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Dual Role of TLR3 in Inflammation and Cancer Cell Apoptosis

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1. Introduction

1.1. The concepts of recognition of non-self by innate immune receptors

Immunity may be regarded as the most sophisticated part of the tissue repair process. In order to reach the *"restitutio ad integrum"* of tissues while minimizing the general consequences of external aggression, multicellular organisms have evolved mechanisms that allow rapid detection of non-self or injured self. Early recognition represents the first stage of protection against pathogens that enables any cell to elicit promptly various forms of protective responses that altogether represent the so-called "innate immune response". In addition, the innate immune response turns on two types of specialized effector immune cells responsible for the adaptive immune response, the T and the B lymphocytes

During the last two decades, two concepts have emerged in an effort to elucidate the basis of the key-initiating step, i.e., the molecular recognition of non-self. On one side, it was first reasoned, and later amply demonstrated, that the structures that are recognized early by the so-called innate immunity must share some important features that allows their recognition as non-self. This led to the model of "Pathogen Associated Molecular Patterns" (or PAMPs), which implies that distinction relies on differences in shape of molecules shared by pathogens, as they have been conserved during the evolution to fulfill important functions. On the other side, the concept of Pathogen Recognition Receptors (PRRs) was built on the assumption that a limited number of germline-encoded receptors should have the capacity to detect the differences in shape displayed by the PAMPs. In agreement with the model, each of the main categories of PRRs displays features that are well suited for discriminating non-self.

The lectins are either soluble or membrane-bound proteins that recognize saccharides through single or multiple Carbohydrate Recognition Domains (CRD). Given the differences

in enzymatic equipment for polysaccharide synthesis, and therefore in the nature (and the shape) of saccharides expressed at the surface of pathogens when compared to eukaryotic cells, those structures represent ideal targets for pathogen detection.

The cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) share domain architecture comprising a nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR) domain. NOD1 and NOD2 recognize distinct building blocks of peptidoglycan (PGN) found in bacterial walls.

The RIG-I (retinoic acid inducible gene-I, DDX58) and MDA5 (melanoma-differentiation-associated gene-5, RH116) represent the RIG-I-like helicases (RLH) family of cytosolic sensors that specifically recognizes double stranded RNA (dsRNA), while AIM2 (absent in melanoma 2) appears to be essential for mediating inflammatory reactions triggered by cytoplasmic DNA that signs the presence of pathogens. Recently, a complex formed of three helicases DDX1-DDX21-DHX36 [1] and a fourth helicase DHX9 [2] have been shown to detect dsRNA in the cytosol of myeloid dendritic cells (mDCs).

Last but not least, the membrane-bound Toll-like receptors constitute a family of 10 members in human (11 in mouse) that share a LRR extracellular domain involved in the binding of PAMPs. TLR1, 2, 4, 5 and 6 are expressed at the cell surface membrane and recognize pathogens-derived lipids, lipopolysaccharides, PGN or proteins. In contrast, TLR3, 7, 8 and 9 recognize (poly)nucleotides in endolysosomes.

2. Toll-like receptor 3: a PRR that activates various types of cells in response to dsRNA

TLR3 is a highly glycosylated type I membrane receptor that appears to be dedicated to the recognition of dsRNA [3] that represents a replication intermediate for many viruses. TLR3 is thus involved in the innate immune response against various viruses [4], and plays a non-redundant role in HSV-1 infection of the CNS [5]. Like all TLRs, TLR3 possesses an extracellular domain made of (23) LRRs and a cytoplasmic toll/IL-1 receptor (TIR) domain required for downstream signaling. Compared with other PRRs also responsive to RNA, the specificity of TLR3 resides in its location at the membrane of endolysosomes and in its affinity for a large range of dsRNA sizes (from > 50 bp to over 2000 bp). In contrast, RIG-I and MDA5 are activated by the presence in the cytoplasm of short or long dsRNA, respectively, while the membrane-bound TLR7 detects the presence of single stranded RNA (i.e. bacterial mRNA).

