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TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis

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Running title: **TLR3 signal in necroptosis**

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Key words: necroptosis, TLR3, TICAM-1 (TRIF), interferon-inducing pathway, RIP signal.

Abbreviations:

CTL, cytotoxic T lymphocytes; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, damage-associated molecular pattern; HMGB1, High-mobility group box 1; HSP, heat shock protein; mDC, myeloid dendritic cells; Mf, macrophages; NK, natural killer; NLR, NOD-like receptor; PAMP, pathogen- associated molecular pattern; PRR, pattern-recognition receptors; RIP, receptor-interacting protein; TICAM-1, Toll-IL-1-homology domain-containing adaptor molecule-1; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR1, TNF α receptor 1;

Abstract

Agonistic stimulation of toll-like receptor 3 (TLR3) is known to lead to oligomerization of the TICAM-1 (TRIF) adaptor, which induces two modes of acute cellular responses, namely, cell survival for type I interferon production, or cell death, either apoptosis or necrosis-like. Selection among these three responses determines the fate of the affected cells, although the switching mechanism of the two cell death pathways in TLR3-stimulated cells remains molecularly unknown. In tumor necrosis factor (TNF) signaling-mediated cell death, apoptosis and an additional pathway, termed necroptosis or programmed necrosis, have been described in detail. Interestingly, a death domain-containing kinase, Receptor-interacting proteins (RIP) are involved in triggering these two cell death pathways. Formation of a RIP1/RIP3 complex (necrosome) without caspase 8 activity is crucial for inducing necroptosis in TNF signaling. On the other hand, RIP1 is known to interact with the C-terminal domain of TICAM-1 and differentially modulate TLR3 signaling independent of the N-terminal-mediating IRF-3 activation. In macrophages and perhaps tumor cell lines, RIP1/RIP3-mediated necroptotic cell death is feasible by stimulation with polyI:C, and should this involve the TLR3/TICAM-1 pathway, innate sensing of viral dsRNA may be linked to cytopathic effect and persistent inflammation that induce damage-associated molecular patterns (DAMPs) and tumor cell death in the microenvironment. Here, we review increasing findings for the possible involvement of the TLR3/TICAM-1 pathway in tumor cell necroptosis and release of DAMPs.

Introduction

Cell death is an important process during development and homeostasis in multicellular organisms. The mode of cell death is closely associated with subsequent events occurring with host biological responses, including inflammation. Cell death has been categorized as apoptotic or necrotic and, until recently, apoptosis had been considered equivalent to programmed cell death (1). Caspases are a family of cysteine proteases that mediate apoptotic cell death in response to agonistic ligands of death receptors, such as TNF α, FasL, and TRAIL, as well as to mitochondrial damage, by inducing BH3-only members of the proapoptotic Bcl-2 family. It is now clear, however, that apoptosis is not the only cellular mechanism that regulates programmed cell death. Necrotic cell death, which has been viewed traditionally as a form of passive cell death, may be executed, at least in part, through a mechanism termed necroptosis or programmed necrosis (2). Necroptosis may be induced by TNF α receptor (TNFR1) agonism, but also by innate pattern-recognition receptors such as Toll-like receptor (TLR) 3 and TLR4 (1,4). These two TLRs can recruit TICAM-1 (TRIF) as an adaptor for IFN-inducing signaling (3). Indeed, a TLR3 ligand, polyI:C, can activate either apoptosis or necrosis depending on the cell lines tested. Cell death induced by activation of TLR3 and its adaptor TICAM-1 may therefore be executed through two distinct modes of cell death pathways (5). The mechanism that dictates the cellular decision to undergo apoptosis or necroptosis in TLR3 signaling, and the mechanism that mediates the execution of necroptosis, are the subject of intense investigation.

Toll-like receptors and other pattern-recognition receptors (PRRs) harbor the ability to recognize or discriminate microbial-specific pattern molecules, termed pathogen-associated molecular patterns (PAMPs) (6). PAMPs trigger maturation of myeloid dendritic cells (mDC) through activation of TLR and/or other pathways to provoke cellular immunity (7). In mDC, nucleic acid-recognizing TLRs (i.e. TLR3, 7, 8 and 9) reside in the endosome and sense their ligands only when the ligands are internalized into the endosome (8). Phagocytozing DNA/RNA of microbial origin therefore allows mDC to induce cross-presentation and NK-activating ligands to work out cellular effectors. Besides this extrinsic mDC maturation route, it is known that the formation of cytosolic autophagosomes may deliver viral nucleic acids in the cytoplasm to the endosome via autophagy (9). In either route, TLR signaling links normal immunological events and pathological cell death.

