# Cytoplasmic Sensing in Innate Immunity

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## Introduction

The vertebrate immune system is composed of two major subdivisions, the innate or inborn immune system and the adaptive or acquired immune system. The innate immune system is our first line of defense against pathogens, has anatomical features that serve as barriers to infection (such as the skin and epithelia or mucous membranes lining the respiratory, gastrointestinal, and urinary tracts) and includes defenses that are constitutively present and ready to be deployed upon infection. The adaptive immune system on the other hand is antigen specific and confers protection against reexposure to the same pathogen. Cells of the innate immune system such as epithelial cells, macrophages, neutrophils, and dendritic cells (DCs) detect pathogens by employing a network of germline-encoded sensors that are present on the cell surface or in the cytoplasm. These are collectively called pattern recognition receptors (PRRs) to denote their ability to recognize conserved microbial structures or pathogen-associated molecular patterns (PAMPS). While evolutionarily believed to sense ligands of microbial origin, some PRRs can also detect endogenous danger signals released from damaged tissues (danger-associated molecular patterns; DAMPS). The major classes of innate sensors include Toll-like receptors (TLRs), nucleotide oligomerization domain leucine-rich repeat receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene I-like receptors (RLRs), as well as several putative sensors of intracellular DNA such as absent in melanoma 2 (AIM2), stimulator of interferon genes (STING) and DNA-dependent activator of interferon regulatory factors (DAI) (Ishii *et al.*, 2008). Upon activation by PAMPS or DAMPS, PRRs initiate innate immune activities and instruct subsequent adaptive immune responses, enabling both short-term effector function as well as long-lasting immunological memory. The PAMPS recognized and the subsequent immune responses initiated vary depending upon the invading microbe and the PRR triggered; however signaling generally converges on activation of nuclear transcription factors such as nuclear factor-$\kappa$B (NF-$\kappa$B) and interferon regulatory factors (IRFs) leading to pro-inflammatory cytokine production, or formation of oligomeric signaling complexes called inflammasomes that activate caspase-1 leading to generation of bioactive interleukin (IL)-1$\beta$, IL-18 and pyroptosis. Exquisite coordination of several innate immune pathways determines the quality, magnitude, and duration of the ensuing host response, ultimately leading to containment of invading microorganisms. Apart from its well appreciated role in host defense, innate signaling is also emerging as a critical factor in human inflammatory disease. Indeed, the unrestrained inflammatory response propagated by innate sensors can be detrimental and has been implicated in the development of a variety of immune disorders including atherosclerosis, Type 1 diabetes, inflammatory bowel disease, and systemic lupus erythematosus (SLE) (Li *et al.*, 2009; Holm *et al.*, 2013; Zhong *et al.*, 2013). In this article we review current information on innate immune signaling with a focus on cytoplasmic sensing. In 'Classes, Structure, and Activation of Innate Sensors,' we introduce the major classes of innate sensors, their signaling pathways and role in mediating protective host defense and/or autoimmunity. In 'Role of Intracellular Organelles and Spatial Relocation,' we discuss the role of subcellular structures and spatial patterns of intracellular movement in generation and/or propagation of the innate immune response. In 'Combinatorial Sensing and PRR Crosstalk,' we discuss the crosstalk between different PRR pathways and integration of signals from multiple ligands displayed by a microbe. A holistic and predictive understanding of innate immunity requires systems-level integration of information from multiple molecular levels, an aspect that is discussed in 'Multiscale Regulation of Cytosolic Sensing Pathways.'

## Classes, Structure, and Activation of Innate Sensors

### CLRs

CLRs are a large, functionally diverse group of soluble and transmembrane proteins that are primarily involved in detecting a wide range of carbohydrate structures on fungal pathogens, but can also recognize a diverse repertoire of structurally dissimilar microbe-associated or endogenous ligands (Hoving *et al.*, 2014). CLRs are characterized by a C-type lectin domain (CTLD) also referred to as a carbohydrate recognition domain (CRD) in cases where carbohydrates are recognized (**Figure 1**). The CLR family encompasses more than 1000 identified members with diverse functions including endocytosis, phagocytosis, cell adhesion, complement activation, tissue remodeling, antimicrobial, pro-inflammatory, and anti-inflammatory responses. However, based on their molecular structure CLRs can be classified into three notable groups: two groups of membrane-bound CLRs namely Type I CLRs that contain multiple CRDs, and Type II CLRs that contain a single CRD, and a group of soluble CLRs (**Table 1**). Prominent examples of CLRs from these groups include dectin-1, dectin-2, DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), mincle, DNGR-1 (CLEC9A), and mannose-binding lectin (MBL). CLRs may be activatory or inhibitory based upon their ability to associate with signaling molecules or the presence of specific motifs in their cytoplasmic tails, which can be one of at least four different types: immunoreceptor tyrosine-based activation motifs (ITAMs), immunoreceptor tyrosine-based inhibitory motifs (ITIMs), a single tyrosine-based motif (hemiITAM), or tyrosine-independent motif. Upon ligand binding of activatory CLRs, the tyrosine residues of an ITAM are phosphorylated by Src family kinases which in turn promote the recruitment of Syk family kinases culminating in the activation of various cellular responses, notably the activation of NF-$\kappa$B and production of reactive oxygen species (ROS).

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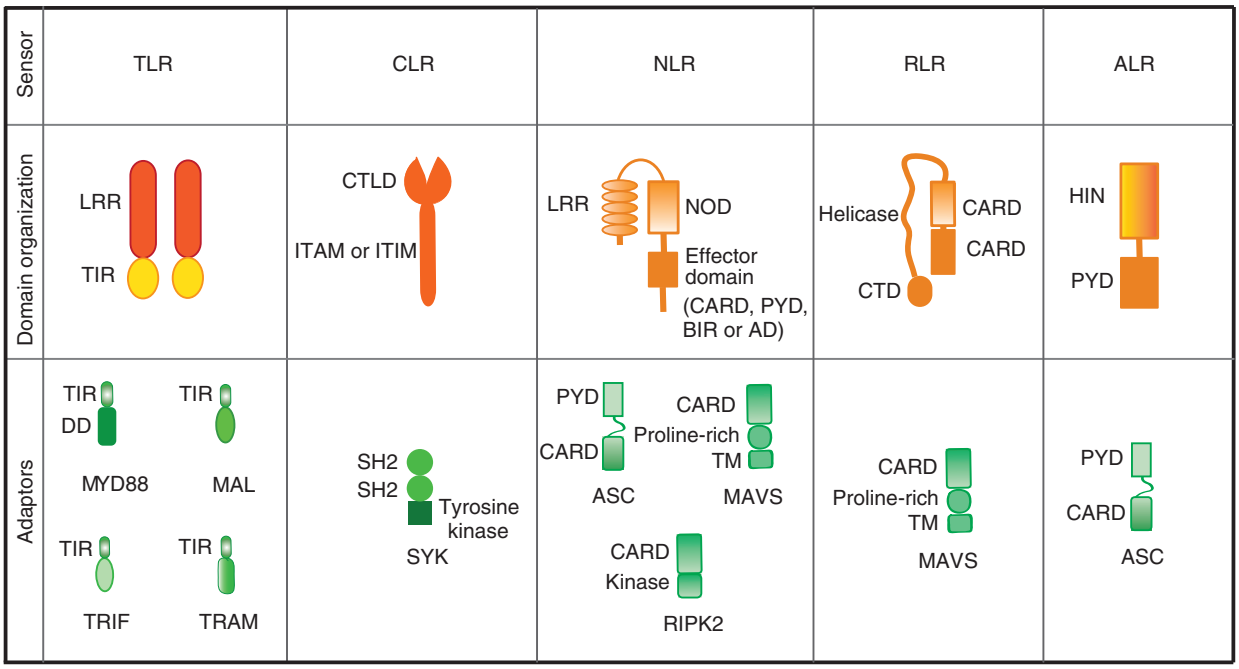



**Figure 1** Chart showing the major classes of innate immune sensors, their general domain organization, and adaptors utilized for signaling. These include the TLRs, NLRs, RLRs, and ALRs. In the case of NLRs, the N-terminal effector region can be a caspase activation and recruitment domain (CARD), pyrin domain (PYD), baculoviral inhibitor of apoptosis repeat (BIR), or transactivator domain (AD) depending upon the NLR. ALR, AIM2-like receptor; ASC, Apoptosis-associated speck-like protein containing CARD; CLR, C-type lectin receptor; CTD, C-terminal domain; CTLD, C-type lectin domain (CTLD); DD, death domain; HIN, hematopoietic expression; interferon-inducible nature; and nuclear localization; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; LRR, Leucine-rich repeats; MAL, MYD88-adaptor-like protein; MAVS, mitochondrial antiviral signaling protein; MYD88, myeloid differentiation primary-response protein 88; NLR, NOD-like receptor; NOD, Nucleotide-binding oligomerization domain; RIPK2, receptor-interacting serine/threonine kinase 2; RLR, RIG-I-like receptor; Syk, Spleen tyrosine kinase; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing interferon-$\beta$.

