innate immune cells control adjacent endothelial cells to enhance permeability, allowing necessary proteins to escape the blood circulation [9]. Other inflammatory mediators act on vascular smooth muscle cells to promote vasodilation, causing an increase in blood flow and helping other immune cells to enter the site of inflammation.

4.3 Transcriptional Regulation

Immune cells, like any other cell type, encompass a vast amount of DNA, which is tightly packed inside the nucleus. This DNA contains all genetic information needed by the cells to produce the necessary molecular structures for pathogenic identification, the enzymes and proteins involved in signal transduction, and the actual chemokines and cytokines produced as a result of immune activation. The DNA is contained in the nucleus in the form of supercoiled antiparallel strands of nucleotides wrapped around core histone proteins, ensuring the tight packing of DNA into structures called nucleosomes. Each nucleosome contains 146 base pairs of DNA wrapped twice around eight histone molecules, such as H2A, H2B, H3, and H4 [10]. This growing chain of nucleosomes along the DNA fiber forms the chromatin. The chromatin is maintained by the negative charge of the DNA strands together with the positive charge of the histone proteins, forming strong bonds that preserve the supercoiled structure. The arrangement of the DNA and histones closely regulates transcription by either allowing RNA polymerase complexes (the transcriptional machinery) to access the DNA strand to search for promoter regions or blocking

DNA access and thus precluding transcription. Euchromatin is the DNA state characterized by loose packaging because the core histone H1 has not yet been added; in contrast, heterochromatin is the DNA state characterized by tight packaging after the addition of H1 [10, 11]. Although transcriptional regulation involves many biological processes, modification of the chromatin state remains one of the most influential methods [12–14]. Many studies have established a direct link between transcriptional activation and altered chromatin states. Such alterations along DNA strands range from the simple addition of methyl groups to specific arrangements of the cytosine pyrimidine rings [15].

4.4 Histone Deacetylases (HDACs) and Transcriptional Regulation

The regulation of histone acetylation and deacetylation is controlled by enzymes, specifically HDACs and histone acetyltransferases (HATs), which are essential for gene regulation [16]. HATs are enzymes that transfer acetyl groups to core histones, with subsequent effects on chromatin remodeling and gene expression. HATs comprise two families [17]: (1) p300/CBP, Taf1, and nuclear receptor coactivators that also possess catalytic acetyltransferase activities and (2) Gcn5 N-acetyltransferases (GNATs) and Morf, Ybf2, Sas2, and Tip60 HATs. HATs are involved in initiating gene expression in macrophages during inflammation [18, 19]. HDACs are enzymes that oppose HAT functions and exert dynamic effects that may derive from other posttranslational modifications. HDAC functions potentially induce further epigenetic changes and alternative gene expression. Eighteen mammalian HDACs have been identified and classified into five groups: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (consists of the NAD+-dependent HDACs), and HDAC11, which constitutes a class of its own (Fig. 4.1) [20]. A single DNA strand consists of a collection of histone molecules that bind to a long chain of nucleotides. The structural binding affinity used by histone molecules to control how tight the nucleotide chain is wrapped around them is dependent on the electrical charge difference between the phosphate groups in the DNA strand and the adjacent N-terminal tails of each histone molecule [21]. Since this electrical charge is manipulated by certain enzymes and chemicals, it is therefore possible to adjust the binding of histone

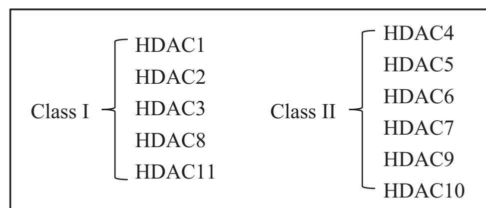

Fig. 4.1 Classification of the two common classes of HDACs

molecules to the DNA strand in individual nucleosomes through the process of acetylation [22]. To maintain electrical balance, the addition of an acetyl group to lysine residues in histone molecules neutralizes their positive charge and thus relaxes their grip on the DNA strand, allowing the nucleosome to be accessed by the transcriptional machinery and all necessary transcription factors, ultimately upregulating the transcription process and producing more mRNA transcripts. This process is very important for active genes for which the promoter regions are actively accessible to the regulatory molecules required for transcription. Alternatively, removal of acetyl groups restores the positive charge of histone molecules, tightening their grip on the DNA strand and rendering the nucleosome inaccessible to the transcriptional machinery. HDACs catalyze the removal of acetyl groups from histone molecules. Many cells harbor this powerful mechanism to regulate the expression of certain genes via specific, similar activities [23]. For example, the cell cycle, a universal biological process that is carried out by most cells in the human body, requires the availability and abundance of certain proteins in a specific time frame. To achieve this goal, cells employ different HDACs to alter the mRNA expression of genes of interest during different phases of the cell cycle [24].

Since the early days of experimental genetics, many scientists have performed experiments using *Drosophila* and *Saccharomyces cerevisiae*, which resulted in the discovery of a variety of biological activities that correlate with their human counterparts [25]. Therefore, eukaryotic and human HDACs have been classified to represent structural homology and catalytic activity. Based on this system, HDACs in humans are classified as class I and class II. Class I HDACs have a closer relationship with the transcriptional regulator RPD3 found in *Saccharomyces cerevisiae*, while class II HDACs share another domain that is more closely related to the HDAC1 protein in the same yeast [26]. Although this classification system has successfully distinguished between closely related HDACs in mammals to date, the overall DNA sequence similarities between eukaryotic enzymes and their prokaryotic counterparts are not sufficiently significant [27]. Eukaryotes are more complex and diverse, and therefore it is expected that different classes of enzymes have more prominent roles in specialized functions, such as cellular differentiation and basic developmental processes [28]. Consequently, some enzymes, such as class I HDACs, are found in almost all cells of the body, while class II HDACs are more restricted to a specialized set of cells [29]. *In vivo* experiments conducted in mouse models have shown that HDACs from both classes control the expression of specific subsets of DNA sequences in both a spatial and temporal manner. Thus, the functional acetylation or deacetylation of only a subpopulation of histones is achieved through the function of members of class I and class II HDACs, either individually or collectively [30, 31].

A very simple representation of HDAC classes shows the members of both class I and class II HDACs. There are more subclasses for some members, such as HDAC9a and HDAC9b. For a detailed and in-depth differentiation of HDAC classes and isoforms, please consult the review by De Ruijter and his colleagues [32].

4.5 Role of HDACs in the Innate Immune System

The cells of the innate immune system are the most crucial line of defense against specific pathogenic invaders as well as cancer cells. Because of their importance in maintaining a healthy state, the regulatory mechanisms controlling the functions and cellular interactions within this system are among the most active areas of medical research [33]. Cells of the innate immune system are controlled by several genetic and epigenetic mechanisms, in addition to external stimuli. This network of regulatory elements influences the overall inflammatory outcome and is essential for defining the epigenetic signatures of individual cells. For example, monozygotic twins may show variable susceptibility to pathological conditions, even while sharing the same environment [34–36]. This finding supports the impact of epigenetic regulation in defining the scope and overall outcome of the inflammatory responses caused by different stimuli. Current studies are focused on HDAC regulation of TLR and type I IFN-mediated signaling pathways.

4.6 HDAC Regulation of TLR-Mediated Signaling

TLRs comprise a very well-known group of genetically conserved receptors that are responsible for the identification of a wide range of pathogens. TLRs are found on the surfaces of many innate cells circulating in the bloodstream and are concentrated in various tissue types. Since their discovery in 2001, many molecular ligands have been identified for each specific TLR [7]. These ligands are molecular activators that are responsible for activating a particular TLR and initiating specific signaling cascades to produce cytokines targeting the pathogen or microbe harboring the activating molecular pattern. Although the contributions of different molecules to the responses of innate cells to different ligands have been established, the actual mechanism underlying this type of regulation remains poorly understood.

As discussed earlier in this chapter, many inflammatory signals activate certain TLRs to produce a required cytokine profile. Cigarette smoking, for instance, increases the production of a mixed group of inflammatory mediators, including MCP-1, TNF-α, and IL-8, which are directly implicated in the development of the classical clinical features of chronic obstructive pulmonary disease (COPD) [37, 38]. The continuous increase in the inflammatory signals that cause mucus accumulation during destruction of the lung parenchyma and ultimately fibrosis and obstruction of the airways has recently been linked to both TLR2 and TLR4 activation [39]. Many of the downstream genes activated by both of these TLRs contain acetylation sites in their promoter regions that attract HDAC1 binding [40]. In another study, HDAC1 was successfully immunoprecipitated along with ATF-3 from LPS-activated macrophages. ATF-3 is known for its function as a negative regulator of TLR4 activation that limits the production of IL-6 and IL-12 post-LPS treatment [41, 42]. The protein complex formed between ATF-3 and HDAC1

modifies histone structures and limits the accessibility of the transcriptional machinery to the IL-6 and IL-12 promoters. HDAC2 forms regulatory complexes with multiple proteins, enabling it to regulate the transcriptional activities of various targets [43, 44]. HDAC2 binds MTA1, a known chromatin modifier implicated in nucleosome remodeling, to form a repressor complex targeting the *MyD88* promoter after TLR4 activation of macrophages. MyD88 is an adaptor protein that is responsible for transferring molecular signals from TLRs to a group of interacting proteins known as nuclear factor-kappa-B (NF-κB), which in turn exerts its effects on multiple genes to determine the scope and amplitude of the immune response and inflammatory reaction. HDAC3 was recently associated with the direct functional inhibition of NF-κB. Nuclear co-localization of HDAC3 with inhibitor of NF-κB-like (IKBL) was observed after TLR stimulation, resulting in a decrease in NF-κB activity [45]. Similarly, the transcriptional inhibitor PLZF recruits HDAC3 to the promoter regions of many NF-κB target genes. These regions contain NF-κB-binding motifs, allowing HDAC3 to modify chromatin and NF-κB to access these DNA regions, ultimately controlling transcriptional activity [46, 47].

Similar to other HDACs, HDAC6 is responsible for the recruitment of the MyD88-GyrB complex after TLR2 and TLR4 activation. HDAC6 negatively regulates those TLR-activated pathways by inhibiting MyD88-TRAF6 formation and attenuating the resulting immune response [48]. HDAC7 is expressed at elevated levels in inflammatory macrophages (thioglycollate-elicited peritoneal macrophages) compared with its expression in bone marrow-derived macrophages and the RAW264 cell line. Overexpression of a specific, alternatively spliced isoform of HDAC7 lacking the N-terminal 22 amino acids promotes the LPS-inducible expression of HDAC-dependent genes (Edn1, IL-12p40, and IL-6) in RAW264 cells [49]. Such regulation is usually carried out by immune cells to limit the cytotoxic and destructive effects caused by strong stimulation of the signaling pathway under specific conditions [50, 51]. Although the exact effects of certain HDACs on various levels of immune signaling pathways have been dissected and elucidated, there is no clear understanding of how many HDACs control the final outcome of the immune response at the molecular level. For example, the effects of HDACs on the polarization and programming of CD4 T lymphocytes following TLR4 activation are not known. Both macrophages and dendritic cells are responsible for activating T lymphocytes to derive a specific adaptive immune response against certain pathogens. This activation may specify T-cell polarization: either Th1, which is pro-inflammatory, or Th2, which is anti-inflammatory [52, 53]. Polarization requires the delicate temporal activation of certain mediators, such as CXCL9, CXCL10, IL-12, IL-15, and MCP-1, to skew the programming toward a certain phenotype. The inhibition of HDAC activation and the subsequent modification of chromatin status change the molecular signatures of these mediators, leading to changes in the inflammatory environment [54].

Another example of how the modification of HDAC activity may influence the immune response against pathogens is seen in the ability of *Candida albicans* or *Saccharomyces cerevisiae* to regulate TLR1 and TLR6 downstream targets in the intestinal mucosa. Both of these fungal species are members of the normal

commensal flora residing in the healthy human intestine. They positively prime the innate immune response against pathogens through the production of IL-8, which exerts a favorable effect by protecting the mucosal surface from intestinal pathogens [55]. The inhibition of HDAC activity, however, causes a marked increase in IL-8, which disturbs the immune balance and leads to the destruction of the commensal flora, creating a niche for pathogenic and opportunistic species to cause further infections [56]. Similarly, chronic and persistent activation of MAP kinases causes articular chondrocyte hypertrophy through the direct stimulation of a group of cytokines and chemokines that includes IL-8 and CXCL1 [57, 58]. Both of these cytokines are upregulated through the induction of TLR1 and TLR6, which increase after treatment with butyric acid, a known class I HDAC inhibitor [59].

4.7 HDAC Regulation of IFN-Mediated Signaling

When a virus infects a cell, the innate immune system employs a wide range of IFN molecules to prepare the surrounding tissues for viral invasion. During this viral invasion, innate immune cells respond in a general way to invading single-stranded or double-stranded DNA or RNA molecules. HDACs control this response by changing the genetic signature, either by amplifying or dampening the outcome. HDAC1 participates in the antiviral response by exerting its deacetylase activity to influence gene expression profiling. Its expression at both the transcriptional and translational levels is significantly reduced after influenza A viral infection. This decreased expression is accompanied by a decrease in the enzymatic activity of HDAC1 and subsequent downregulation of the expression of IFN-stimulated genes (ISGs) such as phosphorylated-STAT1 and IFITM3 [60]. Moreover, type I IFN stimulates the phosphorylation of STAT3, which in turn functions as a transcriptional regulator for many ISGs. The transcriptional activity of STAT3 itself may be modulated by the acetylation of many lysine residues as a direct response to cytokine stimulation [61, 62]. This cross talk between the regulation of STAT3 by IFN and the subsequent regulation of STAT3 by HDACs reveals the complicated regulation of the transcriptional activity underlying a simple viral infection.

The endogenous protein expression levels of HDAC7 are constitutively maintained in cytotoxic T cells (CTLs). Continuous phosphorylation of HDAC7 is actively maintained by CTLs to guarantee the cytosolic accumulation of HDAC7 [63]. Scientists investigating the cause of HDAC7 exclusion from the nucleus discovered that activated CTLs control functional capacity through the genetic signature of many intrinsic pathways [64]. These pathways are the targets of HDAC7 deacetylation [65]. Moreover, the expression of IFN-γ, which is an effector cytokine produced by CTLs in response to T-cell receptor activation, is hindered by the increased expression of nuclear HDAC7 [66] (Fig. 4.2).

The innate immune response is a rapid nonspecific response that is controlled by many molecules. HDACs play a crucial role in controlling the scope and amplitude

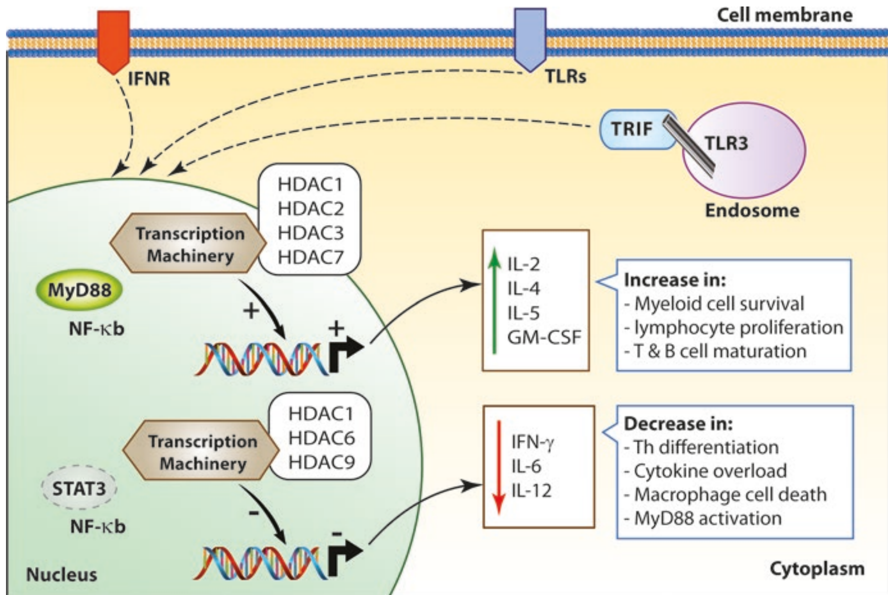

Fig. 4.2 The most important innate immune functions modulated by HDACs

of the reaction, leading to the production of precise amounts of inflammatory cytokines and interleukins.

4.8 HDAC Regulation of Other Immune Mediators

To control infections and inflammatory reactions, the immune system not only modulates the functions of its own cells but also affects many functional and biological aspects of neighboring cells and tissues. Both HDAC1 and HDAC2 regulate the transcription of many genes that are important for cell junction integrity, the overall permeability of the intestinal epithelium and the amount of mucin produced by goblet cells. Both HDACs are also involved in limiting the phosphorylation of the transcription factor STAT3, a major regulator of the inflammatory reaction in the intestinal epithelium. The dysregulation of HDAC1 and HDAC2 functions not only to disrupt the physiological structure of the intestine but also to modify the microbial environment through the effects of STAT3, causing major intestinal inflammatory complications [67].

Earlier in this chapter, we described the cytokine profile following an innate immune response as a major force underlying the cellular response to different immunological stimuli. These cytokines are commonly expressed by many innate immune cells through the transcriptional activation of their respective genes by a transcription factor that is a key player in the immune reaction, NF-kB. When

NF-kB is activated, it binds to promoter regions containing specific kB motifs with downstream signaling targets. Both HDAC1 [68] and HDAC2 [69] control the acetylation of the IL-6 promoter, which in turn controls the accessibility of the transcriptional machinery to chromatin fibers when an immune cell, such as a macrophage, is instructed by internal signaling pathways that such a protein is needed [68]. IL-6 is a key pro-inflammatory cytokine responsible for the differentiation of lymphocytes, monocytes, and B cells. It acts directly on fully mature B cells, T cells, hematopoietic progenitor cells, and many other tissues during inflammation [70]. Dysregulation of its expression during the immune response has been observed in many diseases, such as rheumatoid arthritis [71] and Kaposi's sarcoma [72]. This type of regulation, which affects the immune response during later stages, is not the only way HDACs control immune reactions. HDAC3 deacetylates protein 65 of the NF-kB complex to downregulate the duration of its transcriptional response and its nuclear expression levels [73]. Conversely, HDAC2 is responsible for the deacetylation of class II transactivator, a transcription factor that regulates the expression of the MHC-II complex on the surface of antigen-presenting cells. MHC-II complexes are essential for the recognition and subsequent binding of pathogenic epitopes to innate immune receptors and professional antigen-presenting cells. The deregulation of such proteins may affect the functional capacity of innate immune cells to execute their roles in the immune response [74].

4.9 HDAC Inhibitors and Inflammatory Cytokines

HDAC inhibitors, abbreviated as HDACis, are a class of compounds that specifically target HDACs to functionally inactivate their enzymatic activity or action and therefore prevent histone deacetylation. The collective function of HDACis augments the buildup of nucleosomes with acetylated lysine residues in their core histones, causing chromatin relaxation, attracting the transcriptional machinery, and promoting gene expression. The use of HDACis has been linked to the treatment of many tumors due to their ability to activate genes silenced by the oncogenic transformation of targeted cells and tissues. Many cells in our body are in a constant state of equilibrium between transcriptional activation and inhibition. Cellular proliferation and DNA replication require numerous housekeeping genes that are similar among different tissue types. However, some specialized cells only require certain proteins for selected biological functions, such as inflammatory reactions, or precise amounts of certain proteins during a certain period of time. The ability to change the transcriptional signatures of immune cells is not without precedent [75]. Few studies have delineated the exact mechanisms employed by HDACis to alter the scope of inflammation [76]. Current studies evaluating anti-inflammatory treatments are focused on blocking the signaling pathways of common and nonspecific immunoregulatory proteins. Alternatively, HDACis usually target specific transcription factors or cytokines to control the amplitude of the inflammatory reaction. Several classes of HDACis have been reported to regulate cytokine expression and immunological effects (Table 4.1).

Table 4.1 The functional effects of different HDACis on the activities of cytokines and immune cells

HDAC inhibitor	Immunological effects	Refs.
SAHA	SAHA or vorinostat is a class I inhibitor that also inhibits HDAC5 by directly blocking substrate access to the catalytic site of the enzymes	[87, 88]
	Inhibits the production of IL-6, TNF-α, IL-1-β, NO, and IFN-γ by macrophages in response to LPS stimulation in both mice and humans	
	Inhibits IFN-γ-dependent production of IL-18 and IL-12 by human PMNs	
TSA	TSA, or trichostatin A, is an antimicrobial gene that selectively inhibits class I and II HDACs through the removal of acetyl groups from core histones	[83]
	Inhibits the activity of many TLRs, NODs, RIG-I, Mda-5, and C-type lectins, leading to diminished microbial sensing	
	Inhibits the activity of many immunoregulatory mediators involved in chemotaxis, inflammation, the cell cycle, and tissue repair	
	Inhibits the production of TNF-α, IL-6, and IL-12p40 by macrophages	
LBH589	LBH589, or panobinostat, is a pan-HDAC inhibitor that was approved by the FDA as a treatment for patients with multiple myeloma	[89]
	Inhibits the maturation and immunological activity of dendritic cells, which are required to activate inflammatory T cells and polarization	
	Inhibits the production of IL-12, IL-23, IL-6, IL-10, and TNF-α by activated dendritic cells	
PXD-101	PDX-101, or belinostat, is a hydroxamic acid-type inhibitor that was approved by the FDA as a treatment for patients with peripheral T-cell lymphoma	[90]
	Enhances plasma levels of specific cytokines, such as IL-6, in patients with solid tumors	
MGCD0103	MGCD0103, or mocetinostat, is a selective class I and IV inhibitor that targets the programmed death ligand 1 (PD-L1) pathway (caspase cascade)	[91, 92]
	Induces the cleavage and mitochondrial shuttling of Bax, which results in caspase-9-dependent activation	
Sodium Butyrate	Sodium butyrate is a selective class I inhibitor that is naturally produced in the gut through the dietary consumption of legumes	[93–95]
	Inhibits the IFN-γ-dependent transcriptional activation of NF-κB, which in turn diminishes the production of NO, iNOS, TNF-α, and IL-12p40	
	Inhibits the proliferation of T cells and the motility of macrophages in response to LPS stimulation	

(continued)

Table 4.1 (continued)

HDAC inhibitor	Immunological effects	Refs.
MS-275	MS-275, SNDX-275, or entinostat is a selective HDAC1 and HDAC3 inhibitor that is undergoing clinical trials for the treatment of Hodgkin's lymphoma and breast cancer	[96, 97]
	Downregulates CD1a, CD11c, and CD14 after IL-4 treatment, limiting the differentiation of monocytes into mature dendritic cells	
	Inhibits the functional activity of mature dendritic cells by decreasing their production of TNF-α, IL-6, and IL-12p40	
	Controls CD4+ T helper cell polarization through the downregulation of IL-13 and IL-4 expression and the upregulation of both CXCL10 and IL-12	
FR276457	FR276457 is a selective class I inhibitor that exhibits inhibitory activity toward HDAC4	[98]
	Inhibits T-cell proliferation and prevents the maturation of monocytes into functional macrophages	
	Prevents the differentiation of CD8+ cytotoxic T cells	
Valproic acid	Valproic acid, or Depakene, is a class I inhibitor that was approved by the FDA due to its ability to promote the differentiation of hematopoietic progenitor cells and leukemic blasts	[99, 100]
	Inhibits the production of TNF-α and IL-6 in response to LPS stimulation through modulation of the p50 protein subunit that targets NF-κB activity	
LAQ824	LAQ824, or dacinostat, is a pan class I and II inhibitor	[54]
	Inhibits CD4+ T helper polarization by controlling IL-12, IL-15, CXCL9, and CXCL10 expression by macrophages and dendritic cells	
ITF2357	ITF2357, or givinostat, is a selective class I and II inhibitor	[101, 102]
	Inhibits TNFα, IL-12, IL-1α, IL-1β, and IFN-γ production by PMNs in response to LPS stimulation	

HDACis are a class of biological compounds that limit the activity of histone acetylation, leading to transcriptionally active chromatin. HDACis modulate the inflammatory response by regulating many cytokines and inflammatory mediators

4.10 HDACis and Inflammatory Disease

HDACis have been used to treat cancer in the clinic by functionally regulating antiproliferation, the cell cycle, and apoptosis through gene expression mechanisms. Recently, HDACis have been implemented as an exciting approach to treat inflammatory disease [77]. Additionally, two HDACis (TSA and SAHA) have shown promising results for the treatment of diabetes, which has a substantial inflammatory component. TSA and SAHA suppress cytokines in pancreatic beta cells [78].

Trichostatin A (TSA) also reduces spinal cord inflammation, demyelination, and neuronal and axonal loss and ameliorates disability during the relapsing phase of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS) [79]. The anti-inflammatory activity of inhibitors targeting HDAC1, such as NW-21 and MS-275, reduces the expression of monocyte chemotactic protein 1 (MCP-1), TNF-α, macrophage inflammatory protein 1α (MIP-1α), IL-1, and RANTES in human monocytes stimulated with either TNF-α or lipopolysaccharide for 24 h *in vitro*. Such inhibitors also reduce inflammation and bone loss in a model of arthritis [80]. As shown in animal models, HDACis may exert beneficial effects on colitis by boosting levels of Foxp3+ (forkhead box P3+) T-regulatory cells that dampen inflammation and may also have a potential role in the treatment of inflammatory bowel diseases (IBDs) [81]. Treatment with the HDACi SAHA improved survival by decreasing levels of the pro-inflammatory cytokine IL-6 and increasing levels of the anti-inflammatory cytokine IL-10 in an LPS-induced septic shock mouse model [82]. HDACis also impact host defenses against bacterial infections by reducing the levels of cytokines, chemokines, and, more importantly, microbial sensing molecules (c-type lectins, adhesion molecules) in the innate immune response to TLR treatment [83, 84]. The treatment of murine macrophages with the HDACis TSA or VPA resulted in the reduced killing of *E. coli* and *S. aureus* by impairing phagocytosis and reactive oxygen and nitrogen species generation [84].

Macrophage activation is tightly controlled by the reversible acetylation and deacetylation of histones, and the application of HDACis demonstrates therapeutic effects in animal models of chronic inflammatory disease, depressed macrophage HDAC activity in patients with asthma, chronic obstructive pulmonary (COPD) disease, and inflammatory-mediated rheumatoid arthritis (RA), potentially supporting the administration of therapeutic HDACis [85]. Some studies have directly examined the effects of HDACis on the activation of macrophages derived from the inflamed joints of patients with RA. The inhibition of class I/II HDACs or class III sirtuin HDACs potently blocked the production of IL-6 and TNF-a by macrophages from healthy donors and patients with RA [85]. Two HDACis, trichostatin A (TSA) and nicotinamide, selectively induced macrophage apoptosis by regulating the anti-apoptotic protein Bfl-1/A1, and inflammatory stimuli increased the sensitivity of macrophages to HDACi-induced apoptosis. Importantly, inflammatory cytokine and angiogenic factor induction in RA synovial biopsy explants were also suppressed by HDACis. Although studies have identified important roles for class I/II and sirtuin HDACs in promoting inflammation, angiogenesis, and cell survival in RA, potentially revealing some redundancy between class I and II HDACs, their therapeutic effects must be assessed.

4.11 HDACs and HDACis in Homeostasis and Inflammatory Diseases

Due to the dynamic nature of epigenetic programming, inflammatory signaling events may be reversed by targeting distinct epigenetic enzymes, including HDACs. To gain a better understanding of the roles of specific HDACs in inflammatory disease, we must define the molecular and biological consequences of HDAC inhibition. Although HDACis have been used for clinical trials in cancer, the effects of HDACis on immune regulation are also promising, and these compounds may be applied to treat inflammatory diseases in the future [77]. Various HDACi molecules define specific immune response signaling cascades in different disease models, from acute to chronic inflammation [86]. HDACi molecules primarily block the transcription of key inflammatory modulators to control the full capacity of the inflammatory response after TLR and IFN signaling. The inhibitory effects of HDACis prevent the activation and differentiation of many effector cells, including DCs and macrophages. Some pro-inflammatory cytokines, including TNF-α, IL-6, and IL-12p40, are suppressed by HDACi application. HDACi treatment also enhances the IFN-activated cascade via ISG induction to establish an antiviral state. This phenomenon reveals the potential anti-inflammatory functions of HDACi agents to control the inflammatory signals produced by M1 macrophages in a variety of inflammatory disease models, in addition to their antiviral potential. The successful therapeutic use of HDACi molecules, however, still requires much work given the lack of knowledge regarding the distinct HDACi targeting of pro-inflammatory cytokines or chemokines. Future HDACi therapeutic approaches should focus on the fundamental biological mechanisms underlying the HDACi-mediated inhibition of pro-inflammatory mediators, how to use current sequence and structural information to develop novel HDACi molecules capable of targeting certain HDAC enzymes, and elucidating the regulatory cross talk between HDACis that affects the same class of HDACs. It will also be important to enhance the strategic use of HDACis in combination with biomarkers to assess the response and resistance to an HDACi treatment to demonstrate its effectiveness and rational clinical application.

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Chapter 5
miR-155 Dysregulation and Therapeutic Intervention in Multiple Sclerosis

Claire E. McCoy

Abstract microRNAs play a fundamental role in the immune system. One particular microRNA, miR-155 plays a critical role in hematopoietic cell development and tightly regulates innate and adaptive immune responses in response to infection. However, its dysregulation, more specifically its overexpression, is closely associated with various inflammatory disorders. The purpose of this review is to consolidate how miR-155 underpins a variety of processes that contribute to the pathology of multiple sclerosis (MS). In particular, the impact of miR-155 is discussed with respect to human pathology and animal models. How miR-155 contributes to the activation of pathogenic immune cells, the permeability of the blood-brain barrier, and neurodegeneration in relation to MS is described. Many environmental risk factors associated with MS susceptibility can cause upregulation of miR-155, while many of the current disease-modifying treatments may work by inhibiting miR-155. From this review, it is clear that miR-155 is a realistic and feasible diagnostic, prognostic, and therapeutic target for the treatment of MS.

Keywords miR-155 • MicroRNA • Multiple sclerosis • Experimental autoimmune encephalomyelitis • EAE • Immunopathology • Biomarker • Disease-modifying treatments • Blood-brain barrier • Environmental risk factors • Microglia • Astrocytes • Macrophages

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5.1 Introduction

microRNAs (miRNAs) play a fundamental role in cellular biology. They are critical modulators for a wide range of cellular processes including development, differentiation, proliferation, metabolism, and apoptosis [2, 4]. miRNAs are small ~22 nucleotide RNA sequences that regulate the expression of protein-coding mRNA sequences, often targeting multiple proteins within a particular network or pathway. miRNA targeting is achieved when they are guided to the 3′ untranslated region (UTR) of mRNA sequences, where partial or exact complementary base pairing of the miRNA results in degradation or translational inhibition of the target mRNA molecules. Each miRNA is predicted to bind to more than 200 mRNA target sequences, and as a result, overexpression and/or inhibition of a single miRNA can have a profound effect on cellular function [12, 127]. Major efforts have focused on understanding how miRNAs are induced in the cell, with an overall aim to uncover their specific mRNA target genes. In particular, attention is drawn to understanding which miRNAs or panels of miRNAs are dysregulated in disease. With this in mind, miRNAs are thus emerging as valuable therapeutic targets. Indeed, basic and applied miRNA research has already made a vast contribution in bench-to-bedside application, where >10 miRNA inhibitors (antagomirs) are currently in clinical trials for the treatment of cancer, cardiovascular disease, and hepatitis C virus (HCV) infection [55].