Recent increasing evidence suggests that TLR serves as a receptor not only for foreign PAMP but also for constituents of self cell origin which are liberated from damaged or necrotic cells (10). Pattern-recognition of innate immunity is therefore not merely a mechanism for discriminating pathogen from host but, in addition, is a mechanism for inspecting homeostasis of cell populations, and evaluating the status of cellular damage or necrosis (necroptosis) in this context. Liberated materials with physiological function are called damage-associated molecular patterns (DAMPs) (11). The most popular TLR adaptor MyD88 is known to contain death domains, and some reports have suggested the involvement of TLR signaling secondary to PAMP/DAMPs response in cell death. Necroptotic or damaged cells may represent a result of TLR death signal, and cause a functional complex consisting of the sources of DAMPs and the phagocytic response (11,12).

DAMPs refer to intracellular molecules with inflammation-inducing capacities when it is released from the cell. DAMPs do not belong to the cytokine family but rather resemble PAMP in their functional properties toward mDC and macrophages. Their function may be associated with physical responses regarding regeneration and tumorigenesis. During the past 5 years, necrotic or necroptotic cell death has been closely connected with innate immune responses involving pattern-sensing (12,13). DAMPs include a number of cytosolic or nuclear molecules (Table 1), and surprisingly, nucleic acids from self cells (14). This implies that, like viral DNA and RNA, autologous nucleic acids are patterns for sensors to evoke inflammation. Here we highlight the scope of the immune modulation induced by nucleic acids and necroptotic host cells.

Necroptosis: programmed necrosis induced by TNF-α

TNF-α has been reported to induce two different types of cell death, apoptotic and necrotic-like, in a cell type-specific manner (15,16). TNF-α is implicated in NF-κB activation through its receptor TNFR1, and contributes to maintenance of cell growth in many tumor cell lines. In parallel with the of induced TNF-α, hemorrhagic necrosis has been observed in several tumor lines, however, the molecular mechanism for these differential cellular responses by TNF-α has been a question. Recently, several reports have suggested that formation of the complex consisting of RIP1 and RIP3, named the

"necrosome", is responsible for the switch from apoptosis to necroptosis (17,18). The RIP1/RIP3 complex can assemble only in the absence of functional caspase 8, indicating that this enzyme is acts as a key protease for blocking formation of the necrosome (5,19). Many viral factors, as well as genome instability in tumor cells, can compromise caspase 8 function, thereby facilitating the induction of necroptosis in the affected cells. Hence, TNF promotes cell death by signaling through its receptors and downstream RIP1/3, although the output of TNF signaling is ultimately determined by cell type.

Virus-mediated necroptosis

It is notable that necrosis-like cell death has been observed in polyI:C-stimulated bone marrow-derived mouse macrophages and cell lines (13). TICAM-1 and RIP3 are involved in this process, suggesting the implication of the necrosome pathway in dsRNA-mediated cell death (12,13). It has been reported that viral dsRNA frequently induces apoptosis in infected cells, which is known as a cytopathic effect (20). TICAM-1 and RIP1 may be involved in virus-derived necrotic cell death (5,13), which is rare compared to apoptosis since, consistent with TNF-mediated cell death, necroptosis occurs only when viruses produce caspase 8 inhibitors in addition to dsRNA in infected cells (19). Furthermore, additional setting is required such that virus-derived dsRNA is delivered from cytosol to the endosome of infected cells. This may happen if the dsRNA is encapsulated by autophagosomes which transfer the dsRNA to the endosome where TLR3 is situated. The possible involvement of RIG-I/MDA5 in cell death cannot, however, be ruled out in some cases of viral infection. TNF-α can be produced downstream of the TLR3- and RIG-I-RNA-sensing pathways and may induce necrotic cytolysis. Many RNA viruses induce cell death (20), but the factors determining the induction of necroptosis in virus-infected cells remain to be clarified.

In addition, DNA viruses can induce necroptosis via another pathway, involving DAI (also called DLM-1/ZBP1) (21). DAI is a DNA sensor (22) and directly activates RIP3 without participation of type I IFN induction (21). DNA sensing in the cytoplasm of virus-infected cells is complex however, and it may be that DAI is not the only molecule related to necroptosis. Even if caspase 8 is blocked during infection, it is unknown whether RIP3-mediated necroptosis can be induced through viral DNA

recognition by DAI or through participation of other factors in infected cells (20). In fact, this type of virus-derived necrosis has been reported with DNA viruses bearing caspase inhibitors: vaccinia virus (VV) with B13R/Spi2, pox virus with CrmA, Kaposi's sarcoma-associated herpesvirus (KSHV) with K13 and molluscum contagiosum virus (MCV) with MC159 (20,23). Generally speaking, the modes of cell death secondary to virus infection differ among virus species. The physiological role of TLR3- and DAI-mediated necroptosis should therefore be analyzed in a virus species-specific fashion.

