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Apoptosis Induction by Cytosolic RNA Helicases

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Abstract

Host protection against viruses depends on mounting an effective innate and adaptive immune response. Aside from the production of type I interferon and inflammation activation, a third line of defense against viral infections is the induction of programmed cell death (apoptosis) in infected cells. Recent work has uncovered essential roles for the cytoslic RNA helicases RIG-I and MDA-5 in the induction of apoptosis in diverse cell types. Here we discuss the mechanisms of RLR-induced programmed cell death as a mechanism of antiviral defense.

Introduction

Viruses are infectious particles that consist of a genome, a protein coat, and, in some cases, a lipid envelope. In order to proliferate, they reprogram the cellular machinery of host cells to reproduce the viral genome, which consists of either single (ss)- or double-stranded (ds) DNA or RNA, and synthesize viral proteins, thus enforcing the assembly of viral progeny able to propagate infection. The clinical manifestation of a viral infection depends on the type of virus and the immune status of the host and can range from apparent, subclinical infection over acute life-threatening illness to chronic, immune resistant disease. Since virtually all components of an infectious viral particle are host derived, viral infections pose an exceptional challenge to the immune system.

One important line of defense against infection is to promote survival of host tissues by mounting an inflammatory response with activation of the innate and adaptive immune system. The first step in this process is the detection of viral particles by germ line-encoded pattern recognition receptors (PRRs) which recognize signature molecules of different classes of pathogens, called pathogen associated molecular patterns (PAMPs). The most important viral PAMP is double-stranded RNA, but other unique nucleic acids such as uncapped single stranded RNA or several virus-encoded glycoproteins can also be decoded as PAMPs. Several classes of PRRs are involved in the detection of viruses [1]. As such, Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are transmembrane receptors which reside on the cell-membrane and/or in the endo-lysosomal compartment, screening the extracellular space for the presence of microbial or viral PAMPs. RNA helicases of the retinoic acid-inducible gene (RIG)-I like receptor family (RLRs), NOD-like receptors (NLRs) and the PYHIN/IFI200 family member absent in melanoma 2 (AIM2), on the other hand, are cytosplasmic receptors responsible for the detection of intracellular pathogens [2,3]. Upon recognition of viral PAMPs, these PRRs induce the production of type I interferon and activate an 'antiviral state', a complex, interferon induced global gene expression profile targeted to blunt viral propagation at many levels [4]. Sentinel innate immune cells like dendrite cells and macrophages then instruct and coordinate an innate and adaptive immune response, promoting host cell survival and, ultimately, recovery from viral infection and disease.

A second, less obvious line of defense against viral infection is programmed cell death of infected cells. Viral propagation requires a functional host metabolism. Host cell demise therefore affects the replicative niche and exposes the virus to extracellular immune surveillance, a process sometimes referred to as 'altruistic suicide' [5]. Uptake of dying infected cells by macrophages and dendritic cells promotes activation of the adaptive immune system by presenting viral and microbial antigens to T cells [6].

Programmed cell death is an active cellular suicide mechanism which proceeds along genetically controlled pathways in a cell autonomous manner. Currently, three main cell death signaling pathways are defined, each one characterized by certain distinct molecular and morphological features: *Apoptosis, necrosis* and *pyroptosis. Apoptosis* describes a cell death pathway which depends on the activity of caspases, a set of cysteine proteinases specific for aspartate residues. Caspases are stored in the cytosol of all cells in an inactive pro-form and get activated by proteolytic cleavage. So called initiator caspases (caspase 2, caspase 8, caspase 9, caspase 10) trigger signaling cascades ultimately leading to activation of executioner caspases (caspase 3, caspase 6, caspase 7) which mediate cell death by interfering with vital metabolic pathways such as DNA repair. Cells undergoing apoptotic cell death show cell body shrinkage, plasma membrane blebbing, nuclear condensation and fragmentation, cleavage of chromosmal DNA into internucleosomal fragments, selective cleavage of various cellular proteins, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane and formation of membrane-bound cell fragments (apoptotic bodies) ready for phagocytosis by neighboring cells and resident phagocytes [6,7].

