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REVIEW



## Exploiting poly(I:C) to induce cancer cell apoptosis

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### ABSTRACT

TLR3 belong to the Toll-like receptors family, it is mainly expressed on immune cells where it senses pathogen-associated molecular patterns and initiates innate immune response. TLR3 agonist poly(I:C) was developed to mimic pathogens infection and boost immune system activation to promote anti-cancer therapy. Accordingly, TLR agonists were included in the National Cancer Institute list of immunotherapeutic agents with the highest potential to cure cancer. Besides well known effects on immune cells, poly(I:C) was also shown, in experimental models, to directly induce apoptosis in cancer cells expressing TLR3.

This review presents the current knowledge on the mechanism of poly(I:C)-induced apoptosis in cancer cells. Experimental evidences on positive or negative regulators of TLR3-mediated apoptosis induced by poly(I:C) are reported and strategies are proposed to successfully promote this event in cancer cells.

Cancer cells apoptosis is an additional arm offered by poly(I:C), besides activation of immune system, for the treatment of various type of cancer. A further dissection of TLR3 signaling would contribute to greater resolution of the critical steps that impede full exploitation of the poly(I:C)-induced apoptosis. Experimental evidences about negative regulator of poly(I:C)-induced apoptotic program should be considered in combinations with TLR3 agonists in clinical trials.

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### Introduction

Toll-like receptor 3 (TLR-3) is a member of the Toll-like receptor family, which comprises 10 members in human and is characterized by the presence of an extracellular leucine-rich repeats (LLR) domain that mediates the recognition and binding of pathogen-associated molecular patterns (PAMPs) and a cytoplasmic tail that contains a conserved region, called the Toll/IL-1 receptor (TIR) domain.<sup>1</sup> TLR-3 is expressed by sentinel cells of the innate immune system, such as dendritic cells and macrophages, and by nonimmune cells, including epithelial cells, fibroblasts, and endothelial cells. In contrast, TLR3 is absent from neutrophils and is minimally expressed in T cells.<sup>2,3</sup> TLR3 localizes to the endosomes, where it senses viral and host-derived nucleic acids and initiates inflammatory pathways, activating the innate immune response and establishing an antiviral state to prevent viral replication. Its expression modulates rapidly in response to pathogens, various cytokines, and environmental stress.

TLR3 expression on immune cells has been widely exploited to promote an antitumor immune response, and various TLR3 agonists are being examined in clinical trials for their ability to orchestrate antitumor immunity. Several phase I and II clinical trials are in progress, in which TLR agonists are being used as an adjuvant for antigen-peptide vaccination and in combination with radiotherapy.<sup>4</sup> The antitumor responses that are induced by TLR3 agonists are attributed to their capability to stimulate antigen-presenting cells (APCs), such as DCs, which

in turn activate tumor-specific T cell responses and to their capacity to switch the phenotype of myeloid suppressor cells and tumor-associated macrophages from immunosuppressive to immunosuppressive.<sup>5-7</sup>

TLR3 signaling can also occur on nonimmune cells, contributing to an antitumor response. Many types of cancer express TLR3, including breast carcinoma, oral cell squamous and esophageal carcinoma, cervical carcinoma, ovarian carcinoma, prostate carcinoma, head and neck carcinoma, lung squamous cell carcinoma and adenocarcinoma, hepatocellular carcinoma, and melanoma.<sup>8</sup> Like normal cells, cancer cell lines respond to TLR3 ligands by secreting inflammatory cytokines, type I interferon (IFN I), and chemokines, which enhance the recruitment and activation of immune cells.

Moreover, TLR3 agonists were found to promote the direct inhibition of tumor growth *in vitro* in several murine and human cancer cell models through 2 mechanisms: decreasing proliferation and inducing apoptotic cell death. TLR agonists slow tumor cell proliferation in breast and prostate cancer cell lines, and many studies have reported the apoptotic effects of TLR3 agonists in several tumor histotypes, including breast, melanoma, head and neck, prostate, renal carcinoma, colon, cervical, and lung cancer cells.<sup>8</sup>

Altogether, these studies indicate that several mechanisms contribute to the efficacy of TLR3 agonists in cancer therapy and that targeting TLR3 on tumor cells to induce their apoptosis is a potential therapeutic approach for directly interfering

with cancer progression in patients whose immune systems fail to generate a protective response. This review summarizes our knowledge on the induction of apoptosis by TLR3 agonists and focuses on new strategies to promote this effect in cancer cells.