**Table 1** Summary of select CLRs, their ligands and responses

| *Type* | *Sensor* | *Ligand(s)* | *Response* |
|---|---|---|---|
| Type I | DEC 205 | CpG oligonucleotides, ligands expressed by dying apoptotic or necrotic cells | Antigen presentation, cytokine production |
| | MMR | Mannose, *N*-acetylglucosamines, fucose residues *Pneumocystis carinii, Leishmania donovani* | Phagocytosis, cytokine production, IL-8 in cooperation with TLR2 |
| Type II | Dectin-1 | $\beta$-glucans | Phagocytosis, ROS production, activation of NF-$\kappa\beta$ |
| | Dectin-2 | High mannose-type carbohydrates | and AP-1 through SYK adaptor, ROS production and potassium efflux resulting in NLRP3 inflammasome activation |
| | Mincle | Fungal $\alpha$-mannose, mycobacterial glycolipid trehalose-6′6′-dimycolate | Activation of NF-$\kappa\beta$ and AP-1 through the Fc receptor common $\gamma$-chain (FcR$\gamma$) and SYK |
| | DC-SIGN | Human immunodeficiency virus (HIV)-1, Hepatitis C virus, dengue virus, cytomegalovirus, ebola virus, *Leishmania,* and *Candida* species | Modulation of TLR signaling by acetylation of active NF-$\kappa\beta$ p65 through the serine threonine kinase Raf-1 resulting in anti-inflammatory responses |
| | CLEC9A | DAMPs such as exposed actin filaments of damaged or dead cells | Antigen presentation |
| Soluble | MBL | Repetitive mannose and/or *N*-acetylglucosamine residues, including those on HIV | Phagocytosis, anti-inflammatory cytokines |

Ligand engagement of inhibitory CLRs commonly results in ITIM tyrosine phosphorylation by Src kinases, the recruitment and activation of protein tyrosine phosphatases such as SHP-1 and SHP-2 and the dephosphorylation of substrates leading to the inhibition of cellular activation (Osorio *et al.*, 2011; Redelinghuys and Brown, 2011). CLRs have been well-reviewed elsewhere (Hoving *et al.*, 2014; Osorio *et al.*, 2011) and because they primarily function in recognition of extracellular ligands,

will not be covered in detail in this review. A summary of select CLRs, including their known ligands and signaling responses, is shown in Table 1.

## TLRs

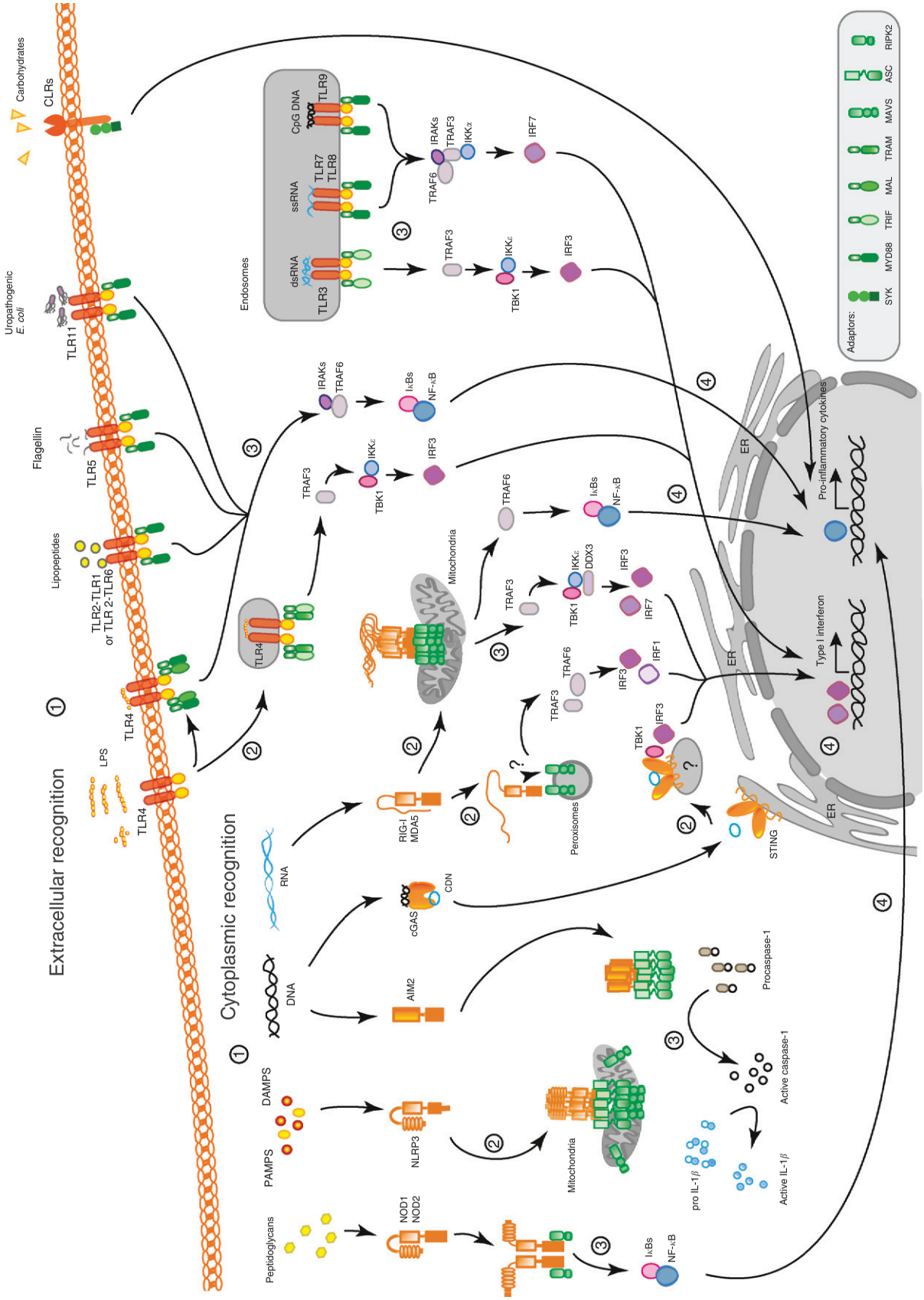
TLRs were the first class of innate sensors to be identified and are the most well characterized PRRs. To date, 13 members of the TLR family have been identified in mammals. These recognize a wide range of PAMPS including LPS (recognized by TLR4); bacterial peptidoglycan, lipoproteins, and fungal zymosan (recognized by TLR2-TLR1 or TLR2-TLR6 heterodimers); bacterial flagellin (recognized by TLR5); unmethylated bacterial or viral CpG DNA (recognized by TLR9); viral single-stranded RNA (recognized by TLR7 and TLR8) and viral double-stranded RNA (recognized by TLR3); and bacterial 23S ribosomal RNA (recognized by TLR13) (Table 2; Akira *et al.*, 2006; Kawai and Akira, 2011). TLRs are type I transmembrane proteins characterized by three distinct domains: an extracellular ligand sensing domain-containing varying numbers of leucine-rich repeat (LRR) motifs that mediate the recognition of PAMPs, a transmembrane region, and a cytoplasmic domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain that activates downstream signaling pathways (Figure 1).

TLRs are expressed on myeloid and lymphoid cells of hematopoietic origin, including macrophages, DCs, B cells, specific types of T cells, and even on non-hematopoietic cells such as fibroblasts and epithelial cells. Moreover, TLRs may be expressed extra- or intracellularly depending upon their PAMP recognition properties. While certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed and bind to their respective ligands on the cell surface, others (TLRs 3, 7, 8, 9, and 13) are found almost exclusively in intracellular compartments such as endosomes where they sense microbial and host-derived nucleic acids (O'Neill *et al.*, 2013; Blasius and Beutler, 2010). TLRs 11 and 12 also localize within endosomal compartments where they are involved in the detection of *Toxoplasma gondii* profilin protein (Yarovinsky, 2014; Andrade *et al.*, 2013; Pifer *et al.*, 2011). All the TLRs are expressed in mice; however, humans lack functional TLR11, TLR12, and TLR13 genes.

TLR signaling is initiated by ligand-induced homodimerization or heterodimerization of receptors. Following dimerization, the TIR domains of TLRs associate with TIR domain-containing adaptor proteins, either myeloid differentiation primary-response protein 88 (MYD88), and MYD88-adaptor-like protein (MAL) also called TIR domain-containing adaptor protein (TIRAP), or TIR domain-containing adaptor protein inducing IFN$\beta$ (TRIF) and TRIF-related adaptor molecule (TRAM). Engagement of these adaptor molecules

**Table 2** Summary of human and mouse TLR ligands, adaptors, responses, and associated human diseases

| *Sensor* | *Ligand(s)* | *Adaptor* | *Response* | *Associated diseases* |
|---|---|---|---|---|
| TLR 1/TLR2 | Triacyl lipopeptides (Pam3CSK4) | MyD88<br>TIRAP/MAL | Inflammatory cytokines | |
| TLR 2/TLR6 | Diacyl lipopeptides<br>Lipoteichoic acid<br>Zymosan<br>Glycolipids<br>GPI anchor<br>Phospholipomannan | MyD88<br>TIRAP/MAL | Inflammatory cytokines<br>Type 1 IFN | Atherosclerosis |
| TLR 3 | Synthetic dsRNA<br>Viral dsRNA | TRIF | Type I IFN | Virus-triggered autoimmune disease |
| TLR 4 | LPS<br>Mannan<br>Glucuronoxylomannan<br>Glycoinositolphospholipids<br>GPI anchor | MyD88<br>TIRAP/MAL<br>TRAM<br>TRIF | Inflammatory cytokines<br>Type 1 IFN | Atherosclerosis |
| TLR 5 | Flagellin | MyD88<br>TRIF | Inflammatory cytokines | |
| TLR 7 | ssRNA<br>imidazoquinoline derivatives (R848) | MyD88 | Inflammatory cytokines<br>Type 1 IFN | Psoriasis, multiple sclerosis, systemic lupus erythematosus |
| TLR 8 | ssRNA<br>imidazoquinoline derivatives (R848) | MyD88 | | Multiple sclerosis |
| TLR 9 | Unmethylated CpG DNA<br>Synthetic CpG oligodinucleotides<br>Hemozoin | MyD88 | Inflammatory cytokines<br>Type 1 IFN | Psoriasis, systemic lupus erythematosus |
| TLR 10 (humans) | Unknown | MyD88 | Activation of NF-$\kappa$B and ENA-789 promoters | |
| TLR11 | *Toxoplasma gondii* profilin protein, uropathogenic *Escherichia coli*, *Salmonella* flagellin | MyD88 | NF-$\kappa$B and IRF8 dependent IL-12 production | |
| TLR12 | *T. gondii* profilin protein | MyD88 | NF-$\kappa$B and IRF8 dependent IL-12 production | |
| TLR13 | Bacterial 23S ribosomal RNA | MyD88 | Activation of NF-$\kappa$B | |