Apoptosis principally proceeds along two distinct but overlapping pathways, the intrinsic/mitochondrial pathway and the extrinsic/death receptor pathway. The intrinsic pathway can be viewed as a mechanism relying upon 'cellular introspection'. Disturbance of cellular homeostasis, as for example during environmental stress by exposition to chemotherapeutic drugs, ultraviolet radiation or microbial infection, leads to activation of BH3-only proteins like Noxa, Puma, Bid, Bim, Bik, Bad, Hrk and others, pro-apoptic members of the Bcl-2-family of proteins. BH3-only proteins induce oligomerization of another subfamily of Bcl-2 poteins called Bax. Oligomerized Bax proteins (Bax and Bak) insert into the outer mitochondrial membrane and permeabilize it for cytochrome c, a highly soluble protein and essential component of the mitochondrial electron transport chain and energy production, and other mitochondrial proteins like second

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mitochondrial activator of caspases (SMAC/Diablo) and HtrA2/Omi. Upon release from the mitochondrial inter membrane space into the cytosol, cytochrome c binds with apoptotic protease activating factor-1 (Apaf-1). Cytochrome c and Apaf-1 form the so-called apoptosome complex and induce activation of the initiator caspase 9, which leads to direct proteolytical cleavage of executioner caspases 3, 6 and 7. These 'execute' the cell through proteolytic cleavage of vital subtrates [6-8]. For full activation, these executioner caspases need to be released from an endogenous inhibitor called X-linked inhibitor of apoptosis (XIAP). SMAC/Diablo and HtrA2/Omi, which leave the mitochondrial intermembrane space along with apoptosome-forming and caspase-activating cytochrome c, reduce the activity of XIAP and thus release the brakes on apoptosis [9].

The extrinsic pathway is triggered upon ligand-binding to death receptors of the tumor necrosis factor (TNF) receptor family. Typical ligands for these transmembrane death receptors are tumor necrosis factor (TNF), Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL), all of which are present under inflammatory conditions. Upon death receptor ligation, the cytosolic receptor domain associates with the adaptor molecule Fas associated death domain (FADD) which in turn recruits procaspase 8 to form a signaling platform called Death inducing signaling complex (Disc). Mature caspase 8 cleaves procaspase 3 and other executioner caspases, inducing the common final pathway of both intrinsic and extrinsic apoptosis leading to cell death [6,8].

Notably, apoptosis does not usually go along with significant inflammation. This can probably be attributed to scrupulous housekeeping performed during the process: Membranes are maintained intact, avoiding spillage of intracellular content into the extracellular space. Nevertheless, it is important to note that apototic cells have in fact been shown to induce immune responses under certain conditions [10].

Apart from apoptosis, other forms of programmed cell death have been described. Both *necroptosis*, a term that denotes a 'programmed' form of necrosis, and *pyroptosis* are considered proinflammatory, immunogenic forms of programmed cell death. A detailed description of these processes is beyond the scope of this article. Excellent articles are available for further information [11,12].

Interestingly, recent findings provide evidence that both arms of antiviral defense, promotion of host cell survival through mounting of a coordinated immune response and host cell altruistic suicide to reduce the viral replicative niche, are activated by RIG-I like receptors. There are three known RLRs: Retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA-5 have both been shown to specifically mediate recognition of a variety of different viruses, whereas LGP2 is believed to function as a regulator of RIG-I and MDA-5 signaling [13]. All RLRs contain a central DExD/H box (DEAD box) helicase domain which binds RNA and hydrolizes ATP, and a C-terminal repressor domain involved in autoregulation. As an N-terminal addition to the DEAD box, RIG-I and MDA-5 have a tandem caspase activation and recruitment domain (CARD), missing in LGP2. The CARD domains serve as signaling hubs to mediate homotypic interaction with the CARD domain of the adapter IFN-β-promoter stimulator 1 (IPS-1), also known as MAVS, VISA or Cardif. In the absence of ligand, the C-terminal repressor domain is believed to cover the CARD domains and physically prevent them from signaling interactions. IPS-1 is a CARD-containing protein anchored to the mitochondrial membrane and other intracellular membranes (e.g. peroxisomes) by its C-terminal transmembrane domain. IPS-1 thereby relocates RIG-I and MDA-5 to the outer mitochondrial membrane and forms an IPS-1 signalosome for type I IFN production (through activation of interferon regulatory factor 3 (IRF3) and IRF7) and proinflammatory cytokine production through activation of CARD9, Bcl10 and the transcription factor NF-κB [14]. RIG-I also activates an ASC dependent inflammasome, inducing caspase 1 dependent processing of proIL-1β and proIL-18 into their mature forms [15]. Thus, virus induced RLR signaling leads to production of IRF3/7 dependent type 1 interferon, NFκB-dependent pro-inflammatory cytokines and caspase 1 dependent IL-1β and IL-18, allowing for a potent antiviral response. RLRs are ubiquitously expressed, and their expression is boosted under inflammatory conditions such as viral infection [14].