### TLR3 signaling pathways

TLR3 is activated by extracellular double-stranded RNA (dsRNA), which is recognized by the receptor in a sequence-independent manner. TLR3 initiates a protective response against dsRNA viruses including PV (polio virus), coxsackievirus group B and serotype 3, encephalomyocarditis virus, and DNA virus infections, such as herpes simplex virus 1 and murine cytomegalovirus.<sup>9–11</sup> In addition, TLR3 recognizes dsRNA that has been transcribed *in vitro* and its synthetic analogs, such as poly (I:C) (polyriboinosinic:polyribocytidylic acid) and poly (A:U) (polyadenylic:polyuridylic acid), which have thus been used to mimic the response to RNA virus infection and are commonly administered in *in vitro* and *in vivo* studies on TLR3-mediated cellular responses.

In myeloid dendritic cells, TLR3 localizes exclusively to the early endosome, whereas macrophages, fibroblasts, and certain epithelial cells also express it on the cell surface. The signal transduction pathway that is mediated by TLR3 begins in the acidic environment of endosomal compartments.<sup>12</sup> Recent evidence has shown that in addition to recognizing dsRNA, cell membrane-bound TLR3 triggers a predominantly proinflammatory response.<sup>13</sup> The ability of exogenous RNAs to induce cellular responses depends primarily on their stability in the extracellular space and their mode of entry into cells. Unlike single-stranded RNA (ssRNA), dsRNA is resistant to degradation, and thus, the viral dsRNA that is released from infected cells can be a potent activator of uninfected surrounding cells, leading to the establishment of an antiviral state.

dsRNA enters cells by clathrin-dependent endocytosis, mediated by Raftlin<sup>14</sup>; or in a complex with the antimicrobial peptide LL-37 through FPRL-1<sup>15</sup>; or through internalization of apoptotic bodies that are derived from virus-infected cells.<sup>16</sup> Once the TLR3-dsRNA species forms in the endosome, TLR3 signaling is then initiated by ligand-induced dimerization of TLR3 receptors. Unlike other TLRs, TLR3 must be tyrosine-phosphorylated after binding to dsRNA,<sup>17</sup> for which the tyrosine kinases EGFR and Src cooperate.<sup>18</sup>

Subsequently, the Toll/interleukin-1 (IL-1) receptor (TIR) domain of TLR3 engage TIR domain-containing adaptor protein inducing IFN $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM). TLR3 is the only TLR that recruits TRIF directly to its TIR domain to initiate signaling. TLR4 can signal independently of MyD88 via TRIF,<sup>19</sup> but it requires the bridging adaptor TRAM. Another peculiarity of TLR3 is that by using TRIF as its principal adaptor, it signals exclusively through a MyD88-independent pathway, whereas TLR4 transduces signals through the MyD88 and TRIF pathways; all other TLRs require MyD88 recruitment (review in<sup>1</sup>).

Recruitment of TRIF to TLR3 is considered the step that dictates downstream signaling processes. TRIF signaling activates the serine/threonine kinase TANK-binding kinase-1 (TBK-1), which phosphorylates the transcription factor interferon regulating factor 3 (IRF3).<sup>20</sup> TNF Receptor Associated Factor 3

(TRAF3) recruitment links TRIF and the TBK-1 kinase complex. IRF3-phosphorylated translocates to the nucleus and activates specific proinflammatory target genes, the most significant of which are type I IFNs.<sup>20</sup> In addition to activating IRF3, TLR3 signaling via TRIF also activates NF- $\kappa$ B.<sup>21,22</sup> The NF- $\kappa$ B branch of TLR3 signaling is activated by TRIF-dependent recruitment of 2 separate pathways—mediated by RIP1 (also referred to as RIPK1)<sup>23</sup> or TRAF6—both of which converge on Transforming growth factor beta-activated kinase 1 (TAK1) and the I $\kappa$ B kinase (IKK) complex. TAK1 phosphorylates IKK alpha and beta, which in turn phosphorylates I $\kappa$ B, the NF $\kappa$ B inhibitor, resulting in I $\kappa$ B degradation and the nuclear translocation of NF $\kappa$ B.<sup>24</sup>

Ultimately, several transcription factors are activated, such as interferon-regulatory factors (IRFs) and cyclic AMP-responsive element-binding protein (CREB), and translocate to the nucleus, where they bind to their respective elements in the promoters of target genes (eg, IFN beta, IL-6, IL-12, and CCL3).<sup>25</sup> Engagement of TAK1 and signaling adaptors also activates mitogen-activated protein kinases (MAPKs) and JUN N-terminal kinase (JNK). p38 MAPK autoactivates on interacting with TAK1-binding protein 1,<sup>26</sup> stimulating AP-1, which induces the transcription of several cytokines and chemokines. Overall, the most significant outcome of all 3 modules of TLR3 signaling is the induction of **inflammatory molecules**, including proinflammatory cytokines (eg, TNF and IL-1), chemokines (eg, CCL2 and CXCL8), endothelial adhesion molecules (eg, E-selectin), and type I IFNs (IFN $\alpha$  and IFN $\beta$ ) crucial for antiviral responses.

In addition to triggering an inflammatory response, dsRNA elicits cell **survival/apoptotic** mechanisms in several mammalian cell types, apparently through disparate pathways.