Necroptosis in inflammation

Apoptosis plays a major role in physiological cell death, however, under pathological conditions, necrosis is very common (1). Necroptosis differs from necrosis in its programmed nature, and differs from apoptosis in that necroptosis is implicated in mediating inflammation. When virus-infected cells undergo apoptosis, apoptotic cells are removed by phagocytosis. Viral genomes, either DNA or RNA, are degraded into small fragments in infected cells, so as neither to stimulate phagocytes including macrophages and dendritic cells, nor to allow the liberation of DAMPs. In contrast, non-apoptotic cell death scatters DAMP and viral products followed by Mf activation (13), which appears to be linked to prolonged infection, in which viruses produce caspase inhibitors or confer resistance to apoptosis in infected cells (24). A typical model of necroptosis evokes two effectors, viral nucleic acids and DAMPs, to modulate bystander host cells and immune-related cells. In the context of necroptosis, these effectors involve amplification of inflammatory responses by myeloid phagocytes (mDC/Mf), which are characterized in inflammation induced by persistent virus infection, and this mode of inflammation confers secondary-released cytokines and mediators to other cells. In addition, viral factors can also result in incipient inflammation, as observed in chronic hepatitis B or C virus infection (24), which might modify the features of infectious milieu in conjunction with viral nucleic acids and DAMPs. Further studies are needed to clarify this process in the context of virus-dependent chronic inflammation, leading to tumor progression.

Necroptosis and oncogenesis

Increasing evidence indicates that inflammatory signals, including NF-κB activation, are crucial for oncogenesis, and studies have demonstrated that DAMPs may be associated with tumorigenesis and opposing tumoricidal immune responses (25, 26). Tumor progression is not always accompanied by virus infection, and it remains unclear whether DAMPs released from non-infected tumor tissue are sufficient to support tumor growth. It has been reported that autologous mRNA acts as a TLR3 ligand (14) and that DNA of self cell origin can stimulate host DNA sensors (22,27). Due to deficits in the identification and functional characterization of DNA sensors and their pathways, however, it is unknown whether host nucleic acids are strong inflammatory inducers compared to viral RNA or CpG DNA. Moreover, the role of RNA sensors in tumor microenvironment has not been clarified yet (Table 2).

DAMPs have recently been identified on the molecular level (11) and representative DAMPs and their receptors (listed in Table 1) include HMGB1 (28), crystalized uric acid (10), S100 proteins (29), naked actin (30,31) and HSPs (32). Their functional features and mechanisms of provoking inflammation have been delineated (11,28,29) and these studies have introduced the concept of the "inflammasome" to the field of innate immunity (33). Caspase 1 is activated by stimulation with NOD-like receptor (NLR) ligands, which contain some DAMPs and crystalized inorganic materials of PAMPs. This, together with up-regulation of preproforms of IL-1 family proteins by TLR stimulation, accelerates the robust release of IL-1β, IL-18 and IL-33 (34). There are many kinds of NLRs as well as TLRs, and the common pathway (including the adaptor ASC) of these NLRs can be activated by a variety of cytoplasmic DAMPs and PAMPs (33,34). These cytoplasmic procytokines are activated by limited proteolysis by caspase 1, and are secreted from the cytoplasm to the cell exterior (34). Hence, IL-1 family proteins require two DAMPs/PAMP signals for their up-regulation and activation (35). The tumorigenic properties of asbestos and silica are in part attributable to inflammasome activation resulting in production of IL-1 family proteins. However, not all DAMPs contain an inflammasome activator, even in the broad sense of this term

Immune response secondary to phagocytosis of dead cells

Phagocytosis of dead cells encompasses not only cell clearance but, in addition, initiation of the immune response. Dead cell antigens are rapidly presented on MHC class II after internalization by dendritic cells, driving the recruitment of CD4 T cells, including Th1/2, Th17 and Treg (Fig. 3). Providing the presence of the second signal of TLRs namely adjuvant, dendritic cells induce cross-presentation, leading to mounting Ag on MHC class I to induce proliferation of CD8 T cells (CTL) (36). The presentation of exogenous Ag by dendritic cells is therefore dependent on the functional features of PAMP/DAMPs (36). In this view, necrotic debris appears to more efficiently cross-prime CTL than apoptotic cells. Cross-presentation is enhanced by molecules such as type I IFN and CD40, and by immune cells including CD4 T, NK and NKT cells. Hence, addition of some adjuvants to the whole body immune system affects many cell types other than antigen-presenting cells, and evaluation of the total output of cross-priming activity is indispensable for adjuvant therapy.

TLR3/TICAM-1 is best illustrated as an *in vivo* inducer of cross-presentation (37). PolyI:C or virus dsRNA are examples of TLR3 ligands, and the cross-presentation-inducing activity of these TLR3 agonists was first described by Schulz et al, in 2005 (38). While effective adjuvancy of polyI:C has since been reported by Steinman and colleagues (37,39), the identity of the DAMPs participating in cross-presentation and possessing latent cross-priming (CTL-inducing) ability has not yet been determined.