Extracellular recognition
Cytoplasmic recognition
Carbohydrates
CLRs
Uropathogenic E. coli
TLR11
Flagellin
TLR5
Lipopeptides
TLR2-TLR1 or TLR2-TLR6
LPS
TLR4
TLR4
Endosomes
CpG DNA
TLR9
ssRNA
TLR7 TLR8
dsRNA
TLR3
IRAKs
TRAF6
TRAF3
IKKε
TBK1
IRF3
IRF7
IκBs
NF-κB
Mitochondria
DDX3
IRF1
ER
Pro-inflammatory cytokines
Type I interferon
Adaptors:
SYK
MYD88
TRIF
MAL
TRAM
MAVS
ASC
RIPK2
DAMPS
PAMPS
Peptidoglycans
NOD1 NOD2
NLRP3
AIM2
DNA
RNA
cGAS
CDN
RIG-I MDA5
Peroxisomes
STING
Procaspase-1
Active caspase-1
pro IL-1β
Active IL-1β

organizes downstream signaling that involves interactions between IL-1R-associated kinases (IRAKs) and the TNF receptor-associated factors (TRAFs), leading to the activation of the mitogen-activated protein kinases (MAPKs), JUN N-terminal kinase (JNK), p38, and to the activation of transcription factors (Figure 2). Two important families of transcription factors that are activated downstream of TLR signaling are NF-κB and the IRFs, but other transcription factors, such as cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1) may also be activated. A major consequence of TLR signaling is the induction of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), IL-1, IL-6, and IL-12, and in the case of the endosomal TLRs, the induction of type 1 interferon (IFN) (Figure 2; Blasius and Beutler, 2010; O'Neill *et al.*, 2013).

Activation of TLRs on antigen-presenting cells such as macrophages, DCs and B cells, strongly influences the development of adaptive immune responses by controlling several essential processes such as the upregulation of co-stimulatory or accessory molecules like CD80 and CD86, enhancement of antigen presentation, B-cell proliferation and maturation, activation of T cells, and suppression of regulatory T cell activity (Iwasaki and Medzhitov, 2010), thereby mediating effective host defense to a wide range of pathogens. However, an unchecked TLR-induced inflammatory response can be deleterious to the host resulting in a variety of immune disorders. TLRs are implicated in the pathogenesis of autoimmune diseases, such as SLE, rheumatoid arthritis, atherosclerosis, hepatitis, diabetes, kidney disease, and certain other disorders (Li *et al.*, 2009). Recent studies have shown that functional TLRs are expressed not only on immune cells, but also on cancer cells, implicating a role of TLRs in tumor biology. Analogous to their favorable and detrimental roles in inflammation, increasing evidence suggests that TLRs act as a double-edged sword in cancer cells too, pointing to a dual function of TLRs as anti- and pro-tumor modulators (Basith *et al.*, 2012). While unrestrained TLR signaling provides a microenvironment conducive for tumor growth and evasion of the immune response, TLRs can also trigger an antitumor response that inhibits tumor progression and promotes tumor clearance. Thus, apart from their protective role against various pathogens, the involvement of TLRs in autoimmune diseases and tumorigenesis indicates the need for further studies to delve into the potential of targeting TLR signaling for therapeutic benefit under these conditions.

## NLRs

While TLRs detect microbial signatures in the extracellular environment or intracellularly in the lumen of endosomes, NLRs are the largest known family of innate sensors that survey the cytosolic environment. In humans, 23 NLRs have been identified (Harton *et al.*, 2002; Inohara and Nunez, 2003; Table 3). Their structural hallmark is a modular organization of three domains with distinct function: a variable C-terminal LRR domain for ligand binding, a conserved nucleotide binding or oligomerization domain (NBD or NOD), and a variable N-terminal effector or protein interaction domain that mediates downstream signaling cascades (Figure 1). The N-terminal domain mediates homotypic protein–protein interactions and can be one of four different types depending upon the receptor subclass: NLRA or Class II transactivator (CIITA) contains an acidic transactivation domain, NLRBs or neuronal apoptosis inhibitor proteins (NAIPs) contain a baculoviral inhibitor of apoptosis repeat (BIR) domain, NLRCs have a caspase activation and recruitment domain (CARD), and NLRPs possess a pyrin domain (PYD). The NLRX1 effector domain bears no homology to the N-terminal region of any of the above four subclasses and is instead categorized as a CARD-related X effector domain. PAMPS and DAMPS are believed to be sensed by the C-terminal LRR domain. Upon ligand binding, the auto-inhibitory LRR undergoes a conformational change, allowing for protein oligomerization through the central NOD domain. This in turn exposes the N-terminal effector domain, allowing for recruitment of downstream signaling adaptors and effector proteins resulting in formation of an oligomeric complex. The activating stimuli, mechanisms of activation, and response to ligand sensing vary within the NLR family (Table 3) and have been well defined only for a few family members; the physiologic functions and relevant signaling pathways of most members of the NLR family are either unknown or poorly defined. Among the NLRs that have been studied intensively, NLRP3, NLRP1, and NLRC4 form a macromolecular signaling complex called the inflammasome that acts as a scaffold for activation of caspase-1, a key step that leads to processing and secretion of the potent pro-inflammatory cytokines IL-1β and IL-18, while NOD1 and NOD2 form signaling platforms that activate NF-κB (Martinon *et al.*, 2009).

**Figure 2** Overview of cytoplasmic innate sensing and signaling mechanisms. Major signaling cascades triggered by extracellular and intracellular PRRs are shown. Pathogen- or danger-associated stimuli are detected by innate sensors (1), often through combinatorial sensing in which multiple receptors sense ligands from the same microbe. Ligand binding results in conformational changes, followed by self-association and/or autophosphorylation of the receptors, allowing them to bind adaptor proteins. Additional trafficking proteins (not depicted) may guide the active oligomeric complexes to secondary compartments within the cell (2). For instance, TLR4 may be trafficked to early endosomal compartments where it binds the adaptors TRIF and TRAM. Activated RIG-I and NLRP3 associate with MAVS on the mitochondrial surface; RIG-I may additionally associate with MAVS on the peroxisomes. STING moves from the ER to a secondary structure that has, as yet, not been fully identified and is therefore depicted with a '?'. Adaptor proteins may allow for assembly of large, oligomeric, multiprotein signaling complexes capable of activating downstream mediators and transcription factors (TFs) resulting in a functional response (3). For example, activated NLRP3 and AIM2 form structures called inflammasomes that serve as platforms for activation of caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18 to produce active cytokines. Activated RIG-I induces polymerization of MAVS into large self-propagating prion-like structures that trigger phosphorylation cascades ultimately leading to activation of the TFs IRF3 and IRF7. The nature of RIG-I and MDA5 interactions with adaptor proteins on the peroxisome is not yet fully understood and is therefore depicted with a '?'. IRF3 and IRF7 translocate to the nucleus and initiate transcription of type 1 IFN (4). The TF NF-κB is also activated by RIG-I-MAVS interaction, ultimately leading to transcription of a number of pro-inflammatory cytokines. Combinatorial sensing of diverse ligands can result in simultaneous or sequential activation of multiple pathways, which may be important for generating a tailored, specific response to a potential pathogen.

**Table 3** Summary of human NLRs, their activators, responses, and associated diseases

| *Effector domain* | *Sensor* | *Activator(s)* | *Response* | *Associated diseases* |
|---|---|---|---|---|
| CARD | CIITA | Unknown | MHC class II transcriptional activation | Bone density defects, Hodgkin's lymphoma, B-cell lymphoma, celiac disease, arthritis, multiple sclerosis, primary adrenal insufficiency, systemic lupus erythematosus, diabetes |
| | NOD1 | Meso-diaminopimelic acid (DAP), $\gamma$-D-glutamyl-meso-diaminopimelic acid (iE-DAP), meso-lanthionine, GM-tripeptide, FK156, FK565 | NF-$\kappa$B activation through IKK complex; MAP kinase pathway activation leading to pro-inflammatory responses | Inflammatory bowel disease, atopic eczema, asthma |
| | NOD2 | Muramyl dipeptide (MDP), M-TRI$_{LYS}$ | NF-$\kappa$B activation through IKK complex; MAP kinase pathway activation leading to pro-inflammatory responses | GVHD, Blau disease, Crohn's disease, asthma, atopic dermatitis, arthritis, sarcoidosis, prostate and endometrial cancer, gastric lymphoma, leprosy |
| | NLRC4 | Flagellin, Type III secretion system components | Inflammasome formation resulting in caspase-1 activation and cleavage of IL-1$\beta$ and IL-18 to their active forms | Susceptibility to bacterial infections |
| | NLRC3 | Unknown | Negative regulator of T cell activation, TLR, and STING signaling | |
| | NLRC5 | Unknown; NLRP3 agonists, including bacterial PAMPs and crystals have been reported as elicitors in cell culture systems | Transcriptional regulator of MHC class I. Other functions are not fully understood because response appears to vary with species, context, and cell type | Susceptibility to viral infections |
| | NLRX1 | Unknown | Activates NF-$\kappa$B and ROS through JNK pathway, negatively regulates RIG-I-mediated IFN response and LPS-elicited TRAF6-IKK signaling pathways | Susceptibility to chronic hepatitis B infection, gastric cancer |
| PYD | NLRP1 | Muramyl dipeptide, *Bacillus anthracis* lethal toxin | Inflammasome assembly resulting in caspase-1 activation and production of active IL-1$\beta$ and IL-18 | Skin autoimmune disorders, vitiligo, Addison's disease, diabetes, celiac disease, autoimmune thyroid disorders, systemic lupus erythematosus, systemic sclerosis, giant cell arteritis, congenital toxoplasmosis, Alzheimer's disease, corneal intraepithelial dyskeratosis, arthritis |
| | NLRP2 | Unknown | | Beckwith–Wiedemann syndrome, GVHD |
| | NLRP3 | Crystals (monosodium urate, calcium pyrophosphate dihydrate, cholesterol, asbestos, silica, hydroxyapatite), amyloid $\beta$, mitochondrial DNA, cardiolipin, ceramides, ATP, ROS, RNA viruses, bacterial toxins | Inflammasome assembly resulting in caspase-1 activation and production of active IL-1$\beta$ and IL-18 | Gout, Cryopyrin-associated periodic fever syndromes (CAPS), diabetes, celiac disease, psoriasis, inflammatory bowel diseases, Alzheimer's disease, atherosclerosis |
| | NLRP4 | Unknown | Unknown, possible suppressor of NF-$\kappa$B | |
| | NLRP5 | Unknown | Unknown | Familial biparental hydatidiform moles |