### TLR3-mediated apoptosis in cancer cells

Salaun B. et al. (2006) first showed that activation of TLR3 effects apoptosis *in vitro* in various breast cancer cell lines and that IFN type I is necessary but not sufficient to activate the apoptotic pathway.<sup>27</sup> Similarly, *in vitro* studies in melanoma models demonstrated that in TLR3-expressing cancer cells, receptor activation by its agonist directly inhibits cell proliferation and induces apoptosis when the agonist is combined with type I IFN.<sup>28</sup> The primary TLR3-mediated mechanism of apoptosis in cancer cells is dependent on **caspase-8** activation. Estornes Y. et al. reported that stimulation of TLR3 in lung tumor cell lines by dsRNA induces the formation of an atypical complex that contains caspase-8 and that interacts with TLR3, although TLR3 lacks a death domain (DD).<sup>29</sup> The model that was proposed by this group considered the requirement of RIP1 for the recruitment of caspase-8 to TLR3. RIP1 is negatively regulated by ubiquitination by the cIAP2 (cellular inhibitor of apoptosis protein)-TRADD-TRAF2 complex.<sup>30–32</sup>

The TRIF-RIP1 axis, which is fundamental for inflammatory processes that originate from TLR3 activation, also governs cell death and survival—RIP1 associates with Fas-associated death domain (FADD) via the death domain.<sup>33</sup> TRIF-RIP1-FADD is central to the assembly of a death-inducing signaling complex that contains caspase-8, the fate of which determines which of the 3 outcomes of TLR3 signaling occurs.<sup>33</sup> Homodimerization

of caspase-8 leads to its autocatalytic activation, RIP1 cleavage and inactivation, and apoptotic cell death.<sup>33</sup> If caspase-8 activity is compromised, RIP1 cleavage is inhibited, allowing RIP1 to interact with RIPK3 and form a necrosome, causing necrotic cell death. In the third outcome, if caspase-8 heterodimerizes with FLIP, a noncatalytically active homolog of caspase-8, partial activation of caspase-8 prevents complete cleavage of RIP1, resulting in cell survival.<sup>34,35</sup>

Paone et al. also described that TLR3 can trigger apoptosis through a PKC alpha-dependent mechanism in prostate cancer cells, which converges on caspase-8. Activation of the downstream targets of PKC alpha—p38 MAPK and c-JUN—increases the autocatalytic activity of caspase-8 and -3.<sup>36</sup>

It has been reported that TLR3 activation can induce apoptosis in cancer cells by the **intrinsic pathway**. Indeed, in melanoma cells, beside TRIF-dependent activation of proapoptotic signaling,<sup>31</sup> Bcl2 expression declines on cotreatment with poly(I:C) and INF- $\alpha$ , suggesting the involvement of the mitochondrial pathway. Moreover, in another study, Bcl2 and Noxa levels are downregulated in endothelial cells that are treated with poly(I:C).<sup>37</sup>

Finally, discrepancy in results in cancer *in vitro* models might be attributed to the use of poly(I:C) with varying lengths,<sup>38,39</sup> moreover, differences between intra- and extracellular administration of such TLR3 ligands implicate other cytosolic dsRNA receptors, such as Melanoma Differentiation-Associated protein 5 (MDA5) and Retinoic acid-inducible gene I (RIG-I), that could affect the poly(I:C) cytotoxic effect.

RIG-I and MDA5 are dsRNA recognition receptors located in the cytoplasm, that concur to mediate anti-viral response together with TLR3, and thus their increase and activation could improve poly(I:C) cytotoxic activity.

### Prognostic significance of TLR3 expression in cancer

Few studies have examined the expression of TLR3 in **cancer specimens** by immunohistochemistry, especially due to the lack of reagents with proven specificity. Moreover, the function of TLR3 on cancer cells must be distinguished from that on immune cells.

In Hepatocellular Carcinoma (HCC), TLR3 positivity was observed in 52.7% of 74 cases by immunohistochemistry.<sup>40</sup> Chew V. et al.<sup>41</sup> confirmed this frequency in 172 HCC patients by qPCR, reporting that TLR3 expression in patients with HCC was associated with greater survival. Moreover, in tumor sections of patients with HCC, TLR3 levels correlated with NK cell activation and an increase in T and NK cell infiltration and was inversely associated with the vitality of tumor parenchyma cells. Similarly, TLR3 activation in *in vivo* models of HCC upregulates chemokines intratumorally and increases the activation of tumor-infiltrating immune cells. *In vitro* evidence from these studies implicates TLR3-induced apoptosis as a mechanism that explains the good prognosis of TLR3-expressing patients.<sup>40,42</sup>

An immunohistochemical study of cancer specimens from 106 patients with gastric cancer showed a positivity for TLR3 expression in 60.4% of the cases. In contrast with evidence in HCC, TLR3 expression was significantly associated with poor overall survival in patients with resectable gastric tumors.<sup>43</sup>