The molecular structure of RLR ligands is becoming increasingly clear. RIG-I is located in the cytosol and recognizes 5' triphosphate RNA (pppRNA) generated by viral RNA polymerases in the cytosol of host cells. Other than carrying 5´ triphosphate ends, optimal RIG-I ligands are short ds RNA molecules from 19 bp to about 1 kb with certain RNA sequences and secondary motifs described to enhance ligand quality. Not all these structures have to be present in one ligand, but strong ligands usually combine more than one of these properties [14] of note, pppRNA does occur physiologically in the nucleus of uninfected cells, but it loses its RIG-I ligand activity before it is released to the cytosol due to processing such as splicing, addition of a 5' cap or additional modifications [16,17]. MDA-5, in contrast, recognizes long ds RNA molecules (<2kb) which can be mimicked by the synthetic ds RNA molecule poly (I:C). Curiously, RIG-I was originally described as a ds RNA-binding protein that triggered IFN production in response to poly (I:C) [18]. This is explained by the finding that shortening of long poly (I:C) molecules progressively converts them from ligands for MDA-5 to ligands for RIG-I. Both short and long ds RNA molecules occur as viral replication intermediates, according to the type of virus involved.

Additionally, short ds RNA is not only a ligand for RIG-I but also for cytosolic RNA activated protein kinase (PKR) [19], and long ds RNA activates not only MDA-5 but also endosomal TLR3 [20] and cytosolic PKR [21]. Differential activation of these receptors requires compartmentalized ligand application, achievable through application of uncomplexed, 'naked' ds RNA molecules for endosomal uptake or liposome complexed ds RNA for access to the cytosol.

Pathogen and host have experienced a common evolution. Viruses have therefore been able to develop escape strategies, such as viral proteins able to modulate apoptosis or IFN pathways. These have complicated the identification of host antiviral signaling pathways. Thus, viral RNA mimetics like pppRNA or poly (I:C) are advantageous for analyzing the basic principles of the antiviral response and have facilitated the characterization of the inflammatory response triggered by RLR ligation. Defined RLR ligands are now used to elaborate antiviral signaling pathways leading to programmed cell death. The following review will focus on RLR induced programmed cell death as a mechanism of antiviral defense.

Induction of apoptosis by cytosolic application of ds RNA

Type I interferons have a general antiproliferative effect on various tissues and are sometimes referred to as 'negative growth factors'. In fact, recombinant IFN α/-β is used for the treatment of some forms of human cancer. IFNs bind to cell surface receptors, inducing downstream signaling which leads to the activation of interferon-stimulated genes (ISGs), among which more than 15 genes have pro-apoptic functions [22]. In order to investigate reduction of tumor growth as treatment

response after application of cystosolic ds RNA, intrinsic pro-apoptic effects must be differentiated from indirect effects mediated by type I interferons, or an adaptive antitumoral immune response.

An early report has shown antiproliferative effects of cytosolic ds RNA on cancer cells [23]. Using cationic liposomes or cellular microinjection to deliver poly (I:C) to the cytosol, a plethora of human cancer cell lines were screened. Cytosolic poly (I:C) inhibited cell growth of a wide variety of malignant cell lines, whereas naked poly (I:C) did not. Growth inhibition was shown to go along with DNA-fragmentation, suggesting that apoptosis was involved. Cancer cells of epithelial or fibroblastic origin were most sensitive, while normal fibroblasts and embryonic or normal liver cells did not die upon such treatment. Variability was also found in the sensitivity of different malignant cells, as leukemia or lymphoma cell lines did not show growth suppression, possibly reflecting inadequate transfection protocols or differing signaling pathways. Interestingly, treatment with type I interferon did not inhibit malignant cell growth, even at high doses, and neutralizing antibodies to IFN α/β did not reduce the antiproliferative effect of cytosolic poly (I:C), pointing towards cell intrinsic, IFN-independent mechanisms. Furthermore, systemic application of cationic poly (I:C)-liposomes inhibited growth of human colon carcinoma cells in a xenograft model of metastatic liver disease, inducing morphologic features of apoptotic cell death in affected cancer tissue. With the concept of pattern recognition receptors yet evolving, the authors had provided early evidence for selective antiproliferative effects of transfected poly (I:C) on various human cancer cell types and postulated cell intrinsic pro-apoptic signaling.