(*Continued*)

**Table 3** Continued

| *Effector domain* | *Sensor* | *Activator(s)* | *Response* | *Associated diseases* |
|---|---|---|---|---|
| | NLRP6 | Unknown | Unknown, possible negative regulator of NF-κB and IL-1β, an NLRP6 inflammasome has been proposed in mouse colonic epithelial cells | Metabolic syndrome-associated abnormalities, colitis, and colon cancer |
| | NLRP7 | Bacterial acylated lipoproteins (acLP) | Unknown, possible negative regulator of IL-1β, inflammasome assembly in response to bacterial acLP | Familial biparental hydatidiform moles, abnormal pregnancies and embryonic development, testicular and endometrial cancer |
| | NLRP8 | Unknown | Unknown | Alzheimer's disease, colorectal cancer |
| | NLRP9 | Unknown | Unknown | Abnormal embryonic development |
| | NLRP10 | Unknown | Negatively regulates caspase-1 activation, regulation of DC emigration from inflamed tissues in mice | Atopic dermatitis |
| | NLRP11 | Unknown | Unknown | |
| | NLRP12 | Unknown | Negatively regulates NF-κB activation | Hereditary fever syndromes, dermatitis |
| | NLRP13 | Unknown | Unknown | Abnormal embryonic development |
| | NLRP14 | Unknown | Unknown | Familial biparental hydatidiform moles, spermatogenic failure |
| BIR | NAIP | Type III secretion system needle proteins | Formation of a large oligomeric complex with NLRC4 resulting in caspase-1 activation and production of IL-1β | Spinal muscle atrophy |

The current paradigm for NLR activation is self-oligomerization followed by recruitment of adaptor proteins that mediate activation of downstream effectors. In the case of NOD1 and NOD2, two well-studied NLRCs that sense different components of peptidoglycan from Gram-negative and Gram-positive bacteria (Elinav *et al.*, 2011), ligand binding allows for the recruitment of the receptor-interacting serine-threonine kinase 2 (RIPK2) adaptor protein through the exposed CARD subsequently leading to polyubiquitination of the inhibitor of NF-κB kinase subunit gamma (IKKγ). This in turn culminates in activation of the IκB kinase (IKK) complex, IκBα phosphorylation followed by its degradation and activation of NF-κB (Windheim *et al.*, 2007; Abbott *et al.*, 2007; **Figure 2**). NOD1 or NOD2 activation also results in the activation of MAP kinases such as ERK-1, ERK-2, JNK, and p38, which coordinate with NF-κB to upregulate the expression of pro-inflammatory molecules (Girardin *et al.*, 2001; Pauleau and Murray, 2003; Park *et al.*, 2007). In addition, activation of NOD2 by single-stranded viral RNA from respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), and influenza virus has been shown to trigger a noncanonical signaling pathway that requires the mitochondrial antiviral signaling protein (MAVS) and induces IRF3 activity, leading to the production of type 1 IFN (Sabbah *et al.*, 2009). In the case of NLRP3, conformational changes in the molecule following ligand sensing expose the PYD domain resulting in interaction with a similar domain on the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), which through homotypic CARD domain interactions associates with procaspase-1 resulting in its auto-catalytic cleavage to enzymatically active caspase-1 (Agostini *et al.*, 2004). The latter then cleaves the pro forms of IL-1β and IL-18 to produce active cytokine. This process is amplified by a second adaptor protein MAVS in response to noncrystalline NLRP3 activators like poly I:C (Subramanian *et al.*, 2013). In contrast to NLRP3, NLRP1, and NLRC4 contain a CARD domain that allows them to associate directly with procaspase-1. NLRC4 and NLRP1 may also recruit ASC, resulting in augmented inflammasome activity; however ASC is not absolutely required for inflammasome assembly (Lamkanfi and Dixit, 2014; Schroder and Tschopp, 2010; Hsu *et al.*, 2008).

Among the inflammasome-forming NLRs, NLRP3 is perhaps the best studied. NLRP3 inflammasome formation appears to require at least two distinct signals. The first signal is an NF-κB activating stimulus, which leads to transcriptional upregulation of pro-IL-1β and NLRP3. This is often referred to as a 'priming' signal and may consist of a TLR activator such as LPS (Bauernfeind *et al.*, 2009). A second signal then activates NLRP3 resulting in inflammasome assembly (Lamkanfi and Dixit, 2014). The nature of this activating stimulus can be diverse. A variety of signals associated with infection or metabolic dysfunction can activate NLRP3, ranging from crystalline substances like monosodium urate, cholesterol, calcium pyrophosphate dehydrate, silica or alum crystals, to noncrystalline activators such as RNA or bacterial toxins, and endogenous danger signals like ATP, mitochondrial DNA, cardiolipin, ceramides, low cytosolic potassium, and increased intracellular calcium (Dostert *et al.*, 2008; Cassel *et al.*, 2008;

Cruz *et al.*, 2007; Lee *et al.*, 2012). How these chemically and structurally distinct stimuli elicit a response from a single sensor is unclear (Gross *et al.*, 2011). One proposed common feature of NLRP3 activators is induction of ROS that may lead to the generation of a potential ligand of NLRP3 or may modify NLRP3 or associated proteins directly (Cruz *et al.*, 2007; Zhou *et al.*, 2010). However, a study also argues that ROS are required for transcriptional induction of NLRP3 expression, but not for NLRP3 activation (Bauernfeind *et al.*, 2011); these divergent conclusions may be due to potentially off-target effects of pharmacological scavengers or inducers of ROS, and their usage at differing doses or duration that may cause varying outcomes depending on the cell type. Another reported pathway involves lysosome disruption by crystals such as those formed by cholesterol, uric acid, or alum, causing release of active cathepsin B that presumably acts as an upstream activator of NLRP3 (Hornung *et al.*, 2008). Interestingly, deubiquitination of NLRP3 can prime NLRP3 nontranscriptionally and is essential for ASC aggregation and inflammasome activity, however the precise purpose of this deubiquitination is not known (Juliana *et al.*, 2012; Lopez-Castejon *et al.*, 2013). The high level of regulation of the NLRP3 inflammasome may be due in part to the prion-like nature of NLRP3 inflammasome assembly. Nucleation of ASC oligomerization through the PYD has been shown to promote energetically-favorable polymerization into large, stable, amyloid-like structures which may serve to quickly amplify signaling (Cai *et al.*, 2014), yet which may be difficult for the cell to reverse once initiated.

The NLRC4 inflammasome has mainly been implicated in the host defense to bacterial pathogens such as *Salmonella typhimurium*, *Legionella pneumophila*, *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Corridoni *et al.*, 2014). Posttranslational phosphorylation of Ser533 in NLRC4 by PKC$\delta$ has been proposed to be crucial for NLRC4 inflammasome function (Qu *et al.*, 2012). NLRC4 is expressed mostly on myeloid cells and activates caspase-1 dependent production of bioactive IL-1$\beta$ upon cytosolic detection of bacterial flagellin, and components of the type III secretion system (T3SS) (Miao *et al.*, 2006, 2010b; Franchi *et al.*, 2006). These bacterial ligands trigger oligomerization of NLRC4 with NAIPs, which have been identified as critical components of the NLRC4 inflammasome. In mice, NAIP5 and NAIP6 in complex with NLRC4 confer specificity for recognition of bacterial flagellin, whereas a NAIP2–NLRC4 complex confers specificity for recognition of the *S. typhimurium* rod protein PrgJ. In humans, NLRC4 and the sole human NAIP serve as sensors for the bacterial T3SS needle protein (Kofoed and Vance, 2011; Yang *et al.*, 2013; Zhao *et al.*, 2011). Activation of caspase-1 through a NLRC4 dependent pathway has also been associated with subsequent cell death, termed pyroptosis, which can take place independent of ASC (Miao *et al.*, 2010a; Broz *et al.*, 2010).

NLRP1 was the first inflammasome-forming NLR to be described (Martinon *et al.*, 2002). NLRP1 is expressed in diverse cell types including myeloid cells, T and B cells. In humans, NLRP1 is a single copy gene which encodes a N-terminal PYD, a NBD/NOD, a LRR domain, a function-to-find domain (FIIND), and a C-terminal CARD. Mice encode three polymorphic forms of NLRP1 proteins (*Nlrp1a*, *Nlrp1b*, and *Nlrp1c*), which lack functional PYD and FIIND domains. Genetic studies have shown that the *NLRP1b* gene is the primary mediator of mouse macrophage susceptibility to *Bacillus anthracis* lethal toxin (LT) in sensitive strains such as BALB/c. Upon exposure to LT, NLRP1 activates caspase-1 resulting in production of mature IL-1$\beta$ and pyroptosis. Some proposed mechanisms of LT-induced cell death include cleavage of mitogen-activated protein kinase kinases (MEKs), impairment of mitochondrial function, ATP leakage, lysosomal membrane permeabilization, and cathepsin B release, proteasome-mediated protein degradation, potassium efflux, caspase-1-mediated macrophage necrosis, and inflammasome activation with release of IL-1$\beta$ and IL-18; however, the mechanism of activation of the NLRP1 inflammasome by LT remains unclear (Alileche *et al.*, 2006; Averette *et al.*, 2009; Muehlbauer *et al.*, 2007; Wickliffe *et al.*, 2008). In addition, both human and mouse NLRP1 can be activated by muramyl dipeptide (MDP). MDP was suggested to induce conformational changes in NLRP1, which enable its oligomerization, thus creating a platform for caspase-1 activation (Faustin *et al.*, 2007). A NLRP1–NOD2 interaction has also been shown to be required for optimal responses to LT and MDP *in vivo* (Hsu *et al.*, 2008).