In 74 breast cancer cases, González-Reyes<sup>44</sup> reported a high percentage of TLR3-positive cases (79%) evaluated by IHC and

that TLR3 expression by tumor cells was significantly associated with a great rate of distant metastasis. Moreover, they noted a link also between elevated TLR3 mRNA levels and tumor recurrence. A randomized clinical trial of 194 women with breast cancer evaluated the effectiveness of the TLR3 agonist poly (A:U), showing that adjuvant treatment with this agonist was associated with a significantly lower risk of metastatic relapse in TLR3-positive tumors.<sup>45</sup> The stratification of breast cancer patients, according to the TLR3 expression, was performed by using an anti-TLR3 antibody (40F9.6) accurately validated by the authors.

Immunohistochemical study on archival tissues of neuroblastomas showed TLR3 positivity in 70/99 (70.7%) patients and that positive TLR3 expression was associated with favorable histology and prognosis.<sup>46</sup>

By PCR array, TLR3 expression has been reported to be high in established human melanoma lines and in human melanoma cells isolate from single-cell suspensions obtained from melanoma tumor biopsies.<sup>47</sup>

With regard to the TLR3 expression and tumor progression, studies on tumor specimens have shown that TLR3 is maintained in the most differentiated neuroblastoma cells in the tumor mass<sup>48</sup>; moreover, tumors with high grades of differentiation generally showed a higher level of TLR3 mRNA expression and positive immunoreactivity suggesting that TLR3 expression in Neuroblastoma (NB) tumor tissues may be correlated with differentiation of NB cells.<sup>46</sup> In contrast, histological grade and TLR3 positivity do not differ in HCC, despite the minor change in TLR3 staining patterns between normal and tumor tissues.<sup>40</sup> In breast cancer, TLR3 was significantly and positively associates with tumor size and tumor stage.<sup>44</sup> Collectively, the prognostic significance of TLR3 remains unresolved by the few studies in cancer patients that are available. Moreover, these studies have not allowed us to determine whether its inverse relationship with the prognosis in various cancer histotypes depends on the balance between apoptosis and survival, as mediated by TLR3 activation, or on other factors. Particularly, to define the potential of TLR3 expression on cancer cells as a therapeutic target, its signaling cascade must be examined for each specific tumor to develop strategies to enhance apoptosis.

### Upmodulation of TLR3 to increase apoptosis

The first level of regulation of TLR3-mediated apoptosis comprises the upmodulation of TLR3 expression. In recent years, many studies have focused on the mechanisms of the transcriptional regulation of TLR3, and several molecules involved in TLR3 modulation has been identified. TLR3 expression is modulated by IFN- $\alpha$ , a cytokine that activates dendritic cells (DCs), NK cells, and macrophages.<sup>49</sup> demonstrated that IFN- $\alpha$  upregulates TLR3 in endothelial and epithelial cells and, following stimulation of TLR3 with its ligand, increases the production of cytokines that mediate the antiviral response, such as IFN- $\beta$ . An IFN-responsive element (ISRE), located approximately -30 bp from the promoter region of human TLR3, drives the expression of IFN- $\alpha$ -induced TLR3.<sup>50</sup> Another study reported that inhibition of the JAK/STAT pathway decreases the induction of TLR3 by IFN- $\alpha$ , implicating JAK/STAT signaling in IFN- $\alpha$ -induced

TLR3 transcription.<sup>51</sup> Moreover, it has been observed that IFN- $\alpha$  treatment can upregulate TRAIL, resulting in cancer cell death.<sup>52</sup> However, death receptors (DRs) are not involved in poly(I:C)-triggered cell death, and Fas, DR4/DR5, and TNFR1 are not associated with caspase-8 after TLR3 activation.<sup>29</sup>

**Histamine** is a biogenic amine that is synthesized and released by mast cells and acts as a vasodilator in several pathological processes. Its function in cancer is not fully understood, because high levels of histamine have been associated with the promotion and inhibition of growth in various tumors.<sup>53</sup> Histamine, a key mediator of allergic inflammation, upregulates TLR3 mRNA and protein in airway epithelial cells and lung cancer cells and enhances the cellular response to poly (I:C), based on IL-8 secretion.<sup>54,54</sup> In contrast, histamine reduces TLR3 levels in cultured human skin fibroblasts.<sup>55</sup>

Priming of esophageal epithelial cells with poly (I:C) induces robust histamine receptor (HR) expression and enhances histamine-induced secretion of GM-CSF, TNF $\alpha$ , and IL-8, implicating the existence of crosstalk between histamine and TLR3 signaling.<sup>56</sup> Moreover, a histamine-forming enzyme, histidine decarboxylase (HDC), is activated in mice that have been primed with various Toll-like receptor agonists, including poly (I:C), resulting in the production of histamine.<sup>57</sup> The response of airway epithelial cells to histamine rises significantly after treatment with poly(I:C).<sup>58</sup> Thus, epithelial sensitivity to histamine might be enhanced during TLR3-mediated inflammation, increasing the expression of HRs, the stimulation of which could in turn upregulate TLR3 expression.