Of significance, miRNAs are critical regulators of the immune system, and their dysregulation clearly impacts the pathogenesis of various inflammatory diseases [80, 81, 116]. One particular microRNA, miR-155, plays a remarkable role in the immune system [79]. In summary, it was first identified when the B-cell integration cluster (BIC) (the gene which encodes miR-155) was found to be highly overexpressed in B-cell-activated lymphomas [22, 52, 118]. Later studies illustrated that miR-155 was not restricted to B cells, and a large-scale sequencing study performed in 26 different organ systems identified that the expression of miR-155 is highly specific for hematopoietic cells [53]. Indeed, several groups noted that miR-155 is potently induced upon activation of both myeloid and lymphoid cells with hematopoietic origin and is critical for the functioning of a healthy immune response to infection [83, 97, 112, 113].

This was further corroborated when mice deficient in miR-155 displayed impaired dendritic cell (DC) and T- and B-cell responses, characterized by faulty antigen presentation, reduced pro-inflammatory cytokines, reduced serum antibody titers, and inappropriate class-switched immunoglobulins, when challenged with infection [97, 113, 121]. Whereas its transgenic overexpression in hematopoietic cells resulted in a myeloproliferative disorder characterized by the gross expansion of myeloid cells, while overexpression in B cells promoted the development of leukemia and lymphoma in mice [15, 82]. Overall, this early data clearly indicates that miR-155 is essential for mounting an appropriate immune response to infection, yet its overexpression can contribute to immune-related disorders [84, 103, 120]. The purpose of this review is to consolidate the current literature with the aim to decode the impact of miR-155 dysregulation in the pathogenesis of multiple sclerosis (MS).

5.2 Multiple Sclerosis as an Inflammatory Disorder

Multiple sclerosis (MS) is the most common inflammatory disease to affect the central nervous system (CNS). It is a demyelinating disease in which the insulating myelin sheaths that surround nerve cells in the brain and spinal cord are damaged. Clinical manifestation is characterized by disturbances in sensory, motor, and cognitive function, with symptoms of pain and fatigue, while the pathology is characterized by lesions detected by magnetic resonance imaging (MRI) within the CNS. MS typically affects young adults where the average age of onset is 30 years old and affects two to three times more females than males [63, 92]. Its prevalence and incidence rate is increasing globally, especially in the northern hemisphere (140 per 100,000) compared to the global prevalence (30 per 100,000) [63, 92]. As well as causing a major personal burden to young adults, diagnosis and treatment requires a highly integrated and complex multidisciplinary approach resulting in significant economic burdens.

Several subtypes of MS have been described and are important for understanding the prognosis and type of treatment decisions. Eighty-five percent of new diagnoses are relapsing-remitting MS (RRMS) which produces attacks followed by periods of remission that can last months or years. However, it usually tends to get worse over time and often progresses to a secondary progressive MS (SPMS) subtype which begins to decline without periods of remission. In primary progressive MS (PPMS), there is an initial attack with a steady decline in disability without any periods of remission [63].

Despite extensive research conducted worldwide, a cause for all subtypes of MS remains unknown but may possibly arise due to genetic predisposition and/or environmental factors. With the advent of genome-wide association studies, 110 distinct genetic regions have been associated with MS [39]. However, only a handful of these gene variants such as human leukocyte antigen (HLA) class II genes (HLA-DQ, HLA-DR), IL-2RA, and IL-7RA have shown functional and correlative association with MS [32, 35, 73]. Environmental factors include a lack of vitamin D, human cytomegalovirus, and Epstein-Barr virus infection, and geographical latitude may have a role [5].

While the cause is not clear, the underlying mechanism is thought to be mediated by the immune system. Particularly in RRMS, it is extraordinarily conclusive that the symptoms and pathology of MS are due to an influx of immune cells crossing the blood-brain barrier (BBB) into the CNS. This immune cell infiltration results in chronic inflammation and the release of inflammatory cytokines and toxins that cause demyelination and neuroaxonal degeneration [21]. Considering the obvious

immunopathology of MS, it was a very natural progression that the impact of miR-155 would be explored in MS.

5.3 miR-155 in Human MS Samples

The first clear indication that miR-155 was dysregulated in MS came about when Meinl and colleagues isolated white matter lesions from paraffin and frozen multiple sclerosis tissue samples. miR-155 was highly upregulated (11.9-fold) in active white matter lesions compared to healthy control white matter [45]. While another study demonstrated that miR-155 was increased in cerebral white matter juxtaposed to active lesions collected from a mixture of relapsing-remitting, primary progressive, and secondary progressive patients [77]. Using laser capture microdissection, miR-155 expression was isolated from individual cell types, namely, myeloid-derived macrophages, microglia, T/B lymphocytes, and astrocytes, suggesting that infiltrating immune cells as well as resident brain cells have the capacity to generate miR-155 ([45, [72]). Interestingly, miR-155 expression was also significantly increased in the neurovascular unit of active lesions from MS brain samples [59]. The neurovascular unit is a sub-anatomical region typically representative of blood-brain barrier comprised of endothelial cells, astrocytes, and neurons and suggests that miR-155 expression is not solely restricted to hematopoietic cells.

Elevated miR-155 in peripheral blood mononuclear cells (PBMCs) isolated from MS patient blood samples has been confirmed in multiple studies [64, 86, 124]. The first of these demonstrated that compared to other miRNAs investigated, miR-155 was remarkably upregulated (two- to threefold) in a cohort of patients with RRMS. Interestingly, the increase of miR-155, combined with miR-146a and miR-142-3p, had an 88.0% specificity in predicting MS disease [124], while another study identified that a subset of (10/24) patients with RRMS had elevated miR-155 expression [64]. Increased expression also correlated with increased IL-17, IFNγ, TNF, and IL-6 and suggests that miR-155 elevation may only occur when cells are in an inflammatory state. Enquiring further, certain studies have elucidated that miR-155 expression was specifically elevated in $CD14^{+}$ monocytes when purified from PBMCs in a cohort of RRMS patients, while others have shown increased miR-155 in sera alone [72, 135].

From these studies, it is clearly evident that miR-155 is overexpressed in a range of human MS samples, yet many more questions remain. For example, what is the source of miR-155 upregulation in MS? Is its overexpression restricted to hematopoietic cells or do other cells play a role? Is it a consequence of inflammation or is it a trigger for the progression and pathology of MS? Can we realistically generate a feasible therapeutic for MS based on miR-155 targeting? Studies from animal models and in vitro studies have greatly contributed to our understanding and are discussed in the following sections.

5.4 miR-155 in MS Mouse Models

The experimental autoimmune encephalomyelitis (EAE) model is the most widely used experimental animal model for MS. Although it is often criticized, it does resemble a model whereby peripheral activation of immune cells by a CNS-originating peptide has the capacity to closely resemble the human MS disease. In this model, an emulsified CNS antigen, typically myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG), is injected together with Freud's adjuvant and pertussis toxin. The result is the activation and presentation of MBP or MOG by dendritic cells to $CD4^+$ T cells in the lymph nodes, which together result in a massive infiltration of differentiated $CD4^+$ T helper (Th1) and Th17 cells, B cells, $CD8^+$ T cells, and innate immune cells infiltrating the CNS, leading to inflammation and tissue damage.

The impact of miR-155 was first implicated when mice deficient in miR-155 were shown to be highly resistant to $MOG_{35\text{-}55}$ peptide-induced EAE. miR-155−/− mice displayed a delayed onset, decreased disease severity and paralysis compared to wild-type mice [74, 79]. Brain histological analysis revealed less inflammation and less demyelination consistent with their reduced disease severity [74, 79]. The beneficial prognosis in miR-155−/− mice was primarily associated with reduced numbers of peripheral Th1 and Th17 in the spleen and lymph nodes, as well as reduced numbers in the CNS [76, 79]. When cultured ex vivo, Th1 and Th17 cells had decreased proliferative responses and reduced capabilities to produce IFNγ and IL-17 upon stimulation, suggesting that they are functionally defective. The progression of EAE was intrinsically linked to $CD4^+$ T cells, because the adoptive transfer of miR-155+/+ $CD4^+$ T cells into RAG1−/− recipients (which lack mature T/B lymphocytes) had a substantially more severe and accelerated disease course compared to mice receiving miR-155−/− $CD4^+$ T cells [79]. Moreover, CNS-isolated $CD4^+$ T cells from EAE-induced rats could be reactivated to produce more miR-155 upon reexposure to MBP in vitro, suggesting that $CD4^+$ T cells have the capacity to further increase miR-155 upon contact with reactive antigens [44]. These studies predict that overexpression of miR-155, typically observed in EAE models and human MS samples, acts to promote a Th1/Th17 phenotype.

The contribution of miR-155 in driving Th1 and Th17 responses was consolidated when wild-type mice were treated with a locked nucleic acid (LNA)-miR-155 oligonucleotide (herein called "antagomir") which reduced the clinical manifestation of disease when administered before or during EAE induction [74, 135]. The antagomir resulted in diminished proliferation and reduced IFNγ and IL-17 secretion in $CD4^+$ T cells isolated from the CNS [74]. In contrast, the intravenous injection of a miR-155 mimic aggravated EAE disease severity caused by the prominent inflammatory infiltration and demyelination in the spinal cord [135]. Additionally, increased frequencies of Th1 and Th17 cells, along with increased IL-17 and IFNγ cytokine production, were observed in the spleen, lymph nodes, and CNS of miR-155 mimic administered mice [135].

Further studies have illustrated how miR-155 mechanistically regulates Th1 and Th17 differentiation and function. For example, O'Connell and colleagues illustrated that miR-155 targets the transcription factor Ets1, a well-established negative regulator of Th17 differentiation [37]. Consistently, miR-155−/− Th17 cells had elevated Ets1 expression and lacked the expression of cytokines essential for Th17 differentiation. Namely, the Ets1/miR-155 axis was necessary for IL-23 responsiveness and was critical for normal expansion of Th17 cells in vivo and during induction of EAE [37]. Escobar and colleagues elegantly demonstrated that miR-155 can regulate the chromatin structure and epigenetic changes in Th17 cells [23]. By performing transcriptome analysis, they identified Jarid2, an RNA-binding protein, was upregulated in miR-155−/− differentiated Th17 cells. An increase in Jarid2 reprograms the epigenome of Th17 cells via H3K27 methylation and results in the silencing of specific genes (such as IL-22) that are required for Th17 differentiation [23]. Defects in Th17 differentiation and cytokine expression in the absence of miR-155 could be partially restored by Jarid2 deletion [23]. Another study demonstrated that miR-155 could control Th1 and Th17 proliferation and tissue migration by directly repressing the enzyme heme oxygenase-1 (HO-1) [136]. HO-1 catalyzes the oxidation of heme to generate carbon monoxide, biliverdin, and iron products and is essential for mediating important anti-inflammatory and antioxidant effects. Thus, when miR-155−/− animals, typically resistant to EAE, were injected with the HO-1 inhibitor ZnPP, disease severity increased [136]. In another study, miR-155-3p was found to be more highly expressed in CNS-isolated $CD4^+$ T cells at the peak of EAE, rather than the typical miR-155-5p variant [75]. In fact, miR-155-3p was shown to drive the upregulation of Th17 marker genes *Rora* and *IL17* compared to miR-155-5p and could specifically promote Th17 differentiation [75].

Altogether, it is particularly evident that miR-155 is critical for driving pathogenic Th1 and Th17 responses, whereas its deletion and/or inhibition results in reduced proliferative, functional responsiveness, and migration into the CNS during EAE. This is extremely important considering Th1 and Th17 cells are key drivers of the human disease [31]. Yet, it must also be emphasized that the contribution of miR-155 in myeloid cells, B cells, or brain-resident cells has not been thoroughly investigated in the context of EAE or other types of MS models. This is intriguing considering miR-155 expression has shown to be specifically elevated in PBMCs and tissue-resident brain cells (microglia and astrocytes) from human MS patient samples. It is critical that we delve deeper into understanding cellular origins of miR-155 in a more thorough manner.

5.5 miR-155 in Peripheral Immune Cells

Intensive research has focused on pathogenic CD4+ T cells as the key participants in the pathogenesis of MS. However from as early as 1990, it was demonstrated that up to 50% of the immune cells that infiltrate the CNS in the EAE model are in fact peripheral myeloid-derived monocytes and macrophages [38]. Importantly, the

depletion of myeloid cells has been shown to completely prevent EAE disease progression [115]. During the early phase of MS, infiltrated monocytes/macrophages are immediately activated to become M1 macrophages, releasing pro-inflammatory cytokines, reactive oxygen species, and toxic metabolites that cause irreversible damage to neurons within the CNS. In fact, M1 polarized macrophages show prolonged periods of apposition within MS lesions, releasing reactive oxygen and nitrogen species that were shown to be particularly toxic to neurons and their axons [38, 44, 76]. During the later phase of disease and during periods of remission, macrophages are less activated and present as M2 or alternatively activated, releasing anti-inflammatory cytokines accompanied by inflammation resolution and tissue repair [96, 131]. For example, selective depletion of M2 macrophages inhibits experimental remyelination, whereas the transfer or enhancement of M2-polarized macrophages suppresses EAE [6, 71, 117]. M2 macrophages have huge capacity in therapeutics; however the molecular mechanisms which drive M2 polarization remain largely unknown. Evidence from the literature suggests that the levels of miR-155 expression in monocytes/macrophages are intimately associated with M1/M2 polarization states.

For example, miR-155 is potently induced by M1 agonists, namely, LPS, IFNγ, TNF, and GM-CSF [83, 84]. The transcription factors required for M1 polarization, such as NF-κB, AP-1, HIF1α, and most recently by us ETS2, required for the sustenance of an M1 phenotype are also absolutely essential for the transcriptional induction of miR-155 [7, 94]. HIF1α has been shown to bind to the miR-155 promoter and enhance its expression [7]. Interestingly, HIF1α plays a central role in the metabolic reprogramming of macrophages, acting to promote glycolysis essential for the maintenance and functional responses required by M1 macrophages [50]. Overexpression of miR-155 in monocytes and macrophages has been shown to increase reactive oxygen species, pro-inflammatory cytokines, and cell surface markers CD80 and CD86 [72, 78, 123, 139]. Furthermore, miR-155 transfected monocytes can enhance T-cell proliferation and IFNγ production [72].

Mechanistically, miR-155 contributes to pro-inflammatory signaling cascades and effector functions in macrophages by inhibiting numerous targets (SHIP1, FADD, SOCS1, IKK, IL13R1, CEBPβ, and SMAD2), which collectively result in the upregulation of the pro-inflammatory cytokines and release of reactive oxygen species [58, 60, 67, 91, 98]. Perhaps the most striking study illustrated that miR-155 control is not restricted to the above cellular targets, when approximately 650 genes required for M1 polarization were shown to be dependent on miR-155 in a whole genome transcriptome array [41].

On the other hand, macrophages treated with M2-polarizing agonists IL4/IL-13 fail to induce miR-155, while the anti-inflammatory cytokine IL-10 can potently inhibit LPS-induced miR-155 expression [68]. Moreover, miR-155−/− display elevated M2-polarizing cytokines such as IL-10 and IL4 and their serum [97]. This suggests that the inhibition of miR-155 can promote an M2 phenotype. Indeed, IL-10 inhibits both the primary miR-155 transcript and mature form in a STAT3-dependent manner [68]. Additionally, IL-10 reduced both ETS2 protein expression and its ability to bind to the miR-155 promoter, required for the transcriptional

induction of miR-155 [94]. Mechanistically, low miR-155 expression can promote the M2 phenotype, by reversing its repression on M2-associated genes, including IL-13R, SMAD2, and CEBPβ. SMAD2 is a transcription factor essential for mediating the anti-inflammatory effects of TGFβ, while CEBPβ is important for the induction of the M2-associated genes Arg1, IL-10, IL-13R, and CD206 [60, 67, 98].

However, the direct implication of miR-155 on monocytes and macrophages during EAE and MS is less established. In the earliest studies, dendritic cells isolated from mice undergoing EAE had reduced pro-inflammatory cytokines, namely, IL-12, IL-1β, IL-6, IL-23, and TNF, that are required for Th1 and Th17 polarization [74, 79]. In human samples, miR-155 expression is elevated in monocyte-derived macrophages within active MS lesions, and others have shown that miR-155 is specifically increased in CD14+ isolated monocytes from MS blood samples [72]. Notably, mice deficient in the M2 agonist, IL-10, have been shown to develop accelerated disease progression following active immunization with CNS autoantigens, whereas IL-10 transgenic mice are resistant to EAE [18, 100]. Gene transfer methods delivering sustained IL-10 expression ameliorated the disease [19, 87]. It would be interesting to determine if the beneficial effects of IL-10 are mediated by its capacity to downregulate miR-155 or whether the conditional deletion of miR-155 can promote M2 macrophage accumulation in the CNS during EAE.

5.6 miR-155 in Brain-Resident Cells

Although the role of miR-155 in microglia and astrocytes has not been directly assessed in EAE, there is overwhelming evidence from other disease models that miR-155 upregulation contributes to neuroinflammation and subsequent neurodegeneration, whereas its deletion has neuroprotective effects. In primary cultured microglia, miR-155 is the most significantly upregulated miRNA under inflammatory M1-skewing conditions, similar to what has previously been shown in periphery myeloid cells [10, 25, 72]. Inhibition of miR-155 decreased the release of pro-inflammatory cytokines and nitric oxide, while the conditioned medium from these microglia could decrease neuronal cell death [10]. One study has demonstrated that IL-1/IFNγ can significantly upregulate miR-155-5p and miR-155-3p in astrocytes. Antagomirs to both isoforms dramatically reduced the production of TNF, IL-6, and IL-8, suggesting that miR-155 is required for polarization of astrocytes into an activated A1 phenotype [111]. This is interesting considering A1 astrocytes can induce the death of neurons and oligodendrocytes and are highly abundant in brain samples from MS, as well as other neurodegenerative disorders including Alzheimer's, Huntington's, Parkinson's, and amyotrophic lateral sclerosis (ALS) [56].

In animal models, sustained transgenic overexpression of miR-155 resulted in aberrations in the proliferation, migration, and differentiation of neural stem cells in the hippocampus. Whereas genetic deletion of miR-155 could restore the neuroinflammation-induced damage [128]. A strong upregulation of miR-155 was

observed within the brain of the Alzheimer's disease model, which occurred simultaneously with increased microglia and astrocyte activation and the appearance of β-amyloid aggregates [33]. miR-155 elevation was also observed in spinal cord microglia from mice with amyotrophic lateral sclerosis (ALS), whereby genetic deletion of miR-155 or treatment with miR-155 antagomirs dramatically increased the survival by 38% in these mice [9]. miR-155 deletion and antagomir treatment were similarly shown to promote the recovery of ischemic stroke as a result of decreased neuroinflammation [89, 125]. Primary miR-155−/− microglia cultures displayed reduced inflammatory responses when treated with α-synuclein, a widespread aggregate found in Parkinson's disease, while a striking neuroprotective effect was observed in miR-155−/− mice with α -synuclein-induced Parkinson's disease [114].

5.7 miR-155 and the Blood-Brain Barrier

The blood-brain barrier (BBB) is a highly selective barrier made up of endothelial cells connected by tight junction proteins. It acts to separate circulating blood from the brain architecture and plays an important role in restricting the diffusion of pathogens, leukocytes, and large molecular weight molecules into the CNS. However, dysregulation of the BBB and the trafficking of peripheral activated leukocytes are among the earliest features observed in MS brains [70]. Although the mechanisms are not fully understood, the release of pro-inflammatory cytokines such as IL-1 and TNF from activated leukocytes and/or activated brain-resident cells can alter the physiology of the endothelial cells that make up the BBB, causing them to increase their permeability and change the dynamics of their tight junctions.

Various studies have confirmed that human endothelial cells can produce miR-155 under inflammatory conditions [51, 59, 93, 109, 130, 140]. Inhibition or overexpression of miR-155 in endothelial cells could either decrease or increase vascular endothelial permeability, respectively [109, 138]. Perhaps the most conclusive data for miR-155 in relation to BBB permeability and its impact on MS was demonstrated in Biozzi mice induced with EAE, an animal model with a predictable relapsing-remitting paralysis course associated with the loss BBB integrity at the spinal cord [59]. When FITC-dextran was injected into wild-type mice undergoing EAE, a high abundance of the marker was located in the spinal cord parenchyma. In contrast, there was a 50% reduction of the marker located in these tissues in miR-155−/− animals [59]. Overexpression of miR-155 increased the leakage of fluorescent dextrans across cultured human brain endothelial cells when challenged with cytokines TNF and IFNγ, whereas miR-155 inhibition reversed this effect. Overexpression of miR-155 could increase the permeability of endothelial cells by targeting tight junction proteins annexin-2 and claudin-1, but also focal adhesion molecules such as DOCK-1 and syntenin-1. In fact, endothelial cells accepted exosome-delivered miR-155, an effect that was shown to destroy tight junctions and

the integrity of the endothelial barrier [138]. Furthermore, miR-155 could increase the adhesion of monocytes and T cells to endothelial cells under shear forces [11].

LPS activation of choroid plexus epithelium (CPE), a unique layer of epithelial cells that form a blood-brain barrier with cerebral spinal fluid (CSF), was shown to release miR-155-containing exosomes [3]. These exosomes were released into the CSF and taken up by astrocytes and microglia but not by neurons in an LPS-induced neuroinflammation model. Primary mixed cortical cultures incubated with miR-155-containing exosomes could potently increase the secretion of pro-inflammatory cytokines IL-6, IL-1, and TNF [3]. This effect was also mimicked in the brain of LPS-injected mice and was blocked when an exosome inhibitor was injected intracerebroventricularly. It is fascinating to consider that the peripheral activation of barrier cells such as endothelium and epithelium could be secreting miR-155-containing exosomes as a form communication that can alter the behavior of cells in the brain.

5.8 miR-155 and Environmental Risk Factors

Numerous large-scale epidemiology studies have been performed to search for MS environmental risk factors. Consistently, infection with EBV, infectious mononucleosis (caused by EBV), smoking, lack of vitamin D, and genetic risk alleles show the strongest correlation with MS susceptibility [5, 39, 102]. In particular, EBV infection has the strongest epidemiological credibility, and there is a large body of evidence to suggest that it plays a major role in the pathogenesis of MS [5, 90]. Although the exact mechanisms are incompletely understood, EBV has been shown to affect multiple immune cell parameters including increased EBV-transformed peripheral B cells with a concomitant increase in EBV viral shedding and production of anti-EBV antibodies, increased autoreactive CD4+ T cells in the CNS, impaired EBV-specific CD8+ T-cell immunity, and activation of innate immune cells in MS patients [90]. Intriguingly, early studies showed that BIC and mature miR-155 were strongly elevated in EBV-infected B lymphocytes [52, 95, 133]. The EBV latency membrane-associated protein (LMP1) is an important activator of NF-kB and the immortalization of B cells. LMP1 could induce BIC transcription and mature miR-155 primarily through the activation of NF-κB, p38, and AP1 transcription factors [30, 95, 134]. Importantly, miR-155 antagomirs could reduce EBV nuclear antigen (EBNA) mRNA expression and EBV copy number in infected cells, as well as inhibit the growth of proliferating lymphoblastoid cell lines [57, 62]. Children with infectious mononucleosis caused by primary EBV infection also displayed elevated miR-155 expression in blood-isolated B cells [29]. Large-scale transcriptome analysis of both viral- and cellular-induced miRNA in EBV transformed cells highlighted that BACH1 is a likely target for miR-155 [106]. In fact, BACH1 has been suggested to play a key regulatory role in EAE and MS [29, 107].

The lack of vitamin D, especially in countries located in latitudes correlated with poor sunlight, has been associated with MS susceptibility. Intriguingly, dendritic cells treated with 1 alpha,25-dihydroxyvitamin D(3) (vitamin D) gave rise to an immature phenotype, characterized by low levels of miR-155 and IL-23 [88]. Subsequent studies showed vitamin D could strongly prevent miR-155 induction in human adipocytes and disrupt the formation of miR-155-containing exosomes in chronic lymphocytic leukemia monocytes [8, 46]. Vitamin D could attenuate LPS-induced signaling through a mechanism that involved inhibition of miR-155 and an increase in the target, SOCS1 [13]. Moreover, the suppressive effect of vitamin D on miR-155 could not be achieved when Ago2, a key protein required for RNA- and miRNA-induced silencing complex, was deleted [40].

Of the 110 genetic risk variants identified from a cohort of 14,496 subjects with MS, 97 of these were associated with immunological function [39]. Although a SNP for miR-155 was not reported, five SNPs (TNFSF14, IL2RA, TNFSF1A, IL12A, and STAT4) accounted for more than 50% of the association. Interestingly, enforced expression of miR-155 in PBMCs could enhance IL-2 expression, while stimulated T cells from miR-155−/− mice have deficient IL-2 production [54, 97]. Similarly, miR-155 inhibition or overexpression could inhibit or promote IL-12 production in DCs [61]. Moreover we have shown that genetic deletion of Ets2 in myeloid cells, a critical transcription factor required for miR-155 induction, failed to produce IL-12 cytokine [94]. It is plausible to consider that hyper-expression of miR-155 in MS patients contributes to the dysregulation of signaling pathways controlled by these risk variants. In fact, in an Italian cohort of 360 MS patients, 4 SNPs were located in close proximity to the BIC gene. Three of these formed a unique haplotype (rs2829803, rs2282471, rs2829806) that was overrepresented in MS patients (13.5%) compared to controls (10.3%) and conferred a 1.36-fold increased genetic risk of developing MS [86]. It will be interesting to see if larger-scale studies can recapitulate this result and find an association of the BIC/miR-155 haplotype with MS.

5.9 miR-155 and Disease-Modifying Treatments

There is no cure for MS. However, there are currently >10 FDA-approved disease-modifying treatments (DMF) for RRMS [20]. Early intervention and treatment with these DMFs significantly slows the progression of the disease, as well as lowering the relapse rate and the formation of new lesions. These medications predominantly act on the immune system as immunosuppressants and can be broadly divided based on their ability to block immune cell infiltration into the CNS (natalizumab, fingolimod, mitoxantrone), to reduce immune cell activity (interferon-β, glatiramer acetate, dimethyl fumarate), or to inhibit immune cell proliferation (teriflunomide, alemtuzumab, ocrelizumab) [17]. Considering miR-155 is elevated in MS patients and in animal models undergoing EAE, it is worth understanding the impact of these treatments on miR-155 expression.

IFNβ and glatiramer acetate were the earliest drugs to be approved for MS and are often used as first-line treatment options [20]. IFNβ helps regulate the immune system, decreasing the amount of immune cells infiltrating the CNS, particularly Th1/Th17 subsets and their respective cytokines. Glatiramer acetate is a synthetic amino acid polymer that mimics myelin basic protein and has been shown to divert the generation of Th1 cells to Th2 cells which can suppress the inflammatory response. In two separate studies, whole blood and PBMC samples isolated from MS patients treated with IFNβ and glatiramer acetate failed to demonstrate any effect on miR-155 expression, whereas other miRNAs were found to be reduced significantly [49, 124]. However, glatiramer acetate did reduce miR-155 expression in urine-isolated exosomes from EAE-induced mice at peak disease [105]. In some respects, the lack of repression on miR-155 in IFNβ-treated patients is not surprising considering IFNβ is an established agonist for miR-155 induction in macrophages [83].

Dimethyl fumarate (DMF) is a methyl ester of fumaric acid and was approved by the FDA as an effective oral treatment for RRMS [20]. Although we still do not fully understand its mechanism of action, studies have shown in MS patients treated with DMF that there is a reduction in Th1/Th17 subsets, an increase in Th2 subsets, and a shift from M1 to M2 macrophages [110, 129], while others have shown that DMF can inhibit microglia and astrocyte inflammation and has neuroprotective effects in vitro and in EAE animal models [1, 126]. Promisingly, miR-155 expression was found to be significantly reduced in monocytes from MS patients receiving DMF [69].

Natalizumab was the first humanized monoclonal antibody approved for MS in 2007 and is classed as a highly effective treatment. It blocks the cellular adhesion molecule α4 integrin on immune cells, inhibiting their ability to bind and migrate through the endothelial BBB. Natalizumab has been shown to reduce miR-155 expression in PBMCs and monocytes isolated from MS patients which also correlated with a decrease in IL-17, IFNγ, and TNF gene expression [64, 69]. Furthermore, patients with the highest expression of miR-155 expression pre-natalizumab therapy had higher levels of anti-EBV nuclear antigen titers in their serum [64]. Fingolimod, although not a monoclonal antibody, works in a similar manner to natalizumab by trapping immune cells in lymph nodes and preventing their migration to the CNS. Fingolimod also significantly reduced miR-155 expression in human monocytes [69].

Alemtuzumab is a humanized monoclonal antibody against CD52, a cell surface receptor expressed on mature T and B lymphocytes, while ocrelizumab, the first approved drug for PPMS, is a humanized monoclonal antibody that binds to CD20, a cell surface marker specifically expressed on B cells. Both treatments result in tagging their respective cells for destruction, and patients have shown very promising improvements in disability and disease progression. Although miR-155 expression has not been explored with either drug, ibrutinib, a B-cell-depleting therapy for chronic lymphocytic leukemia has been shown to significantly decrease miR-155 expression [34].

Overall, this data strongly suggests that miR-155 could be a very effective biomarker for monitoring responsiveness to treatment. Moreover, it indicates that inhibiting miR-155 itself could be a very legitimate and realistic target for the treatment of MS. This is even more pertinent when we consider the astounding efficacy miR-155 antagomirs have had in EAE models, as well as consider the overall impact that miR-155 inhibition has in skewing cells of the immune system to an anti-inflammatory phenotype both in vitro and in vivo [74, 135]. Indeed, judging by the extraordinary number of submitted patent applications for miR-155 antagonists, it strongly suggests that generation of miR-155 antagomirs as a therapeutic treatment is already underway.

5.10 Future Directions

One of the biggest challenges in MS is the lack of a biomarker to effectively stratify different MS subtypes, which is critical when considering the most effective treatment strategy. In addition, a biomarker that aids our understanding of disease severity, responsiveness to treatment, and disease progression is still required. The attractiveness of miRNAs as biomarkers for disease cannot be underestimated [27]. Their presence, not only in cells and tissues but also in readily available body fluids and extracellular vesicles, suggests that they represent a gold mine of noninvasive biomarkers for disease. miRNAs are extremely stable; those that are found in blood are highly resistant to ribonuclease due to their packaging in lipid vesicles, binding to RNA-binding molecules, or their association with high-density lipoproteins [14]. Once expressed, they are long-lived. For example, from a range of immunologically-related miRNAs tested in macrophages, we showed that miRNAs have an average half-life of 5 days [28]. Once isolated from relevant samples, they are resistant to extended storage, freeze-thaw, and extreme pH. The development of sensitive platforms for detection and quantification using methods such as miRNA assays, bead-based assays, NanoString techniques, and deep sequencing has ensured that results are quantifiable, reproducible, and accurate.