The trachea, the pancreas and the placenta are the three organs that show the highest expression of TLR3 mRNA (<http://www.ncbi.nlm.nih.gov/geoprofiles>). However, TLR3 can also be detected by immunohistochemistry in many tissues, including the skin [6], the muscles [7], and the kidneys [8]. At the cellular level, myeloid dendritic cells and macrophages, but not other leukocytes, including monocyte precursors have been found to express TLR3. TLR3 is also present in non-immune cells such as epithelial cells of various origins (lung [9], intestine [10], breast [11], kidney [12], pancreas [13]) but also in

mesenchymal cells [14] and in endothelial cells [15]. Of interest, TLR3 is the TLR that is expressed most strongly in the brain, especially in astrocytes, glia, and neurons[16].

External dsRNA appears to be first internalized by cells through the binding on surface scavenger receptors [17]. In endolysosomes, dsRNA binding leads to TLR3 dimerization and to recruitment through TIR domains homotypic interaction of a single adaptor, TRIF (TIR domain-containing adapter protein inducing interferon beta). TRIF in turns recruits several signaling kinases that activate different transcription factors: 1) through the activation of tumor necrosis factor receptor (TNFR)-associated factor (TRAF6) E3 ubiquitin ligase, TRIF recruits the transforming growth factor- β -activated kinase 1 (TAK1) which mediates downstream NF- κ B activation[18]. 2) Protein kinase R (PKR) is associated with TAK1 and contributes to the activation of the p38 mitogen-activated protein kinase pathway by interaction with MKK6 [19]. 3) TRIF also recruits TBK1 and IKK ϵ through TRAF3, which phosphorylates IRF3 [20, 21] Activated IRF3 translocates into the nucleus and induces expression of Type I IFN [22]. 4) The receptor interacting protein 1 kinase (RIP1K) is also essential for NF- κ B activation but not for IRF3 activation by TRIF [23, 24].

3. Activities of TLR3 ligands on cancer cells

3.1. Inflammatory and proliferative responses of cancer cells

Many types of cancer cells express TLR3. This was established by immunohistochemistry on tumor tissue sections of breast carcinoma [25], oral squamous cell carcinoma [26], cervical carcinoma [27], ovarian carcinoma [28], prostate carcinoma, head and neck carcinoma [29]. Furthermore, the level of TLR3 expression by prostate cancer cells was shown to be significantly associated with higher probability of biochemical recurrence [30]. We have also observed TLR3 staining on lung squamous cell carcinoma and on a portion of HCC (our unpublished data). Furthermore, overexpression of TLR3 has been detected by flow cytometry, by western blot and/or by qPCR in melanoma cells [31, 32], esophageal squamous cell carcinoma[33], head and neck carcinoma cells [34] and multiple myeloma cells [35].

Like normal cells, human cancer cell lines will respond to TLR3 ligand by secreting inflammatory cytokines, IFN-I and chemokines. As an example, we found that NSCLC, OSCC and HCC cell lines could secrete IL-6, IL-8, RANTES, IP-10, and IFN-I, although at different levels depending on the line under consideration (our unpublished data). Likewise, prostate cancer cells secrete IL-8, chemokine (C-C motif) ligand 3, CCL3, CCL5 and IP-10 in response to Poly(I:C) [36], and head and neck cancer cells secrete IL-1 β , IL-6 and IL-8 [29].

Few data have been published regarding the changes of surface membrane protein expression by cancer cells after TLR3 activation. Nevertheless, two reports have shown that CD54 is upregulated, while MHC-I expression remained constant [29, 37].

Regarding cancer cells migration in response to TLR3 stimulation, divergent results have been reported. Studying nasopharyngeal carcinoma (NPC), Zhang *et al.* observed that TLR3

agonist downregulated the expression of chemokine receptor CXCR4 and inhibited cell migration in response to CXCR4 ligand stromal cell-derived factor-1alpha (SDF-1alpha) in chemotaxis assays [38]. Moreover, TLR3 activation reduced the capacity of NPC cells to form metastasis in draining lymph nodes when injected in athymic mice. In contrast, stimulation of TLR3-expressing head and neck OC2 cells with Poly(I:C) was found to induce the secretion of CCL5 and to promote CCL5-mediated migration in OC2 cells [26]. Similarly, Goto *et al.* showed that Poly(I:C) enhanced the migration of melanoma cells *in vitro* [32]. Related to those observations, we regularly observe significant changes in the morphology of cultured cancer cells in the presence of Poly(I:C), but little is known yet on the effects of TLR3 activation on the cytoskeleton.