**IL-27** is another cytokine that potentially regulates TLR3 expression and function in tumor cells. IL-27 belongs to the IL-12 family and is a heterodimeric cytokine that comprises 2 subunits: Epstein-Barr virus (EBV)-induced gene 3 (EBI3) (also known as IL-27B) and IL27-p28 (known as IL-30).<sup>59</sup> IL-27 is produced by (APCs)<sup>60</sup> and governs the activity of B- and T-lymphocytes, based on its ability to induce pro- and anti-inflammatory immune responses. It can induce tumor-specific antitumor and protective immunity through cytotoxic T lymphocyte (CTL) and natural killer (NK) cells, but it has also anti-angiogenic and direct antiproliferative activity against tumors by regulating several chemokines.

Recently, Chiba and colleagues demonstrated that IL-27 enhances the expression of TRAIL and TLR3 in human melanomas and inhibits tumor growth *in vitro* and *in vivo* in cooperation with poly(I:C), partly in a TRAIL-dependent manner. This mechanism might approximate that of IFN- $\alpha$ , which induces cell death in cancer cells by stimulating them to produce TRAIL.<sup>52</sup> IL-27 has been hypothesized to effect IRF-1 expression through WSX-1/STAT1 signaling, resulting in the upregulation of TRAIL. IL-27 also augments TLR3 levels IRF-1-dependently and -independently. Treatment with IL-27 increases RIG-I and MDA5 mRNA and protein levels. However, knockdown of RIG-I or MDA5 does not affect IL-27-mediated suppression of tumor growth, indicating the necessity of the TLR3/TRAIL axis.

### Overcoming TLR3-mediated apoptosis

Several molecules dampen dsRNA-mediated TLR3 activation and impair signaling downstream of TLR3, decreasing apoptosis.

**Mucin 1 (MUC1)** is a pleiotropic molecule that is expressed in nearly all epithelial tissues in the respiratory, gastrointestinal, urogenital, and hepatobiliary tracts; in sebaceous and salivary glands; and in hematopoietic cells.<sup>61,62</sup> Like other membrane-associated mucins, MUC1 hydrates, protects, and provides lubrication to mucosal and epithelial luminal surfaces of ducts, rendering it central to the maintenance of homeostasis and promotion of cell survival in response to harsh environments. In addition, MUC1 functions in the immune system in chronic inflammatory diseases (regulating DCs, monocytes, T cells, and B cells).<sup>63,64</sup>

In cancer, altered MUC1 expression impedes efficient lysis by neutrophils, NK cells, and cytotoxic T cells and limits tumor cell adhesion, allowing them to escape, thus leading to cancer metastasis.<sup>65</sup> Overexpression of MUC1 is associated with tumor progression and a poor prognosis in colon, breast, ovarian, lung, prostate, and pancreatic cancers.<sup>66-70</sup> MUC1 is expressed in airway epithelial cells, extinguishing host inflammatory responses that are initiated by pathogens and protecting tissue from injury. To perform these regulatory functions, MUC1 interacts directly with TLRs to hamper MyD88 or TRIF recruitment and halt the production of inflammatory factors that lie downstream of TLRs. In addition, Kato and colleagues recently demonstrated that similar to what occurs during attenuation of the immune response, MUC1 overexpression impairs poly(I:C)-induced apoptosis,<sup>71</sup> based on evidence that blockade of MUC1 activity decreases TRIF engagement on the TLR3 apoptosome complex.

Another mechanism by which cancer cells contrast poly(I:C)-mediated apoptosis is through **HIF-1 $\alpha$** . HIF-1 $\alpha$  mediates the resistance to several apoptotic stimuli by directly inducing antiapoptotic genes, such as Bcl-xL, survivin, and MCL-1.<sup>72-74</sup> Certain molecular components that are derived from bacteria and viruses, such as dsRNA, activate HIF-1 $\alpha$  under normoxic conditions through TLRs.<sup>75-77</sup> In particular, the I.3 isoform of hif-1 $\alpha$  is upregulated at the mRNA and protein levels following poly(I:C) stimulation of TLR3 in prostate cancer cells. Activation of HIF-1 $\alpha$  upregulates VEGF<sup>78</sup> resulting in angiogenesis in several types of cancer. HIF1 alpha is stabilized and activated by MUC1<sup>79</sup> and the link between these pathways, both of which regulate TLR3 expression, should be considered to propose a therapy including TLR3 agonists. Thus, the levels of HIF-1 $\alpha$  in cancer cells might be critical to the antitumor potential of poly(I:C).