To date, miRNA expression profiles have been conducted in MS patients from a variety of tissues including whole blood, PBMCs, serum, CSF, MS lesions, as well as sorted lymphocyte and myeloid-derived immune cell populations [27, 42]. Interestingly, Jagot and Davoust consolidated 19 miRNA profiling metadata studies conducted in plasma, CNS, and immune cells in an attempt to find the most commonly dysregulated miRNAs in MS patients. miR-155 was identified to be elevated across all MS patient samples tested, along with altered expression of miR-23a, miR-223, miR-22, miR-326, and miR-21 suggesting that these miRNAs may form an important signature in MS [43]. However, it is also important to note that other studies have found no changes in miR-155 expression in PBMCs from RRMS, SPMS, and/or PPMS [16, 36, 47, 48, 66, 85, 108]. In fact, miR-155 was found to be significantly downregulated in $CD4^+$ T cells from a cohort of secondary progressive MS patients [101]. Moving forward, it will be critical to assess if miR-155 elevation

is specific for a particular cell type or subtype of MS, while isolating miR-155 from urine or CSF could provide an alternative avenue of exploration. Overall, generating panels of miRNA signatures to include miR-155, rather than looking at miRNAs individually, could be a more beneficial approach for developing diagnostic and prognostic indicators for MS.

The delivery of stem cells, particularly hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), induced pluripotent stem cells (iPSC), and neural stem cells (MSC), is under intense investigation in many neurodegenerative diseases including MS. In particular, early clinical trials have demonstrated that RRMS patients receiving HSC transplants have had the greatest improvements in disease progression, with a dramatic decrease in relapse frequency and MRI activity [65]. Although the contribution of miRNAs in therapeutic stem cell delivery has not been explored in MS, it has been widely examined in cancer, cardiovascular diseases, arthritis, and neurological disease, where they have been shown to play a fundamental role in stem cell differentiation and therapeutic modulation [24, 99, 104, 119, 132]. It is clearly worth pursuing whether these emerging therapies for MS have a direct impact on miR-155.

In MS, the CNS also has the capacity to remyelinate and repair any damage caused to neuronal axons. Clinicians often observe this as a “shadow plaque” in MRI scans when old lesions have been repaired and remyelinated during the remitting phases of the disease. Remyelination is mediated by a population of oligodendrocyte progenitor cells (OPCs) that can proliferate and migrate to areas of damage, where they differentiate into myelin-producing mature oligodendrocytes. Understanding the molecular mechanisms while identifying novel therapies that can promote oligodendrocyte maturation and remyelination is currently under heavy investigation in the MS field. Recent evidence suggests that microRNAs may govern this process [26]. In particular, miR-219 and miR-338 have been shown to be critical for CNS remyelination after injury, while miR-146a could facilitate remyelination by promoting OPC differentiation in cuprizone demyelinating models [122, 137]. Clearly, the impact of miR-155 in these processes could be an exciting avenue for exploration.

5.11 Conclusion

Although many advances have been made understanding the role of miR-155 in immune cell function and regulation, there are many gaps in our knowledge in understanding its role in non-immune cells. Furthermore, the exact contribution of miR-155 in the majority of relevant cell types has not been directly explored in MS. Generating conditional knockout animals or transgenic overexpressing models will help to answer these questions. Moreover, extending the use of typical MS models to focus on its impact in demyelination and remyelination should be considered. Understanding whether miR-155 is simply a marker for inflammation or whether it plays a predominant role in triggering MS requires further study.

Standardizing miRNA detection methods in various human samples will help consolidate whether miR-155 could be used as a realistic biomarker in MS. Most importantly, therapeutic inhibition of miR-155 and understanding whether it will be most efficacious by delivering it in the periphery or directly to the CNS, or directly to specific cell types or specific MS disease subtypes, remains to be elucidated.

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Chapter 6
Inflammasomes in the Gut Mucosal Homeostasis

Xiaomin Yao and Guangxun Meng

Abstract Inflammasomes are critical checkpoints in inflammation. The activation of inflammasome can cause a series of inflammatory responses including maturation of interleukin (IL)-1β and IL-18 and a specialized form of cell death called pyroptosis. Since its identification in the early 2000s, inflammasomes have been implicated to play multifaceted roles in varied pathological and physiological conditions, especially in the mucosal compartments including the gut. Maintaining gut mucosal homeostasis has always been a remarkable challenge for the host due to both the vast mucosal surface that is exposed to the outside and the enormous amount of local microbiota. To accomplish this challenge, the host mounts a constant dynamic low-grade inflammatory response (physiological inflammation) in coping with insults of microbes in the intestine. This book chapter aims to summarize the current knowledge of how inflammasomes contribute to gut mucosal homeostasis.

Keywords Inflammasome • Gut mucosal homeostasis • IL-1β • IL-18 • Intestinal homeostasis • Pyroptosis

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6.1 Introduction

6.1.1 Characterization of Inflammasomes as a Critical Arm in Innate Immunity

A multicellular organism usually utilizes the barrier system and the immune system to cooperatively segregate body constituents from harmful agents in the surrounding environment while simultaneously leaving some communicable space to absorb benefits such as energy from the outside world. In mammals like humans, the immune system comprises two parts, namely, the innate and adaptive immune systems, which are responsible for non-specific and antigen-specific immune responses, respectively. Although the adaptive immune system is evolutionarily superior and more efficient to target varied intruders, the innate immune system is indispensable for the initiation and full-functioning of the adaptive immune responses. Moreover, since many innate immune cells prefer to reside locally right behind the different barrier systems as well as to patrol in the circulation, innate immunity normally acts as the first line to detect and defend pathogens or organ injuries. Mechanistically, the innate immune system harnesses a set of germ line-encoded pattern recognition receptors (PRR) to recognize different pathogen/danger-associated molecular patterns (PAMP or DAMP). These PRRs are armed with invariable recognition motifs, i.e., each PRR can only target a few limited PAMPs or DAMPs which are ubiquitously presented in different pathogens and tissue injuries, such as lipopolysaccharides(LPS) on gram-negative bacteria cell walls and nucleic acid leaked from dying host cells. Currently, the PRRs contain membrane-associated toll-like receptors (TLRs) and C-type lectin receptors (CLRs), cytosolic NOD-like receptors (NLRs), AIM2-like receptors (ALRs), and RIG-I-like receptors (RLRs) [1]. Of interest, members of the NLRs (NLRP1, NLRP3, NLRP6, NLRC4, and NLRP12) and ALRs (AIM2) can orchestrate a specialized protein complex designated as inflammasomes to license the activation of the profoundly inflammatory caspase-1. The canonical inflammasomes are formed upon either exogenous or endogenous insults, which trigger the PRR molecule to oligomerize and act as nucleate to promote recruited adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 to form prion-like macromolecules, leading to the proximity-induced autoactivation of caspase-1 [2]. Of note, recent studies identified a noncanonical inflammasome formation upon direct binding of cytosolic LPS to caspase-11, which can not only activate caspase-11 itself but also trigger caspase-1 activation through yet unknown mechanisms [3–6]. In addition to members of NLRs and ALRs which are able to form inflammasomes, Shao and colleagues identified a previously neglected PRR, pyrin, to recognize abnormally inactivated RHOA (a small GTPase protein of Rho family) by pathogens and then form classical inflammasome to activate caspase-1 [7]. The activation of inflammatory caspases usually leads to proteolytic maturation and secretion of IL-1β and IL-18, as well as cleavage of pore-forming Gasdermin D (GSDMD), which triggers pyroptosis [2].

6.1.2 A Complex Microbiota-Immune Interaction Underlies the Gut Homeostasis

With more and more newly identified inflammasome-forming PRRs, the landscape of the multifaceted physiological significance of inflammasome has been gradually revealed. Among the many body sites, the intestinal mucosal surface stands out as the major location where the immune system performs its guardian function, since about 70% of immune cells of the entire immune system gather along the intestine [8]. The reason why host deploys such massive immune forces at intestinal mucosal surface is because of the residence of a much more massive amount of microbial flora, namely, microbiota at the same place. It was estimated that a number of 10^{13}–10^{14} microbes reside in the human gut, which is more than ten times of the total number of host cells in our body [9]. Although recently a revised estimation of both the microbial companions and ourselves reduced the ratio from ~10:1 to ~1:1, with each numbering at 3.9×10^{13} and 3×10^{13}, respectively [10], this doesn't affect the magnitude of the huge challenge that gut microbiota has posed to the host. Indeed, in germ-free animals, where no any microbes present in the gut, a substantial shrinkage of the gut local immune system was observed [11]. But surely these tiny bugs have become our neighbors for good reasons during evolution. For example, they help us absorb energy from usually indigestible polysaccharides by degrading and fermenting them to short-chain fatty acid; also, microbiota provides us with precious vitamins to support human health by metabolic processes that we don't possess. More importantly, microbiota can provide vital cues to elicit and shape the sound immune system locally and systematically. Much more than these, microbiota can directly help to expel invasive pathogens trying to penetrate the mucosal surface by competitive "colonization resistance" or by priming the host immune system to better combat infections indirectly [12]. On the other direction, human intestine offers the microbiota with invaluable anaerobic living sphere with exceptionally richness of food for them to prosper, which are rare to find in the inorganic world outside [13]. Such significant mutualism between us and microbiota demands careful maintenance, which is a dynamic status now known as gut homeostasis [13]. As the inflammasomes constitute an important arm of innate immunity, and many mutations identified in inflammasome component genes have been associated with intestinal disorders [14], their roles in keeping gut homeostasis need in-depth investigation. So, in this book chapter, we summarize the current understanding of the inflammasomes' contribution to gut mucosal homeostasis.

6.2 Inflammasome Effectors in Maintaining Gut Homeostasis

The discovery of different inflammasome effector molecules was much earlier than identification of the platform nucleating PRRs [15]. In 1977, Dinarello et al. first purified fever-causing factor, called pyrogen, from human blood [16]. Later on this pyrogen was defined as the first interleukin, i.e., IL-1β [15]. Fifteen years later, the enzyme that is responsible for the maturation of IL-1β, i.e., IL-1β converting enzyme (ICE), a cysteine protease, was cloned and now known as caspase-1 [17–19]. Another interleukin substrate of caspase-1, IL-18, was identified soon after the discovery of caspase-1 [20, 21]. In addition to mediating maturation and secretion of the aforementioned cytokines, caspase-1 activation can also lead to a programed but proinflammatory cell death, which is named as pyroptosis [22]. Since the early identification, the roles of these inflammatory molecules in gut homeostasis have been studied extensively.

6.2.1 IL-1β

As the initial context for characterization of IL-1β is in inflammation, early reports in clinical human inflammatory bowel disease (IBD) patients indicated a pathogenic role of IL-1β in promoting IBD [23]. However, these reports were largely based on studies with patients bearing onset IBD, where it's hard to distinguish whether elevated IL-1β is a result of or the causing factor for IBD. Further studies with animal models trying to clarify this point failed to reach a consensus conclusion, as totally opposite results had been obtained from different researches.

With the antibody blocking approaches to either inhibit or enhance the IL-1 signaling by targeting IL-1R and IL-1R antagonist (IL-1RA), respectively, a positive correlation of IL-1 level with colitis severity was revealed, indicating IL-1β is deleterious for maintaining gut homeostasis [24, 25]. However, in an earlier study, with the same model, they found that pretreatment with low dose of recombinant IL-1β significantly reduced the colitis symptoms [26]. Interestingly, a recent study showed that transplantation of IL-1β-primed mesenchymal stem cells (MSC) can alleviate the chemical dextran sulfate sodium (DSS)-induced colitis [27]. Also in a parallel comparison of IL-1α and IL-1β in DSS-induced colitis using *Il1α*$^{-/-}$ and *Il1β*$^{-/-}$ mice, they found that IL-1β is protective while IL-1α is detrimental [28]. But in a separate research, also using *Il1β*$^{-/-}$ mice, IL-1β was found to be selectively activated by certain bacteria such as pathobiont *Proteus mirabilis* through NLRP3 inflammasome, which contributed to DSS-induced colitis, indicating probably the varied commensal microbiota presented in the intestine that affects disease outcomes [29]. Besides the chemical-induced injury model, there were reports showing that IL-1β can also

protect mice from intestinal infection of *Clostridium difficile* and *Citrobacter rodentium*, by promoting phagocytosis and eradication of bacteria in mononuclear phagocytes [30–32]. But in the study carried out by Alipour et al, treatment with exogenous IL-1β in wild-type mice resulted in exacerbated disease by *C. rodentium*, emphasizing that the balanced IL-1β in the intestine might be the key to gut homeostasis [30]. Another investigation found IL-1β ignited inflammation by promoting the accumulation of innate lymphoid cells in the Helicobacter hepaticus elicited chronic intestinal inflammation [33]. In the genetically predisposed spontaneous colitis model in *Il10*$^{-/-}$ and *Tlr5*$^{-/-}$ mice, IL-1β has also been shown to play a detrimental role [34, 35]. A notable fact is that *Il1β*$^{-/-}$ mice housed under normal condition do not develop spontaneous intestinal inflammation. Taken together, the rather ambiguous data concerning IL-1β's role in the IBD pathogenesis demands further investigation with careful consideration of, first, whether the microbiota (or the specific pathogen used in infection model) presented in the studied subject's intestine has strong or weak ability to trigger IL-1β release and, second, the timing to measure IL-1β's role in IBD, i.e., the initiation stage of inflammation vs after inflammation onset or vs the inflammation resolution stage.

Despite the abovementioned multiple studies focusing on the role of IL-1β in varied intestinal disorders, little information has been revealed about its physiological role under homeostasis. Until recently, an elegant study for the first time revealed the beneficial role of IL-1β under homeostasis, in which IL-1β was shown to be secreted by lamina propria mononuclear phagocytes (LPMP) to signal through IL-1R-myd88 axis on the local type 3 innate lymphoid cells (ILC3), which in return release an important cytokine GM-CSF. GM-CSF helps LPMPs to maintain their ability to secrete regulatory retinoic acid and IL-10 to promote the proliferation of local Tregs, which is crucial to sustain intestinal tolerance to food/commensal-derived microbial antigens [36]. Of great importance, the secretion of IL-1β is dependent on microbiota-associated cues [36]. Another study indicated that IL-1β can directly boost the intestinal epithelium secretion of certain antimicrobial peptide to restrict commensal translocation and to help maintain gut homeostasis [37], which was at least partially supported by an earlier study using ex vivo system, which showed that the intestinal ILCs (Lin(−)c-Kit(+)Sca-1(−) cells) can readily respond to IL-1β stimulation and secrete IL-22 and several antimicrobial peptides [38]. IL-1β has also been shown as critical effector for intestinal eosinophils to supervise local IgA production, thus contribute to gut microbial symbiosis [39]. So accumulating evidences support a beneficial role of IL-1β in gut mucosal homeostasis under resting conditions, which suggests that it is necessary to reexamine any treatment of IBD targeting IL-1β in a clinic to avoid over-medication.

6.2.2 IL-18

The initial identification of IL-18 has pointed out its structural similarity to IL-1β, although functionally it was found to induce interferon (IFN)-γ and promote Th1 response [20, 21]. IL-18's role in inflammatory bowel disease was firstly reported in 1999, by examination of its expression in Crohn's disease (CD) and ulcerative colitis (UC) patients in comparison with control people. These studies indicated that IL-18 was upregulated in IBD patients (more profoundly in CD), and the major contribution of activated IL-18 was from the epithelium, while lamina propria mononuclear cells had limited contribution [40, 41]. Similar to the case of IL-1β, however, these studies failed to tell whether the IL-18 increase in patients was a consequence or causing factor for IBD. Indeed, in a separate study, researchers claimed that the increase of IL-18 in IBD patients was rather heterogenous; only a minor part of investigated CD patients carried accountable elevation for IL-18 [42]. In later researches, polymorphisms in IL-18 genomic locus were claimed for association with increased risk for IBD [43–45], while other studies that either failed to obtain a positive conclusion [46, 47] or identified the IL-18 associated increased risk for IBD were actually dependent on NOD2 polymorphism, which is one of the most prominent genetic predisposing factors for IBD [48].

Earlier researches using neutralization strategies, in the chemical-induced animal model colitis, had shown that IL-18 was proinflammatory, as blockade of IL-18 could significantly improve disease status [49–52]. In addition, another study using a transgenic IL-18 mouse line supported the detrimental role of IL-18 in DSS colitis [53]. Also in the *Il18*$^{-/-}$ mice, TNBS was shown to fail in inducing colitis [52]. In the T cell transfer colitis, administration of antisense RNA against IL-18 mRNA reduced colitis severity, again supporting the notion that IL-18 mediated CD-like colitis pathogenesis [54]. The prevalent blame on IL-18 in fueling IBD in the earlier studies encountered few challenges except a study using *Il18*$^{-/-}$ and *Il18r*$^{-/-}$ mice demonstrated that actually IL-18 could be beneficial in DSS colitis [55]. The lopsided situation started to change only in recent years when studies were carried out to explore the role of inflammasomes in IBD. A series of studies investigating varied inflammasomes in experimental IBD pointed out that IL-18 functioned downstream of inflammasome to provide protection against colitis and/or colitis-associated cancer [37, 56–67]. Based on these discrepancies, Britta Siegmund proposed that the IL-18 activation in the epithelium might be helpful, but activation in lamina mononuclear cells might be pathogenic for IBD [68]. But this hypothesis was challenged by a recent research with careful reexamination of IL-18 signaling in contribution to intestinal inflammation. This study found that in the epithelium, IL-18 was actually playing detrimental roles by deformation of goblet cells (which is responsible for intact mucus barrier) during DSS colitis induction [69]. The debate that whether IL-18 is a friend or foe in IBD seems unsettled yet, but notable clues have been revealed, including that physiological stress can significantly affect experimental colitis severity in wild-type mice through stress-induced IL-18 [70] and that IL-18 deficiency can lead to outgrowth of colitogenic bacteria and dysbiosis, which

could predispose the host to DSS-induced colitis [58, 62]. These intriguing clues enlightened that IL-18's role in pathogenesis of IBD can be an uncertain result of its delicate interaction with local microbiota, neural/hormonal cues, and/or different subsets of cells. Dissecting these relevant pathways may help our understanding, as well as clinical diagnosis of IBD.

Akin to IL-1β, despite the intensive attention being paid to IL-18 in the diseased condition, little effort has been exerted to decipher its physiological role under normal conditions. Except aforementioned studies revealing that IL-18 may help maintain gut microbial symbiosis through supervising local antimicrobial peptide generation [37, 58, 62], currently there is no clear information related to the contribution of IL-18 to gut homeostasis under resting condition, which is in need for future investigation.

6.2.3 Pyroptosis

By studying the *Salmonella typhimurium*-caused caspase-1-dependent cell death, pyroptosis was defined as a new form of programmed inflammatory cell rupture. Since the *S. typhimurium* is a known enteric pathogen, the pyroptosis has been speculated as a countermeasure utilized by infected enterocytes to expose pathogens to be killed by effector cells such as neutrophils [71, 72]. In a spontaneous colitis model caused by TLR2-MDR1A double deficiency, clear activation of inflammasome by commensal bacteria led to myeloid $CD11b^+$ cells to undergo pyroptosis, and similar situation was observed in genetically relevant IBD patients, suggesting that pyroptosis may play a pathogenic role in IBD [73]. Another study using systemic in vivo delivery of flagellin caused NLRC4 inflammasome-dependent but IL-1β- and IL-18-independent rapid pyroptosis, which contributed to the systemic inflammation-induced lethality [74]. Future study is required to clarify the role of pyroptosis in gut homeostasis and IBD.

6.3 Different Inflammasomes in Intestinal Homeostasis and IBD

6.3.1 NLRP1 Inflammasome

NLRP1 inflammasome is the first identified inflammasome ever [75]; it can respond to MDP challenge in human [76] and *Bacillus anthracis* lethal toxin/*Toxoplasma gondii* challenge in mouse [77–80]. However, the role of NLRP1 inflammasome in intestine health and disease has scarcely been reported. The first implication that it plays a role in intestinal homeostasis came from a report showing that a polymorphism in NLRP1 gene is associated with celiac disease, a common intestinal disorder [81], and also another clinical study showing that the NLRP1 Leu155His

polymorphism was more frequent in IBD patients who were unresponsive to glucocorticoid treatment [82]. While in the mouse model study, the only elaborated study carried out with *Nlrp1b*$^{-/-}$ mice showed that NLRP1 was protective in DSS-induced colitis and colitis-associated colon cancer through upregulation of IL-1β and IL-18, with the former providing the majority function [83]. The authors also claimed that NLRP1 mainly functioned in the non-hematopoietic cells to provide protection against experimental IBD [83]. More interestingly, the researchers found that the aggravated colitis was transmissible to WT cagemates through cohousing, which indicated that an outgrowth of colitogenic microbiota was induced in the absence of NLRP1 [83]. Thus, the interaction between NLRP1 and local microbiota (especially certain specific NLRP1 inflammasome-activating bacteria species) and the possible overlapping/complementary relationship between NLRP1 and other NLRs in maintaining gut homeostasis could be of great value that awaits future investigation.

6.3.2 NLRP3 Inflammasome

NLRP3 inflammasome is the most intensively studied inflammasome since its identification to date [84]. The incredibly broad range of stimuli that activate the NLRP3 inflammasome made its exact activation mechanism still foggy; its role in the intestinal homeostasis and diseases is unclear either. First, in the clinical studies for NLRP3 polymorphisms in IBD showed discrepant results, with one group demonstrating that SNPs in the 5-prime region of NLRP3 gene leading to impaired NLRP3 expression and inflammasome activity were associated with CD susceptibility [85], while the other group with a large-scale population-based analysis failed to identify such correlation between NLRP3 polymorphisms and IBD prevalence [86]. The contradictory results in these two studies may be explained by the different genetic background of the subjects they approached, as other two studies revealed that variants in NLRP3 in combination with CARD8 polymorphisms were associated with higher risk to CD [87, 88].

In the animal model study, initially, there were two reports showing that NLRP3 played a protective role in the DSS-induced colitis, as *Nlrp3*$^{-/-}$ mice were more susceptible to DSS challenge [56, 65]. However, these two reports also contradicted with each other upon that in which cell compartment NLRP3 was functioning. By using the same bone marrow transfer strategy, one claimed a profound role of NLRP3 in the epithelium [65], while the other emphasized a function for NLRP3 mainly in the hematopoietic cells [56]. On the contrary, another report showed an even opposite role for NLRP3 in DSS colitis, stating that NLRP3 was pathogenic, as abolishment of NLRP3 protected *Nlrp3*$^{-/-}$ mice from DSS challenge [89]. However, in a successive study, these authors found that the protective effect in *Nlrp3*$^{-/-}$ mice can be blunted by cohousing with WT mice, suggesting that the claimed detrimental effects of NLRP3 in reference [89] are microbiota dependent [90]. Indeed, another study arguing the beneficial role of NLRP3 in both TNBS- and DSS-induced colitis models demonstrated the *Nlrp3*$^{-/-}$ mice contained altered microbiota as compared

with WT mice, indicating that loss of NLRP3 might lead to dysbiosis, hence increased susceptibility to experimental IBD [91]. Although it was claimed that NLRP3 plays a detrimental role in DSS colitis without causing dysbiosis in a relevant study mainly focusing on NLRP6 inflammasome [58], in a recent study by a careful screening of NLRP3 inflammasome-activating mouse fecal bacteria, researchers identified that a pathobiont *Proteus mirabilis* can specifically activate NLRP3 inflammasome in the lamina propria mononuclear phagocytes, which is responsible for aggravating inflammation after epithelium breakage mediated by DSS exposure [29]. This study thus gives a strong example of NLRP3-microbiota interaction in regulating intestinal homeostasis and disease outcome. In another common enteric mucosal infection model, i.e., the *Citrobacter rodentium* infection of mice intestine, it was also shown that NLRP3 has an important role in the epithelium, but with unexpected data showing that *Nlrp3*$^{-/-}$ mice had intact intestinal caspase-1 activity [92], which indicated that some hidden facts fill the gap of our current understanding in terms of NLRP3's anti-infection function in the gut. Under resting conditions, in addition to the aforementioned report showing that NLRP3 plays a role in maintaining gut microbial symbiosis [91], a recent report showed that through short-chain fatty acid (SCFA)-GPR43/GPR109A signaling axis on the epithelial cells, potassium efflux was triggered and NLRP3 inflammasome was activated, which promoted the integrity of the intestinal epithelium [93].

In summary, the role of NLRP3 in regulating gut homeostasis and diseases has been increasingly appreciated; however, several key matters still require future studies to be addressed, which include where (epithelium vs lamina propria hematopoietic cells) and how NLRP3 regulates microbiota under homeostasis and diseases (injury vs infection) and how these interactions determine the homeostatic status/final disease outcome.

6.3.3 NLRC4 Inflammasome

NLRC4 was initially characterized with caspase-1-activating ability upon *Salmonella typhimurium* challenge. It forms inflammasome upon Naip-dependent detection of either cytosolic flagellin or type 3 secretion system (T3SS) component proteins [2]. Its role in intestinal homeostasis was implicated by the identification of mutation in NLRC4 causing infantile enterocolitis and autoinflammation syndrome [94]. In mouse models, *Nlrc4*$^{-/-}$ mice showed increased susceptibility to colitis-instigated colon tumorigenesis (AOM + DSS-induced colon cancer (CAC) model) [95, 96]. However, these two reports showed discrepant results in explaining NLRC4's role in the acute DSS colitis model, with Hu et al. arguing a dispensable role while Carvalho et al. pointed out a protective role for NLRC4 in this model [95, 96]. Contradictory results have also been shown, as another study showed *Nlrc4*$^{-/-}$ mice had similar susceptibility to CAC as WT mice [56]. In supporting for NLRC4's protective role, another study using *Naip1–6*-deficient mice showed increased colitis-associated colon tumor formation, but unexpectedly, in the acute colitis

model, *Naip1–6* seem to be detrimental, which was speculated to be an NLRC4-independent event [97]. In accordance to its primary function in dictating *Salmonella typhimurium* infection, local or systemic administration of either flagellated bacteria or flagellin alone leads to NLRC4-dependent intestinal inflammation, which would either help expel the pathogen or lead to a severe acute inflammation-induced rapid death of mice [74, 96]. Recently, the epithelium-intrinsic NLRC4 inflammasome activity has been proven essential and sufficient for the expulsion of *S. typhimurium*-infected (or cytosolic flagellin-alerted) intestinal epithelial cells, which highlighted the critical role of NLRC4 in the surveillance of flagellated gut microbes [98, 99]. Thus, despite the in-depth understanding of NLRC4 in restricting specific pathogens such as *S. typhimurium*, its role in the intestinal homeostasis and IBD is quite preliminary and controversial. The reasons behind these contradictory conclusions from different laboratories can vary, but surely the gut microbiota needs to be taken into consideration for future studies.

6.3.4 NLRP6 Inflammasome

Unlike other inflammasomes, the molecular characterization of NLRP6 inflammasome is currently lacking. Nonetheless, in an overexpression system, NLRP6 was shown to interact with ASC to promote caspase-1 activation in vitro [100], but the upstream ligand or signals responsible for NLRP6 activation are not clear, as in the primary *Nlrp6*$^{-/-}$ macrophages, inflammasome activation in response to *Salmonella typhimurium*, *Listeria monocytogenes*, or LPS + ATP was unaffected [101]. However, the role of NLRP6 in the intestinal homeostasis has been well studied. The expression of NLRP6 is constantly high in the intestinal epithelium, and deficiency of this gene leads to dysbiosis, as well as aberrant goblet cell-derived mucus layer [58, 102]. In the intestinal monocyte, a beneficial role of NLRP6 was also revealed during DSS colitis induction, which is mediated by IL-18 in an autocrine manner [64]. NLRP6's role in goblet cell function was supported by a delicate study showing that NLRP6 inflammasome was found critical in licensing sentinel goblet cell activation upon bacterial penetration of the epithelium [103]. In a successive study, the NLRP6 inflammasome was shown to promote gut homeostasis through a positive feedback loop, wherein NLRP6 regulates local antimicrobial peptides production via IL-18, thus supervising microbiota to produce more metabolites such as taurine to support NLRP6 inflammasome activation [62]. Another study indicated that NLRP6 has a pivotal role in detecting intestinal virus through DHX15-MAVS-induced type 1 interferon induction [104]. Thus, NLRP6 has a multifaceted role in regulating intestinal homeostasis and gut diseases.

6.3.5 AIM2 Inflammasome

The AIM2 inflammasome was initially identified in detecting abnormal translocation of cytosolic DNA, mainly from invading pathogens or damaged host cells [2]. The intestine is a place where continuous opportunistic bacterial/viral invasion or physical/chemical damages occur, all of which may lead to aberrant DNA presence in the cellular space, suggesting a potential involvement for AIM2 inflammasome in the gut. Indeed, recent reports using AIM2-deficient mice have revealed such important function of AIM2 in the intestine in varied experimental and clinical disease contexts. In clinical analysis of mutations in small bowel carcinogenesis patients, a high prevalence of AIM2 frame shift was detected [105], and the absence of AIM2 expression in colorectal cancer patients was associated with poor prognosis [106], indicating its importance in tumor control. In addition, an increase of PYHIN-200 family members AIM2 and IFI16 was detected in the mucosa of IBD patients [107]. In the animal study, AIM2-deficient mice showed increased susceptibility to both DSS colitis and colitis-associated colon cancer [37, 63, 108]. But in the tumor patients as well as animal models receiving radiation/chemotherapy, AIM2 in the intestine detects the resultant DNA damage and induces inflammasome-dependent cell death and proinflammatory responses, which leads to severe damages to the host. Thus, targeting AIM2 in such circumstances represents a promising countermeasure to relieve suffering of patients [109, 110].

6.3.6 Noncanonical Inflammasomes: Caspase-11 and Pyrin Inflammasome

Recently, the caspase-11 mediated noncanonical inflammasome, and the pyrin inflammasome has been characterized [3–7]. The role of pyrin in the intestinal homeostasis and disease is still an open question. Unlike pyrin, the noncanonical inflammasome-forming caspase-11 has been studied in the context of intestinal inflammatory processes. Three studies all showed that caspase-11 protects against DSS-induced colitis and epithelial and hematopoietic caspase-11 is required [111–113]. Mechanistically, two reports emphasized IL-18 and IL-1β in mediating the protective effects [111, 112], while the third study denied cytokines' major effects, since they observed normal or even increased IL-18/IL-1β production in caspase-11-deficient mice after DSS challenge. Instead, they attributed the protective effects to pyroptosis downstream of caspase-11 inflammasome [113].