Another report demonstrated similar effects with a mouse B16 melanoma model. Peritumoral injection of complexed poly (I:C) showed major suppression of tumor growth on subcutaneous B16 allografts. The authors described a tumor specific immune reaction with activated infiltrating dendritic cells and tumor specific CD8 T-cells in peritumoral tissue and draining lymph nodes. Possible direct pro-apoptic effects of cytoslic ds RNA were not investigated [24].

In this respect, Peng et al. [25] found that poly (I:C) treated human hepatocellular carcinoma cells shrunked, detached and showed activation of caspase 3 and 7, while caspase 8, a crucial initiator caspase of the death receptor associated extrinsic pathway of apoptosis, was not activated. The pan-caspase inhibitor Z-VAD reduced cell death induced by poly (I:C) transfection. Suggesting involvement of the mitochondrial pathway of apoptosis, involvement of caspase 9 and cytochrome c release were not formally addressed [25]. Inao et al. [26] demonstrated reduced viability of various human breast cancer cell lines after transfection with poly (I:C).

Treated cells shrank in size and displayed positivity for Annexin V. Apart from activation of caspase 3/7; the mitochondrial initiator caspase 9 was shown to be activated in all cell lines [26]. Taken together, these studies pointed towards RLR dependent antiproliferative effects on cancer cells mediated by the activation of canonical signaling pathways of apoptosis and the induction of an antitumoral immune response.

Mechanisms of RLR induced apoptosis

Evidence for a connection between RLR activation and the induction of apoptosis initially came from over expression studies in transfection-permissive cell lines. MDA-5 was first described as a gene whose expression was induced in type 1 IFN-treated human melanoma cells and had melanoma growth-suppressive properties [27].

Over expression of MDA-5 in human melanoma cells was then shown to induce apoptosis [28]. Regarding RIG-I, one group reported activation of caspase 3 upon forced expression in a human keratinocyte cell line. In addition, over expression of IPS-1, the essential adapter for RIG-I and MDA-5 induced proinflammatory cytokine and interferon responses also induced caspase 3 activation [29]. Additionally, over expression of IPS-1 in HEK 293 cells lead to caspase dependent induction of mitochondrial apoptosis in the absence of interferon signaling. Upstream receptors were not directly defined, but over expression of RIG-I or MDA-5, in contrast to the data obtained with keratinocytes and melanoma cells, did not induce apoptosis in HEK 293 cells, probably reflecting cell type specific differences in apoptosis regulation or lack of crucial adapter molecules for RLR-dependent signaling [30]. An IPS-1 dependent, IRF3 independent activation of caspase 3 and induction of apoptosis via the mitochondrial pathway was independently confirmed using as mouse neuroblastoma cell line [31].

First direct evidence for RIG-I induced apoptosis came from a study investigating effects of defined RIG-I ligands on B16 melanoma cells. Poeck et al. [32] showed RIG-I dependent induction of apoptosis in B16 cells after transfection of short ds RNA molecules with a 5' triphosphate end (pppRNA). In B16 cells, this pro-apoptic effect was dependent on interferon signaling, since siRNA-mediated down regulation of IFNAR1, which together with IFNAR2 composes the interferon α/β-receptor IFNAR, strongly diminished cell death. In a mouse model for metastatic growth of B16 melanoma cells in the lung, i.v. injection of RIG-I ligands strongly reduced the number of lung metastases. This effect was shown to be mediated by RIG-I and depended on both interferon signaling and the activity of natural killer (NK) cells in immunocompetent mice [32]. These data pointed towards an immunogenic aspect of RIG-I induced apoptotic cell death.