Recent studies have uncovered host regulatory mechanisms and pathogen immune-evasion strategies targeting inflammasome function, further emphasizing the importance of inflammasomes in the antimicrobial response. For instance, mouse effector and memory CD4+ T cells have been shown to suppress NLRP1 and NLRP3 inflammasome-mediated caspase-1 activation and subsequent IL-1$\beta$ release by select TNF family ligands in a cognate manner (Guarda *et al.*, 2009), and type 1 IFNs repress the activity of NLRP1 and NLRP3 inflammasomes through a STAT-1-dependent mechanism (Guarda *et al.*, 2011). Human NLRP1 is regulated by interactions with the anti-apoptotic proteins Bcl-2 and Bcl-$X_L$, which bind and suppress NLRP1, reducing caspase-1 activation and IL-1$\beta$ production (Bruey *et al.*, 2007). NLRP1 is also targeted by viruses to evade innate immunity. For instance, Vaccinia virus F1L protein, a viral homolog of the cellular Bcl-2 protein, has been demonstrated to bind and inhibit NLRP1 through a small hexapeptide in F1L thereby promoting viral virulence (Gerlic *et al.*, 2013). In another example, Kaposi's sarcoma herpes virus (KSHV) Orf63 was found to be a viral homolog of human NLRP1 that interacts with NLRP1 and blocks NLRP1-dependent innate immune responses, thereby contributing to reactivation and generation of progeny virus. KSHV Orf63 was also shown to inhibit the NLRP3 inflammasome (Gregory *et al.*, 2011).

Many newly emerging aspects of the biology of NLR proteins point to functions that transcend pathogen detection and inflammation to include autophagy (e.g., autophagic responses to bacteria are regulated by NOD2, NLRC4), tissue homeostasis (e.g., NLRP3 functions as a key regulator of intestinal homeostasis), transcriptional regulation (e.g., CIITA and NLRC5 function as transcriptional regulators of major histocompatibility complex I and II respectively), as well as nonimmune functions such as embryonic development (NLRP5, NLRP2), and reproduction (NLRP7, NLRP14) (Kufer and Sansonetti, 2011). NLRs can act as negative or positive regulators of a variety of signaling pathways. For instance, NLRC3 acts as a negative regulator of T cell activation, NLRP2,

NLRP4, NLRP6, NLRP12, and NLRC5 inhibit NF-$\kappa$B activation, and NLRC5 and NLRX1 negatively regulate type 1 IFN signaling pathways. On the other hand, activating roles have been reported for NLRP12 and NLRP2 in activation of caspase-1, NLRX1 in ROS production, and NLRC5 in IFN-dependent antiviral responses (Lich and Ting, 2007). Thus NLRs may have diverse roles depending upon the cell type and the infectious context (**Table 3**).

The critical role of NLRs in physiology is further underscored by their clear implication in several human autoinflammatory and autoimmune diseases. Mutations in NLRP3 and NLRP12 lead to hereditary periodic fever syndromes, while mutations in NOD2 are linked to inflammatory bowel disease, Crohn's disease or Blau syndrome (Zhong *et al.*, 2013). The NLRP3 inflammasome has also been implicated in diseases associated with metabolic dysfunction such as obesity, gout, type 2 diabetes and insulin resistance, atherosclerosis, and Alzheimer's disease due to increased activation of caspase-1 and IL-1$\beta$ downstream of NLRP3 triggered by diverse danger signals depending upon the disease context (Vandanmagsar *et al.*, 2011; Masters *et al.*, 2010; Duewell *et al.*, 2010; Heneka *et al.*, 2013). Polymorphisms in NLRP1 are associated with a variety of autoimmune disorders including vitiligo, celiac disease, type 1 diabetes, autoimmune thyroid disorders, systemic lupus erythematosus, rheumatoid arthritis, and Alzheimer's disease (Zhong *et al.*, 2013). Genome-wide association studies (GWAS) have identified linkage of polymorphisms and/or mutations in NLR genes with a host of other human diseases; these are summarized in **Table 3**. Given the essential role of NLRs in human disease, future studies investigating the activating ligands, physiological roles and signaling pathways triggered by diverse NLRs will be key to providing fundamental insights into not only how NLRs normally function but also for addressing the molecular basis of aberrant NLR function in patients.

## RLRs

The RLRs serve as cytosolic sensors of RNA. RLR family members include retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) which differentially regulate viral RNA detection (**Table 4**). They are characterized by three distinct domains: a C-terminal domain (CTD), a central DExD/H box helicase domain for RNA binding, and two N-terminal CARD domains, that mediate downstream signaling; these N-terminal CARDs are present in RIG-I and MDA5 but not in LGP2 (**Figure 1**). RIG-I preferentially binds to short ($<300$ bp) double-stranded (ds) RNAs that have blunt ends and a 5′ triphosphate (5′-ppp) moiety while MDA5

**Table 4** Summary of select human nucleic acid sensors, their ligands, responses and associated diseases

| *Sensor class* | *Nucleic acid sensor* | *Ligand(s)* | *Response* | *Associated diseases* |
|---|---|---|---|---|
| RLR | RIG-I | 5′ppp-dsRNA<br>Viruses: Sendai, Influenza, Newcastle disease, Japanese encephalitis, measles, Vesicular stomatitis, Dengue, Hepatitis C, Rabies | Type 1 IFN | Multiple sclerosis, Type 1 diabetes, psoriasis, rheumatoid arthritis |
| RLR | MDA5 | dsRNA, Poly(I:C)<br>Viruses: Picornavirus, Sendai, Encephalomyocarditis, Rotavirus, Dengue, Rabies | Type 1 IFN | Type 1 diabetes |
| Other | DAI | B-form dsDNA<br>Pathogens: Human cytomegalovirus, HSV-1, *Streptococcus pneumoniae* | Type 1 IFN through IRF and TBK1 pathways. | |
| Other | DDX41 | B-form dsDNA<br>Viruses: Adenovirus, HSV-1, Vaccinia | Type 1 IFN through STING pathway | |
| Other | RNA pol III | B-form dsDNA<br>Pathogens: Epstein–Barr virus, Adenovirus, *Legionella pneumophila* | Type 1 IFN production through RIG-I pathway | |
| ALR | AIM2 | B-form dsDNA<br>Pathogens: Vaccinia virus, HSV-1, *Francisella tularensis*, *Listeria monocytogenes* | Inflammasome formation resulting in IL-1$\beta$ and IL-18 and cell death by pyroptosis; NF-$\kappa$B activation | Systemic lupus erythematosus |
| ALR | IFI16 | dsDNA, sequence-independent, $70 \gg 50$ bp<br>Viruses: HSV-1 | Type 1 IFN through STING pathway, inflammasome formation in nucleus of Kaposi-sarcoma associated herpes virus (KSHV) infected endothelial cells | Systemic lupus erythematosus |
| ALR | PYHIN1 | | STING-dependent IFN production | Asthma in African Americans |

preferentially recognizes long dsRNA strands (>1000 bp) with no end specificity (Kato *et al.*, 2008; Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). Both may recognize chemically synthesized dsRNA such as poly I:C or RNA analogs. While 5′ppp-dsRNA exhibits maximal binding affinity, RIG-I is also capable of binding single-stranded (ss) RNA molecules, although with lower affinity (Wang *et al.*, 2010). RIG-I and MDA5 have been reported to have both differential and redundant roles in sensing RNA viruses. RIG-I recognizes mainly negative-sense ssRNA viruses or positive-sense ssRNA/dsDNA viruses including those in the paramyxoviridae, orthomyxoviridae, rhabdoviridae, bunyaviridae families, and some flaviviruses such as Japanese encephalitis and hepatitis C virus; in contrast, MDA5 was shown to primarily detect the dsRNA intermediate form generated during replication of positive ssRNA viruses such as those in the picornaviridae family (Kato *et al.*, 2005, 2006; Loo *et al.*, 2008). However, both are required for an optimal response to West Nile virus, dengue virus, reoviruses, and some paramyxoviruses such as Sendai and measles virus. While RIG-I and MDA5 may differentially detect RNA viruses, they are thought to trigger similar downstream signaling cascades (Yoneyama *et al.*, 2005). In both cases, the ligand-bound RLR engages the mitochondrial or peroxisomal adaptor protein MAVS to activate the cytosolic protein kinases IKK and TANK-binding kinase 1 (TBK1), which in turn activate the transcription factors NF-κB and IRF3, resulting in production of type 1 IFN and other inflammatory antimicrobial cytokines (**Figure 2**).