Another molecule that impair dsRNA-induced signaling is ADAM15. ADAM15 belongs to the disintegrin and metalloproteinase (ADAM) family. ADAM15 is overexpressed in several types of solid tumors, including breast, bladder, lung, colorectal, ovarian, and prostate cancer<sup>80-83</sup>; however, its function in promoting or suppressing cancer progression differs between tumor types. High expression of ADAM15 correlates with a poor prognosis in NSCLC and bladder cancer patients, in which it contributes to metastatic tumor progression through various mechanisms, including direct activation of MMP9.<sup>84</sup>

Recently, **ADAM15** was identified as a TRIF-interacting protein by liquid chromatography (LC)/mass spectrometry (MS) following immunoprecipitation of the TRIF complex in HEK293 cells that were transfected with TLR3 and stimulated

with poly(I:C).<sup>85</sup> ADAM15 mediates the proteolytic cleavage of TRIF, resulting in the downregulation of NF $\kappa$ B and IFN $\beta$  activity. Accordingly, its suppression increases proinflammatory cytokine production. Similar to other molecules that govern TRIF recruitment to dsRNA-activated TLR3, the assembly of the apoptosome complex is expected to be impaired by ADAM15-catalyzed cleavage of TRIF. The demonstration of the relevance of ADAM15 in TLR3-mediated apoptosis could provide the rationale for combining ADAM15 inhibitors and poly(I:C) to boost cancer cell apoptosis.

### Strategies to improve poly(I:C)-induced apoptosis

A dissection of TLR3 signaling would contribute to greater resolution of the critical steps that impede full exploitation of the poly(I:C)-induced apoptosis. Based on comprehension of the elements in the poly(I:C)-induced apoptotic program, strategies have been proposed to increase its cytotoxic effect in cancer cells.

Several lines of evidences highlight the importance of receptor-interacting protein 1 (RIP1) and inhibitor of apoptosis proteins (IAPs) in the TLR3 apoptotic cascade. Apoptosis is tightly regulated at several levels, and IAPs are key negative regulators of the intrinsic and extrinsic apoptosis pathways.<sup>86</sup> cIAP1 and cIAP2 are E3 ubiquitin ligases that suppress apoptosis by directly inhibiting the activities of caspases and mediating the ubiquitination of several substrates, including RIP1-4.<sup>87,88</sup> RIP1 is necessary for poly(I:C)-induced apoptosis, serving as a docking site for caspase-8 recruitment to the TLR3/TRIF apoptosome complex.<sup>29</sup> On TLR3 activation, RIP1 associates with TRIF and caspase-8, and subsequently, E3 ubiquitin ligases, including cIAP2, complex with RIP1 that undergoes polyubiquitination, resulting in the negative regulation of death complex formation and then apoptosis. RIP1 ubiquitination by cIAP1 and cIAP2 is a critical event, because it prevents RIP1 from forming a cytosolic complex with caspase-8 and Fas-associated protein with death domain (FADD) (complex II) and thus the subsequent activation of caspases.<sup>89,90</sup> This regulatory mechanism evolved to protect immune cells from death due to pathogenic microorganisms. Conte et al. induced c-IAP2 expression in macrophages with LPS and found that depletion of c-IAP drove apoptosis in them.<sup>91</sup> Cancer cells exploit this mechanism to circumvent poly(I:C)-induced apoptosis.<sup>92</sup> c-IAP2 is overexpressed in many types of cancer and is associated with disease progression, poor prognosis, and chemoresistance.

**SMAC mimetics** are small-molecule drugs that mimic Smac, a mitochondrial inhibitor of cIAPs, and were developed to fight cancer.<sup>93</sup> The goal of SMAC mimetics is to suppress cIAPs, re-establishing the extrinsic apoptotic pathways to induce cancer cell death. Suppression of cIAP2 by SMAC mimetics increases TLR3-mediated apoptosis in lung cancer cells.<sup>29</sup> Considering that SMAC mimetics have demonstrated their efficacy as anticancer agents in the clinic, the combination of poly(I:C) and SMAC mimetics is a promising strategy to enhance TLR3-mediated apoptosis.<sup>30,94</sup>

Based on the crosstalk between the TLR3 and retinoic acid receptor (RAR) pathways, 2 separate studies suggested the co-administration of **retinoids** as a promising approach to increase the cytotoxic effects of poly(I:C). Bernardo and

colleagues observed that dsRNA receptors are retinoic acid (RA)-inducible genes, demonstrating that treatment of breast cancer cells with RA upregulates RIG1, MDA5, PKR, and TLR3.<sup>95</sup> Accordingly, the levels of all of the main downstream mediators of such dsRNA receptors rose, enhancing poly(I:C)-induced apoptosis. RA also stimulates type I IFNs, which ultimately upregulate TRAIL. More recently, the same group reported RA/poly(I:C) cotreatment in breast cancer cell lines synergized in activating **IRF3** through TLR3.<sup>96</sup>