Lastly, rare examples of cancer cells proliferating in response to TLR3 activation have been published. For example, one multiple myeloma cancer cell lines showed an NF- κ B-dependent proliferation in response to Poly(I:C) [39]. Moreover, indirect evidences led to the conclusion that TLR3 might support the proliferation of some head and neck cancer cell lines proliferation through c-Myc upregulation [40], and of papillary thyroid carcinoma [41].

3.2. Anti-proliferative effects on cancer cell

Direct inhibition of tumor growth by TLR3 agonists has been reported *in vitro* for human breast, melanoma, prostate, head and neck, multiple myeloma, clear renal carcinoma, colon, lung, and cervical cancer cells [11, 31, 42-51]. Two mechanisms contribute to the inhibition of tumor growth upon TLR3 activation; (i) decrease of proliferation and (ii) induction of apoptotic cell death.

3.2.1. TLR3 decreases proliferation of cancer cells by blocking progression through the cell cycle

Decrease of tumor cell proliferation in response to TLR3 activation by Poly(I:C) dsRNA has been demonstrated by BrDu incorporation experiments for breast and prostate cancer cells [11, 46], and by Ki-67 staining in prostate cell lines [51], and likely participates to the dsRNA anti-tumoral effect in the other types of cancers listed above. The blockade of cell cycle appears to result from the combined downregulation of cyclin D1 and upregulation of cyclin-dependent kinase inhibitor p27 [11, 46, 52] and/or the inhibition of the Akt signaling pathway [51].

3.2.2. TLR3 triggers the apoptosis of cancer cells

a. General considerations on apoptosis

Apoptosis is an evolutionarily programmed cell death that was first described by Kerr and colleagues in 1972 [53]. It is crucial for successful embryonic development and for the maintenance of normal cellular homeostasis in adult organisms. Deregulation of apoptosis is

involved in an extensive variety of diseases such as cancer and autoimmunity, but also in immunodeficiency, degenerative diseases, or infertility.

Apoptotic cell death results from the dismantlement of the cell by the sequential activation of cysteine proteases, called caspases, that cleaves numerous proteins in the cell. Two major pathways of apoptosis have been identified: the “extrinsic pathway” and the “intrinsic pathway”. The first one is typically triggered by ligation of cell surface Death Receptors of the TNFRI superfamily (such as TRAIL-R or FAS) which allows the formation of a supramolecular complex called DISC (for Death Inducing Signaling Complex) in which FADD plays a key role in the recruitment and the activation of the initiator caspase-8 (and also caspase-10) inside this platform. Inactive caspase-8 monomers are forced to dimerize when in close proximity inside the DISC, which triggers their catalytic activity leading to autocleavage and stabilization of caspase-8 in its active form. The “intrinsic pathway”, also called the “mitochondrial pathway” is typically initiated by a diverse range of stress condition such as DNA damage, ER stress, or withdrawal, and leading to mitochondria alterations and cytochrome C release, and activation of the initiator caspase-9 in a molecular platform called Apoptosome. The “extrinsic” and “intrinsic” pathways are tightly regulated by FLIP and BCL-2 family proteins, respectively, and converge to the activation of the executioner caspase-3 and -7 that cleave essential proteins required for cellular homeostasis.

In “Type I cells”, such as lymphocytes, activation of caspase-8 directly catalyzes the maturation of caspase-3 and triggers cell death. In other cells, such as hepatocytes, caspase-8 activation cleaves the BH3-only protein BID, generating a mitochondrion-permeabilizing fragment (t-BID for truncated BID) which creates an amplification loop of the death signal that is required for cell death to occur. These cells are called “Type II cells”.

b. TLR3 activates the extrinsic pathway of apoptosis in cancer cells

The first demonstration that TLR3 activation by dsRNA Poly(I:C) can directly induce apoptotic death of cancer cells *in vitro* was recently achieved by our group in 2006, in a model of breast carcinoma cell lines [11]. Since this first observation, an increasing number of studies has been started, and to date, the direct inhibitory effect of TLR3 ligands on tumor cell survival has been reported on melanoma, head and neck, prostate, clear renal carcinoma, multiple myeloma, colon, cervical, and lung cancer cells. Moreover, the relevance of TLR3 expression in cancer cells for dsRNA antitumor effects has now been demonstrated in immunodeficient mouse models and has been validated as a biomarker for the therapeutic efficacy of dsRNA on metastatic relapse [25]. This indicates that TLR3 targeting could represent an opportunity for the development of novel cancer therapy strategies.