Recent structural studies of RIG-I bound to dsRNA have contributed toward a better understanding of ligand binding and activation of RIG-I. In its inactive form, RIG-I is in an auto-repressed, open conformation whereby the RIG-I CARD motifs sequester the helicase domain. Upon binding of the helicase and CTD with dsRNA and ATP, the protein rearranges into a closed conformation that exposes the CARDs which then interact with the N-terminal CARD of the adaptor protein MAVS to induce NF-κB and IRF3 activation and establish an antiviral state (Kowalinski *et al.*, 2011). The CARDs of RIG-I have been shown to nucleate the aggregation of MAVS into self-propagating prion-like structures that activate IRF3 (Hou *et al.*, 2011). It is hypothesized that such MAVS polymers may serve to quickly amplify signaling. The prion-like nature of MAVS polymerization bears resemblance to ASC aggregation observed during inflammasome assembly discussed previously, and is under tight regulation to achieve the optimal balance between the antiviral response and unchecked tissue damaging inflammation (Reikine *et al.*, 2014). These regulatory mechanisms include posttranslational modifications such as polyubiquitination and phosphorylation/dephosphorylation of RIG-I and MDA5 that are critical for their immune signaling ability (Gack *et al.*, 2010; Zeng *et al.*, 2010; Wies *et al.*, 2013). Furthermore, signaling may be modulated by virally encoded factors that manipulate these posttranslational modifications (PTMs) or cause proteolytic degradation and cleavage of MAVS (Gack, 2014), as well as by numerous host proteins including the third RLR LGP2 that has been reported to have stimulatory and inhibitory effects on MDA5 and RIG-I signaling respectively (Zhu *et al.*, 2014). Further investigations are needed to clarify the role of LGP2 in antiviral immunity.

## AIM2-Like Receptors and Other Intracellular DNA Sensors

Cytoplasmic DNA derived from infectious agents like viruses that replicate in the cytosol or from dying host cells can act as a danger signal to alert the immune system to infection or altered cellular homeostasis. A number of proteins that mediate cytoplasmic surveillance of host- or pathogen-derived DNA have been identified in recent years, however their role in host defense and/or autoimmunity and the mechanistic aspects of their activation are just beginning to be elucidated. These include the AIM2-like receptors (ALRs) (Hornung *et al.*, 2009), DAI (Takaoka *et al.*, 2007), and multiple DNA sensors that engage the ER-localized adaptor protein STING (Ishikawa and Barber, 2008; Holm *et al.*, 2013) resulting in the production of type 1 IFN.

STING binds cyclic dinucleotides (CDNs), second messengers synthesized by bacteria or by the receptor cyclic GMP-AMP synthase (cGAS), which synthesizes noncanonical CDNs from dsDNA (Yin *et al.*, 2012; Burdette *et al.*, 2011; Zhang *et al.*, 2014). STING contains an N-terminal transmembrane region and a cytosolic C-terminal domain (CTD) that interact in a dimerized complex in the absence of CDNs. Upon ligand binding, the CTD is released allowing it to interact with TBK1 and IRF3 (Ouyang *et al.*, 2012). Phosphorylated IRF3 in turn binds to the *Ifnβ* promoter, resulting in IFN-β synthesis (**Figure 2**). Several DNA sensors have been shown to act through the STING pathway, including several members of the ALR family discussed below.

DAI was the first TLR-independent cytosolic DNA sensor to be reported. It recognizes long strands (~500-1 kb in size) of dsDNA in its canonical B-form. Upon sensing of dsDNA by DAI, a TBK1 and IRF3-dependent signaling cascade is initiated, resulting in the production of type 1 IFN (Takaoka *et al.*, 2007). Whether or not STING is also required has not yet been fully elucidated. Activated DAI has also been shown to recruit the receptor-interacting protein kinases RIP1 and RIP3, leading to NF-κB activation (Rebsamen *et al.*, 2009). Subsequently, the DDX41 DExD/H box helicase was also shown to bind intracellular B-form DNA or CDNs leading to a type 1 IFN response (Parvatiyar *et al.*, 2012; Zhang *et al.*, 2011). The signal is transduced by direct binding of activated DDX41 to STING which results in phosphorylation of IRF3 through TBK1. Cytosolic DNA may also be detected through the actions of RNA Polymerase III, which transcribes dsDNA to 5′-ppp RNA. The RNA is in turn sensed by RIG-I, resulting in a RIG-I-mediated IFN and NF-κB response (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). This pathway has been implicated in sensing intracellular bacteria, like *L. pneumophila*, and DNA viruses, like Epstein–Barr virus, Herpes simplex virus (HSV)-1, and adenovirus.

Five AIM2-like receptors (ALRs) have been identified in humans and 13 in mice. These are members of the Pyrin and HIN domain (PYHIN) family encoded by an IFN-inducible gene cluster on chromosome 1. In humans, they comprise of AIM2, IFN-γ inducible protein 16 (IFI16), myeloid cell nuclear differentiation antigen (MNDA), pyrin and HIN domain family member 1 (PYHIN1), and the recently identified pyrin domain only protein 3 (POP3) (**Table 4**). They are characterized by a N-terminal PYD and/or a C-terminal hematopoietic IFN-inducible nuclear protein (HIN)

domain (Figure 1). The HIN domain contains partially conserved repeats that allow for binding of DNA ligands resulting in activation of the inflammasome, type 1 IFNs or both depending upon the ALR. For instance, detection of DNA by AIM2 leads to assembly of an inflammasome resulting in activation of caspase-1 and production of active IL-1$\beta$ (Hornung *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009), PYHIN1 activates STING-dependent IFN production (Brunette *et al.*, 2012), while IFI16 can lead to inflammasome activation or STING-dependent activation of type 1 IFN (Kerur *et al.*, 2011; Unterholzner *et al.*, 2010). AIM2 is perhaps the best studied of the ALRs. Recent structural studies have advanced our understanding of how AIM2 binds DNA to activate immune responses. Electrostatic interaction between positively charged HIN domain residues and the dsDNA sugar-phosphate backbone allows for nonsequence-specific DNA recognition by AIM2 (Jin *et al.*, 2012). In the inactive state, the HIN and PYD domains complex to maintain the molecule in an autoinhibited conformation. Binding of dsDNA to the HIN domain opens the protein conformation releasing the PYD domain, which is then free to engage the adaptor protein ASC resulting in assembly of the AIM2 inflammasome that culminates in production of active IL-1$\beta$ and IL-18. However, the AIM2 inflammasome differs from the NLR inflammasomes in that the protein scaffold centers not on protein oligomerization domains but on multiple AIM2 molecules binding to the same dsDNA ligand. Nevertheless, AIM2 interaction with the adaptor ASC may initiate prion-like self-polymerization of ASC into large, stable filaments which have been shown to be both necessary and sufficient for AIM2 inflammasome responses (Cai *et al.*, 2014; Lu *et al.*, 2014). Given the polymeric nature of inflammasome assembly, it is anticipated that AIM2 activation must be under strict regulation; however, the regulatory mechanisms are poorly understood to date. Recently POP3, a newly identified member of the ALR gene cluster has been shown to negatively regulate DNA virus induced activation of the AIM2 and IFI16 inflammasomes by inhibiting recruitment of ASC to these ALRs (Khare *et al.*, 2014). Additionally, activation of IFI16 is also regulated by viral proteins that degrade IFI16 or block its oligomerization thereby interfering with the antiviral response (Orzalli *et al.*, 2012; Li *et al.*, 2013b).

Emerging data suggest that apart from host defense, DNA sensors may also be involved in development of autoimmune disease resulting from persistent triggering of these sensors by accumulated endogenous DNA (Holm *et al.*, 2013). Inefficient removal of chromatin due to defects in deoxyribonuclease 1 is associated with SLE (Napirei *et al.*, 2006), while mutations in the gene encoding three prime repair exonuclease 1 (TREX1), a negative regulator of STING signaling, result in Aicardi–Goutières syndrome (AGS), a severe neurological brain disease (Crow *et al.*, 2006). Thus, innate immune detection of DNA results in different functional outcomes, i.e., either host defense or pathology, depending upon the biological context. The field of DNA sensing is still burgeoning, and it will be interesting to gain a fuller understanding of the mechanisms underlying the beneficial and pathological consequences of innate recognition of DNA, while exploring the prospect of using nucleic acids as agents to promote antiviral immunity.

## Role of Intracellular Organelles and Spatial Relocation

Activation of innate sensors following ligand binding may be manifested not only through changes in protein conformation and phosphorylation, but also through their spatial relocalization (**Figure 2**). Following activation, the cytoplasmic sensors RIG-I, MDA5, and NLRP3 move from the cytoplasm to the mitochondria where they associate with MAVS (Seth *et al.*, 2005; Subramanian *et al.*, 2013). This relocalization is essential for signal propagation; dislocation of MAVS from mitochondria abolishes RIG-I-dependent type 1 IFN and NLRP3-dependent inflammasome activation in response to RNA viruses (Seth *et al.*, 2005; Park *et al.*, 2013). AIM2 relocalizes from the cytosol to ASC foci upon activation by DNA. Likewise, all other murine ALRs translocate from the nucleus to puncta containing the adaptors ASC, STING, or both in discrete patterns under co-expression conditions (Brunette *et al.*, 2012). STING itself relocates within the cell following activation. In its inactive state, STING is present on the endoplasmic reticulum (ER), the mitochondria, and/or the mitochondria-associated ER membranes, which tether the ER to the mitochondrion (Ishikawa and Barber, 2008; Zhong *et al.*, 2008). Upon activation, STING moves to 'concentrated puncta' within the cell. These foci have been proposed to be associated with autophagy; however the association between STING activation and autophagy remains unclear (Burdette and Vance, 2013).