The induction of a specific antitumoral immune response by RLR ligands was also suggested by two publications of Barchet and collegues who investigated the effect of RLR stimulation on human epithelial ovarian cancer (EOC) cells. When transfected with pppRNA or poly (I:C), activation of RIG-I or MDA-5, respectively, induced apoptosis in EOC cells from ascites of treatment-naive disseminated ovarian cancer patients. RLR-activated EOC cells upregulated MHC-I, proinflammatory cytokines, chemokines and type I IFN. When co-cultured with monocytes, the predominant APC in peritoneal fluid of EOC patients, RLR ligand treated EOC cells were phagocytosed and induced monocytes to express co-stimulatory molecules, chemokines and type I IFN, at the same time suppressing IL-10 production. These latter findings were in contrast to monocytes co-cultured with EOC cells treated with conventional chemotherapeutic agents, which did not induce type I IFN production and upregulated release of immunosuppressive IL-10. The hypothesis drawn from these studies was that RIG-I and MDA-5 induce an immunogenic form of programmed cell death [33,34].

In a subsequent study, the pro-apoptic properties of RIG-I and MDA-5 were investigated in molecular detail [35]. Transfecting human melanoma cells with pppRNA resulted in positivity for Annexin V and activation of caspase 3, all bonafide markers for apoptotic cell death. Treatment with poly (I:C) induced apoptosis to a similar extent. Using siRNA knockdown, apoptosis was shown to be mediated by RIG-I or MDA-5, respectively, but was independent of TLR3 and PKR. Both pppRNA and poly (I:C) induced up regulation of IFNβ mRNA. Apoptosis and IFN induction by pppRNA and poly (I:C) were dependent on the adapter molecule IPS-1. Contrary to the finding in mouse B16 melanoma cells, apoptosis induction in human

melanoma cell lines was independent of IFN and IRF3, indicating cell type specific differences in RLR mediated apoptosis regulation. To separate intrinsic pro-apoptotic effects of RLR ligands on tumor cells from secondary immune mediated effects such as NK cell activation, NOD/SCID immune-deficient mice were used for a lung metastasis model with human melanoma cells. These animals have a strongly impaired cellular immune system including B cells, T cells, and NK cells. Both size and number of human melanoma lung metastases were significantly reduced after i.v. treatment with pppRNA or poly (I:C), providing strong support to the hypothesis that RIG-I and MDA-5 can induce cell intrinsic apoptosis of tumor cells, leading to tumor regression independent of a secondary antitumoral immune response. Dissecting apoptotic pathways responsible for RLR induced cell death on a molecular level, transfection of pppRNA or poly (I:C) was shown to depend on caspase 9 and Apaf-1, followed by cytochrome c release. Caspase 8 and FADD were dispensable for efficient apoptosis induction but caspase 8 was secondarily activated by caspase 9. These data clearly indicated that RLR induced apoptosis is mediated by the intrinsic, mitochondrial pathway of apoptosis. In order to elucidate the link between RLR induced, IPS-1 dependent signaling and the induction of mitochondrial apoptosis, expression and activity of BH3-only proteins, central inducers of intrinsic apoptosis, were evaluated.

Although both Noxa and Puma were shown to be induced on the protein level, only knockdown of Noxa but not Puma reduced the amount of apoptotic cells. Contrary to interferon induced sensitization to apoptosis, RLR induced apoptosis by RIG-I and MDA-5 was not to be mediated by p53 [35].

Cell Type Specific Sensitivity for rlr Induced Apoptosis

A growing body of evidence seems to suggest that tumor cells may be more susceptible to programmed cell death than nonmalignant cells. Primary human melanocytes, fibroblasts and keratinocytes isolated from healthy skin were less sensitive for apoptosis induction by pppRNA or poly (I:C) than mouse and human malignant melanoma cells. No apoptosis was induced in various lymphocyte subsets exposed to the same stimulus [32,35]. Certain common alterations of tumor cells, such as the phenomenon referred to as 'oncogene addiction', may result in increased vulnerability to certain apoptotic stimuli. Yet, despite phenotypic resistance of nonmalignant skin cells to apoptosis, transfection of these cells with pppRNA and poly (I:C) induced expression of Noxa and Puma to a similar extent as in malignant melanoma cells. The difference was instead found downstream of pro-apoptotic BH3-only proteins: Expression analysis revealed that the pro-apoptic Bcl2-family protein Bcl-x_L was upregulated in nonmalignant skin cells but not in malignant melanoma cells after RLR-ligand transfection, and that specific siRNA knockdown of Bcl-x_L in nonmalignant skin cells strongly increased the sensitivity to apoptosis, leading to cell death after RLR-ligand transfection [35]. This showed that RIG-I and MDA-5 activate pro-apoptic signaling in both tumor cells and nonmalignant cells, but nonmalignant cells are protected from cell death by activation of the antiapoptotic Bcl2-familiy member Bcl-xL. Nonmalignant skin cells but not malignant melanoma cells therefore seem to be able to counterbalance pro-apoptic stimuli of activated Noxa, selectively protecting healthy cells from programmed cell death.