Several studies have clearly demonstrated that TLR3-induced apoptosis in cancer cells is dependent on caspase-8 activation [44, 50, 54, 55], suggesting that TLR3 activation triggers the “extrinsic pathway” of apoptosis. Interestingly, caspase-8 activation and apoptosis triggering in response to TLR3 activation is independent of the classical Death Receptors since invalidation of these receptors by siRNA or by neutralizing antibodies do not block TLR3-mediated caspase-8-dependent apoptosis [54, 55].

The canonical activation of caspase-8 by Death Receptor relies on a particular domain shared by these receptors at their C-terminal side, and called Death Domain (DD). This DD is crucial for the assembling of the DISC through its association with the DD of the adapter FADD which in turn recruits caspase-8 through homotypic interaction between their respective Death Effector Domain (DED). However, TLR3 does not contain such a DD, and the mechanism by which TLR3 activates caspase-8 remained unexplained until recently.

c. TLR3 behaves like a Death Receptor in cancer cells

Clues to understand how TLR3 activates caspase-8 came from cell death models of ectopic TRIF transfection [56, 57]. Genetic modifications of TRIF allowed to conclude that the RHIM (RIP Homotypic Interaction Motif) C-terminal domain of TRIF is crucial for TRIF-induced caspase-8 activation. This domain was previously shown to be required for homotypic association with the RHIM domain of RIP1 kinase and for NF- κ B signaling triggering [23]. Interestingly, RIP1 contains also a DD, and the hypothesis of a molecular platform containing TRIF/RIP1/FADD/caspase-8 and mediating apoptosis was born. However, evidences of the molecular assembly of this platform to TLR3 in physiologic conditions were lacking.

Our group and that of Martin Leverkus recently highlighted the molecular mechanism of TLR3-mediated cell death [54, 55]. In these two independent studies, TLR3 activation by dsRNA Poly(I:C) lead to the formation of a DISC-like complex containing caspase-8/FADD/FLIP/RIP1 and TRIF - RIP1 playing a crucial role in the formation of this complex - confirming at a physiologic level the previous studies. Generation of new anti-TLR3 monoclonal antibodies allowed us to establish that TLR3 was also present in the complex [55], indicating that even in absence of a DD in its C-terminal side, TLR3 is able to directly engage the “extrinsic pathway” of apoptosis by recruiting the initiator caspase-8 to itself, a characteristic initially observed for the death receptors TRAIL-R or FAS. We propose that dsRNA-mediated TLR3 dimerization allows the recruitment of TRIF through TIR homotypic interaction which in turn allows the recruitment of the DD-containing RIP1, the adapter FADD, and caspase-8 to trigger apoptosis (Figure 1a).

However, when we investigated the role of FADD in TLR3-mediated caspase-8 activation we were struck by the fact that invalidation of FADD by siRNA transfection did not prevent TLR3-induced caspase-8 activation and apoptosis whereas FAS- or TRAIL-R-dependent apoptosis were prevented [55]. FADD possesses both a DD and a DED, and was therefore expected to provide the molecular link between RIP1 and caspase-8. Additional works are required to elucidate the role of FADD in the TLR3-dependent caspase-8-containing complex, but we can hypothesize that another adapter (such as FAF1 [58]) might exert a redundant function, or that RIP1-caspase-8 association is direct and does not require an adaptor molecule (which was previously observed *in vitro* with purified proteins [59])

d. Several molecular checkpoints negatively regulates TLR3-induced apoptosis

When screening the effect of dsRNA on lung tumor cell lines, we observed that not all the cells were sensitive to dsRNA-induced apoptosis, even when they express a functional

TLR3. Moreover, normal lung epithelial cells were also resistant to apoptosis, indicating that physiologic negative regulators of the TLR3 apoptotic pathway exist in the cells. Two major and complementary checkpoints can be inferred from the literature.