6.4 Concluding Remarks

Our current understanding of inflammasomes' role in intestinal mucosal homeostasis and disease has been summarized in Fig. 6.1, which is complex and severely unequilibrated. On the one hand, due to the relatively long history of identifying different inflammasome components and effectors, the effector molecules characterized earlier such as IL-1β and IL-18 have been studied intensively and controversially, while the latter identified inflammasome orchestrator molecules such as NLRs and AIM2 are still to be investigated, with pyrin even left unstudied. On the other hand, these insights were largely obtained from studies under diseased conditions, while the physiological working model of inflammasome under resting

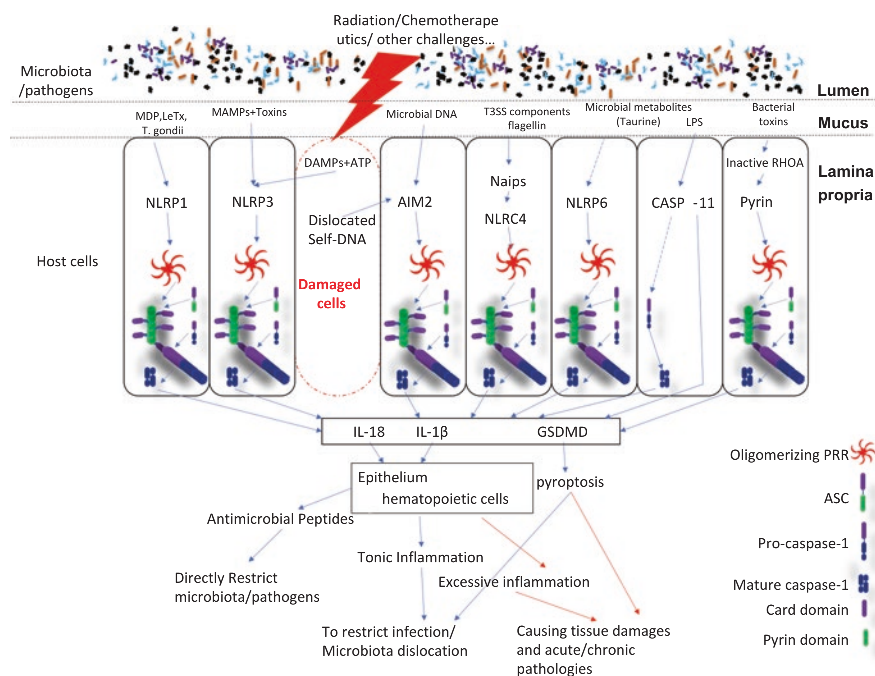

Fig. 6.1 The complex network of different inflammasomes in regulating gut mucosal homeostasis and diseases

The intestinal lumen contains a diverse repertoire of commensal/pathogenic microbes, which often opportunistically impedes the intestinal barrier (mucus layer); the intestine as well will experience injuries caused by ingestion of harmful matters or radio-/chemotherapies, which leads to tissue damages. The varied inflammasome-forming pattern recognition receptors (PRR) in either epithelial or lamina propria cells detect these microbial or danger signals to initiate inflammasome formation, hence activating the downstream effector cascade. The three major effector molecules, IL-1β, IL-18, and GSDMD, would either target the downstream epithelial/hematopoietic cells to regulate inflammatory and antimicrobial responses or form large pores on the cell membrane to mediate pyroptosis, which would both help the expulsion of infected pathogens and boost local inflammation

conditions is underinvestigated, probably due to the lack of practical models or inefficient examination tools. For better comprehension of current knowledge and explanation of the contradictories, and for further broadening of our knowledge concerning the physiological role of inflammasomes in intestinal homeostasis, future studies should include more newly developed methods to monitor inflammasome activity. Moreover, full consideration of multiplayers in the gut disease process, including host immune cells, barriers, and intestinal contents (food and microbiota), as well as neural/humoral cues locally and systematically, is also necessary.

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Chapter 7
Microbial Factors in Inflammatory Diseases and Cancers

Hong Sheng Ong and Howard Chi Ho Yim

Abstract The intestinal microbes form a symbiotic relationship with their human host to harvest energy for themselves and their host and to shape the immune system of their host. However, alteration of this relationship, which is named as a dysbiosis, has been associated with the development of different inflammatory diseases and cancers. It is found that metabolites, cellular components, and virulence factors derived from the gut microbiota interact with the host locally or systemically to modulate the dysbiosis and the development of these diseases. In this book chapter, we discuss the role of these microbial factors in regulating the host signaling pathways, the composition and load of the gut microbiota, the co-metabolism of the host and the microbiota, the host immune system, and physiology. In particular, we highlight how each microbial factor can contribute in the manifestation of many diseases such as cancers, Inflammatory Bowel Diseases, obesity, type-2 diabetes, non-alcoholic fatty liver diseases, nonalcoholic steatohepatitis, and cardiovascular diseases.

Keywords Microbiota • Inflammation • Inflammasome • TLR • NLR • Microbial metabolites • Inflammatory diseases • Cancers • Dysbiosis • GPCR • Inflammatory Bowel Diseases (IBD) • Obesity • Type 2 diabetes • Non-alcoholic fatty liver diseases (NAFLD) • Non-alcoholic steatohepatitis (NASH) • Atherosclerosis

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7.1 Introduction

Millions of microbes inhabit the surfaces of the human body. Most of them reside in the gastrointestinal tract and exist in a microbial community named as microbiota. Amazingly, they do not cause any harmful effect to the host. Rather, these microbes foster a symbiotic relationship with their host. While the host provides them a favorable habitat, they participate in the host's digestive process by providing an extra range of enzymatic systems to harvest energy for themselves and the host. They also interact bidirectionally with the host immune system to shape their own ecology and the development of the host immune system, achieving a symbiosis to cope with environmental challenges. This symbiosis plays an important role in maintaining human health.

Many diseases such as cancers, Inflammatory Bowel Diseases (IBD), and metabolic diseases including obesity, type-2 diabetes, non-alcoholic fatty liver diseases (NAFLD), non-alcoholic steatohepatitis (NASH), and atherosclerosis have been linked to different degree of inflammation. Interestingly, this inflammation is associated with an alteration of the symbiotic relationship between the microbiota and its host. This altered relationship is called a dysbiosis. In this chapter, we focus on how metabolites (Table 7.1), cellular components (Table 7.2), and virulence factors (Table 7.3) of the microbiota interact with the host to influence the dysbiosis and involve in the etiology of these inflammatory diseases and cancers.

7.2 Microbial Metabolites

7.2.1 Short-Chain Fatty Acids (SCFAs)

The human intestine does not have enzymes that are capable of degrading most complex carbohydrates and plant polysaccharides [1]. Thus, humans rely heavily on the microbiota present in the colon to ferment undigested carbohydrates including cellulose, xylans, and resistant starch into short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate [1]. The SCFAs undergo passive diffusion into the host, acting as an important source of energy for the host [1, 2]. For example, butyrate acts as an energy substrate for colonic epithelial cells, while acetate and propionate provide energy to the peripheral tissues [3].

Apart from its role of supplying energy, SCFAs can regulate the host physiology by acting as signaling molecules that bind G-protein-coupled receptors (GPCRs) in cells [2]. For example, SCFAs induce secretion of glucagon-like peptide 1 in the colon via GPR43 activation, leading to the enhancement of insulin sensitivity [4]. SCFAs activate GPR41 to induce the intestinal expression of Pyy which inhibits gut motility, raises intestinal transit rate, and reduces the harvest of energy from the gut [5]. In the adipocytes, SCFAs, via GPR43, inhibit insulin signaling, leading to the reduction of fat accumulation in adipose tissue and the increase of metabolism of

Table 7.1 Involvements of microbial metabolites in inflammatory diseases and cancers

Microbial metabolites	Signaling receptor involved	Associated malignancy/physiology
SCFAs	GPR45	↓ Experimental colitis
	GPR43, GPR109A	↓ Experimental colitis
	GPR43	↑ Gout-associated inflammation
		↑ Insulin sensitivity
		↓ Fat accumulation in adipose tissue
		↑ Lipid and glucose metabolism in non-adipose tissues
	GPR41	↓ Gut motility
		↑ Intestinal transit rate
		↓ Energy harvest from gut
		↑ Intestinal gluconeogenesis
		↓ Allergic airway inflammation
	GPR109A	↓ Colitis-associated colon cancer
		↑ Colon cancer in a genetic model
AHR ligands	AHR	↑ Epithelial integrity
		↓ Experimental IBD
H_2S		↑ IBD
		↑ Colorectal cancer
Primary bile acids	FXR	↓ NAFLD
Secondary bile acids	TGR5	↑ Insulin sensitivity and glucose tolerance
		↓ Obesity
		↓ Hepatic steatosis
		↓ Intraplaque and adipose tissue inflammation
		↓ Vascular lesion formation
		↓ Atherosclerosis
		↑ Obesity-associated hepatocellular carcinoma
Taurine	NLRP6	↓ Experimental colitis
TMAO		↑ Atherosclerosis
Spermine	NLRP6	↑ Experimental colitis
N^1,N^{12}-diacetylspermine		↑ Colorectal cancer

lipids and glucose in other tissues [6]. Moreover, butyrate and propionate activate intestinal gluconeogenesis via cyclic AMP (cAMP)-dependent mechanism and via a GPR41-dependent gut-brain circuit, respectively [7].

An optimal level of SCFAs is required for maintaining a balance between energy harvest and expenditure. However, obesity is closely linked to a higher level of SCFAs. In genetically obese mice, the level of acetate and butyrate is higher in their cecum as compared to their lean littermates [8]. This is attributed to the presence of microbiota in obese mice that is able to degrade dietary polysaccharides into SCFAs [8]. Similarly, overweight human subjects also have higher concentration of SCFA

Table 7.2 Involvements of microbial cellular components in inflammatory diseases and cancers

Microbial components	Signaling receptor	Associated malignancy/physiology
LPS	TLR4	↓ Experimental colitis
		↑ Epithelial integrity
		↑ Obesity
		↑ Type 2 diabetes
		↑ NAFLD and NASH
		↑ Pancreas tumorigenesis
		↑ Colon tumorigenesis
		↑ Liver tumorigenesis
Flagellin	TLR5	↓ IBD
		↑ Insulin sensitivity
		↓ Hyperlipidemia
		↓ Hypertension
		↓ Adiposity
		↑ Tumor progression in a mouse model of sarcoma
		↓ Tumor progression in a mouse model bearing the breast carcinoma cell line
PSA	TLR2	↓ Experimental colitis
		↓ A mouse model of multiple sclerosis
Bacterial DNA	TLR9	↓ Experimental colitis
		↑ Sensitivity to glucose and insulin
		↓ Obesity
		↑ NASH during inflammasome malfunction
MDP	NOD2	↓ Experimental colitis
		↑ Insulin sensitivity
		↓ Colon cancer tumorigenesis

Table 7.3 Involvements of microbial virulence factors in inflammatory diseases and cancers

Microbial virulence factors	Signaling receptor	Associated malignancy
FadA adhesin	E-cadherin	↑ Colon tumorigenesis
PKS islands		↑ Colon tumorigenesis
BFT		↑ Colon tumorigenesis

than the lean subjects [9]. Consistent with the observations in mice, the obese human individuals have enriched microbiota that is able to process carbohydrate for energy [10] and have a higher level of ethanol in their breath [11]. As SCFAs can act as energy source for the host, these findings suggest that the energy harvest in obese people is elevated by the enrichment of SCFA-producing microbiota, which may eventually contribute to the etiology of obesity.

SCFAs act as a double-edged sword in inflammatory diseases and cancers. For example, SCFAs, via GPR45, inhibit histone deacetylase in regulatory T (Treg) cells thereby increasing the frequency and function of these cells, thus alleviating

the disease severity of experimental colitis [12, 13]. Furthermore, SCFAs, via GPR43 and GPR109A, induce membrane hyperpolarization and Ca^{2+} ion mobilization in colonic epithelial cell, to activate the NOD-like receptor protein 3 (NLRP3) inflammasome [14]. This leads to the activation of caspase-1 and interleukin (IL)-18, which maintain the integrity of colon epithelium and thus the protection of mice from experimental colitis [14]. As protein kinase R (PKR) has recently been shown to inhibit NLRP3 inflammasome activity and has suggested to regulate the colitis development [15, 16], it would be interesting to investigate if SCFAs activate NLRP3 inflammasome by suppressing PKR activity as well. SCFAs which help reduce the severity of experimental colitis are produced by 17 strains of *Clostridia* in cluster XIVa, IV, and XVIII [17, 18]. Consistently, these bacteria and SCFA level also reduce the disease severity in IBD patients [19–22]. While not limited to the gut, the anti-inflammatory effect of SCFAs is also observed in the airway. SCFAs, via GPR41, suppress allergic airway inflammation by augmenting the generation of dendritic cell precursors in the lung and subsequently decreasing T helper type 2 (Th2) cell function [23]. On the other hand, SCFAs can also exacerbate inflammation. For example, acetate potentiates the monosodium urate crystal-activation of inflammasome possibly via GPR43, leading to the induction of IL-1β and CXCL1 expression and the subsequent exacerbation of gout-associated inflammation [24].

SCFAs activate GPR109A to induce the differentiation of Treg cells and IL-10-producing T cells by triggering anti-inflammatory responses in macrophage and dendritic cells, leading to the protection against colitis-associated colon cancer [25]. In contrast, the butyrate derived from an altered gut microbiota promotes tumorigenesis in a genetic mouse model of colon cancer by inducing colon epithelial cells hyperproliferation [26]. Hence, the role of SCFAs varies among different types of colon cancer.

7.2.2 Aryl Hydrocarbon Receptor Ligands

Tryptophan is an essential amino acid that is found in red meat, fish, eggs, and cruciferous vegetables [27, 28]. Gut microbes such as *Lactobacillus* can catabolize tryptophan into indole-3-aldehyde which is a ligand for the aryl hydrocarbon receptor (AHR) [29]. Upon binding of its ligand, AHR is transported into the nucleus and binds to the AHR nuclear translocator, leading to the transcription of genes including IL-22 in Th17 cells and group 3 innate lymphoid cells [27]. IL-22, in turn, induces the expression of antimicrobial peptides and production of mucus, thereby maintaining the epithelial integrity of the intestine [27]. Interestingly, absence of AHR or AHR ligands in the diet increases the disease severity in a murine IBD model [30]. This suggests that AHR ligands may play a role in preventing IBDs via maintenance of the epithelial integrity.

7.2.3 Hydrogen Sulfide

Food, such as dried fruits, nuts, fermented beverages, and brassica vegetables, and the host colonic mucus layer provide rich sources of inorganic and organic sulfate for the sulfate-reducing bacteria to extract energy [31, 32]. This energy extraction involves oxidation of organic compounds or hydrogen and reduction of sulfate into hydrogen sulfide (H_2S) in the colon [32]. Of note, these sulfate-reducing bacteria in the human colon mainly belong to the genus of *Desulfovibrio* in the class of *Deltaproteobacteria* [33].

Although previous studies have shown contradictory results on the amount of sulfate-reducing bacteria present in the mucosal layer and feces of IBD patients [34–40], the level of H_2S has been shown to be consistently higher in IBD patients as compared to the healthy individuals [34, 41]. There are two hypotheses on how H_2S induces inflammation. It has first been proposed that H_2S causes inflammation in IBDs by inducing injury in colon epithelium via its genotoxic effect and by suppressing the effect of SCFA [42–44]. Recent studies, however, have held a different view that the elevated level of H_2S in IBD patients may degrade the outer and inner mucus layer of the colon of the patients, leading to the invasion of gut microbes into the epithelium and the subsequent colonic inflammation [32, 45].

H_2S has also been associated with colorectal cancer. This is supported by the fact that the level of fecal H_2S is higher in patients who had surgical removal of sigmoid colon cancer and later developed neoplasia as compared to the healthy individuals [46]. In addition, H_2S activates mitogen-activated protein kinase and induces DNA damage [47, 48] which together induce colonic mucosal hyperproliferation [43, 49], thereby leading to colorectal cancer.

7.2.4 Bile Acids

There are two classifications of bile acids: primary and secondary. Primary bile acids include chenodeoxycholic acids and cholic acids in human and cholic acids and muricholic acids in mice and rats [50]. They are synthesized from cholesterol in liver and then conjugated with glycine or taurine in human, or only with taurine in rodents [50]**.** They are subsequently released into the duodenum to perform its function of solubilizing cholesterol, dietary fats, and fat-soluble vitamins for absorption [1, 50]. Most of these conjugated bile acids are reabsorbed in the ileum and transported back to the liver via the hepatic portal vein, thereby preserving 95% of the bile acid pool [50]. However, a portion of these preserved bile acids are deconjugated by bacteria including *Lactobacillus*, *Bifidobacteria*, *Clostridium*, and *Bacteroides* [50]. To perform such deconjugation, these bacteria utilize the bile salt hydrolase [50]. Deconjugated bile acids are not absorbed by the small intestine but are metabolized into secondary bile acids via 7α/β-dihydroxylation in the colon by bacterial members in genus of *Clostridium* including *C. scindens*, *C. hiranonis*, *C.*

hylemonae, and *C. sordellii* [27]. These secondary bile acids include lithocholic acid deconjugated from chenodeoxycholic acid and deoxycholic acid deconjugated from cholic acid in human, and murideoxycholic acid deconjugated from muricholic acids in mice [50].

Bile acids can act as ligands which bind to cell surface receptors responsible for regulating different metabolic processes. For example, primary bile acids mainly bind to the farnesoid X receptor (FXR) which is highly expressed in the liver, ileum, and kidney [51, 52]. In healthy individuals, FXR controls the synthesis of bile acids via a negative feedback mechanism [50]. However, the dysregulation of primary bile acid activation may result in metabolic diseases. For example, FXR-deficient mice fed on chow diet develop hyperglycemia and hypercholesterolemia [53, 54]. Similarly, mice with double deficiency of FXR and Apolipoprotein E which were fed on a high-fat and high-cholesterol diet, gained weight and increased the severity of atherosclerosis with elevated expression level of inflammatory genes [55]. In contrast, mice with double deficiency of FXR and low-density lipoprotein receptor on a high-fat diet have improved lipid profile and protect against diet-induced development of obesity and atherosclerosis [56]. Similarly, FXR-deficient mice which are fed on a high-fat diet or have genetically obese background are protected against obesity and have improved glucose homeostasis [57–59]. Hence, the role of FXR in obesity and obesity-associated diseases such as atherosclerosis and type-2 diabetes remains controversial. This controversy may have resulted from the different microbiota present across different animal facilities. Further studies are required to identify the specific microbiota which fine-tune the effect of FXR on these diseases. Moreover, patients with NAFLD have reduced expression of hepatic FXR which is associated with the elevated level of liver X receptor, sterol regulatory element-binding protein 1C (SREBP-1C), and enhanced hepatic triglyceride synthesis [60]. This elevation of SREBP-1C is associated with the severity of hepatic steatosis in the NAFLD patients [60]. Therefore, these findings suggest that the primary bile acids play a role in regulating the development of metabolic diseases. However, the role of gut microbiota in these diseases requires further investigation.

Secondary bile acids bind to TGR5 (GPR113). Upon activation, TGR5 induces the release of GLP-1 in enteroendocrine L cells by promoting mitochondrial oxidative phosphorylation and Ca^{2+} influx [61]. This improves the insulin sensitivity and glucose tolerance in obese mice [61]. The TGR5 activation also enhances the energy expenditure in brown adipose tissue, preventing obesity, insulin resistance, and hepatic steatosis [61, 62]. This is partly mediated by the cAMP-dependent thyroid hormone-activating enzyme type 2 iodothyronine deiodinase [62]. In addition to its effect on metabolism, TGR5 also affects the immune system. TGR5 activation by synthetic bile acid inhibits NFκB-dependent expression of cytokines including IL-1β, TNF-α, and IL-6 via cAMP signaling in macrophages [63]. This reduces intraplaque inflammation and vascular lesion formation, thereby preventing the development of atherosclerosis [63]. This macrophage-specific TGR5 activation also inhibits the adipose tissue inflammation by suppressing chemokine expression and macrophage infiltration, leading to the protection against insulin resistance [64]. This suppression is mediated by AKT-mTOR-dependent induction of

differential translation of the dominant-negative C/EBPβ isoform [64]. Hence, the binding of TGR5 with secondary bile acids can prevent metabolic diseases including obesity, atherosclerosis, hepatic steatosis, and type-2 diabetes.

Taurine, derived from the deconjugation of primary bile acid, has been shown to activate the NLRP6 inflammasome to induce IL-18 and antimicrobial peptides, providing protection against colitis [65]. However, the clinical relevance of this finding requires further examination.

Development of cancer can be associated with either the dysregulated bile acid secretion or certain types of bile acid. Colorectal cancer patients have increased level of bile secretion and fecal bile acid concentration [66, 67]. Furthermore, the fecal concentration of secondary bile acids in African Americans with a high risk of colon cancer is higher than that in rural native Africans with a low risk of the disease [68]. On the other hand, the deoxycholic acid has suggested to provoke the senescence-associated secretory phenotype in hepatic stellate cells which in turn induces various inflammatory and tumor-promoting factors such as IL-1β in the liver, thus promoting the obesity-associated hepatocellular carcinoma development in mice [69].

*7.2.5 Trimethylamine-*N*-Oxide (TMAO)*

In our diet, trimethylamine-*N*-oxide (TMAO) is directly obtained from fish, or indirectly from meat and high-fat diets that are typically rich in choline and L-carnitine [70]. TMAO can be directly absorbed and transported to the blood for normal function of the body, while in the precursor form, choline and L-carnitine have to first be converted into trimethylamine (TMA) by the gut microbiota. Once absorbed, TMA is then converted into TMAO in the liver by the host hepatic enzyme flavin-containing monooxygenase 3 [70]. The normal function of TMAO includes counteracting the destabilizing effect of urea on proteins and nucleic acids in kidney, and acting as a chaperone that promote protein folding [70].

Diets play a role in shaping the composition of the gut microbiota which in turn determines the plasma level of TMAO. For example, the plasma level of TMAO increases in human subjects who ingest phosphatidylcholine [71]. This elevation is inhibited by antibiotic treatment but reappears after antibiotic withdrawal [71]. Similarly, people who are omnivores have a stronger ability to convert L-carnitine into plasma TMAO than people who are vegans or vegetarians [72]. Such difference in L-carnitine metabolism depends on the differences in specific bacterial taxa in the human gut between these two groups of people [72]. These findings suggest that the long-term dietary habit shapes the composition of microbiota to determine the metabolism of the ingested food.

The increased plasma level of TMAO has been associated with the elevated risk of adverse cardiovascular events including overall mortality, myocardial infarction, or stroke in human [71–73]. This induction of TMAO, which is suggested to be a

result of the altered gut microbiota [72], promotes macrophage cholesterol accumulation and foam cell formation [74]. This leads to the promotion of atherosclerosis [74]. On the other hand, the reduced level of available phosphatidylcholine leads to NAFLD in mice [75]. This is caused by the dysregulated enzymatic systems of the altered gut microbiota and the stronger ability of the host to produce excretory methylamine from phosphatidylcholine [75]. Hence, the levels of TMAO and its precursors play a role in regulating metabolic diseases including cardiovascular diseases and NAFLD.

7.2.6 Polyamine

Polyamines are polycationic molecules that are present in living cells to carry out different cellular functions including gene transcription, translation, cell growth, and cell death [76]. These polyamines including putrescine, spermidine, and spermine can be generated by the host cells or the gut microbes to maintain the integrity of the epithelial barrier [76]. However, the upregulation or downregulation of the polyamine level has been associated with diseases. For example, spermine level is increased during colitis caused by NLRP6 deficiency [65]. The elevated spermine inhibits the NLRP6 inflammasome induction of IL-18 and antimicrobial peptide, promoting the disease severity in an experimental colitis model [65]. Intriguingly, the levels of polyamine metabolites including N^1,N^{12}-diacetylspermine are higher in biofilm-positive colon cancer tissues [77]. When the colon cancer patients are treated with oral antibiotics, the isolated colon cancer tissues have no biofilm or culturable bacteria and a reduced level of N^1,N^{12}-diacetylspermine [77]. As biofilm has been associated with the enhanced crypt epithelial cell proliferation in colon mucosa [78], biofilm may act synergistically with the host cancer to generate polyamines that may promote colorectal cancer development [77].

7.3 Microbial Components

7.3.1 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a cell wall component of the Gram-negative bacteria. It is a ligand of Toll-like receptor 4 (TLR4) and is present in many gut bacteria. Commensal bacteria maintain an optimal TLR4 activation to ensure the gut epithelial integrity, leading to the protection against the intestinal injury-induced colitis [79]. This protection is mediated by MyD88, a downstream signaling molecule of TLR4 [79], which in turn induces the expression of cyclooxygenase 2 from intestinal epithelial cells and macrophage, leading to the enhanced mucosal production of prostaglandin E2 and the subsequent maintenance of gut epithelial survival [80–83].

However, when there is over-activation of TLR4 possibly by LPS from microbiota, the gut barrier is disrupted, and gut bacteria invade the mucosa, leading to the exacerbation of chemically induced colitis [84].

Apart from its role in IBD development, LPS also regulates metabolic inflammation. Mice fed on a high-fat diet have an increased plasma LPS level, elevated intestinal permeability and a higher proportion of LPS-containing gut microbiota, resulting in metabolic endotoxemia [85]. Subcutaneous infusion of LPS into mice leads to insulin resistance and obesity [85]. Consistently, antibiotic treatment decreases the metabolic endotoxemia and the cecal content of LPS in mice that are genetically obese and fed on a high-fat diet, resulting in the reduction of glucose intolerance, body weight gain, fat mass development, and inflammation [86]. Indeed, the metabolic endotoxemia is mediated by CD14, a co-receptor for LPS binding to TLR4 [86]. In support of these studies, patients with metabolic syndrome and type-2 diabetes have endotoxemia [87, 88]. Furthermore, NLRP3 and NLRP6 inflammasomes suppress the progression of metabolic diseases including NAFLD and NASH via IL-18-dependent changes of the gut microbiota [89]. These changes of gut microbiota correlate with the increased hepatic steatosis and inflammation via the influx of TLR4 and TLR9 ligands into the portal circulation, leading to enhanced hepatic TNF-α expression and the subsequent NASH progression [89]. In agreement with the data from mouse models, NAFLD patients have elevated levels of TNF-α, IFN-γ, and IL-6, disruption of microvilli structure in small intestine, and enhanced gut permeability [90–92]. Hence, it is possible that the endotoxemia, which is resulted from the increased gut permeability by the loss of inflammasome activity, is crucial for the development of these metabolic diseases.

LPS, possibly leaked from the gut microbiota, has been shown to promote the development of pancreatic and liver cancer via a TLR4-dependent signaling pathway [93, 94]. This role of LPS for tumorigenesis can also be observed in colitis-related colon cancer. TLR4-deficient mice are protected against the development of such cancer [95], while constitutive activation of TLR4 enhances it [95, 96]. Similarly, overexpression of TLR4 is observed in tumors of the IBD patients [95]. Whether the LPS promotion of colitis-related colon cancer development originated from the gut microbiota requires further studies. Moreover, the intestinal mucus secretion is impaired in tumor in a genetic mouse model of colorectal cancer, leading to the increased gut permeability and translocation of LPS of the gut microbiota into the portal circulation [97]. This in turn activates the myeloid cells to induce IL-17 and IL-23 via the TLR4-MyD88 pathway, promoting colon cancer development [97]. Similarly, in another genetic mouse model of colon cancer, microbiota has been suggested to activate the MyD88 signaling pathway in intestinal epithelial cells which inhibits the degradation of the c-Myc oncoprotein, leading to the promotion of colon tumorigenesis [98]. As c-Myc has been shown to activate IRAK1 which is a signaling molecule downstream of MyD88 [99], it would be interesting to study whether this c-Myc stabilization acts as a positive feedback mechanism to amplify this microbiota-MyD88-dependent tumor formation. In summary, it is suggested that LPS translocates into circulation from the gut microbiota by a leaky gut during the development of liver, pancreatic, and colon cancers. This, in turn, promotes the

development of these cancers. However, the causes of this gut leakiness require further examination.

7.3.2 Flagellin

Flagellin is a structural component of flagella which allows the pathogenic bacteria to adhere and invade host tissues [100]. It is recognized by TLR5 [100]. Interestingly, TLR5-deficient mice develop spontaneous colitis with increased bacterial load in the colon as compared to wild-type mice [101]. This inflammation may be mediated by the increased colonic expression of hematopoietic cell-derived pro-inflammatory cytokines [101]. Deletion of TLR4 rescues the colitis of TLR5-deficient mice but not the bacterial load in the colon [101], suggesting that TLR4 ligands are involved in induction of mucosal inflammation when TLR5 is absent. This notion is supported by the finding that the polymorphisms in TLR5 gene are associated with ulcerative colitis and simultaneous polymorphisms in TLR4 and TLR5 are correlated with decreased level of pro-inflammatory cytokines [102]. In addition, TLR5 is associated with metabolic syndrome. TLR5-deficient mice exhibit hyperphagia and develop hyperlipidemia, hypertension, insulin resistance, and increased adiposity [103]. These metabolic changes correlate with changes in the composition of the gut microbiota in these mice [103]. Transfer of this microbiota into wild-type germ-free mice results in similar metabolic changes [103]. Similarly, mice lacking TLR5 in intestinal epithelial cells have a low-grade inflammation in the spleen and colon and a delayed clearance of pathobiants, leading to the development of metabolic syndrome and colitis [104]. This inflammation is correlated with the increased localization of gut microbiota into the colonic mucosa and the increased levels of fecal LPS and flagellin [104]. Loss of TLR5 in dendritic cells does not lead to the development of inflammation or the change in the composition of the microbiota but only dampens the flagellin-induced IL-22 production [104]. Consistently, a study using germ-free mice has shown that the gut commensal microbiota is responsible for the gut inflammation when TLR5 is absent [105]. Hence, it is possible that flagellin from the commensal microbes activates TLR5 to maintain the gut epithelial integrity. When this barrier is impaired in the case of TLR5-deficiency such as TLR5 gene polymorphisms, some gut commensal microbes become pathobiants and induce mucosal inflammation via TLR4, contributing to the development of IBD and metabolic diseases.

Depending on the cancer type, commensal bacteria can either potentiate or suppress the extraintestinal cancer progression via interaction with TLR5. Rutkowski et al. has shown that gut commensal microbiota induces systemic IL-6 serum level via TLR5, hence promoting tumor progression in a mouse sarcoma model [106]. This elevation of IL-6 expands the granulocytic myeloid-derived suppressor cells, leading to the secretion of galectin-1 from γδ T cells [106]. The secreted galectin-1 suppresses the antitumor immunity and enhances malignant progression [106]. On the contrary, when the tumor is unresponsive to IL-6, gut commensal microbiota

activation of TLR5 suppresses tumor growth by inhibiting IL-17 production in mice bearing the breast carcinoma cell line [106]. Consistently, breast cancer patients with TLR5 R392X polymorphisms have poorer survival and higher IL-17 levels than the patients with wild-type TLR5 alleles [106]. Whereas in the case of ovarian cancer, patients with the same polymorphisms have improved survival [106]. As discussed above, TLR5 can maintain the epithelial integrity. Hence, it is possible that the gut microbiota modifies the outcome of cancer malignancy only when there is an impairment of gut lining caused by the loss of function of TLR5.