In a parallel study, Soengas and collegues drew a very similar picture of RLR induced programmed cell death, focusing on the effects of poly (I:C). Similar to pppRNA, poly (I:C) induced MDA-5 dependent apoptotic cell death in mouse and human melanoma cells leading to induction of initiator and executioner caspases 8, 9, 3 and 7. Expression- and knockdown studies also singled out the BH3-only protein Noxa as mediator of apoptosis induction in melanoma cells. Induction of compensatory anti-apoptotic Bcl2-proteins like MCL-1 did not occur. Although poly (I:C) induced type I IFN production in an MDA-5 dependent manner, exogenous IFN did not recapitulate poly (I:C) induced cell death of human melanoma cells even at doses 100 times higher than those secreted after MDA-5 activation, again indicating that type I interferon was not the central mediator of apoptosis after MDA-5 activation in human melanoma cells. In the *in vivo* setting, both immunocompetent (WT) and immunodefficient (SCID) mouse strains displayed a significant reduction in numbers and size of lung metastases from mouse B16 melanoma cells after i.v. treatment with poly (I:C). Furthermore, in a mouse model of coetaneous melanoma that shares key features with the human disease, i.v. treatment with poly (I:C) significantly reduced number, size and metabolic activity of melanoma lesions.

In total, these studies identified an IFN independent, cell autonomous, pro-apoptic signaling pathway activated by RIG-I and MDA-5 in cancer cells (Figure 1).

RLR induced apoptosis in nonmalignant cells

Despite the finding of increased sensitivity of cancer cells for RLR induced apoptosis, there is evidence that cell autonomous apoptosis after RLR stimulation is not an exclusive feature of malignant cells. The group of Zhu and coworkers showed that RIG-I mediated signaling is active during normal murine myelopoiesis. Disruption of the RIG-I gene in mice lead to the development of a progressive myeloproliferative disorder characterized by marked granulopoiesis [36]. Mechanistically, the same group reported in a following study that RIG-I intrinsically induced activation of STAT1 and downstream interferon-stimulated genes (ISGs), leading to cellular growth inhibition and terminal differentiation of acute myeloid leukemia (AML) cells. Interestingly, these antiproliferative effects of RIG-I, which was originally discovered

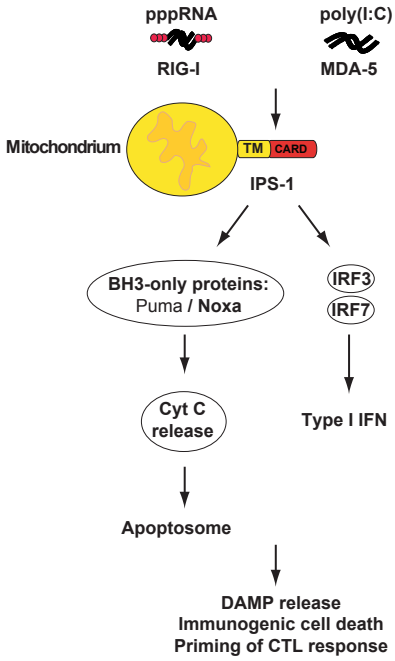

Figure 1: Apoptosome activation by RLRs.

by this group in a screen for retinoic-acid inducible genes [37], were triggered by application of retinoic acid, independent of ds RNA ligation and the canonical IPS-1 pathway [38].

These data hint towards intrinsic homeostatic, non-canonical functions of RIG-I protein in myelopoiesis, while the signaling pathways mediating these antiproliferative effects remain to be determined in detail. Dwelling on the idea of a role for PRR-activity in hematopoiesis, Liu et al. [39] investigated the effect of ds RNA on human CD34+ hematopoietic progenitors. Administration of naked poly (I:C) induced caspase dependent apoptosis in CD34 cells. Of the BH3-only proteins, Expression of Noxa was strongly induced, and effects were independent of type I IFN.