It is known that phagocytosis induces functional modulation of mDC and Mf (Fig. 3): phagocytes are skewed to a regulatory phenotype by producing IL-10 and TGF-β in response to PAMP during phagocytosis of apoptotic cell debris (40,41). This suggests that uninternalizable material exerts different effects than internalizable material on mDC during their phagocytic interactions. Phagocytes undergo cytoskeletal rearrangement when they phagocytose debris, which integrates cell adhesion molecules that accelerate interaction between phagocytes and dead cell debris. Opsonization of dead cells further enhances phagocytosis as well as induction of immune modulation (42). Complement-mediated opsonization of dead cells strongly triggers immune modulation of mDC/Mf (43). Yet, apoptotic and necroptotic cells have been studied on this issue without exact discrimination between them (44) and for this reason, the mechanism whereby necroptotic cells initiate immune modulation through phagocytosis

by mDC/Mf compared to apoptotic cells remains largely uncharacterized. Elucidating the role of necroptotic cells and DAMPs as adjuvants for NK activation and Ag-presentation is relevant to antitumor therapy. Since phagocytosis of dead cells by mDC usually induces tolerogenic mDC, additional adjuvants are necessary to stimulate presentation of tumor Ag on the MHC of mDCs, which would be important for induction of an effective immune response against cancer.

Termination of inflammation

Inflammation directs tissue repair and regeneration in the damaged lesion, and the microenvironment formed during inflammation serves a base for assembling stem cells to initiate tissue development and reorganization (Fig. 3). The inflammatory environment simultaneously confers signals for genome instability and cell growth on the damaged region and facilitates the accumulation of gene mutations. Furthermore, incipient inflammation compromises the immune system to the extent that abnormal proliferation of malignantly transformed cells is tolerated. The malignant cells construct a kind of organ that involves tumor-associated macrophages serving a scaffold for invasion and metastasis (45). In this context, a region harboring DAMPs-mediated persistent inflammation provides a nidus for tumor progression (Fig. 3). Therapeutics for suppressing inflammation, such as aspirin, may be a kind of immune therapy irrespective of the presence or absence of infection (46). We surmise that two types of inflammation exist, namely tumor-supporting and tumor-suppressing, implying that inflammation is a complex consisting of many differential molecular bases. We have shown that certain adjuvants can induce tumor-suppressing inflammation, thereby limiting tumor proliferation by DAMPs (47). The redirection by adjuvant of cell death/inflammation signals to antitumor properties is a potentially intriguing therapy for cancer, particularly so since the mechanism of adjuvant-induced signaling is being increasingly well characterized on the molecular level (48,49). Clarification of the role of adjuvant signaling in compromising tumor progression will lead to the discovery of non-toxic synthetic tumor-regressing adjuvants with potential as novel cancer therapeutics (50).

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Figure legends

Fig. 1. TLR3 signaling for cell death and effector induction in myeloid cells.

Cell maintenance (left panel) and cell death (right panel) signals are schematically depicted. TLR3/TICAM-1 assembles a molecular complex around the oligomerized TLR3 in the endosome for effector induction. The complex (named Speckle) translocates to the cytoplasm, dissociating from the TLR3/endosome. IRF-3 and NF-κB are activated in Speckle, leading to their nuclear translocation and induction of type I IFN and inflammatory cytokines, respectively. In dendritic cells, NK-activating ligands and factors for cross-presentation are induced downstream of IRF-3/7 (left panel). In contrast, cell death signal occurs with apoptosis and/or necrosis depending on the TLR3/TICAM-1 signaling (right panel). TLR3-dependent apoptosis has been reported in several cancer cell lines (49), and TLR3-dependent necroptosis has been reported in mouse bone marrow-derived Mf (13). These events occur based on RIP1/RIP3 activation, signals similar to those in TNFR1. Whether or not relocation of the TICAM-1 complex is required for the cell death signal, as well as the mechanism determining either the IFN/cytokine or the cell death signal, remains unknown.