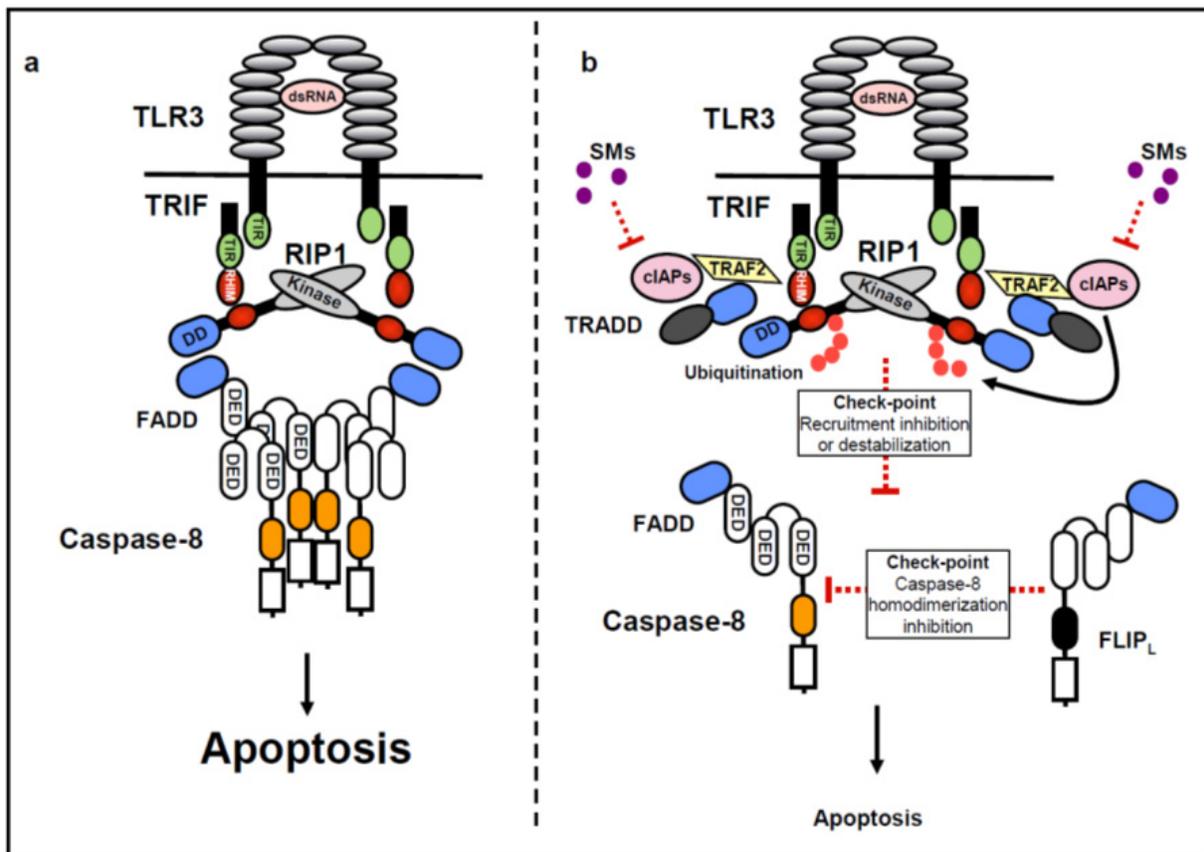


Figure 1. Hypothetical model of TLR3-triggered apoptosis: mechanisms. **a**, TLR3 activation by dsRNA induces the formation of an atypical caspase-8-activating complex containing caspase-8/FADD/RIP1/TRIF and TLR3. Successive homotypic interactions are required for TLR3 to recruit caspase-8. TLR3 possesses a TIR domain that binds to the adaptor TRIF through homotypic TIR domain interaction, while TRIF possesses a RHIM (RIP Homotypic Interaction Motif) domain in its C-terminal side allowing its association with the RHIM domain of RIP1. Then, RIP1 can recruit FADD through homotypic interaction between their Death Domain (DD), and FADD recruits caspase-8 through their respective Death Effector Domain (DED). **b**, Regulatory mechanisms of caspase-8 recruitment and activation by TLR3. In addition to RIP1, the adaptor TRIF recruits an ubiquitin ligase complex containing the adaptor TRADD and the ubiquitin ligases TRAF2 and cIAPs which drives ubiquitination of RIP1, a post-translational modification required for NF- κ B activation, that limits its association with caspase-8 by directly preventing and/or destabilizing the binding. In absence of cIAPs, which can be achieved by smac mimetics (SMs) treatments that triggers cIAPs auto-ubiquitination and degradation by the proteasome, RIP1 is not ubiquitinated which favours the recruitment of caspase-8. In presence of FLIP at sufficient level, the affinity of FLIP for caspase-8 favours the formation of FLIP-caspase-8 heterodimers, hence preventing the formation of apoptotic caspase-8 homodimers. cIAPs and FLIP may constitute two different molecular checkpoints acting at two different levels for the negative regulation of caspase-8 recruitment by TLR3.

d.1. The upstream antiapoptotic IAPs-dependent checkpoint

The mammalian Inhibitor of Apoptosis (IAP) proteins, c-IAP1, c-IAP2, and XIAP, are critical regulators of cell death through their direct activity towards caspases. IAPs are also well known modulators of inflammatory signaling and immunity. These proteins consist of three N-terminal Baculovirus IAP Repeat (BIR) domains, a C-terminal Really Interesting New Gene (RING) domain that confers E3 ubiquitin ligase activity, and a Caspase-Recruitment Domain (CARD) – in c-IAP1 and c-IAP2 – required for