7.3.3 Polysaccharide A (PSA)

Polysaccharide A (PSA), which is a cell wall component of the symbiont *Bacteroides fragilis*, shapes the maturation of the developing immune system including the development and function of T cells [107]. PSA signals through TLR2 on $CD4^{+}$T cells to activate the Treg cells to secrete IL-10, leading to the protection against inflammation in mouse models of IBD and multiple sclerosis [108–111]. In addition, PSA can be released by *Bacteroides fragilis* in outer membrane vesicles (OMV) [112]. This PSA-containing OMV is sensed by TLR2 present in intestinal dendritic cells to promote Treg cells and anti-inflammatory cytokine production, leading to the protection against experimental colitis [112].

7.3.4 Bacterial DNA

Cytosine-guanine dinucleotide (CpG) motifs of bacterial DNA are present in many commensal bacteria. They are recognized by TLR9. Activation of TLR9 at apical surface of intestinal epithelial cells suppresses the NF-κB activation and expression of TNF-α and IL-8, leading to the protection against experimental colitis [113]. This protection is also mediated by TLR9 activation of hairy enhancer of split 1 and expression of vascular endothelial growth factor that promote the intestinal wound healing and CpG-TLR9 enhancement of Treg cell function [114, 115]. Moreover, TLR9 reduces glucose intolerance and insulin resistance, thus protecting against high-fat diet-induced obesity [116]. This is possibly mediated by the TLR9 suppression of M1 macrophages and Th1 cells and TLR9 inhibition of pro-inflammatory cytokine and chemokine expression in the adipose tissue [116]. In contrast, TLR9 ligands promote NASH progression when the permeability of gut is increased by inflammasome malfunction [89]. Hence, the gut integrity may determine whether TLR9 ligands promote or suppress the inflammation in IBD and metabolic diseases.

7.3.5 Muramyl Dipeptide (MDP)

Muramyl dipeptide (MDP), which is a peptidoglycan motif, is present in a wide range of Gram-positive and Gram-negative bacteria including those present in our gut microbiota. It is recognized by nucleotide-binding oligomerization domain protein 2 (NOD2). Previous studies have shown that polymorphisms in NOD2 are associated with Crohn's disease (CD) which is one of the IBD subtypes [117, 118]. The ileal mucosa of CD patients with homozygosity in NOD2 mutation has higher load of *Bacteroidetes* [119]. An elevated load of *Proteobacteria* and a lower load of *Firmicutes* are also associated with the NOD2 mutation [119]. In mice, however, conflicting results have been reported on the role of NOD2 in altering the composition of the gut microbiota, and the mechanism of NOD2 regulation on these changes [117, 119]. Regardless of this inconsistency, translocation of Gram-negative bacteria, Gram-positive bacteria, and yeast *Saccharomyces cerevisiae* on gut Peyer's patches in the ileum is increased in NOD2-deficient mice [120]. These mice are also more susceptible to the experimental colitis [120]. In addition, more adherent-invasive *Escherichia coli* (*E. coli*), which is associated with CD, are attached to and translocate across the Peyer's patches of the NOD2-deficient mice via their long polar fimbriae [121]. Apart from colitis, NOD2-deficient mice have elevation of inflammation in adipose tissue and liver, and insulin resistance when they are fed on a high-fat diet [122]. This is caused by the bacterial translocation from the gut into the adipose tissue and liver [122]. These findings suggest that NOD2, possibly activated by MDP, maintains the gut lining integrity to prevent the translocation of gut microbes, leading to the protection against IBD and metabolic diseases. This integrity maintenance could be mediated by the MDP-NOD2 inhibition of the gut-microbe-activated TLR2 and TLR4-dependent pro-inflammatory responses and by the NOD2 modulation of TGF-β-producing Treg cells in colonic lamina propria [123–126]. However, the origin of the MDP and the mechanism of its translocation across the mucus layer of the gut require further investigation.

NOD2-deficient mice are more susceptible to colitis-associated colorectal cancer [127]. This is mediated by the increased IL-6 secretion resulted from the changes of the gut microbiota in these mice [127]. In addition, variant R702W in NOD2 has been associated with colorectal cancer [128]. These findings suggest that NOD2 plays a role in ensuring a healthy gut microbiota possibly via the above-proposed mechanism of gut integrity maintenance.

7.4 Virulence Factors

7.4.1 FadA Adhesin

Fusobacterium nucleatum (*F. nucleatum*) is an opportunistic commensal bacterium which is present in the oral cavity [129] and is associated with different periodontal diseases [129]. FadA, a virulence factor of *F. nucleatum*, is a surface adhesin that defines the invasiveness of this bacterium [129]. Interestingly, the level of FadA gene in colon tissue from patients with adenomas and adenocarcinomas is higher than that from the healthy individuals [129]. Furthermore, FadA binds to E-cadherin on the gut epithelial cells, leading to the activation of β-catenin induction of epithelial cell proliferation [129]. This results in the *F. nucleatum* potentiation of the tumor growth in a genetic mouse model of colon cancer [130]. Consistently, higher frequency of *F. nucleatum* has also been found in the samples of patients with colorectal cancers [131, 132].

7.4.2 Polyketide Synthases (PKS) Islands

E. coli strains are one of the Gram-negative bacteria that constitute a healthy gut microbiota. However, there is an increased attachment of *E. coli* to the colon mucosa in patients with adenocarcinoma as compared to healthy controls [133–135]. Some strains of *E. coli* have a 54 kb polyketide synthases (PKS) genotoxicity island in their genome. This island encodes multiple enzymes to synthesize colibactin, which is a genotoxin [136]. *E. coli* that contain PKS island have been found in the mucosal lesions of patients with IBD and colorectal cancer [137–139]. They also enhance tumor development in two mouse models of colitis-associated colon cancer and a genetic mouse model of colon cancer [137, 138, 140]. Several mechanisms have been proposed on how colibactin promote colon cancer development. Firstly, it induces cellular senescence by conjugating p53 with small ubiquitin-like modifier, leading to the enhanced tumor growth [140]. Secondly, it induces the colon epithelial cell proliferation [138]. Lastly, it cross-links DNA by its warhead, leading to the induction of DNA damage and the subsequent cell cycle arrest and genomic instability [141–143].

7.4.3 **Bacteroides fragilis** *Toxin (BFT)*

Bacteroides fragilis (*B. fragilis*) are anaerobic symbionts living in the colon [136]. They are classified into non-toxigenic *B. fragilis* (NTBF) and enterotoxigenic *B. fragilis* (ETBF) [136]. While both NTBF and ETBF can colonize mouse colon, only ETBF can promote colitis and tumor development in a multiple intestinal neoplasia

mouse model via the activation of Th17 cell responses [144]. This is possibly mediated by *B. fragilis* toxin (BFT) derived from ETBF that can induce the expression of spermine oxidase to catabolize polyamine in colonic epithelial cells, leading to DNA damage [145]. Consistently, higher percentage of colonoscopic biopsies and fecal samples containing the bft gene is found in colorectal cancer patients than in the controls [146, 147]. Hence, both BFT and the entire ETBF are crucial in potentiating the development of IBD and colon cancer.

7.5 Concluding Remarks

Microbial metabolites, microbial components, and virulence factors interact with the host tissues and cells both locally and systemically, contributing to the pathogenesis of inflammatory diseases and cancers. These interactions are made possible by the ability of microbial factors to cross the gut epithelial barrier. While microbial metabolites or secretory toxins can diffuse passively across this barrier, the gut epithelia contain a mucus layer that blocks the passage of intact gut microbes. Thus, it is reasonable to propose that the cellular components of these microbes can only interact with the host cells and tissues when this barrier has been breached during disease development. However, studies have implicated that these microbial components can activate tolerogenic immune responses to maintain this barrier integrity without compromising it in the first place. The mechanisms taken by the gut microbiota or the host to maintain the gut epithelial barrier require further investigation. In addition, the rapid advancement of meta-omic technologies will facilitate the discovery of more microbial factors that contribute to the development of inflammatory diseases and cancers. Once identified, they will provide insights into the establishment of preventive measures and intervention strategies to tackle these diseases.

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Chapter 8
cGAS-STING Activation in the Tumor Microenvironment and Its Role in Cancer Immunity

Geneviève Pépin and Michael P. Gantier

Abstract Stimulator of interferon (IFN) genes (STING) is a key mediator in the immune response to cytoplasmic DNA sensed by cyclic GMP-AMP (cGAMP) synthase (cGAS). After synthesis by cGAS, cGAMP acts as a second messenger activating STING in the cell harboring cytoplasmic DNA but also in adjacent cells through gap junction transfer. While the role of the cGAS-STING pathway in pathogen detection is now well established, its importance in cancer immunity has only recently started to emerge. Nonetheless, STING appears to be an essential component in the recruitment of immune cells to the tumor microenvironment, which is paramount to immune clearance of the tumor. This review presents an overview of the growing literature around the role of the cGAS-STING pathway in the tumor microenvironment, with a specific focus on the role that cancer cells may play in the direct activation of this pathway, and its amplification through cell-cell transfer of cGAMP.

Keywords cGAS • cGAMP • STING • Interferon • Connexin • Cancer

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8.1 Introduction

Detection of pathogen-associated molecular patterns (PAMPs) by the immune system is the foundation of innate immunity and is one of the first lines of defense against infections. Viral nucleic acids and bacterial cell wall components (such as lipopolysaccharides or flagellar proteins) are PAMPs selectively detected by different types of innate immune receptors. These include membrane-bound Toll-like receptors (TLRs)—located at the surface of the cells or in the endosomes (e.g., TLR3, TLR4, and TLR7/8/9)—or cytoplasmic sensors such as retinoic acid-inducible gene I (RIG-I)-like helicases, which detect foreign RNAs [1, 2]. Activation of such innate immune sensors results in the production of antiviral and antibacterial proteins, including cytokines such as TNF-α or type I interferons (IFNs). Type I IFNs exert a strong antiviral effect through transcriptional induction of more than 2000 genes [3].

8.1.1 Stimulator of Interferon Genes: STING

Stimulator of interferon genes (STING, also known as MITA, ERIS, MPYS, or TMEM173) was independently discovered by four different groups in 2008 in an attempt to characterize mechanisms of DNA recognition resulting in the production of type I interferon (IFN), independently of TLR9 [4–7]. STING is an ER-localized protein containing N-terminal transmembrane helices and a large C-terminal cytosolic domain. Its activation promotes signal transduction through the TBK1-IRF3 axis and production of type I IFNs [8, 9]. STING is directly involved in intracellular bacterial detection through sensing of cyclic dinucleotides (CDNs). During infection, CDNs are produced by bacteria such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* [10, 11], which activate STING to promote strong immune responses to these pathogens.

8.1.2 Cyclic GMP-AMP Synthase: cGAS

Despite the initial demonstration of STING's involvement in intracellular DNA sensing [12], a lack of evidence for a direct dsDNA-STING interaction prompted the community to suggest the existence of an upstream DNA sensor. Several potential cytoplasmic DNA sensors had been described (e.g., DAI, IFI16, DDX41, DNA-PK, MRE11, Sox2, and PQBP1, reviewed in [13]), before the discovery of cyclic GMP-AMP synthase (cGAS) by the Chen laboratory in 2013 [14]. Following cytoplasmic DNA detection, cGAS produces an endogenous second messenger, 2′3′-cyclic GMP-AMP (2′3′-cGAMP), that binds directly to STING to promote its activation [15–19]. It is now clear that cGAS is an important immune receptor for

DNA viruses and retroviruses. As such, its capacity to sense cytoplasmic DNA is not restricted to standard Watson-Crick DNA, and it can also sense DNA-RNA hybrids [20]. The DNA cGAS senses can originate from various sites; in addition to bacterial and viral DNA, there is now good evidence that it senses self-DNA leaked from the nucleus [21] or mitochondria [2, 22].

By activating STING, DNA detection by cGAS culminates in the production of pro-inflammatory cytokines and the recruitment of the IFN response. Soon after the discovery of this pathway, the role of cGAS-STING in sterile inflammation (i.e., in the absence of any pathogen) was investigated. Mutations promoting a gain-of-function of STING have been shown to result in chilblain lupus [23], a rare type of cutaneous auto-inflammation. In addition, *cGas* and/or *Sting* genetic deletion was found to rescue animals from lupus-like diseases [24–26].

8.1.3 cGAS-STING in Tumor Development

Beyond its critical role in innate immunity, the cGAS-STING pathway is rapidly emerging as a critical player in the control of tumor development. Clearance of tumorigenic cells by the immune system initially relies on type I IFN production by dendritic cells (DCs) and the recruitment of $CD8^+$ T cells, which promote the targeted death of such aberrant cells. Accordingly, chemically induced tumors develop better in mice lacking type I IFN signaling compared to their wild-type (WT) counterparts; similarly, a deficiency in type I IFN signaling results in poorer rejection of transplanted immunogenic tumors [27, 28].

Recruitment of $CD8^+$ T cells in the tumor microenvironment is the key step in antitumor immunity and is directly dependent on type I IFN production by DCs [29, 30]. Critically, STING appears to play a unique, non-redundant role in the recruitment of $CD8^+$ T cells to the tumor microenvironment [31, 32]. Indeed, genetic loss of a number of the most important signaling molecules known to date in innate immunity did not have any impact on $CD8^+$ T cell infiltration in the tumor microenvironment in a syngeneic melanoma model [31, 32]. Conversely, the loss of STING abolished the spontaneous infiltration of $CD8^+$ T cells in this melanoma model [32, 33].

8.1.4 STING Agonists as Antitumoral Adjuvants

The use of pathogen-associated molecular pattern (PAMP)-like molecules to potentiate DC activation upon radiation therapy and help tumor clearance is well established, with several clinical trials currently underway [34]. Specifically, there has been a great deal of enthusiasm around the concept that synthetic STING ligands could be designed to facilitate tumor clearance [35, 36]. One STING ligand that showed early promise was DMXAA, a flavonoid compound comprising two phenyl

rings and a heterocyclic ring. DMXAA was found to exert strong antitumoral activities in mice and to act as a direct ligand for mouse STING [37]. However, DMXAA failed in human clinical trials [38] and was found not to be a direct ligand for human STING [39]. Other research has focused on cGAMP, the second messenger that activates STING. Although cGAMP can be synthesized in vitro, its application as an adjuvant is partially limited by the fact that it is not membrane permeable. Nonetheless, cGAMP injection has been found to synergize with radiation therapy to control local and distant tumors in pancreatic cancer [40] and to decrease chemotherapy toxicity in colon cancer [41].

To remedy the limitations of natural cGAMP, various types of synthetic STING ligand based on the structures of CDNs have already been synthesized and tested in various cancer models (reviewed in [39]). Most of these ligands have been designed to target human STING and have the potential to be used in clinical trials, as exemplified by compound MIW815 (ADU-S100). In line with a role for STING in $CD8^+$ T cell recruitment and considering prior evidence with DMXAA, intratumoral injection of these CDN molecules into different types of cancer (melanoma, colon, glioma, and breast carcinomas) caused rapid tumor regression and mediated lasting and systemic antigen-specific T cell immunity [37, 35, 39]. The action of STING agonists may not be limited to antitumor immunity, and they could also promote direct apoptosis of cancer cells, as suggested by observations in malignant B cell leukemia [36].

In addition to designing synthetic ligands, loading of otherwise non-permeable cyclic di-GMP into liposomes was found to stimulate clearance of murine melanoma [42]. This suggests that experimentation with packaging and delivery of STING agonists may help enhance the therapeutic potential of these molecules.

8.1.5 STING and Immunotherapy

The tumor microenvironment plays a critical role in the control of $CD8^+$ T cells to kill tumor cells. The specificity of $CD8^+$ T cells relies on the surface expression of different receptors and ligands, creating an immune checkpoint to limit aberrant killing by $CD8^+$ T cells. Programmed cell death 1 (PD-1) expression on $CD8^+$ T cells inhibits their activation upon binding of its ligand, PD-L1. Expression of PD-L1 in the tumor microenvironment promotes inhibition of $CD8^+$ T cell clearance. Consequently, strategies to block PD-L1 or its binding to $CD8^+$ T, ultimately aimed at reigniting immune clearance of tumor cells, have shown great therapeutic potential in several types of cancers [43]. Critically, the cGAS-STING axis appears to be very important in the capacity of PD-L1 blocking strategies to reactivate $CD8^+$ T cells, as evidenced by cGAS- and STING-deficient animal models [44], and in studies using co-administration of PD-L1 with STING ligands [45, 46].

Conversely, there has been evidence that STING activation can dampen immune activity through induction of the immune checkpoint indoleamine 2,3-dioxygenase

(IDO) [47–49]. Further, in a subtype of head and neck cancer where the tumor was in a repressed immune state, STING activation could not mediate recruitment of CD8+ T cells [45].

With these lines of evidence taken together, the role of STING in immunotherapy is context dependent, and further studies are needed to better understand the signaling pathways initiating the different outcomes of its activity. How STING is activated within tumor cells is not fully understood either and is a subject for further study, although the role of STING in immune cells during tumor immunity and tumor clearance is well documented. In this review, we will focus on the role of cGAS and STING within tumor cells. We will also address the role of cell-cell communication in STING activation within the tumor microenvironment.

8.2 Mechanisms Underlying IFN Production in Immune Cells Within the Tumor Microenvironment

8.2.1 STING Activation by Tumor DNA

The current model of IFN production and CD8+ T cell priming by tumor cells indicates a possible role for the activation of antigen-presenting cells (APCs) by DNA released from the tumor as a result of apoptosis and taken up by APC via phagocytosis. This is supported by the demonstration that DNA from the tumor could be observed in APCs where STING was activated and type I IFN produced [32, 31, 50]. In this model, sensing of apoptotic cell-derived nuclear DNA by DCs recruits the STING-IRF3 axis to promote type I IFN production, thereby enhancing the functionality of DCs in an autocrine loop. Activated DCs further mediate the activation and the clonal expansion of CD8+ T cells favoring tumor clearance [31].

However appealing, this model does not account for how the tumor DNA would be released from the endosome/phagolysosome of APCs to reach the cytoplasm, where cGAS is located. In fact, previous work indicates that phagocytosis of apoptotic cells does not result in IFN-I activation, due to sequestration of DNA in the phagosome [51]. Similarly, despite tumor DNA being found in APC cells *in vivo*, dead tumor cells incubated with APCs did not engage an IFN response in vitro [32]. Another limit of the phagocytosis model is that it restricts the role of tumor cells in initiating an immune response to only dying/apoptotic cells.

There is also emerging evidence that the growth and spread of tumors, such as melanoma tumors, which display very little cell death, are regulated by STING. Indeed, mouse melanoma cells transplanted in mice lacking STING were found to develop significantly more lung metastases than WT mice [31]. Collectively, these examples indicate that the essential role of STING in tumor restriction is probably not limited to the activation of cGAS-STING in APCs upon phagocytosis of dead/apoptotic tumor cells.

8.2.2 Cytosolic DNA Activation of cGAS-STING

STING is indirectly activated by cytosolic DNA, through upstream cGAS engagement. Following cytoplasmic DNA recognition, cGAS produces cGAMP which acts as a second messenger between cGAS and STING. Critically, once synthesized, cGAMP has the capacity to be transferred between cells and mediate STING activation in adjacent cells [52], provided they form gap junctions with the cell making cGAMP. In homeostasis, physical restriction of DNA in the nucleus or phagosome (when the DNA is phagocytosed) ensures that self-DNA is not accessible to cytosolic cGAS. However, it is now apparent that self-DNA can escape from its original localization and activate the cGAS-STING pathway in certain contexts. To prevent aberrant cGAS recruitment, several key nucleases are at play to degrade DNA molecules escaping nuclear retention. Accordingly, defects in such DNases—e.g., TREX1 [53], DNase2a [54], or the nuclease-like SamHD1 [55]—lead to cytoplasmic DNA accumulation and cGAS-STING activation by self-DNA. In addition, certain chemical modifications of DNA, such as 8-OHG, protect DNA from DNase degradation (such as by TREX1) thereby favoring cGAS activation [56, 25]. Decreases in genome stability or faults in DNA repair pathways can also lead to STING activation. Loss of ATM, which results in an impaired DNA damage response, increases genomic instability and causes spontaneous type I IFN production through STING activation [57]. In line with this, a lack of RPA and/or RAD51, which normally bind to damaged DNA and sequester it in the nucleus, results in cGAS detection of leaked cytoplasmic DNA [58]. Therefore, the cGAS-STING pathway is indirectly involved in the response activated upon DNA damage.

Genome stability is directly dependent on the capacity of the cell to stop and repair its DNA. During each cell division, multiple mutations are made but are also constantly being repaired. Oncogenes and tumor suppressors regulate cell cycle and proliferation. In cancer, these genes are often mutated to cause sustained proliferation. This constant pressure to proliferate leads to genomic instability by forcing the cells to divide even in the presence of damaged DNA [59]. Accordingly, cytosolic DNA can be observed in B cell lymphomas, but not in normal B cells [60]. Despite being predominantly produced by infiltrating immune cells, some growing tumors can also produce type I IFNs [50]. Such type I IFN production can directly relate to cGAS-STING activation as recently suggested using a model of breast cancer with genomic instability [61]. Although a detailed understanding about how nuclear DNA can be leaked to the cytoplasm remains elusive, there is data to suggest that such leakage results from an active process. Overexpression of RNaseH1 (that degrades the R-loops of DNA-RNA hybrids) reduces the level of cytosolic DNA and type I IFN production and hampers the rejection of the lymphoma tumors [62]. Conversely, in prostate cancer cells, the MUS81 endonuclease cleaves DNA at stalled replication forks, promoting export of DNA products into the cytoplasm [63]. This results in type I IFN production through STING activation by the cancer cells, which consequently enhances the rejection of the tumor *in vivo* [63]. All together, these data strongly suggest that the cGAS-STING can be activated in select tumor cells, independently of APC phagocytosis.

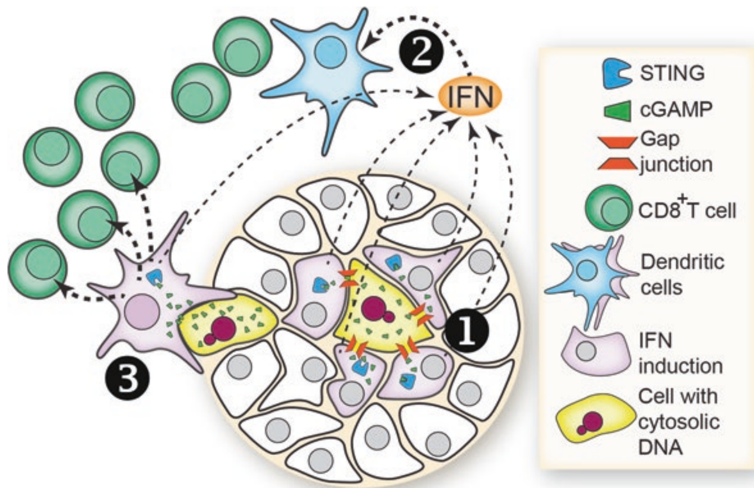

Fig. 8.1 Engagement of cGAS in tumor cells can amplify type I IFN production
Genomic instability, DNA damage, and accelerated cell proliferation can mediate leakage of DNA into the cytoplasm in tumor cells. The released DNA can then be detected by cytosolic cGAS to result in cGAMP production. cGAMP acts as a second messenger, which can directly activate STING in the cGAS engaged cells (if present—in this schematic the cells with cytosolic DNA do not express STING). Independent of intracellular STING activation, cGAMP can transfer to adjacent cells through gap junctions and activate STING in these recipient cells (*1*). Such cells, although not exhibiting cytosolic DNA, respond to cGAMP through STING engagement and produce type I IFN. This amplification of type I IFN production and associated cytokines by adjacent cells promotes the recruitment and activation of dendritic cells (*2*). Recruited dendritic cells scan tumor cells and can be further activated by cGAMP transfer through immune synapse or phagocytosis (*3*). Ultimately, activation of dendritic cells results in CD8^{+} T activation and tumor immunity

8.2.3 cGAS-STING Activation in Tumor Cells

To date, most reports addressing the *in vivo* role of the cGAS-STING pathway in the tumor environment have relied on the use of *Sting*$^{-/-}$ tumor-bearing mice compared to WT mice [33, 32, 35]. These models, although supporting a critical role for STING in the tumor microenvironment, are unable to define the source of STING activation in the tumor itself. Given that about 50% of tumor cell lines express cGAS (e.g., 5/11 colorectal cancer cell lines [64] and 7/11 melanoma cell lines [65]), cGAMP production by tumors is likely to be a frequent occurrence, assuming they also have defective DNA repair capacity. Whether such tumor-derived cGAMP activates type I IFN production by cancer cells is dependent on the presence of STING [64, 65], but this cGAMP can also modify the tumor microenvironment through horizontal transfer of the second messenger to adjacent cells [52].

With this in mind, at least two different scenarios involving cGAMP expression in tumor cells can be envisioned as illustrated in Fig. 8.1. First, tumor-derived

cGAMP production could lead to type I IFN production by the tumor cells or STING-competent adjacent tumor cells and mediate the subsequent recruitment of immune cells. Alternatively, tumor-derived cGAMP could directly engage STING in immune cells during phagocytosis or during immune synapse formation. These scenarios are not mutually exclusive and could possibly happen simultaneously; they place the focus on tumor-derived cGAMP, rather than immune-derived cGAMP, and illustrate how cGAMP transfer to immune cells better equipped for type I IFN production could be used to amplify the local detection of cytoplasmic DNA, indicative of aberrant cellular replication in this case.

In support of the origin of non-hematopoietic-derived cGAMP, it should be noted that *Trex1*$^{-/-}$ mice initially produce type I IFN in their non-hematopoietic compartment. This primary induction mediates recruitment of inflammatory cells that produce even more type–I IFN [66]. Importantly, cGAMP may also potentiate local type I IFN production through nonimmune adjacent tissues. As such, in an *in vivo* model of melanoma engraftment, cGAMP passage from the tumor to the vasculature may explain the observation that the primary source of IFN was endothelial cells [33].

It is tempting to speculate that cGAS activation in tumor cells could explain why some patients develop spontaneous leukocyte infiltration and antitumor T cell responses. Such spontaneous T cell tumor infiltration has, for instance, been reported in melanoma [67] and ovarian [68], breast [69, 61], and colorectal cancers [70]. Whether such infiltration depends on cGAS activation in the tumor and how such activation would take place are not currently defined. Future studies investigating the correlation between tumor-derived cGAMP and leukocyte infiltration may help refine disease prognosis—leukocyte infiltration is already a powerful prognostic factor in colorectal cancer patients [71]—while being informative about the best therapeutic approach to be selected.

8.2.4 Loss of cGAS-STING Expression in Tumor Cells

Many bacteria and viruses have evolved to block innate immune pathways, thereby facilitating their intracellular survival. Similar selection pressure constantly operates on tumor cells, which attempt to evade clearance by the immune system. Type I IFN is crucial to immune cell recruitment into the tumor microenvironment, and STING is a key factor in such type I IFN production, as discussed previously. One could speculate that selective pressure operates to block the cGAS-STING pathway in tumor cells to facilitate immune evasion. Accordingly, cGAS-STING inhibition has been recently described in human cancers.

The first extensive study on the loss of the cGAS-STING pathway comes from the field of colorectal adenocarcinoma (CA). In that research, cGAS-STING activity was decreased in the vast majority of CA cell lines, which lacked the capacity to produce type-I IFN in response to cytoplasmic DNA [64]. In some cell lines such as HT29 cells, the pathway was altered but still functional [64]. In support of this

in vitro data, one third of 48 clinical samples of adenocarcinoma analyzed showed a loss of cGAS expression [64]. Interestingly, upregulation of cGAS expression in the early stages of cancer and disruption of the STING pathway in advanced stages were reported in a similar study [72]. These data indicate that engagement of the pathway during early stages of tumor development, but inhibition of the pathway later in disease, favored tumor growth. Similarly to CA, melanoma cells displayed recurrent loss of cGAS-STING expression, ultimately inhibiting type I IFN production [65]. Epigenetic repression was found to relate to the cGAS and STING inhibition proposed in these studies [64, 65], but other factors are most likely at play. In another study, ovarian cancer cells (serous, clear cell, and endometriosis) had lost responsiveness to DNA transfection via STING-IRF3 activation [73]. These reports collectively suggest that loss of expression or activity of the cGAS-STING pathway may favor the development of the tumor cells.

Therapeutically, such a loss of the cGAS-STING response can be harnessed to obtain clinical benefit. Indeed, cGAS-STING-deficient tumors are more susceptible to viral oncolysis—where modified DNA viruses like HSV-1 have been used to target and kill cancer cells [65, 64].

8.2.5 cGAS-STING in Pathogen-Driven Carcinogenesis

Whether the cGAS-STING pathway is involved in pathogen-driven carcinogenesis is a question that remains elusive. Human papillomavirus (HPV) is the causative agent of cervical cancer and other types of cancer. HPV is a DNA virus, making it a potential target of cGAS sensing [74]. Accordingly, and although not detailed in the current literature, the cGAS-STING pathway may play a role in HPV infection and cancer development. In line with this hypothesis, a single nucleotide polymorphism (SNP) in cGAS (rs311678) has been recently associated with a reduced risk of cervical precancerous lesions. This SNP was found to modulate cGAS expression in vitro, and higher cGAS was associated with a reduced risk of HPV infection [75]. As described for other viruses, HPV can also counteract the activity of the innate immune system. In vitro expression of the HPV E2 protein in human primary keratinocytes downregulates the expression of STING and several other innate immune genes [76]. In addition, STING repression has been shown in HPV^{+} low-grade squamous intraepithelial lesions, when compared with HPV^{-} controls. Furthermore, viral oncogenes such as HPV E7 and E1A of human adenovirus A5 were proposed to bind to the N-terminal region of STING to reduce its downstream signaling [77].

In line with a role for the cGAS-STING pathway in reducing the risk of early stages of pathogen-driven cancer, STING expression is significantly decreased in gastric cancers when compared to non-tumor tissues [78]. *Helicobacter pylori*, the main causative agent of gastric cancer, can activate STING and promote inflammation [78]. These data collectively suggest a role for cGAS and STING in pathogen-mediated carcinogenesis.

8.3 Connexin Expression in Tumor Cells and Its Impact on Tumor Development

Beyond the inhibition of tumor-derived type I IFN production by cGAS, one could argue that loss of cGAS expression by a significant number of tumor cells is also important for stopping the propagation of cGAMP within the tumor microenvironment. As mentioned previously, cGAMP transfers horizontally through gap junctions to activate STING in adjacent cells [52]. With this in mind, it is tempting to revisit previous works on the role of gap junctions in tumorigenesis.

8.3.1 Connexins and Gap Junctions

Gap junctions are formed through the interaction of connexins from both interacting cells to promote the intercellular circulation of ions and small molecules such as cGAMP. Among the family of connexins, connexin (CX) 43 and CX45 were found to be essential in human embryonic kidney 293T (HEK293T) cells for cGAMP horizontal transfer [52]. Critically, transfer of cGAMP to HEK293T CX43/45-deficient cells could be restored through the expression of human CX26, CX31, CX32, CX40, CX43, and CX62 and mouse CX43 and CX45, suggesting that most connexins are able to transfer cGAMP (noting that human CX50 overexpression did not restore transfer) [52].