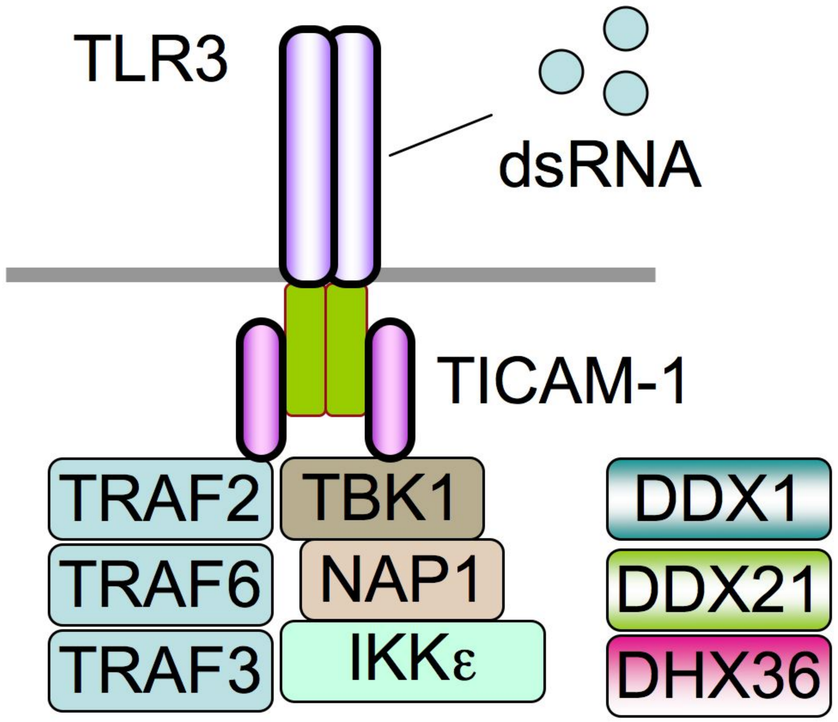
Fig. 2. Necroptosis induced by the DAI pathway.

Cell maintenance (left panel) and cell death (right panel) signals induced by DAI are schematically depicted. The cell maintenance signal induces activation of IRF-3 and NF-κB to support antiviral responses (left panel). Type I IFNs and inflammatory cytokines are the main effectors induced by IRF-3/NF-κB activation. In contrast, DAI activates RIP3 to induce necroptosis in DNA virus infections in which caspases are inhibited. When viruses express caspase inhibitors, the RIP1/RIP3 necrosome signal plays a dominant role in the compensation of cell death with necroptosis (right panel). If caspase 8 functions for RIP3 inactivation, apoptosis may a dominant phenotype, though the scheme has not yet been experimentally confirmed. The mechanism discriminating between the two signaling events is unknown.

Fig. 3. Inflammation provides the environment for infection-related cancer.

Immune cells infiltrating the tumor mass may modulate the tumor microenvironment by recognition of PAMP/DAMPs. Cancer cells undergoing necrosis liberate DAMPs and debris containing nucleic acids, which recruit immune cells involved in the inflammatory response. Tumors in some cases benefit from the inflammatory response and in other cases regress in response to inflammation, and the mechanism determining this switch remains to be clarified.

TLR3 signal for inducing effectors



Speckle formation in cytoplasm

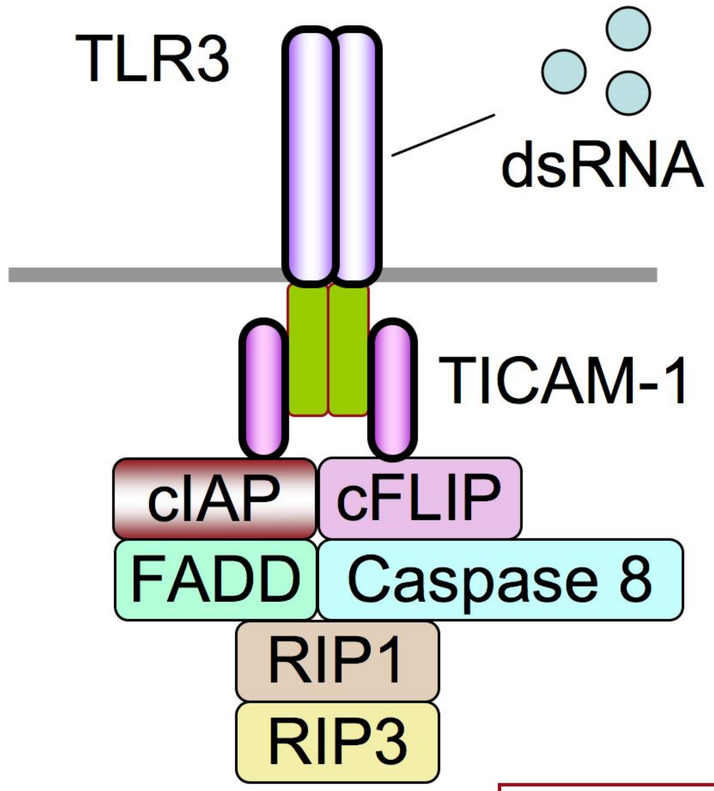
Activation of NF-κB/IRF-3

NK cell activation
CTL induction

Interferons
cytokines

Host protection

TLR3-mediated cell death



Relocation

DISC (Comp Ⅱa)