Galli et al. described an unexpected relationship between the RA- and poly(I:C)-activated pathways through miRNAs.<sup>97</sup> Poly(I:C) increases the expression of 4 miRNAs (microRNA-29b, -29c, -148b, and -152) that target DNA methyltransferases, effecting the demethylation of several genes, including RAR $\beta$ . Through this mechanism, they demonstrated that epigenetic silencing of retinoic acid receptor beta-2 (RAR $\beta$ ) could be reverted in prostate and breast cancer cells by poly(I:C). Accordingly, the reconstituted expression of silenced RAR $\beta$  sensitizes cancer cells to RA-induced apoptosis. This coregulation between the poly(I:C) and RAR pathways could be examined further to evaluate the combined administration of retinoids and dsRNA to induce apoptosis in cancer cells.

IL-24 is a cytokine that belongs to the IL-10 family. Adenoviral expression of IL24 (Ad-IL-24) was initially developed as an antiviral agent, but interest in its antitumor potential has risen, based on its ability to induce apoptosis in several types of cancer, including lung, liver, kidney, melanoma, breast, and glioma. Ad-IL-24 appears to control cell survival and proliferation by rapidly activating several proapoptotic molecules, such as Bcl-2, Bax, p-38 MAPK, and ERK.

Discrepancies have emerged with regard to the antitumor efficacy of nonvirally administered IL-24 and Ad-IL-24. IL-24 requires a secondary stimulus to induce apoptosis, which in Ad-IL-24 is provided by viral structural components that are necessary for its intracellular delivery.<sup>98</sup> In Ad-IL-24 administration, active replication of virus occurs solely to present dsRNA in the cell and activate TLR3. The cytotoxic effects of Ad-IL-24 are exerted specifically through the TLR3/TRIF complex, as demonstrated by the decrease in cell death in the presence of a TRIF inhibitor or TLR3 siRNA. Expression of cFLIP and cIAPs were did not observed in the TLR3 complex in cells treated with IL-24, enabling it to initiate the apoptotic signaling cascade. These results indicate that IL-24 does not mediate the cytotoxic effects per se but sensitize cancer cells to TLR3-mediated apoptosis. Further, RPKs are upregulated in glioma and lung cancer cells by Ad-IL-24<sup>99</sup>—a mechanism that contributes to dsRNA-induced apoptosis. These data suggest that Ad-IL-24 is a promising anticancer therapy, although the mechanisms by which it acts requires further study.

The tumor suppressor gene **TP53** is a transcription factor for TLR3 in several epithelial and hematopoietic cancer cell lines.<sup>100,101</sup> The consensus sequence for p53 lies in the promoter of the TLR3 gene; consequently, p53 status in cancer has emerged as a fundamental determinant of TLR3 pathway activation by dsRNA agonists. There is little or no expression of TLR3 transcripts in p53  $-/-$  cells or cells that carry p53 loss-of-function mutations. Mutations that do not alter p53 function were recently demonstrated to induce the expression of TLR3 and its downstream partners.<sup>102</sup> Accordingly, the apoptotic

response that is elicited by poly(I:C) depends on functional p53. The relationship between p53 and TLR3 is further complicated by findings that p53 is in turn activated by transfection with poly(I:C)<sup>103</sup> and that the regulation of its transcriptional activity depends strictly on other components, such as p21.<sup>104</sup>

There are several clinical considerations that must be made with regard to the link between p53 and TLR3 when developing a dsRNA-based cancer therapeutic. p53-inducing chemotherapies can upregulate TLR3 expression and activation, and consequently, poly(I:C) can synergize with genotoxic agents. Many anticancer treatments, such as 5-fluorouracil (5-FU), cisplatin, etoposide,<sup>51</sup> doxorubicin (DXR), UV and ionizing radiation,<sup>101</sup> potentiate *in vitro* poly(I:C)-induced cancer cell death. However, approximately 50% of tumors bear loss-of-function p53 mutations or are p53 null, which could explain the failure of dsRNA-based therapy without stratification of patients, based on p53 status.

As discussed, TLR3 levels are tightly regulated by IFNs, and IFN- $\alpha$ , - $\beta$ , and - $\gamma$  can increase TLR3 expression and the apoptotic efficacy of dsRNA through signaling pathways other than p53. Accordingly, IFN- $\alpha$  additively enhances the apoptotic effects of poly(I:C) in p53+/+ and p53-/- cells; thus, it has become a more applicable drug candidate for overcoming the need for p53 activation. However, the combination of p53-inducing agents, IFN- $\alpha$  and poly(I:C) has been demonstrated *in vitro* to be the most effective strategy.