In addition to signal transduction, spatial relocalization may also influence the outcome of signaling by determining the nature of the downstream response. Perhaps the best studied example is that of TLR4, which senses extracellular LPS. Activation of TLR4 on the plasma membrane results in its dimerization followed by binding to MyD88 and TIRAP/MAL adaptor proteins. This complex is trafficked to lipid rafts ultimately resulting in activation of NF-$\kappa$B and expression of pro-inflammatory cytokines. However, TLR4 may also engage the adaptors TRIF and TRAM resulting in phosphorylation of IRF3 and ultimately in production of type 1 IFN. For this latter pathway to be initiated, complex assembly must occur in early endosomes (Gangloff, 2012). Thus movement of TLR4 from the plasma membrane to the endosomes switches the outcome of signaling from production of NF-$\kappa$B-dependent cytokines to type 1 IFN. Likewise, TLR9 in most cells traffics from the ER through the golgi to proteolytically active early endosomal compartments where it is activated by cleavage of its ectodomain, facilitating signaling through NF-$\kappa$B to activate pro-inflammatory cytokines. In plasmacytoid DCs however, cleaved TLR9 in endosomes interacts with the adaptor protein (AP)-3 complex which delivers it to specialized lysosome-related organelles, where TLR9 can engage the TRAF3 and IRF7 signaling pathway resulting in induction of type 1 IFN (Honda *et al.*, 2005; Sasai *et al.*, 2010). A third example is that of the cytoplasmic RNA sensor RIG-I that can engage its adaptor MAVS from both peroxisomal and mitochondrial locations. Following viral infection, peroxisomal MAVS induces IRF1-dependent but type 1 IFN-independent rapid expression of IFN-stimulated genes (ISGs) and defense factors that may provide immediate protection, whereas mitochondrial MAVS activates IRF3 and type 1 IFN-dependent expression of ISGs

with delayed kinetics, that may serve to amplify and stabilize the antiviral response (Dixit *et al.*, 2010). More recently, it has been demonstrated that signaling via mitochondrial MAVS induces IFN-$\beta$ and IFN-$\lambda$, while peroxisomal MAVS selectively activates an IFN-$\lambda$ response (Odendall *et al.*, 2014). In all these instances, the intracellular movement of the innate sensor and/or its signaling adaptor to specific subcellular compartments determines the nature of the response.

The significance of this spatial relocalization is not completely understood. It seems possible that the cellular location of ligand detection or pathogen replication is not always the best location for signal propagation. Nuclear ALRs, for instance, may be appropriately positioned to sense dsDNA in the nucleus where viral DNA is undergoing replication, however once activated move to a site where the downstream signaling components are located (Brunette *et al.*, 2012). Organelle membranes could provide ideal platforms for solid-phase oligomerization of innate sensors and/or their adaptors leading to assembly of active complexes, thereby allowing the input signal to be quickly amplified to ensure a robust innate response. Additional, as yet unidentified, molecules on organelle membranes might also be required for a complete functional response. Mitochondria, for instance, have been shown to directly contribute to bactericidal activity through the generation of ROS upon stimulation of a subset of TLRs. This response involves translocation of the TLR signaling adaptor, TRAF6, to mitochondria where it engages evolutionarily conserved signaling intermediate in Toll pathways (ECSIT), a protein that participates in mitochondrial respiratory chain assembly, resulting in increased mitochondrial ROS generation (West *et al.*, 2010). We speculate that relocalization of innate immune signaling components to organelles such as mitochondria may contribute to or enhance microbicidal and/or other yet unknown responses. Mitochondria also play a role in regulating the apoptotic response to stress signals (Wang, 2001), and localization of innate signaling complexes on mitochondria could allow for the integration of signals from innate immune and cell death pathways.

## Combinatorial Sensing and PRR Crosstalk

Innate sensing of pathogens is a complex, interconnected system of receptors, adaptors, and signaling pathways. A single pathogen may be detected by multiple receptors; for example, *Mycobacterium paratuberculosis* infection is sensed by TLR2, TLR4, and NOD2 (Ferwerda *et al.*, 2007) while rhinovirus infection of bronchial epithelium is sensed by TLR3, RIG-I, and MDA5 (Slater *et al.*, 2010). Activation of each of these receptors initiates distinct as well as common signaling cascades, which must be synthesized by the cell into an effective immune response. Crosstalk between innate immune signaling pathways is therefore an essential determinant of the quality and magnitude of the ensuing host response. Cooperation between multiple signaling pathways may be important for mounting an appropriate response to an elicitor. For example, concomitant activation of the CLR Dectin-1 and TLR2, both of which sense fungal cell wall components, is required to induce optimal cytokine responses in macrophages. This collaboration requires Dectin-1 signaling through the Syk pathway, and can also occur with TLRs 4, 5, 7, and 9 that like TLR2, signal via the adaptor MyD88. Activation of Dectin-1 alone induces no TNF and activation TLR2 induces a low level of TNF. However activation of both Dectin-1 and TLR2 results in sustained degradation of I$\kappa$B, enhanced nuclear translocation of NF-$\kappa$B, and a synergistic TNF response (Dennehy *et al.*, 2008). In another study, collaboration between TLR2 and TLR9 was required for optimal host resistance to *Mycobacterium tuberculosis* (Bafica *et al.*, 2005). Signal integration from multiple pathways may thus be important for the cell to mount a scaled, robust, specifically tailored response to a pathogen, while decreasing potential 'noise' from commensal microbes and/or homeostatic cellular functions.

Sequential activation of multiple PRR pathways may enable the cell to generate an optimal response to contain a potential pathogen while minimizing immunopathology. For instance, in conventional DCs, a subset of herpes simplex viruses is first detected by cell surface TLR2 interacting with the virions, and then by intracellular TLR9 that senses viral genomic DNA (Sato *et al.*, 2006). In another study, sequential activation of MyD88-independent and MyD88-dependent cytokine responses by the intracellular bacterium *Listeria monocytogenes* was required for monocyte recruitment to infected tissue and monocyte activation respectively (Serbina *et al.*, 2003). Finally, NLRP3 inflammasome activation requires successive activation of the TLRs and NLRP3 (Netea *et al.*, 2009; Bauernfeind *et al.*, 2009). A TLR-activating stimulus first induces NF-$\kappa$B-dependent upregulation of pro-IL-1$\beta$ and NLRP3. A second NLRP3-activating stimulus then triggers assembly of the inflammasome resulting in processing of procaspase-1 to its active form, which in turn cleaves the pro form of IL-1$\beta$ to generate active cytokine. Both TLR and NLR pathways are therefore essential for induction of a complete IL-1$\beta$ response in macrophages.

Innate immune signaling pathways may also negatively regulate each other. For instance, the RLR-stimulated response has been shown to interfere with TLR signaling. The RLR-activated transcription factor IRF3 dominantly binds to the promoter region of the interleukin-12b (*Il12b*) gene, outcompeting TLR-induced IRF5. As a consequence, activation of RLRs in mice attenuates TLR-induced T helper type 1 (Th1) and interleukin 17-producing helper T cell (Th17) responses, and pre-infection of mice with a virus reduces their ability to respond to bacterial pathogens effectively (Negishi *et al.*, 2012). Likewise, NLRX1 has been proposed to interact with TRAF6 thereby inhibiting NF-$\kappa$B signaling in response to LPS stimulation. Additionally, NLRX1 acts as a negative regulator of RIG-I signaling by interfering with RIG-I–MAVS interaction. Consequently, *Nlrx1* $-/-$ mice exhibit increased expression of antiviral signaling molecules and pro-inflammatory IL-6 after influenza virus infection, and this increased inflammation is associated with marked morbidity and tissue pathology in *Nlrx1* $-/-$ mice (Allen *et al.*, 2011). Negative regulation may also occur by sensors within the same PRR family. For example, within the TLR pathway, the adaptor MyD88 inhibits the TLR3-TRIF dependent response, which may protect the host from immunopathology associated with excessive production of IFN-$\beta$ (Johnson *et al.*, 2008; Siednienko *et al.*, 2011). Within the NLR family, NLRP7 has been shown to negatively regulate the NLRP3 inflammasome by

direct interaction with procaspase-1 and pro IL-1$\beta$ (Radian *et al.*, 2013). Finally, although not as well characterized, several members of the NLR family have been proposed to have anti-inflammatory functions. NLRP6, NLRP10, NLRP12, NLRC3, and NLRX1 have all been reported to negatively regulate canonical or noncanonical NF-$\kappa$B activation and subsequent cytokine responses (Allen, 2014).

Crosstalk occurs not only between innate signaling pathways, but also between innate and other cellular pathways. For example, mTOR, which is usually involved in cell growth and metabolism, has also been shown to limit pro-inflammatory responses to bacterial stimuli by blocking NF-$\kappa$B activation and conversely increase anti-inflammatory cytokines like IL-10 by enhancing STAT3 activity, thereby limiting the priming of Th1 and Th17 cells (Weichhart *et al.*, 2008). Moreover, in the context of an infection, crosstalk also occurs between the host and pathogen. Many pathogens can modulate the host response, allowing them to overcome or bypass host defense mechanisms. For instance, the human cytomegalovirus actively cleaves host DNA and disrupts the ability of the infected cell to repair it, while selectively maintaining nucleotide excision repair of the viral genome (O'Dowd *et al.*, 2012). Likewise, many viral pathogens actively cleave or sequester components of innate immune pathways to disrupt signaling, while intracellular bacterial pathogens such as *Salmonella enterica* and *Mycobacterium tuberculosis* have developed strategies to avoid, modulate, or hijack the host immune response in their favor (Forrellad *et al.*, 2013; Gack, 2014; de Jong *et al.*, 2012; **Figure 3**). Interplay between PRR and other cellular pathways as well as between the host and the pathogen, are therefore essential parameters that influence the outcome of infection.

## Multiscale Regulation of Cytosolic Sensing Pathways

Successful host defense against pathogens requires development of an appropriate, scaled immune response commensurate with the nature of the threat, a process that involves dynamic, feedback-controlled interactions between immune system components at multiple molecular and cellular levels. Information is integrated from the genomic, transcriptional and translational levels, posttranslational controls, protein–protein interactions, assembly of functional multiprotein complexes and their appropriate cellular localization, to coordinate a successful effector response against the potential pathogen, followed by induction of appropriate negative regulatory mechanisms to restore cellular homeostasis (**Figure 3**). Information from heterogeneous cells must further be integrated into an organ- and organism-level response. Regulation at each level affects the response at many others; therefore a functional immune response is the result of an orchestrated set of events, with combinatorial regulatory potential. Deciphering the intricate physical and functional interactions that underlie innate immunity will therefore require a global, systems-level understanding of the molecular and cellular networks that comprise the processes at all levels (Subramanian *et al.*, 2015).