8.3.2 Loss of Intercellular Cell Communication in Early-Stage Tumors

Loss of intercellular communication by cancer cells was first described over half a century ago [79]. Since their discovery, connexins have been shown to exert both pro- and antitumoral activities, making it a controversial field of research [80]. The overall view is that retention of connexin expression benefits antitumoral activities at early stages but can later favor metastasis. Arguably, loss of connexin expression early in tumor development would be expected to reduce cGAMP horizontal transfer to adjacent cells and inhibit type I IFN induction—thereby favoring tumor initiation and immune evasion. Accordingly, CX43 expression is decreased in prostate cancer patients compared to controls. In line with what is observed with the loss of cGAS, the reduction of CX43 expression was found to correlate with advanced stages of cancer [81]. Similar trends were observed in breast cancers and head and neck squamous cell carcinomas (HNSCC), where low expression of CX43 correlated with a negative prognosis [82]. Given that HPV is also often detected in HNSCC tumors [83], it is of interest to note that HPV-E6 protein expression was associated with a reduction of gap junction formation [84].

Further supporting an antitumoral effect of connexins in early tumor growth, overexpression of CX43 was found to reduce melanoma tumor growth in vivo [85], while its downregulation stimulated the growth of prostate cancer cells [86]. Re-expression of connexins in breast cancer cell lines implanted *in vivo* also reduced tumor growth [87]. Qigesan, a molecule that increases the expression of connexins, reduced cell migration and invasion in esophageal cancer cells [88]. Critically, in a model of chemically induced mammary tumors, enforced expression of connexin decreased the incidence of tumor formation, while no difference was observed on the tumor growth [89]. These findings suggest that connexins may play a greater role in tumor initiation than tumor development [89]. It should, however, be noted that the latter tumor model relied on tumoral expression of the Cre recombinase—which we linked recently to cGAS-STING engagement [90]—opening the possibility for a direct role for the cGAS-STING pathway in the initiation of tumors in this model.

8.3.3 The Role of Connexin in Metastasis

Conversely, there are instances where increased expression of connexins was found to enhance metastasis. Indeed, in a mouse melanoma model, the metastatic capacity of the cancer cells was found to be dependent on CX26 expression [91]. Furthermore, CX43 expression is induced in CA cell lines that display greater metastatic potential, while CX43 levels are almost absent in other tumor cell lines [92].

Critically, direct evidence for the role of cGAMP and gap junction in metastasis was recently reported. When breast and lung cancer metastatic cells migrated to the brain, they increased connexin expression, allowing passage of tumor cell-derived cGAMP to adjacent astrocytes [93]. cGAMP transfer to astrocytes promoted astrocyte activation and the subsequent secretion of pro-inflammatory cytokines favoring tumor growth and chemotherapeutic resistance [93].

8.4 cGAS-cGAMP: Connexins-STING in Chemotherapy

The role played by intercellular cell communication during chemotherapy is also controversial: there are reports of both positive and negative roles for connexins on the outcome of chemotherapy. For example, increased expression of connexins enhanced the sensitivity of RKO colon cancer cells to diverse chemotherapeutic agents such as fluorouracil, oxaliplatin, and irinotecan [94]. Critically, increased sensitivity was observed in vitro when the cells were more confluent or when they were treated with retinoic acid, which induces the expression of connexins [94]. In addition, low expression of connexins correlated with reduced sensitivity of hepatocarcinoma cells to oxaliplatin [95]. On the other hand, inhibition of connexins was

reported to sensitize glioblastoma cells previously shown to be resistant to chemotherapy [96].

Chemotherapy, which mostly relies on the greater sensitivity of tumor cells to DNA damage, often induces type–I IFN production [97]. We recently discovered that the DNA intercalating agent acriflavine could promote cGAMP synthesis in SV40T immortalized mouse embryonic fibroblasts (MEFs), in association with increased cytoplasmic DNA levels [21]. Despite previous evidence of cGAS and STING activation following DNA damage and cytoplasmic DNA leakage [56, 57, 98], there had been no prior demonstration of the direct engagement of cGAS and cGAMP production in these contexts. Critically, the capacity of the topoisomerase I inhibitor, topotecan (TPT), to restrict breast cancer cell proliferation *in vivo* was abrogated in mice lacking STING [99]. This, however, does not tease out whether cGAMP was generated by the tumor after chemotherapy treatment or by phagocytes. Similar results were found using irradiation of tumors [100]. Interestingly, single-stranded DNA (ssDNA) leaking in the cytoplasm of the tumor cells following chemotherapy was shown to be a by-product of BLOOM syndrome helicase (BLM) and the exonuclease-RNase EXO1 [101]. Along with the discovery that RPA and RAD51 normally work to retain ssDNA generated by BLM and EXO1 during DNA repair [58], these findings suggest that cytoplasmic DNA leakage is likely related to a saturation of the cell's capacity to retain it in the nucleus [101]. Although ssDNA is not supposed to activate cGAS, it can bind to it weakly, and it is likely that its modification upon DNA damage (such as by 8-OHG) somewhat favors cGAS activation [56, 101]. Further work is clearly warranted to better define the modalities of cytoplasmic DNA leakage and cGAS engagement upon DNA damage by chemotherapy.

8.5 DNA Damage Engages cGAS Activity and Horizontal STING Amplification

We have previously demonstrated that DNA damage can mediate cGAMP production [90, 21]. When using inducible Cre recombinase-mediated DNA damage, we observed that only a proportion of cells displayed the hallmarks of DNA damage (through γH2AX staining) [90]. Critically, we demonstrated that the capacity of damaged cells to generate a widespread type I IFN production in the cell monolayer was strongly dependent on cell density—which we attribute to a connexin-dependent transfer of cGAMP [90]. This illustrates the capacity of healthy adjacent cells to amplify the signal of selected damaged cells and suggests that a similar feedback loop could operate in the tumor microenvironment upon induction of DNA damage by chemotherapy. Surprisingly, cGAS-depleted bone marrow-derived DCs can be activated after co-incubation with irradiated tumor cells, albeit modestly, while STING depletion completely thwarted the effect. This residual activation in cGAS-deficient cells suggests that engagement of cGAS in phagocytes is not essential for

STING activation by irradiated tumor cells [100]. From this point of view, the capacity of tumors cells to generate cGAMP and transfer it to adjacent cells may play an important role in the outcome of chemotherapy.

8.6 Conclusion

In summary, the cGAS-STING axis is crucial to cancer immunity. There is accumulating evidence for a role for STING in tumor DCs and cross priming of $CD8^{+}$ T cells. These findings clearly suggest that STING ligands have strong therapeutic potential. Nonetheless, therapeutic STING activation may also contribute to the tumor expressing interferon-stimulated genes previously linked with chemoresistance [97, 101, 102]. As such, critical questions regarding the modalities of activation of the pathway in the tumor microenvironment and its impact on chemotherapies are still to be answered. For instance, while DCs have a central role in mediating the recruitment of an antitumor immune response through STING, how other cell types like macrophages and neutrophils contribute to this pathway should also be addressed. Critically, the source of cGAMP activating STING in APCs and its putative modalities of transfer to APCs are not defined. In light of the current literature reviewed herein and our own experiments, we propose a model, illustrated in Fig. 8.1, in which cGAMP can be synthesized by tumor cells to play a role in immune cell activation through immune gap junctions. While this is supported by the demonstration that metastatic breast cancer cells could transfer cGAMP to astrocytes in the brain [93], direct evidence of cGAMP production by tumor cells and its transfer to APCs remains to be found. Defining if and how cGAMP can be made by tumors has the potential to help understand why select tumors are devoid of infiltrating immune cells. Given that the efficacy of preferred chemotherapies has been associated with STING signaling *in vivo* (e.g., irinotecan), these may be particularly effective when used in patients with active cGAS and functional connexin tumors.

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Chapter 9
TLR Agonists as Adjuvants for Cancer Vaccines

Ji-Kun Li, Jesse J. Balic, Liang Yu, and Brendan Jenkins

Abstract Toll-like receptors (TLRs) are one of the best characterised families of pattern recognition receptors (PRRs) and play a critical role in the host defence to infection. Accumulating evidence indicates that TLRs also participate in maintaining tissue homeostasis by controlling inflammation and tissue repair, as well as promoting antitumour effects via activation and modulation of adaptive immune responses. TLR agonists have successfully been exploited to ameliorate the efficacy of various cancer therapies. In this chapter, we will discuss the rationales of using TLR agonists as adjuvants to cancer treatments and summarise the recent findings of preclinical and clinical studies of TLR agonist-based cancer therapies.

Keywords Toll-like receptor • Agonist • Adjuvant • Clinical trial

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9.1 Toll-Like Receptors

TLRs are a class of single membrane-spanning, catalytically inactive receptors. Ten human and 13 mouse TLRs have been classified to date [1, 2]. This protein family is best known for their ability to detect conserved microbial components, so-called PAMPs (pathogen-associated molecular patterns) [3]. The well-characterised TLR microbial ligands include: bacterial lipopolysaccharide (LPS) and its derivatives which activate TLR4; lipoteichoic acid and lipoprotein from bacterial cell wall and fugal zymosan which stimulate TLR1, TLR2 and TLR6; and bacterial flagellin which is sensed by TLR5. Additionally, unmethylated bacterial DNA stimulates TLR9; double-stranded RNA activates TLR3; and single-stranded RNA (ssRNA) is recognised by both TLR7 and TLR8. The cellular localisation of TLRs largely reflects their function and mode of ligand interaction. For example, the TLRs that recognise viral and bacterial nucleic acids such as TLR3, TLR7, TLR8 and TLR9 are mainly localised endosomally, while TLRs on the cell surface such as TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are involved in detection of bacterial components in the extracellular space. Several TLRs, particularly TLR2 and TLR4, have been shown to detect not only exogenous PAMPs but also host-derived endogenous "damage-associated molecular patterns" (DAMPs) [4]. Many such ligands are increasingly being identified and include heat-shock proteins, high mobility group box 1 (HMGB1), various metabolic products such as reactive oxygen species (ROS) and uric acid as well as extracellular matrix components such as fibronectin and hyaluronan fragments [4].

Early studies suggested that TLRs are preferentially expressed on innate immune cells where the types of TLRs and level of expression are governed by cell-type specificity and function, which is associated with specific cytokine production [5–8]. More recent data demonstrate that TLRs are also expressed on epithelial cells of the gastrointestinal, urogenital and respiratory tracts where they play important roles in the first-line defence against infection. Additionally, they may also function to preserve epithelial barrier integrity [9–11]. Ligand binding of TLRs induces dimerisation and conformational change which activates two major signalling cascades – the MyD88-dependent or MyD88-independent pathways. Ultimately, this results in the activation and transduction of numerous downstream pathways including nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (IRFs) to induce the upregulation of type I interferons (IFNs) and various pro-inflammatory cytokines (e.g. type I IFN, IL-1, IL-6, TNFα, etc.) and chemokines (e.g. MCP-1, CCL and CXCL chemokines). Given the canonical outcomes of TLR activation, the past decade has seen a considerable effort in ways to engage or modulate TLR signalling as potential therapeutic targets capable of enhancing antitumour effect by orchestrating innate responses and activation of the adaptive immune system [12, 13]. The basic immunology of TLR recognition of ligands and mechanisms of signal transduction has been extensively reviewed elsewhere. Rather, this chapter focuses on TLR agonists as adjuvants for cancer immunotherapy in preclinical and clinical studies.

9.2 Cancer Vaccine and Adjuvant

Cancer vaccines, being prophylactic or therapeutic, aim to stimulate or restore the immune system's capacity to protect against persistent infection that initiate and drive oncogenesis. Prophylactic vaccinations against human papillomavirus-induced genital cancers with Gardasil® or Cervarix® vaccines or hepatitis B-induced hepatic cancers with Engerix®-B or Recombivax HB® vaccines have been used worldwide and play an important role in public health [14, 15]. Despite several decades of intense research and clinical evaluation, only one therapeutic vaccine (Provenge®) has been approved by FDA for the treatment cancer – androgen-independent metastatic prostate cancer [16]. In addition to drug safety profiles, there remain a few challenges that impact on vaccine efficacy that must be overcome. For example, the major drawbacks for currently used purified tumour-associated antigens (TAA) such as DNA/RNA, recombinant protein and peptides have poor immunogenicity and may cause inappropriate immune response that elicit no benefit or protection against the targeted infection or malignancy [17–19]. To overcome this and to be more effective, adjuvants are often co-administrated within the vaccine. In most cases, adjuvants were designed to augment the magnitude of an adaptive response to the vaccine administered.

Based on their perceived mechanism of action, adjuvants in the clinical settings can be divided into two main classes: immunostimulators and delivery system adjuvants [18]. Many natural and synthetic agents can be used as adjuvants such as emulsions of liposomes or PAMPs [18]. A carefully designed formulation of an adjuvant-vaccine combination is required for directing appropriate types of responses and for achieving synergism, which is otherwise difficult to achieve with a single adjuvant. For example, Cervarix, the prophylactic cancer vaccine against various types of human papillomavirus (HPV), contains purified virus-like particles (VLPs) of the major capsid (L1) protein of HPV types 6, 11, 16 and 18. Purified VLPs are then absorbed on aluminium hydroxy sulphate particles which act as the first adjuvant, the delivery system as aluminium typically induces Th2 immune profiling. Monophosphoryl lipid A (MPL), the TLR4 agonist, is then added as the second adjuvant which broadens the immune responses [20]. Substantial evidence showed that using natural ligands or synthetic agonists for PRRs as adjuvants can activate multiple elements of innate immunity. A number of TLR agonists are now in clinical or preclinical studies either stand-alone or with many different combinations for improving therapeutic cancer vaccines. So far, three TLR agonists have been licensed by the FDA for use in human cancers, including BCG (the bacillus Calmette-Guerin), a mixed TLR2/TLR4 agonist originally developed as an anti-tuberculosis vaccine but is currently approved for in situ bladder cancer vaccine; MPL (monophosphoryl lipid A), also a mixed TLR2/TLR4 agonist derived from LPS that is used as an immunostimulatory adjuvant as part of Cervarix; and, finally, imiquimod, an imidazoquinoline derivative that can function via TLR7-dependent or TLR7-independent mechanisms [21] that have been tested in many human malignancies (Table 9.1).

Table 9.1 The representative TLR agonists used in clinical trials for cancer therapy

Agent	TLR	Compound source	Indication	Notes	References
852A	TLR7	A synthetic imidazoquinoline	Leukaemia, Lymphoma, Melanoma	Sustained tolerable using prolonged s.c schedule [22]	NCT00276159, NCT00319748, NCT00091689
Ampligen (rintatolimod)	TLR3	poly-IC12U	Breast Ca, Ovarian Ca, Colorectal Ca	Th1 response [23]	NCT01355393, NCT01312389, NCT01545141
BCG	TLR2,4	*Mycobacterium bovis*	Bladder Ca	Intravesical use, FDA approved [24]	NCT02365207, NCT02015104
Cadi-05	polyTLR agonist	*Mycobacterium indicus pranii*	Melanoma	IFNγ-dependent antitumour effect [25]	NCT00675727
CBLB502 (entolimod)	TLR5	*Salmonella enterica flagellin*	Colorectal Ca, SCHNC	Radioprotective and organ-specific immunoadjuvants [26]	NCT02715882, NCT01728480
CpG7909 (Promune)	TLR9	Class B unmethylated CpG oligonucleotides	Renal cell Ca, NSCLC, Breast Ca	Stat1-dependent cell death [25]	NCT00043407, NCT00070629, NCT00043394
DIMS0150 (Kappaproct)	TLR9	ssDNA-based synthetic ODN	UC	Enhance the steroid sensitivity [27]	NCT01493960
Hiltonol	TLR3 MDA5	poly-ICLC	Non-melanoma Skin cancer, Glioma, Lymphoma	Stable and heat tolerant [19]	NCT02423863, NCT01188096, NCT01976585
Imiquimod	TLR7	Imidazoquinoline	Basal cell Ca, CIN	FDA approved [17]	NCT01264731, NCT02917746, NCT02917746

IMM-101	polyTLR agonist	*Mycobacterium obuense*	Melanoma, Unresectable Ca, Pancreas Ca	Restore Th1 and downregulate Th2 [28]	NCT01559818, NCT03009058, NCT01303172
IMO-2055 (EMD1201081)	TLR9	Phosphorothioate oligodeoxynucleotide	Renal cell Ca, NSCLC	Impair EGFR signalling [29]	NCT00729053, NCT00633529
ISS 1018	TLR9	Phosphorothioate oligodeoxynucleotide	Lymphoma	Synergic effects with rituximab [30]	NCT00251394
OM-174 (CRX-527)	TLR2,4	*Escherichia coli*	Solid tumours	Well tolerated in phase I [31]	NCT01800812
Picibanil (OK-432)	TLR2,4	*Streptococcus pyogenes*	Pancreas Ca, AEC	DC maturation [32]	NCT00795977, NCT00291473
Resiquimod	TLR7, TLR8	Imidazoquinoline	Various cancer type	Testing in numerous combination therapy [17]	NCT00821652, NCT00470379, NCT01676831
SD-101	TLR9	Phosphorothioate ODN	Lymphoma, SCCHN	Resensitise to PD-1 blockade [33]	NCT02521870, NCT02927964
TMX-101	TLR7	Imiquimod	Bladder Ca *in situ*	IL-6 and IL-18 induction [34]	NCT01731652
VTX-2337 (motolimod)	TLR8	A small molecule agonist	Ovarian Ca, SCCHN	Strong IL-12 and TNFα production [35]	NCT02431559, NCT02124850

Abbreviation: *pDC* plasmacytoid dendritic cell; *s.c* subcutaneously; *BCG* bacillus Calmette-Guerin; *poly-ICLC* particular formulation of poly-IC that includes carboxymethyl cellulose and poly-L-lysine as stabilising agents; *NSCLC* non-small cell lung cancer; *ODN* oligodeoxyribonucleotide; *SCHNC* squamous cell dead and neck cancer; *UC* ulcerative colitis; *CIN* cervical intraepithelial neoplasia; *AEC* antigen-expressing cancers

9.3 TLR Agonists as Adjuvants

A rationale for adjuvanted vaccines is to induce the synchronous activation of dendritic cell (DC)-presenting antigen and promote Th1 and $CD8^{+}$ T cell responses with minimal adverse effects. Extensive mouse studies and human trials using synthetic TLR agonists have demonstrated that adding TLR agonists to cancer treatments profoundly influences the extent of adaptive immune responses to tumour antigens. Such enhancing effects include direct activation of DC subsets, type I IFN production, enhanced cross-presentation, augmented $CD8^{+}$ T cell responses and increased antibody titres; reinvigorated immunosurveillance in patients whose immune system is compromised or in less immunogenic patients such as elderly and children (change the tumour environment); sensitised conventional chemotherapy or radiotherapy; and dose sparing, either minimising the number or amount of antigen introduced or reducing the vaccine schedule for optimal effects [20, 36]. The key effects of TLR agonists on immune system are summarised in Fig. 9.1.

9.3.1 TLR2

Phylogenetic analysis identified TLR2 along with TLR1, 6 and 10 with highly similar primary sequences that cooperate with each other during PAMP recognition. [37, 38]. TLR2 can functionally heterodimerise with TLR1, TLR6 and possibly TLR10 to specifically recognised products from gram-positive bacteria, including triacyl lipopeptides and *Mycoplasma fermentans* macrophage-activating lipopeptide (MALP-2). Pam3Cys, a synthetic triacylated lipoproteins, is widely used ligand to activate TLR2 in the laboratory [39].

SMP-105 is a TLR2 agonist developed from the insoluble fraction of the cell wall skeleton (CWS) of *Mycobacterium bovis*. One study has demonstrated that the activation of TLR2 by SMP-105 significantly increased IFNγ-producing cells and tumour-specific cytotoxic lymphocytes (CTL) in mice inoculated with Lewis lung cancer cells, resulting in a growth suppression of implanted tumours [40]. However, SMP-105 has not yet been trialled, and the use and development of TLR2 agonists as an adjuvant in anticancer immunotherapy has not been investigated. This may be at least partly because of regulatory T cell (Treg) activation by TLR2 agonists and resultant production of the anti-inflammatory IL-10 [41–43]. In contrast, recent studies have shown that TLR2 activation by Pam3Cys can suppress Treg activity and promote a Th17-like phenotype shift in multiple sclerosis patients. Overall, this suggests that the outcomes of TLR2 signalling may be contextually and therefore immunobiologically diverse [44].

CBLB612 is a synthetic lipopeptide that specifically binds to TLR2 and TLR6. However, this molecule was evaluated as only a bone marrow protective agent for cancer patients before and after chemotherapy. A phase II double blind, multicentre study (NCT02778763) was completed on efficacy and safety of this molecule as a

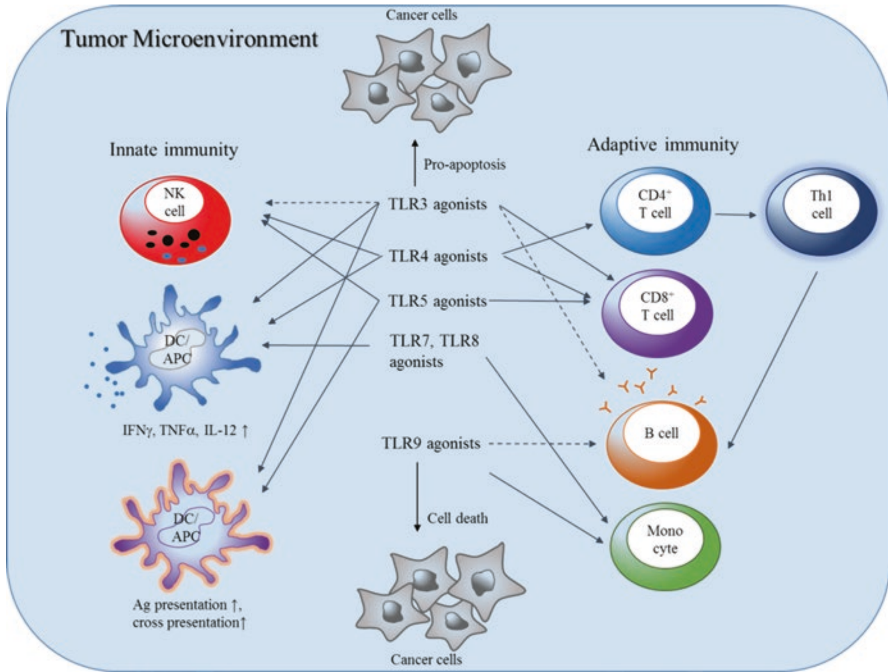

Fig. 9.1 Antitumour responses by TLR ligands through direct and indirect effects

monotherapy for neutropenia prophylaxis in breast cancer patients receiving myelo-suppressive chemotherapy.

9.3.2 TLR3

TLR3 is an endosomal receptor that recognise double-stranded RNA molecules. The primary TLR3 ligand is viral dsRNA. Polyinosinic-polycytidylic acid (poly-IC), first developed in 1967, is a synthetic dsRNA molecule used in many studies to activate TLR3 and MDA5 [19, 45]. It has been shown that stimulation with poly-IC induces strong type I interferon production, humoral immunity and Th1 responses. However, the significant toxicity and the degradation by serum nucleases limit its development as a clinical dsRNA adjuvant.

To improve the safety and therapeutic potential, another two poly-IC derivatives, poly-IC12U (Ampligen®) and poly-ICLC (Hiltonol®), were developed and tested in numerous clinical trials as effective adjuvants. The former is a modified poly-IC with shortened half-life and shown to induce lower absolute levels of type I interferon than poly-IC [19]. Early studies showed that Ampligen not only activates NK cell and converts M2 macrophage to M1 counterparts but also elicits direct cytotoxic effects on cancer cells [19, 23]. However, more interest is currently focused on test-

ing this TLR3 agonist against HIV infection, myalgic encephalomyelitis and chronic fatigue syndrome [46].

Hiltonol® is designed to be highly resistant to serum nucleolytic hydrolysis. Its denaturation temperature can be as a high as 40 °C. When administered, the prolonged and enhanced activity of this compound has been shown to induce changes in immune-related gene profiles indicative of the activation of multiple canonical innate immune pathways [47]. In early studies, this drug was initially proposed to be used in clinical trials for treating children with acute leukaemia and neuroblastoma [48]. Due to high dose and systemic side effects, more recent clinical trial has transitioned to local administration such as intramuscular (i.m) or subcutaneous (s.c) route. Current studies have demonstrated that inclusion of poly-ICLC with overlapping long peptides (OLP) from a human tumour self-antigen NY-ESO-1 and Montanide ISA-51 was well tolerated and elevated the antigen-specific antibody titre and T cell responses in ovarian cancer patients immunised subcutaneously (NCT00616941) [49]. A further study of a sensitive CD154 expression-based assay characterised that the major effect of poly-ICLC was achieved through enhancing OLP vaccine-induced $CD4^+$ T cell as an increased IFNγ/IL-4 ratio was detected. However, this study did not assess the influence of using poly-ICLC as a standalone adjuvant [50]. Another very recent study showed that NY-ESO-1-specific IFN-producing $CD8^+$ T cells were significantly increased in patients immunised with poly-ICLC than controls without poly-ICLC treatment [51] (UMIN000007954). Consistent findings were reported in a study of treating low-grade glioma (LGG) patients using a subcutaneous emulsified vaccination of glioma-associated antigen (GAA)-derived peptide with concurrent intramuscular injections of poly-ICLC (NCT00795457). This project was initially to explore an appropriate strategy for treating immunocompetent patients with slow growth rate gliomas as these patients may exhibit more immunogenicity and gain greater benefit from immunisations than those immunocompromised subjects with high-grade gliomas [52]. These results suggest TLR3 agonists as a promising adjuvant to cancer vaccines that target various tumour-associated antigens. Nevertheless, large-scale trials are required to evaluate the safety and efficacy of TLR3 agonist-based cancer therapies.

9.3.3 TLR4

TLR4 was the first TLR identified in mammals and can be activated classically by LPS (also known as endotoxin) from gram-negative bacteria [13]. TLR4 also recognises DAMPS, including heat-shock protein 70 [53] and nonhistone chromatin-binding nuclear constituent HMGB1 [54]. These proteins can be released by cancer cells following heat stress or chemo-/radiotherapy and act as danger signals promoting antitumour immunity by activation of TLR4 in DCs. Patients carrying single nucleotide polymorphisms (SNP) in TLR4 affecting the interaction between TLR4 and HMGB1 relapsed earlier after chemo-/radiotherapy than those with the normal TLR4 allele [54].

Monophosphoryl lipid A (MPL), a derivative of lipid A from gram-negative *Salmonella minnesota* endotoxin with reduced LPS (TLR4 ligand) toxicity in humans, was found to have antitumour activity in vivo early in the 1960s. Since 1984, this molecule as an adjuvant has been extensively investigated for cancer vaccines in the clinical setting. AS04 (Adjuvant System 04), an aluminium salt and MPL-based adjuvant, was endorsed by FDA in 2009 as part of Cervarix®. AS04 was also developed by GSK Biologicals in different formulations and used as proprietary adjuvant in numerous trials of cancer vaccine targeting specific TAAs (MAGE-3, MUC-1, sialyl-Tn and Ras mutant) expressed on multiple cancer types [6, 55].

Bacillus Calmette-Guérin (BCG) is a vaccine primarily developed for the prevention of tuberculosis worldwide [24]. Its anticancer potential began to be evaluated in the clinical settings in the 1970–1980s [24]. Testing BCG as monotherapy in human bladder cancer demonstrated either no clinical benefit or was proven inconclusive due to the small cohort sizes. Since 1977 intravesical instillation of BCG has been the "gold standard" treatment for patients with in situ or non-muscle invasive bladder cancer. Several clinical trials listed were designed to compare BCG as a single agent with combination therapy. Successful trials using BCG for melanoma therapy demonstrated better prognosis overall when BCG was combined with a melanoma cell vaccine or in combination with topical treatment of 5% imiquimod, a cream-formulated TLR7/8 agonist [56]. The non-specific protection by BCG was shown to be mainly through activation of TLR2 and TLR4 in macrophages and DCs, inducing strong cytokine and chemokine production such as IFNγ, IL-2 and TNFα. However, BCG has been found to activate $CD4^+$ $CD25^+$ Treg cells and promotes TGFβ and IL-10 secretion, which could be the reasons of unfavourable results in some trials [56–58].

Picibanil (OK-432) is a lyophilised preparation of *Streptococcus pyogenes* that is approved in Japan for the treatment of cervical cancer, gastric cancer and oral cancer [59]. Studies using this compound in other malignancy appear still active [60–62].

GLA-SE (G100), a synthetic glucopyranosyl lipid A (GLA) which is an oil-in-water emulsion (ES), is a novel TLR4 agonist, and this particular formulation allows to maximise the activation of multiple immune-related signalling pathways [63]. A recent preclinical study showed that intratumoural injection of G100 three times a week significantly suppressed tumour growth and resulted in 60% CR (complete tumour regression) in an A20 lymphoma tumour model via a $CD8^+$ T cell-dependent manner. Gene profiling analysis further demonstrated upregulation of broad immune-related genes, though also including T cell exhaustion marker such as CTL4, LAG3 and PD-L1 in G100-treated tumour. These data provide a rationale for co-administration of this TLR4 agonist with checkpoint blockade therapy to gain a potential synergistic effect [64] and support the current ongoing clinical trial of G100 for patients with non-Hodgkin's lymphoma as a single agent or in combination with pembrolizumab, a humanised antibody against PD-1 (NCT02501473). Furthermore, a single injection of G100 subcutaneously prior to tumour inoculation resulted in reduction of metastatic development of a mammary adenocarcinoma and a colon cancer cell model in both rats and mice with no adverse effects.

The antitumour effect of G100 appeared to be mainly associated with enhanced activation of NK cells [65]. A pilot clinical trial of intratumoural injection of G100 as monotherapy for patients with resectable Merkel cell carcinoma in a neoadjuvant setting exhibited an acceptable tolerability and increased $CD8^+$ T cell antitumour activity [59, 66]. Additionally, in order to trigger a potent tumour antigen-specific antitumour response, G100 was also formulated with recombinant NY-ESO-1 protein and developed to be another immunogenic agent named ID-G305. A novel "priming-boost" combination approach called CMB305 is to sequentially dose LV305 (an in vivo DC-targeting vector expressing the NY-ESO-1 gene) and ID-G305. This recipe allows to synergistically induce multiple level of antitumour immune effects. Of note, CMB305 does not require patient-specific manufacturing or ex vivo manipulation of patient samples. The phase Ib open-label, multicentre trial designed to evaluate the safety, tolerability, immunogenicity and preliminary clinical efficacy of CMB305 in patients with NY-ESO-1-positive tumours is currently recruiting (NCT02387125).