Transfected poly (I:C) was shown to have more pronounced pro-apoptotic effects. The authors suggest RIG-I and/or MDA-5 as pro-apoptotic receptor candidates for both naked and transfected poly (I:C), but do not provide experimental proof [39]. Considering the fact that all data on induction of apoptosis after RLR stimulation in nonmalignant cells was so far derived from bone marrow cells, it is tempting to speculate that nonmalignant cell types sensitive for RLR induced programmed cell death may be found preferentially in compartments with an endogenously high proliferation index, reminiscent of tumor cells.

Conclusion

Cytosolic RNA helicases of the RLR family have emerged as central pattern recognition receptors for the detection of viruses. Apart from type I IFN production and the initiation of an innate and adaptive immune response, RLR-induced apoptosis is now being recognized as another pillar of antiviral defense. Direct stimulation of the RNA helicases RIG-I or MDA-5 by either pppRNA or poly (I:C) induces signaling that activates the mitochondrial pathway of apoptosis. Available data suggests the following model: RNA helicases RIG-I and MDA-5 use the adapter molecule IPS-1 to transmit the signal to Noxa, one of the BH3-only proteins as central inducers of intrinsic apoptosis. P53 seems not to be involved in this process.

Noxa induces cytochrome c release and consequent formation of the apoptosome with Apaf-1, thereby activating caspase 9 which then leads to proteolytic cleavage of caspases 3 and 7. Evidence is clear that cytosolic RLR-mediated intrinsic apoptosis is independent of interferon or other indirect, immune mediated effects. However, most healthy cells, due to a long common evolution between pathogen and host, have developed escape strategies enabling them to avoid the execution of apoptotic suicide mechanisms. Upregulation of members of the pro-apoptotic Bcl2-family or inhibitor of apoptosis (IAP) family of proteins [40] have been proposed to convey relative protection of these cells against RLR mediated apoptosis. Interestingly, cancer cells have lost this mode of protection and suffer the unmitigated effects of RLR induced mitochondrial apoptosis. Currently, other than malignant cells, only certain types of highly proliferative bone marrow cells have shown suicide-sensitivity after RLR stimulation. The fact that RLR stimulation in nonmalignant cells unleashes signaling pathways which activate both pro-apoptotic and anti-apoptotic effects mirrors the delicate coordination of antiviral defense. In response to viral infection, prosurvival innate and adaptive defense mechanisms need to be balanced with cellular suicide programs that help to diminish the viral replicative niche. It is obvious that this balance must be differentially regulated among diverse cell types, and the varying expression of pro- and anti-apoptotic proteins is an important factor which helps to fine-tune the antiviral response.

The finding that tumor cells have lost parts of the capacity to counter-regulate pro-apoptotic effects of RLR stimulation has clear implications for tumor therapy. The increased sensitivity of cancer cells for RLR induced cell death makes RLR ligands like pppRNA or poly (I:C) candidates for mono- or combination tumor therapy, urgently sought after considering the limitations of classic cytotoxic chemotherapy, namely varying response rates and important off-target side effects in the treatment of most cancers.

Other than induction of tumor cell apoptosis, treatment with liposome complexed RLR ligands will inevitably activate IPS-I and IRF3 dependent type I interferon production in cell culture or *in vivo*, contributing to a parallel, targeted immune response. Additionally, antigen presenting cells, as part of the immune surveillance, will phagocytose RLR treated apoptotic tumor cells and potentially help to instruct an adaptive immune response against tumor components. Such a mechanism has recently been described for chemotherapy induced cancer cell death. NLRP3 dependent inflammasome activation in dendrite cells through danger associated molecular patterns (DAMPs) released from dying cancer cells was proposed to be a crucial component for the induction of an antigen specific cytotoxic T cell response, mediating antitumor activity [41]. However, data showing induction of adaptive immunity upon recognition of RLR-killed cancer cells are still missing. Nevertheless, the full potential of RLR-ligands for cancer therapy may lie in a combination of direct and indirect, e.g. interferon mediated pro-apoptotic effects and the instruction of a specific antitumor immune response, presenting an interesting topic for future research which may allow the development of new anticancer therapeutics.

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