FADD Caspase 8

RIP1

cFLIP

Apoptosis

Necrosome (Comp Ⅱb)

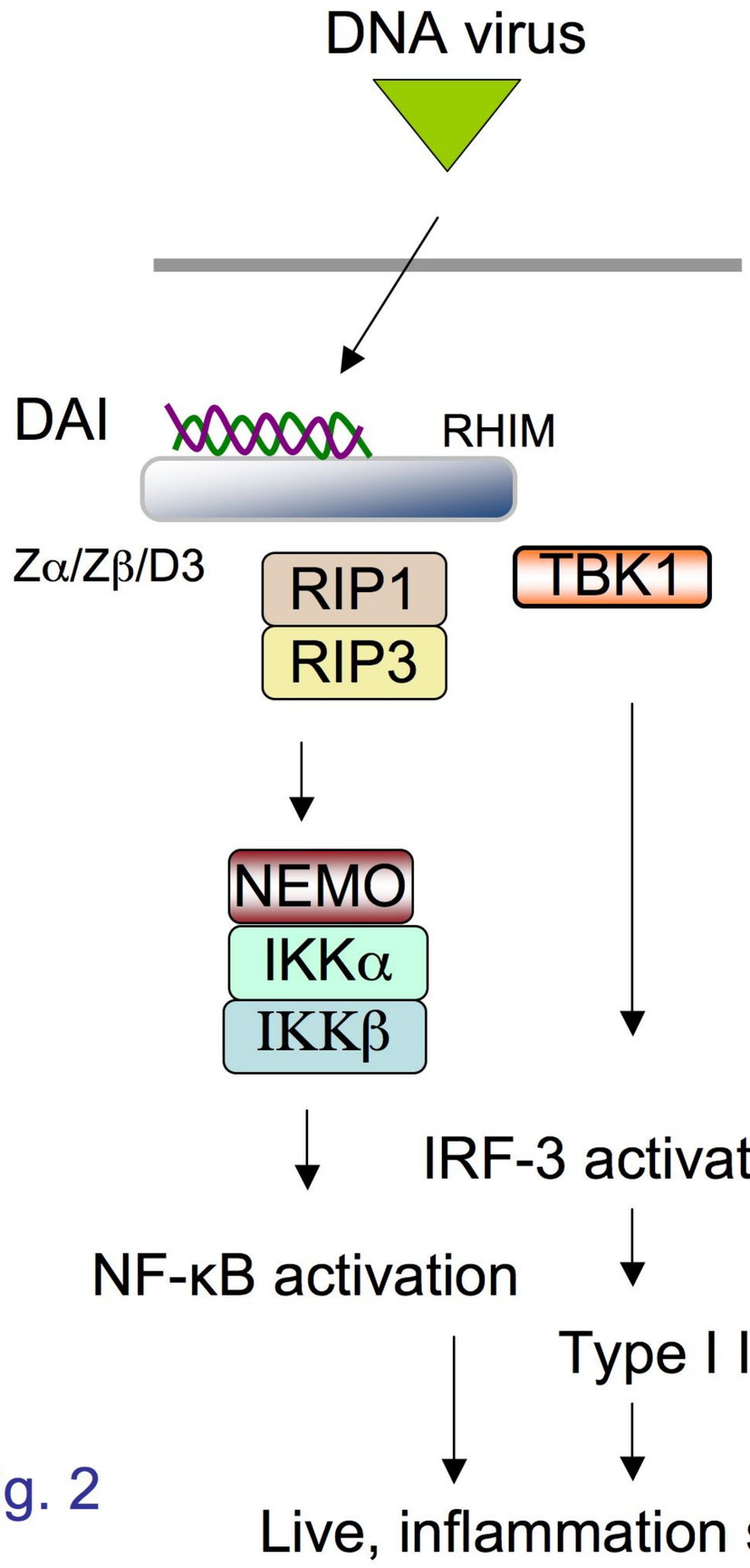
P RIP1

? RIP3 P direct ?