9.3.4 TLR5

Flagellin protein, a constituent protein of bacterial flagella, is the only known natural ligand to activate TLR5. Formulation using liposomal engrafted synthetic peptide containing flagellin fragments can induce DC maturation in vitro and in vivo [25]. In a study employing a mouse xenograft melanoma model, a vaccine formulated with both N-terminal (9Flg and 42Flg) and C-terminal (10Flg and 11Flg) flagellin peptide engrafted ovalbumin (OVA) liposomes suppressed lung metastasis in mice inoculated with B16-OVA cells compared to control mice [67]. More interestingly, TLR5 agonists lack induction of self-amplified driving cytokines, such as TNFα, IL-1β and IL-2, which make its fingerprint as a safe adjuvant for systemic administration [26].

Entolimod (CBLB502), a pharmacological optimised flagellin derivative, has revealed antitumour effects in numerous mouse tumour models [26, 68–71]. Craig et al. also showed that activation of TLR5 signalling by systemic administration of entolimod as a single agent inhibited at least two types of murine tumour metastases to high TLR5 expression organs such as the lung and liver. These antitumour effects were initiated through a CXCR3-dependent NK-DC-$CD8^+$ T cell axis [26]. One phase I clinical trial has been completed to determine the safety and preliminary evidence of efficacy using entolimod (i.m or s.c) in patients with late-stage solid tumours (NCT01527136). Overall, the treatment with entolimod was well tolerated with only common adverse events such as fever, transient hypotension and hyperglycaemia.

Interestingly, entolimod has been shown a protective effect against renal dysfunction in a murine model of acute renal ischemic failure as well as in an ulcerative colitis model [72, 73]. Furthermore, this compound showed radioprotective activity in mouse and primate models without reducing tumour radiosensitivity [74]. Further

preclinical study identified the liver and gastrointestinal tract as the major target organs of this molecule [75]. These evidence supported current clinical studies and raise considerable interest to test entolimod in development as a radiation countermeasure in emergency severe condition such as acute radiation syndrome or radiation sickness from radiotherapy against cancer. These findings allow entolimod to be a versatile player in cancer therapy.

Another TLR5 agonist, M-VM3 (Mobilan), is currently in two clinical trials (NCT02654938, NCT02844699) for prostate cancer. Mobilan was designed as a recombinant non-replicating adenovirus encoding human TLR5 and its specific agonistic ligand, flagellin. Delivery of this system into tumour cells would render an autocrine activation of TLR5 and result in subsequent strong adaptive antitumour immune responses. Certain human tumours expressing the coxsackievirus and adenovirus receptor (CAR) such as prostate cancer and several tumours of female reproductive system will be the primary targets using this TLR5 agonist-based system [76].

9.3.5 TLR7/8

TLR7 and 8, both expressed in the endosomes/lysosome, are receptors for ssRNA, especially U or GU-rich oligoribonucleotides [77]. They share high sequence homology and predominately overlap in the ligands they can each respond to. In humans, these TLRs are abundantly expressed in multiple subsets of human DCs. Stimulation of TLR7 and 8 with their agonists significantly augments multiple subset DC maturation, Th1 cellular immunity, cross-presentation and humoral immunity [78, 79]. Of note, conjugation of TLR7/8 agonist rather simply mixing with antigens has been demonstrated more effective to generate $CD8^+$ immunity [17, 80].

Resiquimod, a TLR7/8-bispecific agonist, is a prototypical imidazoquinoline molecule [17]. Early studies showed that soluble molecules like resiquimod distribute quickly from the site of injection throughout the body and fail to induce local immune activation, thereby limiting its clinical utility. To resolve this problem, prototypical imidazoquinolines were formulated as a dermal cream [17]. Aldara®, imiquimod 5% cream, is the one of the only three FDA-approved commercialised small molecule TLR agonists for HPV-mediated external genital warts, superficial basal cell carcinoma and actinic keratosis [24]. In recent clinical trials, imiquimod cream has been further exploited as stand-alone or in combination with various antitumour therapies including chemo-/radio- or laser therapy in various human cancers [17]. A study of topical imiquimod in breast cancer patients with skin metastasis reported that 2 of 10 patients achieved a partial response with histologic evidence of immune-mediated tumour regression. The treatment was well tolerated with exception of frequently local adverse events [81]. In contrast, another randomised controlled trial (NCT00066872) conducted at 12 centres in the UK on 501 participants reported that patients with nodular and superficial basal cell carcinoma treated with imiquimod 5% cream were superior to excision surgery [82].

Considering the diversity of subtype of this skin cancer, to determine surgical or non-surgical modalities alone or combination needs to be optimised by future investigation [83, 84]. Furthermore, in a completed two-part randomised trial on high-risk melanoma patients, NY-ESO-1 antigen emulsified in Montanide was intradermally injected, followed by topical application of 0.2% resiquimod gel or placebo to the vaccine site. Although this formulation with topical resiquimod was safe, the clinical outcome reveals no significant differences between study groups as addition of topical resiquimod was not sufficient to induce consistent specific $CD8^+$ T cells. The reason could be that topical application of the TLR agonist may fail to absorb adequately and activate diverse DC populations in deeper skin layers [85], highlighting the selection of administration route for optimal efficacy using TLR agonists.

3M-052, a novel lipid-modified imidazoquinoline, was developed and evaluated as part of a conventional vaccine formulation. Compared to resiquimod, 3M-052 induced a prolonged response locally with diminished systemic inflammation. It was also evaluated as an adjuvant in many vaccine models such as alum suspensions, liposome formulation and oil-in-water emulsion [17, 86, 87]. In a preclinical study, Singh et al. showed that intratumoural injection 3M-052 in mouse melanoma and prostate tumour models suppressed local and distant tumour growth via not only promoting tumour-specific $CD8^+$ T cells but also shifting M2 macrophages to M1 phenotype. 3M-052 has also exhibited synergic effects with checkpoint inhibitor therapy using CTLA4 and PDL-1 antibodies [88].

9.3.6 TLR9

TLR9 is expressed in the endosome of specific immune cell types – plasmacytoid DC and B cells – where they recognise bacterial or viral DNA containing unmethylated cytosine-guanine (CG) dinucleotides motifs [89]. Activation of TLR9 through the signalling of MyD88 leads to activation of interferon regulatory factor (IRF) 7, resulting in expression of type I IFNs [90].

CpG-7909 (PF-3512676, Promune®), a class B CpG, is the most extensively studied single-stranded CpG ODN. Unfortunately, two phase III clinical studies for advanced non-small cell lung cancer reported that addition of CpG-7909 to chemotherapy gained no improvement to either overall or progression-free survival [91, 92]. Although phase I/II studies examining CpG-7909 in combination therapies were completed in various human cancers such as B-cell lymphoma (NCT00185965), metastatic breast cancer (NCT00824733) and oesophageal cancer (NCT00669292), no trials further assessing this molecule for cancer therapy are active.

MGN1703, a covalently closed natural DNA molecule, is a novel TLR9 agonist, which belongs to a different family called dSLIM (double stem loop immunomodulatory) [89, 93]. While CpG-7909 mainly stimulates B cells and may cause several CG motif-independent immune responses like IL-8 induction, dSLIM elicits significant IFN-α induction and broad activation of human immune cells in vitro [89]. In

a phase II study of 59 patients with metastatic colorectal cancer treated with the first-line chemotherapy bevacizumab (anti-VEGF-A), patients who also received MGN1703 showed a superior progression-free survival (PFS) by (NCT01208194) [94–96]. A pivotal phase III trial has been designed to further investigate these data and is currently recruiting patients (NCT02077868). This compound has also been tested in a phase I trial to determine the highest tolerable dose in combination with ipilimumab (anti-CTLA-4) to patients with advanced solid tumours (NCT02668770) and another study in patients with small cell lung cancer (SCLC) (NCT02200081).

SD-101, another synthetic CpG molecule, was tested by intratumoural co-administration with ipilimumab after local radiation therapy in patients with low-grade, recurrent B-cell lymphoma (NCT02254772). The primary objective was to identify the best dose of intratumoural ipilimumab with TLR9 agonist in patients to augment different phases of antitumour immune responses as shown in previous preclinical studies [97, 98]. However, the result has not been released yet. Another trial using this molecule combined with ibrutinib (also known as Imbruvica, a targeted inhibitor for Bruton's tyrosine kinase) and intratumoural radiotherapy for low-grade follicular lymphoma is currently listed as recruiting (NCT02927964) in order to re-evaluate adverse event during this heavy combination therapy.

9.4 Conclusion Remarks

Coupled with chimeric antigen receptor therapy [99] and immune checkpoint blockade [100], engagement of manipulating TLR signalling has drawn considerable interest as a treatment modality in the cancer immunotherapy field. Particularly, synthetic TLR agonists have been actively exploited for their safety and clinical efficacy in various therapeutic settings. Substantial evidence demonstrates that TLR agonists are potent immunostimulators and enhance natural or therapy-triggered antitumour immune responses. Despite the wealth of research in the cancer immunotherapy field, the past few years has witnessed a steady decrease in the number of clinical trials using TLR agonists as cancer treatments, be that unimodal or as adjuvants to cancer vaccines [59]. There may be some reasons for this. Firstly, most TLR ligands initiate complex signalling cascade and may influence various cell types in cell-dependent manners, of which we haven't fully elucidated. Immune cells, cancer cells and tumour stromal cell differ in their specific TLR expression and may all contribute to biological consequences of TLR activation [101]. Noninflammatory roles of TLRs in tumour progression such as regulation of apoptosis and proliferation in context of chronic inflammation and carcinogenic condition have been addressed [9]. Secondly, recent studies reveal that TLR activation can exert not only immunostimulatory effects but also immunosuppressive effects by regulating IL-10, Treg activity and PD-L1 expression [102–104]. In addition, as with many other antitumour agents in general, the underperformance of TLR agonist used in some trials may relate to the fact that patients recruited in most studies are in late-stage disease. Metastatic tumours, which are aberrant in multiple signalling pathways and

have mutations in various key genes regulating cellular functions, may all contribute to drug resistance. Additionally, immune system depression as a consequence of late-stage cancer may impede any effective induction of antitumour responses. Last but not least, the delivery system, type of tumour-associated antigen, type of TLR agonist and/or other adjuvants, schedule, route and site of administration need to be carefully considered and further investigated to acquire optimal activation and specificity [19]. Recent convergence of large-scale sequencing, cancer biology and bioinformatics allows researchers to tailor the strategy of priming the immune system against multiple patient-specific neoantigens (tumour mutation-derived antigens) [105, 106]. Additionally, there is scope for these formulations of specific TLR agonists to be personalised for specific patients' tumours. Combination therapies based on stratification of patients by their immune state may lead to better targeted trials using TLR agonists.

Overall, basic research and clinical trials provide a strong rationale for using TLR agonists as adjuvants to cancer treatments.

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Chapter 10
Telomere Damage Response and Low-Grade Inflammation

Lihui Wang, Xianhua Yu, and Jun-Ping Liu

Abstract Telomeres at the ends of chromosomes safeguard genome integrity and stability in human nucleated cells. However, telomere repeats shed off during cell proliferation and other stress responses. Our recent studies show that telomere attrition induces not only epithelial stem cell senescence but also low-grade inflammation in the lungs. The senescence-associated low-grade inflammation (SALI) is characteristic of alveolar stem cell replicative senescence, increased proinflammatory and anti-inflammatory cytokines, infiltrated immune cells, and spillover effects. To date, the mechanisms underlying SALI remain unclear. Investigations demonstrate that senescent epithelial stem cells with telomere erosion are not the source of secreted cytokines, containing no significant increase in expression of the genes coding for increased cytokines, suggesting an alternative senescence-associated secretory phenotype (A-SASP). Given that telomere loss results in significant alterations in the genomes and accumulations of the cleaved telomeric DNA in the cells and *milieu externe*, we conclude that telomere position effects (TPEs) on gene expression and damage-associated molecular patterns (DAMPs) in antigen presentation are involved in A-SASP and SALI in response to telomere damage in mammals.

Keywords Telomere dysfunction • Cytokines • Immune cells • Inflammation • Tissue senescence

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Abbreviations

A-SASP	alternative senescence-associated secretory phenotype
DAMPs	damage-associated molecular patterns
DDR	DNA damage response
SALI	senescence-associated low-grade inflammation
TPE	telomere position effect

10.1 Introduction

Telomeres are comprised of tens of thousands of repetitive DNA sequences (TTAGGG) at the end of chromosomes to prevent deterioration or fusion by losing some sequences during cell division. Telomerase is a ribonucleoprotein complex that operates to maintain telomeres (chromosomal ends), counteracting cell division-associated telomere shortening in the stem cell compartment and cancer [1, 2]. In the absence of telomerase in most differentiated cell types, consecutive shortening of telomeres in a chromosome replication-dependent fashion occurs, and the cell cycle-dependent, unidirectional catabolism of telomeres constitutes a mechanism with critically damaged telomeres terminating chromosome replication and initiating cellular senescence [3]. Loss-of-function gene mutations on telomerase subunits occur in a number of diseases [4, 5], including dyskeratosis congenita [6, 7], aplastic anemia [8, 9], liver cirrhosis [10, 11], and idiopathic pulmonary fibrosis (IPF) [12–15].

Experimental studies on animals have demonstrated certain causal roles of telomerase deficiency compromised in tissue homeostasis and dysfunctional organs [16–19]. Knockout of telomerase RNA component (TERC) or telomerase reverse transcriptase (TERT) causes the phenotypes to resemble dyskeratosis congenita [20, 21] and bone marrow failure [17, 21, 22]. In the lungs of TERC-deficient mice, elevated apoptosis of alveolar stem cells (alveolar epithelial type II cells, AECII) [23] hindered tissue growth in partial pneumonectomies [24]. The mechanisms of telomerase deficiency-associated diseases have not been fully elucidated, including the signaling of telomerase and telomeres in different conformations. The potentials of telomerase as antigens and in putative responses to vaccinations have been demonstrated under various conditions including immune resistance, evasion, and tolerance in animals [25].

Recently, deficiency in TERC or TERT causes a remarkable elevation in various proinflammatory cytokines, including IL-1, IL-6, CXCL15, IL-10, and TNF-α, and monocyte chemotactic protein 1 (chemokine ligand2, CCL2), decrease in TGF-β1 and TGFβR1 receptor in the lungs, and spillover of IL-6 and CXCL15 into bronchoalveolar lavage fluids [26]. Intriguingly, mice with TERC deficiency and short telomeres do not show obvious pulmonary fibrosis [27], whereas mice that developed pulmonary fibrosis from bleomycin insult require telomerase activity [28–30], mirroring the finding that the majority of IPF lung samples showed increased telom-

erase activity [30]. In the following sections, we will discuss the phenotypes of telomerase deficiency and telomere dysfunction-associated cellular responses, especially pulmonary alveolar stem cell senescence-associated low-grade inflammation (SALI) [26].

10.2 Telomerase Deficiency, Telomere Shortening, and Lung Epithelial Aging

Evidence indicates that telomerase operates in normal AECII [23, 31, 32] and is stimulated by silica inhalation and bleomycin instillation causing pulmonary fibrosis in rodents, suggesting that telomerase activity is involved in pulmonary fibrotic lesion [33, 34]. This scenario is in a sharp contrast to the relationship between telomerase gene mutations and IPF in humans [12–15]. Since genetic disruption of *TERC* or *TERT* leads to loss of telomerase activity and shortening of telomeres in mouse AECII cells [23, 35], we directly tested the hypothesis that the damaging effect of telomere ablations contributes to IPF in mice in vivo. Interestingly, disruption of *TERC* or *TERT* initiates telomere DDR with decreased telomere length; increased TIFs; increased p15, p16, and p21 and inductions of AECII replicative senescence characteristic of reduced total numbers of AECII; and increased populations of AECII positive for HP1γ and β-Gal staining [26]. Previous studies showed that telomerase deficiency renders pulmonary tissues susceptible to damage caused by cigarette smoke, potentially underpinning emphysema with or without pulmonary fibrosis [27, 36, 37]. It is thus feasible that telomerase is required for AECII alveolar stem cell renewal and repair of damaged differentiated cells in pulmonary interstitium [38, 39]. However, IPF myofibroblast markers were not evidenced in the telomerase-deficient mouse lungs, suggesting that telomerase deficiency and telomere shortening are responsible for pulmonary senescence but not alone sufficient in causing pulmonary fibrotic lesion [26]. It is possible that under the telomere dysfunctional pressure, additional signals are required for senescence-associated myofibroblast transdifferentiation in pulmonary fibrotic lesion.

Recent studies indicate that regulation of AECII stem cells by extracellular signaling predominantly dictates AECII response to injury signals and that only about 1% of mature AECII cells divide intermittently, with about a 40-day doubling time, supporting an overall renewal rate of 7% of alveoli per year [39]. Injuries to the AEC1 epithelial cell population, and together with EGF receptor activation, induce AECII broad stem cell functions [39]. Whereas activation of the Ras-ERK pathway stimulates proliferation [39] and inhibits differentiation [40] of AECII, inhibition of the Ras-ERK pathway is essential for TGF-β1-induced AECII differentiation [40]. Interestingly, previous studies by us and others showed that whereas mitogens upregulate telomerase activity by Ets transcriptional activation of the *TERT* gene [41, 42], TGF-β family members downregulate telomerase activity by Smad3 tran-

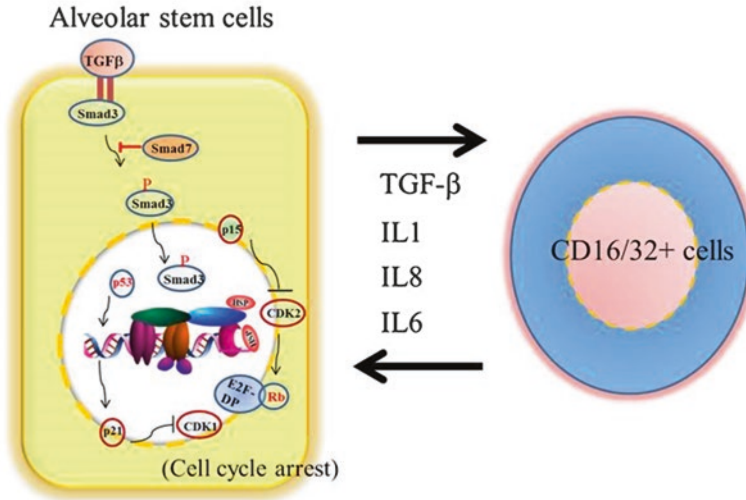

Fig. 10.1 Schematic of alveolar stem cell aging triggered by TGF-β intracellular signaling and p53- and Rb-mediated cell cycle arrest in response to cytokine-mediated cellular interaction with innate immune cells

scriptional repression of *TERT* in epithelial cell lines [43–45]. These data together suggest that the EGF-Ets and TGF-β-Smad3 pathways regulate the *TERT* gene and proliferative potential of AECII in a reciprocally opposing manner. Thus, TGF-β signaling induction of telomerase inhibition, cell proliferative suppression, and myofibroblast transdifferentiation could be a key event in initiating fibrotic lesion.

TGF-β is a key player in antagonizing AECII proliferation but stimulating AECII differentiation [46–53]. To date, it is still unclear which transcriptional targets downstream of TGF-β signaling mediate AECII differentiation and whether TGF-β-induced Smad3-mediated telomerase downregulation plays a part in suppressing AECII proliferation and allowing AECII to undergo differentiation (Fig. 10.1). In the telomerase-deficient mice where we found no evidence of significant fibrotic lesion, TGF-β and its receptor are downregulated [26]. It is possible that activation of the TGF-β signaling pathway may boost α-SMA and Col1α1 increases in the telomerase-deficient lungs. Previous studies showed increased expression of α-SMA in AECII as a marker of AECII undergoing the gene expression involved in myofibroblasts by EMT [40, 49, 54]. TGF-β stimulates α-SMA gene expression through Smad3 interaction with β-catenin on the α-SMA gene promoter in AECII [55]. In addition, TGF-β activates the transient receptor potential vanilloid 4 (TRPV4) channels and actin polymerization, resulting in the formation and nuclear translocation of the myocardin-related transcription factor (MRTF-A)/serum response factor complex and the subsequent stimulation of the α-SMA gene transcription [56]. Since anomalous expression of α-SMA and Col1α1 in AECII is among the features of AECII transdifferentiation to myofibroblasts and suggestive of a fibrotic lesion of

pulmonary fibrosis [49, 54], further investigations are required to characterize the regulatory mechanisms underlying the limited temporospatial processes and scales of AECII transdifferentiation triggered by telomere dysfunction.

10.3 Telomere Dysfunction and Inflammation

Aging tissues and age-related diseases are intimately associated with low levels of chronic inflammation [57, 58]. In this regard, inflammation has been demonstrated to contribute to aging in mouse skin [59] and in mouse model of DNA damage-driven progeria [60–62], suggesting synergistic interactions between DNA damage responses (DDRs) and inflammatory signals. Telomere dysfunction-induced DDR is a major cause of cellular senescence [63]. Severe telomere dysfunction is induced by telomere shortening in late-generation telomerase ($terc^{-/-}$) knockout mice, where it compromises the function of tissue-specific stem and progenitor cells, limits tissue regenerative capacity, and accelerates aging [64]. Since factors secreted by senescent cells participate in mediating chronic inflammation [65–67], it is anticipated that telomere shortening that causes replicative senescence is associated with SASP directly or indirectly. It is noteworthy that chronic low-grade inflammation enhances telomere dysfunction by increasing ROS-mediated DNA damage and thus accelerates accumulation of senescent cells [68]. A recent study also found that GATA4 is a key regulator of crucial senescent phenotypes and connects DDR to senescence and inflammation through IL1A and TRAF3IP2 activation of NF-κB [69].

However, telomere dysfunction in AECII triggered an inflammatory response with upregulations of cytokine signaling pathways known to provoke inflammation in the lung [26, 70]. A marked increase in proinflammatory cytokines occurs to the telomerase-deficient lung tissues, resulting in a significant spillover into the BAL fluids [26]. The concentrations of IL-6, CXCL15, and TNF-α in the pulmonary parenchyma are increased several folds with spillovers into the BAL fluids significantly in telomerase-deficient mice [26]. These findings of telomere dysfunction-caused SASP profiles of altered cytokines and growth factors in the mouse pulmonary tissues are consistent with persistent telomere DDR in both human and mouse studies [71–73]. Furthermore, the findings of markedly increased IL-1α and IL-1β are consistent with their regulatory roles in increased IL-6 and IL-8 [73], and increased CCL2 in the microenvironment of telomere-induced AECII senescence is consistent with a fundamental role in the recruitment of NK cells [74]. Among the differentially upregulated genes are Il17c, which encode interleukin 17c, and MIF encoding macrophage inhibitory factor, consistent with their known roles in epithelial-derived innate immune responses in the lung [70]. Thus, consistent with recruitment of inflammatory cells in the innate immune pathways, the proinflammatory cytokines constitute a signature of chronic low-grade inflammation incurred by telomerase deficiency and telomere damages [26].

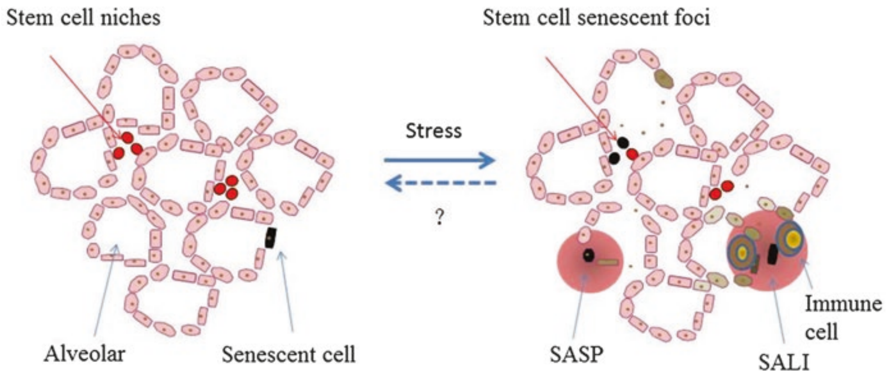

Fig. 10.2 Graphic representation of SALI foci in pulmonary tissue. SALI involves both inflammatory cytokines and cells in the foci of senescent stem cells, by contrast to SASP that defines secreted molecules from the senescent cell. SALI functions to mediate senescence spreading with the fundamental effectors of innate immune cells that interact with senescent cells and generate a progressive radial pattern of cytokine gradients

10.4 Molecular Mechanisms of Telomere-Associated Inflammation

Although senescent AECII cells show no significant increase in proinflammatory cytokine gene expressions, an alternative pathway that leads to SASP cannot be ruled out as an intermediate event in the sterile SALI [26]. Consistently, a large heterogeneous population of mononuclear inflammatory cells is positive for CD45 and/or CD16/32 in the TERC-deficient lungs [26]. It is possible that telomere attrition is a primary damage involved in the low-grade inflammation, a novel phenotype we preferred to term telomere-induced, stem cell senescence-associated, low-grade inflammation (tSALI) [26]. Since telomere DDR triggers the permanent cell cycle arrest of the stem cells lacking telomerase, SALI may generally serve as a potential mechanism to spread cellular senescence (Fig. 10.2). In line with this hypothesis, recent studies have shown that chronic inflammation suffices to induce telomere dysfunction and accelerate aging in mice [68]. Thus, as a primary means of prompting AECII senescence and undermining AECI repair, SALI represents a cellular mechanism of the focal circuit that mediates the spreading of cellular replicative senescence from the original senescent foci in pulmonary aging (Fig. 10.2).

In mediating senescence transmission, SALI assumes a fundamental feature of innate immunity which requires recruitment and activation of inflammatory cells. The participation of inflammatory cells in SALI may be central to the process of senescence progression (Fig. 10.2) and play an obliging role in the full development of aging-related pathologies by provoking cellular transition from senescence to transdifferentiation, immortalization, and transformation. Therefore, by bridging and mediating the development of tissue pathological changes, SALI corresponds to a critical window of intervention in aging-related disease.

Although it remains unknown how telomere-induced alternative SASP (tSASP or A-SASP) and subsequent tSALI are evolved during senescence progression, it is possible that at least four pathways are involved through which A-SASP progresses to tSALI. First, telomere shortening unchecks the telomere position effect (TPE) on the transcriptions of the genes encoding inflammatory cytokines and pathogenic factors [75]. Second, telomere shortening renders epigenetic alterations of heterochromatin formation resulting in activation of a specific group of gene expressions [76]. Third, telomere DNA fragments shedding off chromosomes into the *milieu externe* serve as damage-associated molecular patterns (DAMPs) that perpetuate the tSALI response, by analogy to mitochondrial and mammalian DNA molecules acting as DAMPs [77, 78]. Fourth, compromised NF-κB signaling results in depressed regulatory factors in controlling innate immune response (see below). It has previously been indicated that telomere shortening is associated with telomere DNA and protein accumulations that are involved in complex molecular signaling [3].

Premature telomere erosion in peripheral blood mononuclear cells is a common characteristic of autoimmune syndromes [79], additional to aging-associated diseases including chronic obstructive pulmonary diseases (COPD), neurodegeneration, obesity, and vascular diseases. Telomere loss has been shown to increase the susceptibility to autoimmune disease as telomerase overexpression in T cells serves as a promising therapy for the treatment of autoimmune disease [80]. Several studies have shown that hTERT overexpression in T cells extends their replicative lifespan while maintaining normal cell function [81]. Controversially, two studies have demonstrated chromosomal abnormalities in hTERT-transduced T cells [82, 83], and long-term culture of hTERT-transduced T cells results in accumulation of the cyclin-dependent kinase inhibitors p21 and p16 Ink4a, molecules that mediate cell cycle arrest and replicative senescence [84].

In addition, chronic inflammation aggravates telomere dysfunction and cell senescence, decreases regenerative potential in multiple tissues, and accelerates aging of mice. Anti-inflammatory or antioxidant treatment, specifically COX-2 inhibition, rescued telomere dysfunction, cell senescence, and tissue regenerative potential, indicating that chronic inflammation may accelerate aging at least partially in a cell-autonomous manner via COX-2-dependent hyperproduction of ROS [68]. Persistent inflammation aggravates telomere dysfunction by increasing oxidative stress at least partially through COX-2 activation [68]. This then accelerates accumulation of senescent cells, which intensifies proinflammatory and prooxidant signaling by the SASP response and by induction of mitochondrial dysfunction [85, 86], spreading DNA damage and senescence toward bystander cells [87]. As the cardinal transcriptional regulator of inflammation-related genes including proinflammatory interleukins, chemokines, cytokines, adhesion molecules, and others, NF-κB is not only activated by proinflammatory, stress, and cell senescence signals [88] but also regulated by telomerase. Mice lacking functional telomerase are defective in mounting an acquired immune response following an LPS challenge [89]. Binding directly to the NF-κB p65 subunit, telomerase regulates NF-κB p65 recruitment to a subset of NF-κB promoters such as those of IL-6 and TNF-α [89]. Furthermore, NF-κB transcriptionally upregulates telomerase activity, suggesting a

feed-forward regulation between NF-κB and telomerase [89]. How loss of telomerase in AECII would compromise NF-κB-dependent gene expressions and which NF-κB downstream gene(s) might be involved in mediating tSALI require further investigations. Consistent with telomerase involvement in proinflammatory response, a recent report shows that circulating peripheral blood mononuclear cells in patients with metabolic syndromes produce enhanced levels of TNF-α and IL-6 and have high levels of telomerase activity [90].

10.5 Perspectives

Prevention of SALI from occurring would be of significant importance in prohibiting tissue premature aging, including the case of IPF. A major interceptive step to intervene senescence spreading by SALI would be to arrest the trigger of such self-antigen as telomeric DNA, thereby disallowing the generation of cytokine gradients and immune cell chemotactic selection. It is foreseeable that the mechanisms underlying A-SASP will be uncovered to provide the key molecular interfaces for targeting. Investigations on the mechanisms by which tSALI takes place with the involvement of tSASP will include characterization of the molecules that bind with telomeric DNA and telomere-associated DAMPs and the molecules that are involved in innate immune response unchecked by compromised NF-κB signaling in the absence of telomerase as an upregulator. New discoveries of the mechanisms mediating telomere molecular processing and recognition will also involve the cellular receptors and presenters for telomere-triggered innate immune responses. Antagonizing these sites will provide novel molecular targets for effectively designing prophylactic and therapeutic modalities.

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Chapter 11
The Development and Diversity of ILCs, NK Cells and Their Relevance in Health and Diseases

Yuxia Zhang and Bing Huang

Abstract Next to T and B cells, natural killer (NK) cells are the third largest lymphocyte population. They are recently re-categorized as innate lymphocytes (ILCs), which also include ILC1, ILC2, ILC3, and the lymphoid tissue inducer (LTi) cells. Both NK cells and ILC1 cells are designated as group 1 ILCs because they secrete interferon-γ (IFN-γ) and tumor necrosis factor (TNF). However, in contrast to ILC1 and all other ILCs, NK cells possess potent cytolytic functions that resemble cytotoxic T lymphocytes (CTL). In addition, NK cells express, in a stochastic manner, an array of germ line-encoded activating and inhibitory receptors that recognize the polymorphic regions of major histocompatibility class I (MHC-I) molecules and self-proteins. Recognition of self renders NK cell tolerance to self-healthy tissues, but fail to recognize self ('missing-self') leads to activation to neoplastic transformation and infections of certain viruses. In this chapter, we will summarize the development of NK cells in the context of ILCs, describe the diversity of phenotype and function in blood and tissues, and discuss their involvement in health and diseases in humans.