Another approach to improving the efficacy of TLR3-mediated apoptosis is the combination of a dsRNA agonist with p53-reactivating drug RITA (reactivation of p53 and induction of tumor cell apoptosis). This small molecule rescues the apoptosis-inducing functions of mutant p53 in several cancer cell lines.<sup>105,106</sup> RITA can pharmacologically restore p53 activity in hotspot mutants that have lost the ability to interact with p53 binding sequences and then increase poly(I:C)-mediated apoptosis. Genotoxic agents, such as DXR and 5-FU, can not affect alone TLR3 expression in p53-/- cells. Whereas, the combination of RITA and DXR or 5-FU upregulates TLR3 expression, and the addition of poly(I:C) further increases cell death.

Complicating the relationship between TLR3 and p53, TAp63 is a crucial regulator of TLR3-induced apoptosis in human umbilical vein endothelial cells (HUVECs).<sup>37</sup> P63 belongs to the p53 family, and TAp63 is a transactivation domain p63 isoform, which does not differ structurally from p53 and acts as a tumor suppressor.<sup>107,108</sup> Sun and colleagues reported the upregulation of TAp63 followed TLR3 activation and, after the translocation of TAp63 into the nucleus, its binding to p53- or p63-responsive elements to initiate extrinsic and intrinsic apoptosis. Moreover, in immortalized HUVECs, p53 does not contribute to poly(I:C)-induced apoptosis—pretreatment with the p53 inhibitor pifithrin and p53 shRNA does not repress the cell death that is induced by poly(I:C). These results are consistent with the relevance of p53 to TLR3-mediated apoptosis, because p63 and p53 are not functionally redundant and because the former has been implicated in many p53-independent pathways. Thus, we speculate that the apoptosis that was observed by Taura et al. in p53 -/- colon carcinoma cells that were treated with IFN- $\alpha$  and poly(I:C)<sup>51</sup> is attributed to the involvement of TAp63.

To incorporate a TLR agonist, such as poly(I:C), into a therapeutic antitumor strategy, the **interaction** between TLRs and their agonists must be considered, so that the appropriate combinations of TLR ligands are used. Preclinical studies have suggested that various TLRs can stimulate the same signaling pathway to enhance the desired effect; yet, the functions of each TLR might counteract each other when they are activated together. CpG-ODNs are recognized by Toll-like receptor 9 (TLR9) leading to strong immunostimulatory effects correlated with NK cell expansion at tumor site.<sup>109,110</sup> Since CpG-ODN can enhance the antitumor activity of DNA-damaging chemotherapy and radiation therapy in preclinical mouse models,<sup>111</sup> it is largely used in anti-cancer regimens. Zhang and colleagues showed that simultaneous cotransfection of poly(I:C) and ODN M362, a TLR9 agonist, had less proapoptotic effect on HCCs than transfection with poly(I:C) alone. Further studies indicated that these effects were due in part to the phosphorothioate modification to CpG-ODN, which blocked the entry of poly(I:C) into tumor cells. By administering poly(I:C) after CpG-ODN this restriction was lifted and the poly(I:C)-mediated proapoptotic effects were increased *in vitro* and *in vivo*.<sup>112</sup>

Forming a therapeutic strategy that is based on TLR3-mediated apoptosis also requires the effects on **normal cells** to be taken into account. No studies have systematically compared the apoptotic effects of TLR3 ligands on cancer cells and their normal histotype counterparts. The little data that exist suggest that normal cells are less sensitive to poly I:C-induced apoptosis than transformed cells.<sup>113,114</sup>

## Conclusions

TLR3 recognizes dsRNA and its synthetic ligands and primarily drives the response against viral infections. Although TLR3 was first identified in immune cells, several studies have shown that it is also expressed by tumor cells. The TRIF-dependent pathway of TLR3 signaling might contribute to counteract tumor growth triggering apoptosis in cancer cells—an effect that could be exploited to promote the direct killing of tumor cells and the generation of a specific memory response against apoptotic tumor cell-derived antigens.

Based on the ability of TLR3 to activate the immune system, TLR3 agonists (Ampligen<sup>®</sup>, Hiltonol<sup>®</sup>, poly IC-LC) are already being examined in clinical studies on cancer therapies as single agents and in combination with chemo- or immunotherapeutic drugs. Considering the capacity to mediate tumor cells apoptosis, TLR3 ligands have become an even more attractive therapeutic option for the treatment of cancer and novel therapies might incorporate TLR3 agonists with molecules that enhance their direct cytotoxic effects against tumors, still not taken into account.

We have provided an overview of the molecules that are positively linked to the activation of the TLR3 apoptotic program, including the modulation of its expression and signaling—both of which are potential targets for therapeutic interventions to improve the direct apoptotic effects of TLR3 on cancer cells. We have also focused on the critical negative regulators of TLR3-mediated apoptosis that could extinguish its activation and on approaches for overcoming this event. Attention should be paid to combinations of TLR3 agonists

and proven antitumor molecules to implement TLR3 ligand-based therapies in clinical trials.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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