Necroptosis

Fig. 1

DAI-dependent live signal



DAI-mediated cell death

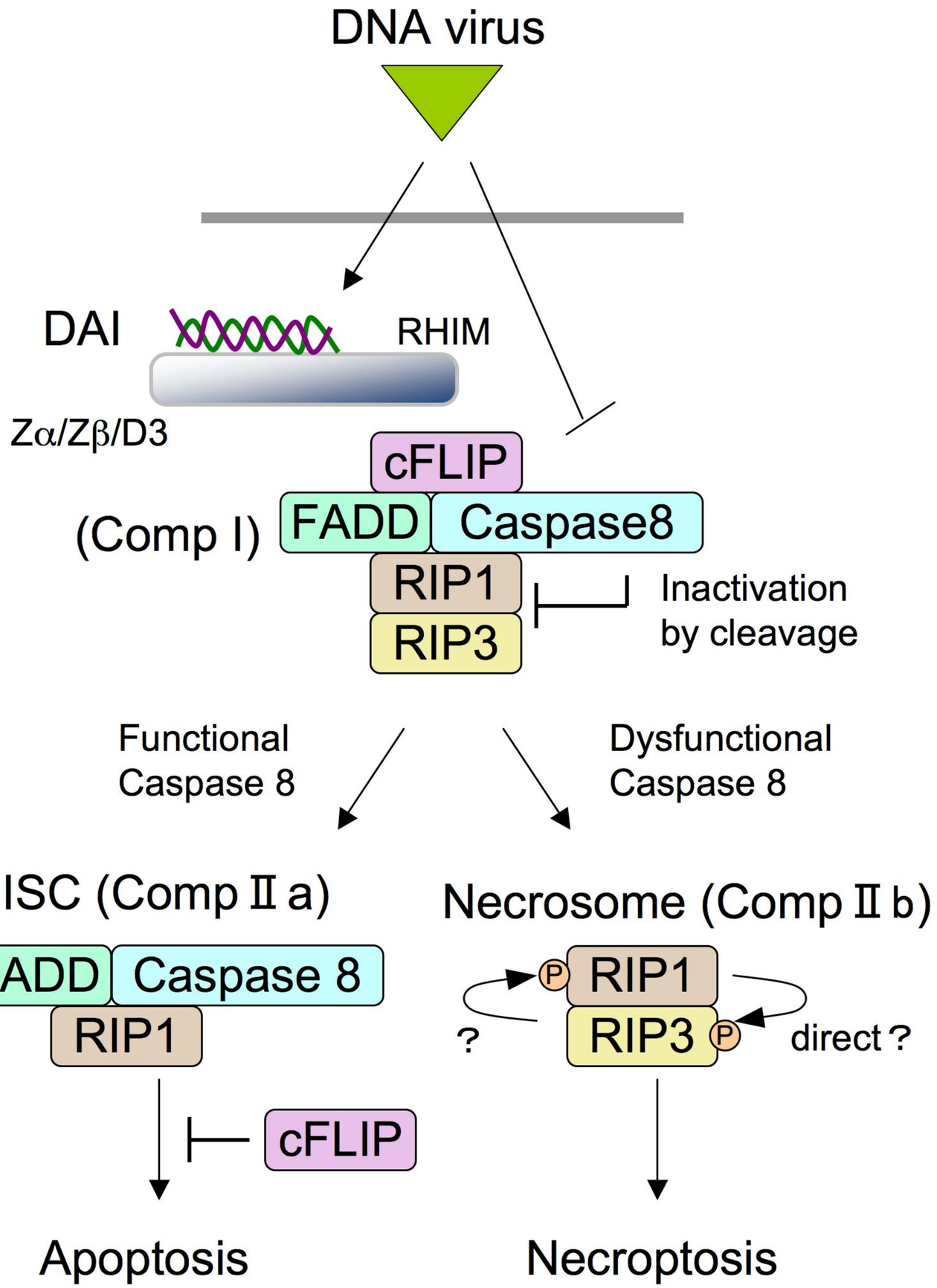


Fig. 2

Fig. 3

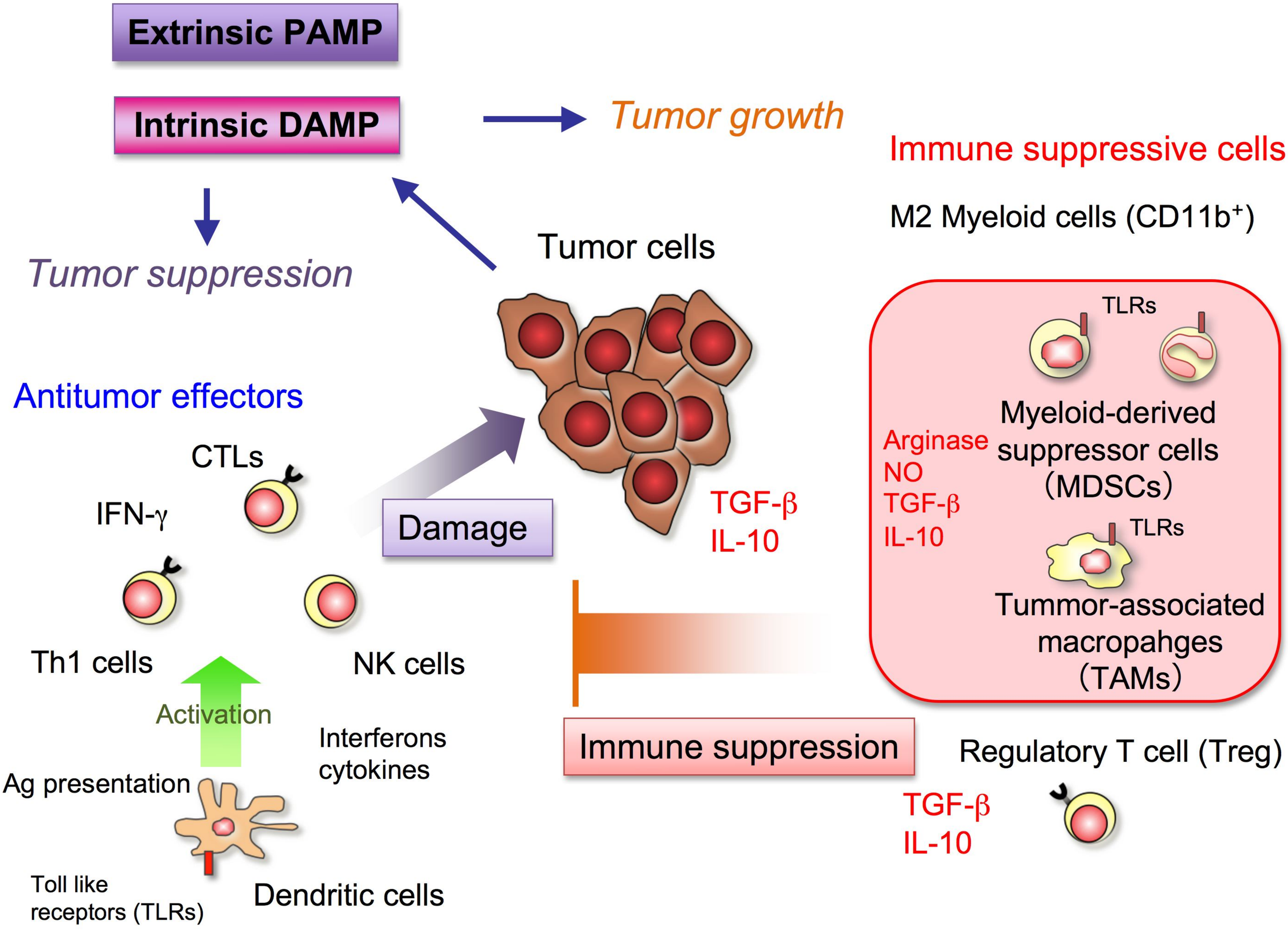

Extrinsic PAMP
Intrinsic DAMP
Tumor growth
Tumor suppression
Tumor cells
Immune suppressive cells
M2 Myeloid cells (CD11b+)
TLRs
Myeloid-derived suppressor cells (MDSCs)
Arginase
NO
TGF-β
IL-10
TLRs
Tummor-associated macropahges (TAMs)
Antitumor effectors
CTLs
IFN-γ
Damage
TGF-β
IL-10
Th1 cells
NK cells
Activation
Interferons cytokines
Immune suppression
Regulatory T cell (Treg)
TGF-β
IL-10
Ag presentation
Toll like receptors (TLRs)
Dendritic cells

Table 1 . Host response to nucleic acids and other DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids (PAMP)	
cytosolic long dsRNA	MDA5
cytosolic 5'-PPP-RNA	RIG-I
endosomal >140bp dsRNA	TLR3