This seems a daunting endeavor; however, recent technological advances have allowed for the collection of comprehensive, large-scale 'omics' datasets at multiple molecular and cellular levels. Coupled with major developments in computational modeling and network analysis methodologies, they hold the potential to greatly advance our understanding of innate immune regulation by enabling a systems approach to innate immunity. Genomics, which involves sequencing of entire genomes, has enabled GWAS that permit unbiased association of genetic variants commonly arising due to single nucleotide polymorphisms with disease traits (Xavier and Rioux, 2008). Such studies have revealed association of polymorphisms in cytosolic sensors, particularly the NLRs, with a plethora of autoimmune and autoinflammatory diseases (Zhong *et al.*, 2013; **Table 3**). A notable example is the association of NOD2 genetic variants with susceptibility to Crohn's disease (Xavier and Rioux, 2008). Transcriptomics, the global analysis of gene expression pathways, has resulted in the identification of novel transcription factors, *cis*-regulatory elements, and previously unknown signaling modules involved in innate immune responses, while ChIP-Seq (chromatin immunoprecipitation followed by sequencing) approaches have allowed for identification of genome-wide binding sites for these transcription factors (Gilchrist *et al.*, 2006; Elkon *et al.*, 2007; Ramsey *et al.*, 2008; Gottschalk *et al.*, 2013). Together with network perturbation strategies such as RNAi, these technologies have allowed for elucidation of gene-regulatory networks controlling the innate immune response (Amit *et al.*, 2011). Transcript profiling has also been used to elucidate immunological responses to vaccine antigens, with the goal of predicting vaccine efficacy and potentially optimizing the immunogenicity of vaccines (Li *et al.*, 2013a). For instance, systems biology approaches have been used to determine the mechanisms involved in conferring immunity to yellow fever (Querec *et al.*, 2009; Gaucher *et al.*, 2008), seasonal influenza (Nakaya *et al.*, 2011) and Human immunodeficiency virus (HIV) (Zak *et al.*, 2012) and to develop computational models that can predict vaccine efficacy in subsequent, independent trials.

Advances in mass spectrometry have further enabled collection of large-scale proteomic datasets for quantification and characterization of proteins and posttranslational modifications (PTMs) in response to various stimuli. For instance, new developments in proteomics technologies have resulted in exciting discoveries of phosphorylation sites and led to an increased understanding of the role of phosphorylation in the immune response (Gottschalk *et al.*, 2013). Formation of functional multiprotein complexes (involving receptor dimerization/oligomerization, interaction with adaptor proteins, and other protein–protein interactions necessary for assembly of the signaling complex) is an essential step that controls signal propagation and critically determines the quality and/or magnitude of the innate response. Protein expression, protein–protein interaction, and PTM data can provide insights into assembly of such functional complexes, and when analyzed using network analysis tools, can also help elucidate complex signaling circuits underlying the innate response (Subramanian *et al.*, 2015). Finally, metabolomics, the comprehensive analysis of metabolites such as metabolic intermediates, hormones, and secondary metabolites, has been used to better understand host–pathogen interactions (Olszewski *et al.*, 2009) and to develop metabolic biomarkers for diseases (Monteiro *et al.*, 2013). Although metabolomics has not been widely employed in the study of innate immunity, it is

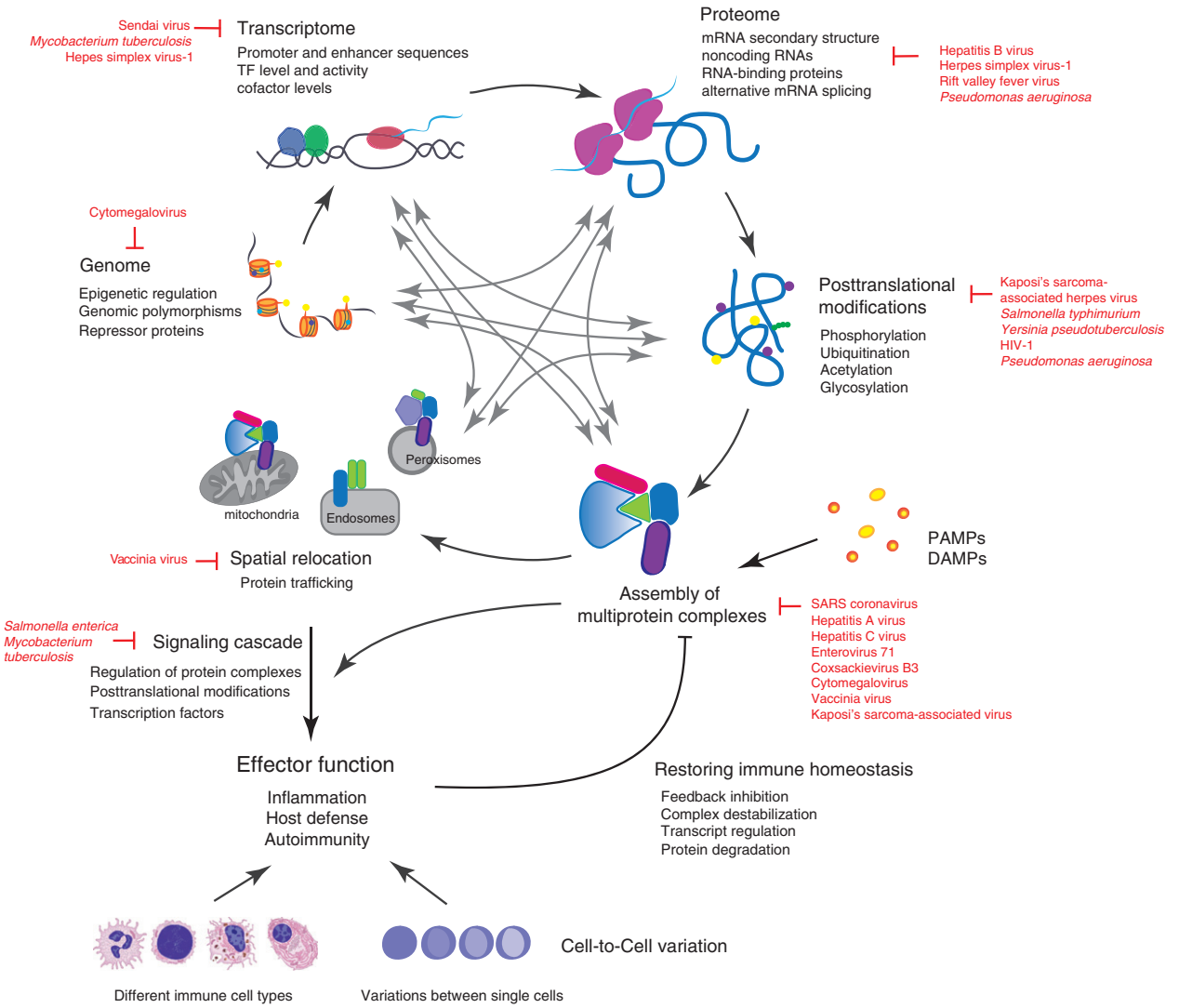



**Figure 3** Systems-level overview of innate immunity. Development of a functional immune response is dependent on interactions between cellular components and processes at multiple levels. Modifications at the genomic level, such as genetic polymorphisms, affect the sequence of the gene being transcribed, while epigenetic modifications or other effects on DNA regulation influence the amplitude of transcription. An RNA transcript may be regulated by miRNAs, lncRNAs, and other noncoding RNAs, mRNA secondary structure or splicing events that may have profound effects on translation and protein expression. A protein may be further modified posttranslationally by addition of biochemical functional groups (such as phosphates or acetates) or through structural changes (such as formation of disulfide bonds). Sensing of PAMPs and DAMPs by innate sensors initiates the assembly of multiprotein signaling complexes, and the signal may be further propagated through trafficking or spatial relocalization of the protein(s) or protein complex to intracellular compartments. Formation of functional signaling complexes is therefore a highly regulated process that results from an integrated set of intracellular events like transcription, translation, posttranslational modifications (PTMs), proper protein trafficking, and localization. The nature and magnitude of the response may also vary depending upon the type of innate immune cell encountered (e.g., macrophage, neutrophil, and B cell) and stochastic variations between single cells of the same type. Pathogens may further modulate or evade the immune response by interfering with host defense mechanisms at multiple levels; some examples are indicated in red. The regulation and integration of these events occurring at various levels determines the eventual effector response that may either result in host protection and restoration of immune homeostasis or unrestrained inflammation leading to tissue damage and/or autoimmunity.

now appreciated that cytosolic sensors, particularly the NLRs play an unequivocal role in sensing danger signals produced during metabolic stress. A role for metabolism in controlling inflammation has also emerged (Kominsky *et al.*, 2010), and future studies should provide greater insight into the metabolic networks controlling innate immunity.

Integrative analysis of high-dimensional data acquired from multiple molecular levels in this manner will be key to elucidating the complex spatiotemporal interactions that underlie the development of a functional immune response. Advances in network biology have facilitated representation of the intricate interactions at each level as physical or functional networks of interconnected components, which enables recognition of emergent properties arising from these interactions (Subramanian *et al.*, 2015). When substantiated by focused experimentation, this approach holds great promise for accelerating

our understanding of the transcriptional, gene-regulatory, and signaling networks controlling the innate immune response. Integration of the aforementioned global approaches with advances in network biology, computational, and mathematical tools, may thus in future endeavors permit the generation of quantitative and predictive models of innate immunity, which in turn, will empower vaccinology, drug discovery, and other therapeutic possibilities for treatment of human immune disorders.

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*See also*: Cellular Immunology: Innate Immunity: Scavenger Receptors. Cellular Immunology: Overview: Introduction to Functional Cell Biology of Immunity

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