Keywords NK cells • Development • NK receptors • Human disease

Abbreviations

CHILP	Common helper ILC precursor
CLP	Common lymphoid progenitor
CTL	Cytotoxic T lymphocytes
EILP	Earliest ILC progenitors

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Eomes	Eomesodermin
Ets1	ETS proto-oncogene 1
Gata3	GATA-binding protein 3
ID2	Inhibitor of DNA binding 2
IFN-γ	Interferon gamma
ILC	Innate lymphocyte
ILCP	ILC progenitors
JAK1/3	Janus kinase 1/3
LTi	Lymphoid tissue inducer cells
MCMV	Murine cytomegalovirus
Mef	Myeloid elf-1-like factor
MHC-I	Major histocompatibility class I
mTOR	Mechanistic target of rapamycin
NFIL3	Nuclear factor interleukin 3
NK	Natural killer
PD-1	Programmed cell death-1
PDK1	3′-Phosphoinositide-dependent kinase 1
PLZF	Promyelocytic leukemia zinc finger
S1P1	Sphingosine-1-phosphate receptor 1
T-bet	T-cell-specific T-box transcription factor
TCF-1	T-cell factor 1
TNF	Tumor necrosis factor
TOX	Thymocyte selection-associated high-mobility group box
TRAIL	TNF-related apoptosis ligand
Zeb2	Zinc finger E-box-binding homeobox 2

11.1 NK Cells Are a Group of Innate Lymphocytes that Secrete Adaptive Immune Cytokines

The innate immune system is constituted with granulocytes, monocytes, macrophages, and dendritic cells that secrete inflammatory cytokines, as well as innate lymphocytes that secrete adaptive cytokines such as IFN-γ, interleukin (IL)-4, and IL-17. NK cells are the prototypic ILCs, and they were first described in 1975 as being able to naturally kill mouse leukemia cells [1]. Since 2008, the concept of ILCs [2] has been expanded and now includes the related subsets of NK, ILC1, ILC2, ILC3, and the lymphoid tissue inducer (LTi) cells [3]. ILCs are characterized as having lymphoid morphology but lack rearranged antigen-specific receptors and myeloid and dendritic cell phenotypical markers. ILCs develop initially from progenitors in the fetal liver [4, 5] and, later, in the adult bone marrow [6–8]. They subsequently seed mucosal tissues, where they continue to proliferate and become tissue-resident cells and maintain tissue homeostasis. ILCs and T cells share similar transcription factors that govern their differentiation and produce similar key cytokines [2, 9]. Thus, in analogy to T cells, ILCs are subdivided into cytotoxic (NK)

and all other "helper"-like subsets that resembles IFN-γ/Th1-, interleukin 4 (IL-4)/Th2-, and IL-17/Th17-secreting $CD4^+$ T helper cells [10].

11.2 ILCs Are Generated from Progenitors Downstream of the Common Lymphoid Progenitor

All ILCs initially derive from the common lymphoid progenitor (CLP). The transition from CLP to ILC-specific transcriptional program is accompanied with differential expression of over 400 genes [4, 5, 11], with temporal requirements for Nfil3 (nuclear factor interleukin 3, also known as E4bp4), TCF-1(T-cell factor 1, encoded by *Tcf7*), and ID2 (inhibitor of DNA binding 2). Nfil3 expression is essential for the development of ILC progenitors prior to their commitment, and it is induced by mesenchymal-derived IL-7 [12–14]. NFIL3 also directly activates ID2 [14, 15]. TCF-1 represses genes critical for stem cell (*Hhex* and *Lmo2*) and pro-B cell (*Spib, Irf8, Ly6d*) function [11], and its loss affects the differentiation of both NK and other ILC subsets [16–18]. ID2 induces a major regulatory shift with broad repression of progenitor cell transcription factor genes and upregulation of critical regulators including Tox (thymocyte selection-associated high-mobility group box) and Gata3 (GATA-binding protein 3) [11]. Thus, immedicably downstream of the CLP, the earliest ILC progenitor (EILP) is TCF-1^+ [17], which further becomes $ID2^{hi}$ common helper ILC precursor (CHILP) when NK cell potential is lost [6, 14, 19, 20]. After acquisition of promyelocytic leukemia zinc finger protein (PLZF, encoded by *Zbtb16*), ILC progenitor (ILCP) loses the capacity to differentiate into LTi cells [5, 6]. Programmed cell death-1 (PD-1) is co-expressed with PLZF and can be used as a cell surface marker to identify ILCP [11] (Fig. 11.1).

11.3 NK Cells Develop Through Immature and Mature Stages

In the adult mouse bone marrow, pre-NK cell progenitor (pre-NKP) downstream of CLP (Lin^-Flt3^+ $CD27^+CD244^+$ $CD127^+CD122^-Ly6D^-$) has a $Lin^-Flt3^-CD27^+CD244^+CD127^+CD122^-$ surface phenotype, which further develop into rNKP (recently re-defined NK progenitor) that expresses CD122 [21, 22]. CD122 couples with the common γ-chain (CD132) and forms the IL-2/IL-15 receptor, allowing NK cells to respond to IL-15 and activate JAK1/3 and STAT5 [23–25]. IL-15 also activates 3′-phosphoinositide-dependent kinase 1 (PDK1)-mTOR and regulates Nfil3 and CD122 expression [26]. rNKP develops through an immature NK cell (iNK) stage to become mature NK (mNK) cells. iNK expresses NK1.1 but does not express CD49b (antigen to DX5). The expression of Ly49 receptors on the developing iNK cells is critical for NK cell education and maturation and for the detection of

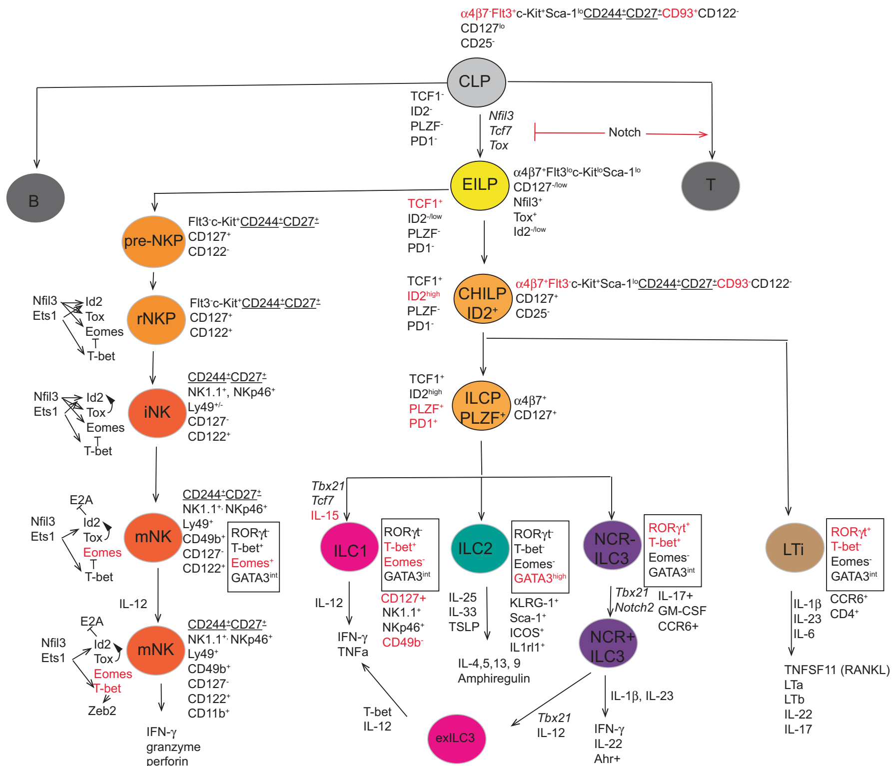

Fig. 11.1 NK and helper ILCs development in mice
T, B, NK and all other helper ILCs develop from common lymphoid progenitor (CLP). NK and ILCs development accompany with sequential differential acquirement of hundreds of transcription factors: Nfil3 and Tcf1 are required for the development and commitment of early ILC progenitors (EILP). Expression of ID2 leads to the commitment of common helper ILC precursor (CHILP), which is not able to further develop into NK cells. When PLZF is expressed, ILC progenitor (ILCP) is formed and its LTi potential is lost. Downstream of EILP, pre-NK progenitor (pre-NKP) develops into re-defined NK progenitor (rNKP) that expresses CD122, which couples with CD132 to form the IL-2/IL-15 receptor, allowing NK cells to respond to IL-15. rNKP then develops through an immature stage (iNK) to become mature NK cells (mNK). Nfil3, Tcf1, Ets1, Id2, Eomes, T-bet and Zeb2 governs NK cell development from EILP to mNK cells

invading pathogens, such as murine cytomegalovirus (MCMV) [27, 28]. The most iNK-cell-proximal mNK cells are $CD27^+CD11b^-$, produce IFN-γ and TNF-α when activated, but are not yet fully cytotoxic effector cells. Cytotoxic capacity improves with NK cell maturation by type I interferons (IFN-α or IFN-β) or proinflammatory cytokines IL-2, IL-15, IL-12, and IL-18, which upregulate CD11b through T-bet and zinc finger E-box-binding homeobox 2 (Zeb2) [29, 30]. Of note, iNK cells in the bone marrow differentiate through four stages sequentially as $CD27^-CD11b^-$, $CD27^+CD11b^-$, $CD27^+CD11b^+$, and $CD27^-CD11b^+$ [31, 32]. Besides CD11b and Dx5, mature NK cells also highly express KLRG1, CD62L, and CD43 [32].

Apart from Tcf1 and Nfil3 [8, 13–15, 17, 33] required for EILP commitment, Ets1, Id2, Eomes, and T-bet are transcription factors essential for NK cell development. Ets1, required for early NK cell lineage commitment, induces *Id2*, *Tbx21*, and *Il2rb* (CD122) expression [34–36]. Id2 suppresses E protein target genes (e.g., *Socs3*, *Tcf7*, *Cxcr5*), and the suppression of Socs3 promotes NK cell response to IL-15 [37, 38]. IL-15 is crucial for NK cell survival through the induction of the anti-apoptotic protein Bcl-2 [39, 40]. Eomesodermin (Eomes) and T-bet are members of the T-box family of transcription factors and are required by iNK and mNK cells [41]. However, tissue-resident NK cells may exhibit different developmental reliance on T-bet and Eomes [42].

NK cell maturation and function are regulated by an additional group of transcription factors. These include the Ets family protein myeloid elf-1-like factor (Mef, also known as ELF4) [43] and PU.1 (encoded by *Spi1*) [44], which respectively regulate perforin expression and NK cell proliferation in response to IL-2 and IL-12. PR domain zinc finger protein 1 (Blimp1, encoded by *Prdm1*), induced by IL-15 in a T-bet-dependent manner during early NK cell development, promotes granzyme B expression but inhibits NK cell maturation and proliferation to low concentrations of IL-15 [45]. Tox regulates mNK development partially through the induction of Id2 [46]. The Ikaros family member Aiolos (encoded by *Ikzf3*) promotes IFN-γ expression; however, its absence enhances the ability of NK cells to control tumor cells [47]. Kruppel-like factor 2 (Klf2) restricts iNK cell proliferation but is required for migration of NK cells toward IL-15-rich microenvironment [48]. IFN regulatory factor 2 (Irf2) is required for NK cell maturation in the periphery and survival in bone marrow. At homeostatic state, Gata3 is required for bone marrow NK cell maturation from $CD27^{+}CD11b^{-}$ stage and for bone marrow egress, liver migration, and IFN-γ expression. In the face of infection, Gata3-deficient NK cells demonstrated inferior control of *Listeria monocytogenes* burden in the liver [49]. However, Gata3-deficient NK cells exhibited superior activity toward MCMV due to increased CD25 expression [50]. Discrepancies regarding forkhead box protein O1 (Foxo1) exist in the literature. In one report, Foxo1 was shown to be required for iNK cell survival by inducing autophagy that removes damaged mitochondria and intracellular reactive oxygen species (ROS) [51]. In another report, however, Foxo1 inhibited late-stage NK cell maturation and function by downregulating *Tbx21* expression [52].

11.4 Tissue-Resident NK Cells Acquire Unique Phenotype and May Have Distinct Developmental Pathways

Tissue-resident NK (trNK) cells often express CD69, CD103 (αE integrin), and CD49a (α1 integrin), which are involved in retaining NK cells in the tissues. CD69 inhibits type I interferon-induced expression of sphingosine-1-phosphate receptor 1 (S1P1). S1P1 and S1P5 on NK cells binds to sphingosine-1-phosphate (S1P), which

forms a gradient with the highest concentration in peripheral blood and, thereby, promotes egress of lymphocytes from tissues into the blood [53, 54]. CD103 forms a heterodimer with β7 integrin and binds to E-cadherin on epithelial cells [55]. CD49a forms a heterodimer with β1 integrin and binds to collagen [56]. The expression of both CD103 and CD49a is regulated by transforming growth factor-β (TGF-β1) [57]. Development of trNK cells may be different from conventional blood NK cells. $CD49a^{+}$ $DX5^{-}$ $Trail^{+}$ trNK cells in the mouse liver express higher amount of TNF-α and GM-CSF than blood and spleen conventional NK cells, and they develop in a T-bet-dependent manner in the absence of Nfil3 [41, 42]. $CD49a^{+}DX5^{-}$ NK cells that resemble liver trNK cells are also observed in the mouse uterus and skin [42]. In contrast, salivary glands [58] and uterine NK cells [59–61] develop require Eomes in the absence of Nfil3. In addition, a population of $CD127^{+}$ NK cells develop in Gata3- and IL-7-dependent manner independently from T-cell precursors in the mouse thymus, and thymic trNK cells demonstrate reduced granzyme B but increased IFN-γ, GM-CSF, and TNF expression [62, 63].

11.5 NK Cell Diversity and Activity Are Regulated by Variegated Surface Receptors

The activities of NK cells are regulated by various germ line-encoded activating or inhibitory receptors (Table 11.1), many of which are expressed in stochastic patterns, resulting in many subsets of functionally distinct NK cells [64–66]. The families of NK receptors that recognize MHC class I include the murine Ly49 receptors, the primate killer cell immunoglobulin-like receptors (KIRs), and the CD94-NKG2 receptors in both rodents and primates [65]. Inhibitory receptors in humans and rodents normally contain one or more intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) that can activate downstream SHP-1, SHP-2, and SHIP phosphatase [67, 68]. Many of the activating receptors lack intracellular signaling motifs and transduce signals via the association with immunoreceptor tyrosine-based activating motif (ITAM)-containing adapters DAP12, FcεRγ, and CD3ζ, which recruit and activate Syk or ZAP70 tyrosine kinases [69]. NKG2D ligands are self-proteins related to MHC class I molecules. They are generally absent on the cell surface of healthy cells but are frequently upregulated upon cellular stress [70]. NKG2D recruits DAP10 and mediates signaling through the activation of PI3K [71, 72] and ERK [73]. Human KIRs contain either two (KIR2D) or three (KIR3D) extracellular immunoglobulin (Ig)-like domains. They are designated as KIR2DL or KIR3DL, respectively, if they possess a long cytoplasmic domain containing ITIM motif. KIR2DS and KIR3DS have short cytoplasmic domains lacking ITIM but associate through a charged residue in their transmembrane regions with DAP12 or FceRIγ, respectively. KIR2D receptors typically recognize human HLA-C alleles,

Table 11.1 The activating and inhibiting NK receptors on mouse and human NK cells

Activating NK receptors				Inhibiting NK receptors			
Gene	Common name	Ligand	Species	Gene	Common name	Ligand	Species
Klra4	Ly49D	H-2D^{d}	NOD Hamster	*Klra1,3,7,9*	Ly49A, C, G2, I	H-2D^{d} H2-M3	NOD B6 BALB
Klra8	Ly49H	MCMV-m157	NOD B6	*Klrb1b, d*	NKR-P1B, D	Ocil (Clr-b)	mouse
Klra16	Ly49P	MCMV	129	*Lilrb4*	Gp49b1	αvβ3 integrin	Mouse
Klrb1c	NK1.1 NKR-P1C		Mouse	*Pilra*	PILRa	O-glycosylated CD99	Mouse
Klrb1f	NKR-P1F	Clrg	Mouse	*SIGLEC-E*		Sialic acid	Mouse
Pilrb1	PILRβ	o-glycosylation CD99	Mouse	*CD244*	2B4	CD48	Mouse
ITGAL	LFA-1 CD11a	ICAM-1, 2, 3	Mouse Human	*KLRG1*	Mafa	E-, N-, R-cadherins	Mouse Human
KLRD1-KLRC2,3	CD94-NKG2C, E	Mouse Qa-1^{b}; Human HLA-E	Mouse Human	*KLRD1-KLRC1*	CD94-NKG2A	Mouse Qa-1^{b} Human HLA-E	Mouse Human
KLRK1	NKG2D	Mouse Rae-1, H60, MULT1; Human MICA, MICB, ULBP1-6	Mouse Human	*LAIR1*	LAIR-1 CD305	Collagen XVII	Mouse Human
NCR1	NKp46	Hemagglutinin	Mouse Human	*KLRB1*	NKR-P1A, CD161	LLT1 (CLEC2D)	Human
FCGR3	CD16	IgG	Mouse Human	*LILRB1*	ILT2 LIR1 CD85j	HLA-class I	Human

(continued)

Table 11.1 (continued)

Activating NK receptors				Inhibiting NK receptors			
Gene	Common name	Ligand	Species	Gene	Common name	Ligand	Species
CD226	DNAM-1	CD112 CD155	Mouse Human	*KIR2DL1-3,5*	CD158	HLA-C	Human
CD2	LFA-2	CD58	Human	*KIR3DL1,2*	CD158	HLA-Bw4 Some HLA-A	Human
CD244	2B4	CD48	Human	*CEACAM1*	CD66a	CD66	Human
CD48		2B4	Human	*SIGLEC7*	CDw328	Ganglioside GD3	Human
KLRF2	NKp65	KACL	Human	*SIGLEC9*		Sialic acid	Human
KLRF1	NKp80	AICL	Human	*SIGLEC10*		CD52	Human
NCR2	NKp44		Human	*TIGIT*		CD155/PVR CD112/Nectin-2/ CD112	Human
NKC3	Nkp30	B7-H6	Human	*CD96*		CD155	
KIR2DS	CD158	HLA-class I	Human				
KIR3DS	CD158	HLA-class I	Human				
SLAMF7	CRACC	CRACC	Human				
SLAMF6	NTB-A Ly108 CD352	NTB-A					

whereas KIR3D receptors recognize HLA-B or some HLA-A alleles [74, 75]. The NKG2 family contains one inhibitory NKG2A and two activating members NKG2C and NKG2E. The CD94-NKG2 receptors recognize nonclassical MHC-I that is HLA-E in humans and its ortholog Qa-1 in mice [76–78]. A subset of human NK cells express KIR-related inhibitory receptor, LILRB1, which recognizes a shared epitope in all human MHC class I proteins [79].

NK cells also express activating and inhibitory receptors that recognize non-MHC ligands [80]. For example, murine CD244 (2B4) recognizes CD48, an interaction essential for the IL-2-driven expansion and activation of NK cells [81]; human NKR-P1A (CD161) recognizes the lectin-like transcript-1 (LLT1, encoded by *Clec2d*), which is expressed on activated dendritic cells and B cells and inhibits NK cell cytotoxicity and IFN-γ expression [82, 83]; killer cell lectin-like receptor G1 (KLRG1) recognizes cadherins and mediates 'missing-self' education [84]; Gp49B1 recognizes αvβ3 integrin and inhibits IFN-γ expression [85, 86]. The activating DNAX accessory molecule-1 (DNAM-1, also known as CD226) [87–89] and the inhibiting T-cell immunoreceptor with Ig and ITIM domains (TIGIT) [90, 91] receptors both recognize poliovirus receptor (PVR, also known as CD155) and poliovirus receptor-related 2 (PVRL2, also known as nectin-2 and CD112), which are frequently expressed on transformed or stressed cells.

During NK cell development, the expression of self-MHC class I-reactive inhibitory receptors 'licenses' NK cells. Under physiological conditions, licensed NK cells engage through the Ly49 and KIR inhibitory receptors with MHC class I and prevent NK cells from attacking self, and this self-tolerance is mediated through the recruitment of SHP-1, SHP-2, and SHIP phosphatase [67, 68]. Interestingly, licensed NK cells are more potent in their cytotoxicity toward MHC class I-deficient target cells and secrete more IFN-γ and TNF-a under noninflammatory conditions [92, 93]. During infection, however, inhibitory receptor engagement impairs the ability of licensed NK cells to control cytomegalovirus (CMV) infection [93]. The absence of inhibitory receptors on NK cells can have a beneficiary effect in human leukemia patients receiving irradiation therapy followed by bone marrow transplantation. The absence or mismatch of donor NK inhibitory KIR receptors with recipient MHC-I was associated with better leukemic cell clearance and graft acceptance [94].

Activating receptors have the ability to recognize 'altered-self', which is often induced on malignant or stressed cells [95], and trigger NK cells to kill their targets. NK cells mediate target-cell killing by a number of mechanisms, including (1) the secretion of cytokines, (2) exocytosis of cytoplasmic granules containing perforin and enzyme, (3) FAS ligand and TNF-related apoptosis ligand (TRAIL)-mediated induction of apoptosis, and (4) CD16 cross-linking and antibody-dependent cell-mediated cytotoxicity (ADCC) [94]. However, when NK cells are chronically exposed to endogenous, as well as foreign ligands recognized by their activating receptors, they are tolerated through either receptor downregulation or hyporesponsiveness [65]. NK cell tolerance mediated by activating receptors is reversible and can be broken in the presence of inflammatory cytokines or infection. For instance, in C57BL/6 mice receiving MCMV infection, initially both licensed and

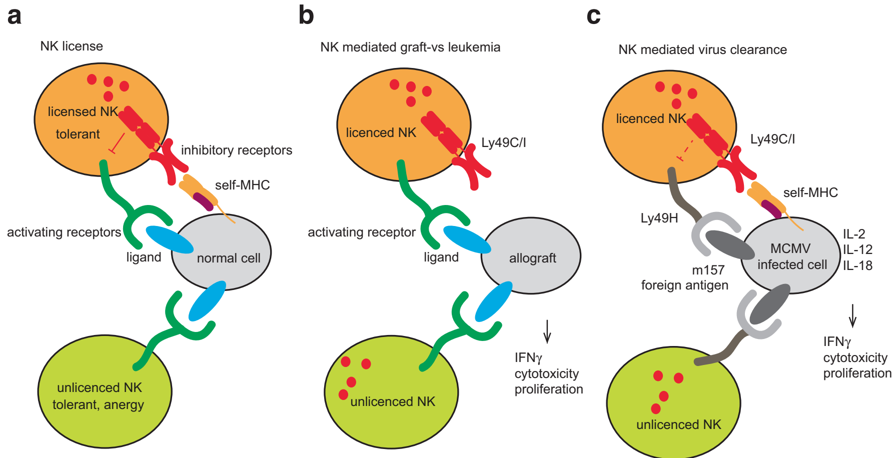

Fig. 11.2 NK cell license, activation and inhibition
(**a**) NK cell license occurs with the expression of self-MHC class I-reactive inhibitory receptors, Ly49 in mice and KIR in man. This prevents NK cells from attacking self. In the absence of inhibitory receptors, chronic exposure of activating receptors with their ligands can also render NK cell hyporesponsive. (**b**) NK cell activation takes place under instances of human leukemia patients receiving irradiation therapy followed by bone marrow transplantation. The absence or mismatch of donor inhibitory NK receptors with recipient MHC-class I promotes leukemic cell clearance by both licensed and unlicensed NK cells. (**c**) During viral infection, inhibitory receptors on licensed NK cells inhibit their proliferation burst, and under these circumstances, unlicensed NK cells are the main mediators of viral clearance

unlicensed NK cells expressed CD69 and upregulated IFN-γ and granzyme B at similar level, but, subsequently unlicensed NK cells predominated in numbers and were the main mediators of viral clearance. The engagement of the activating Ly49H receptor with MCMV-encoded glycoprotein m157 on infected cells promoted unlicensed NK cells to undergo a proliferative burst, but the inhibitory receptors on licensed NK cells restrained the proliferation through SHP-1 phosphatase signaling [68, 93] (Fig. 11.2).

11.6 NK Cells Participate in Tissue Remodeling in Humans and Undergo Clonal-Like Expansion During Viral Infection

A mouse analog of human NK progenitor has been defined as $Lin^{-}CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$, which selectively gives rise to NK cells *in vitro* and *in vivo* [96]. Circulating human NK cells are a diverse population. In any given individual, the diversity is generated by the developmentally distinct NK cell subsets, KIR gene content, polymorphisms, and copy number variations [64], with differentiation and reprogramming in response to tissue-specific environment and infections [97]. Transcriptional, telomere length, and transfer of human NK cells

into NOD/SCID/$\gamma c^{-/-}$ mice have demonstrated that circulating NK cells in human blood display sequential $CD56^{bright}$ $CD62L^{+}$, $CD56^{dim}CD62L^{+}$ $CD94^{high}$, and $CD56^{dim}CD62L^{-}$ $CD94^{low}$ developing stages [98, 99]. $CD56^{bright}$ $CD62L^{+}$ NK cells are mostly KIR^{-} $NKG2A^{+}CD27^{dim}$ $CD57^{-}CD16^{+/-}$ but express CD127 and CD117 (also known as KIT and SCFR), which are also hallmarks of non-NK ILCs [2, 100]. Upon stimulation with combinations of IL-12, IL-15, and IL-18, $CD56^{bright}$ $CD62L^{+}$ and $CD56^{dim}CD62L^{+}$ NK cells strongly proliferate and produce significantly greater amount of IFN-γ than $CD56^{dim}CD62L^{-}$ NK cells. However, engagement of the activating receptors evokes more prominent chemokine (MIP-1α, MIP-1β and RANTES) and cytokine (IFN-γ) expression and NK cell cytotoxicity in $CD56^{dim}CD62L^{+}$ and $CD56^{dim}CD62L^{-}$ cells. [98, 101, 102]. $CD56^{dim}$ NK cells can further develop with the sequential loss of NKG2A and the acquisition of KIRs and CD57 [103]. $CD56^{dim}CD57^{+}$ NK cells have increased cytotoxic capacity than $CD56^{dim}CD57^{-}$ NK when they are activated through CD16 [104].

In parallel to mice, human tissue-resident NK cells also express CD69, CD103, and CD49a, and they may derive directly from progenitors that reside within the tissues [97]. NK cells are found at high frequencies in the endometrium of human uterus and decidua in the first trimester of pregnancy. Throughout the second half of the menstrual cycle, progesterone from the ovaries acts on uterine stromal cells, which in turn secrete IL-15 and support uterine NK cell proliferation [105]. During pregnancy, a key role for $CD56b^{right}$ uNK cells is to promote trophoblast invasion and maternal spiral artery remodeling, which is mediated through the production of IL-8, interferon-inducible protein-10 (IP10), and an array of angiogenic factors including vascular endothelial growth factor A (VEGF-A), VEGF-C, and angiopoietins [99, 106]. Critically, fetal trophoblasts, which come into direct contact with maternal blood and tissues during pregnancy, are exempt from uNK-mediated cell killing. Uterine $CD56^{bright}$ $CD49a^{+}CD103^{+}CD9^{+}$ NK cells express perforin, granzymes A and B, and the activating receptors NKp30, NKp44, NKp46, NKG2D but are unable to form mature activating synapses and thus are not cytotoxic [107, 108]. Furthermore, the high expression of inhibitory KIRs (KIR2DL1, KIR2DL2, KIR2DL3), the CD94-NKG2A receptor complex, and the LILRB1 inhibit NK cell activation through the recognition of HLA-C, HLA-E, and HLA-G expressed on the extravillous trophoblasts, respectively [107, 109]. Interestingly, primary villous trophoblasts do not express HLA, and extravillous trophoblasts are devoid of HLA-A and HLA-B.

In liver sinusoids, NK cells represent up to 30–40% of all hepatic lymphocytes [110], and $CD56^{bright}$ and $CD56^{dim}$ cells are present in equal proportions [111, 112]. Hepatic $CD56^{bright}$ NK cells express CD69 and are tissue resident [113, 114]. Liver resident macrophages (Kupffer cells) interact with NK cells to keep immune tolerance to nonpathogenic antigens from food and LPS from gut commensal bacteria, but remain alert to infections by pathogens and viruses. In recognition of bacterial cell wall products via TLR2/4 -MyD88, Kupffer cells secrete IL-10 and blunt NK cell activation [115]. However, when DNA or RNA viruses activate the TLR3-TRIF-IRF-3 [115] or TLR8 pathways [116], Kupffer cells elicit potent IFN-γ and TNF expression in $CD56^{bright}$ trNK cells. Intrahepatic NK cells also mediate target-

cell killing through the expression of TRAIL, whose expression is correlated with the control of hepatitis C virus (HCV) infection [117]. But during HBV infection, TRAIL also causes liver damage and can eliminate antigen-specific T cells [118, 119].

Clonal-like expansion and memory formation of NK cells have been observed in humans with cytomegalovirus (HCMV) [120–123], chikungunya virus (CHIKV) [124] and hantavirus [125] infections. Clonal-expanded cells are characterized by the expression of NKG2C, CD57, and activating KIRs (KIR2DS4, KIR2DS2, KIR3DS1), a general lack of the expression of inhibitory NKG2A and KIR3DL1 receptors (in individuals expressing its HLA-Bw4 ligand), and the decreased expression of CD161 (also known as KLRB1), NKp30, NKp46, and CD7. A subset of clonal-expanded NK cells can further acquire adaptive phenotypes that resembles more with cytotoxic $CD8^+$ T lymphocytes than conventional NK cells. The intronic region of *ZBTB16* in adaptive NK cells is hypermethylated, which is correlated with the decreased expression of PLZF and its target genes encoding FcεRγ, SYK, and EAT-2. Adaptive PLZF-deficient NK cells are distinct from clonal-expanded NK cells expressing CD57, NKG2C and PLZF, and produce less IFN-γ upon cytokine stimulation with IL-12 and IL-18 [126].

11.7 Conclusion

NK cells are a heterogeneous population of innate lymphocytes that develop from the common lymphoid progenitors. Tissue-resident NK cells may have different developmental origins and are phenotypically distinct from their blood counterparts. NK cells employ both inhibiting and activating receptors for 'missing-self' education, activation, and terminal differentiation. In humans, NK cells are critical for the implantation of the embryos and for the control of neoplastic transformation and viral infections, but they may also induce collateral damages to the tissues. Despite lacking rearranged antigen-specific receptors, NK cells can acquire adaptive T-cell features by clonal-like expansion and alteration in their DNA methylation profiles during viral infections.

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