nonmethylated CpG DNA	TLR9
cytosolic dsDNA	DNA sensors*
Self molecular patterns (DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4,**
S100 proteins	RAGE
Self nucleic acids (DAMP)	
Self DNA	DNA sensors*
Self mRNA	TLR3

*See Table 2; ** D40, CD91, Scavenger receptors etc.

Table 2. RNA・DNA recognition molecules in vertebrates

Receptors	Adaptors	Ligands	Induction of type I IFN
TLR family			
TLR3	TICAM-1	dsRNA, stem RNA	+
TLR7/8	MyD88	ssRNA	+
TLR22	TICAM-1	dsRNA	+
PKR	?	dsRNA	--
RLR family			
RIG-I	MAVS	5'-PPP RNA, dsRNA	+
MDA5	MAVS	dsRNA (long)	+
NLR family			
NALP3	ASC	dsRNA	+
NOD2	MAVS	ssRNA	+
DDX family			
DDX1	TICAM-1	dsRNA	+
DDX21	TICAM-1	dsRNA	+
DHX36	TICAM-1	dsRNA	+
DNA sensors			
TLR9	MyD88	CpG DNA	+
DAI	TBK1	dsDNA	+
Pol3/RIG-I	MAVS	dsDNA	+
IFI16	TBK1	dsDNA	+
DDX41	STING	dsDNA	+
DHX9	MyD88	dsDNA	+
DDX36	MyD88	dsDNA	+
ZAPS	?	dsDNA	+