autoinhibition of their ligase activity at steady state. Notably, c-IAP1 and c-IAP2 regulate ubiquitin-dependent innate immune signaling in a way of TLRs or TNF-R, such as the activation of nuclear factors NF- κ B, through their ubiquitin ligase activity toward key molecules of the signaling pathways. Based on the contribution of IAPs in cancer cell survival, small pharmacological inhibitors have recently been developed. These antagonist molecules, dubbed Smac-mimetics (SMs), mimic the N-terminal IAP-binding motif of SMAC (an endogenous mitochondrial IAP inhibitor), and selectively bind the BIR2 and BIR3 domains of IAPs. In particular, interaction of SMs with c-IAP1 and c-IAP2 results in auto-ubiquitination activity and rapid proteasomal degradation [60-62].

The use of SMs shed new light on cIAPs functions. Notably, it has been demonstrated that non-degradative K63-linked ubiquitination of RIP1 by cIAPs is required for efficient NF- κ B activation and prosurviving signaling in response to TNFR-I activation [61, 63, 64]. Moreover, RIP1 ubiquitination by cIAPs prevents RIP1 from binding caspase-8 and blocks apoptosis after TNF stimulation [63, 65, 66]. Hence, cIAPs dependent RIP1 ubiquitination functions as an early checkpoint to protect from TNF-RI-induced cell death until a later checkpoint take place via the expression of pro-survival genes through the NF- κ B pathway (reviewed in [67]). Ubiquitination of RIP1 is also important for TLR3-induced NF- κ B activation, and like for TNF signalling, the adaptor TRADD and the ubiquitin ligase TRAF2 are required for efficient RIP1 ubiquitination [68, 69].

Recently, two groups described a new RIP1-mediated death platform, termed the ripoptosome, which is formed upon downregulation of cIAPs and XIAP by SMs treatment or genotoxic stress [54, 70]. They showed that invalidation of IAPs allows the self-assembling of a cytosolic molecular complex containing RIP1, FADD and caspase-8, independently of Death Receptor signaling, and mediating apoptotic cell death.

Interestingly, IAPs inhibition by SMs treatment sensitizes a variety of cancer cells (melanoma, nasopharyngeal carcinoma, cervix, NSCLC...) to TLR3-mediated apoptosis [44, 49, 50, 54, 55]. cIAP1 and cIAP2 play non-redundant roles in this apoptotic process since specific invalidation of cIAP1 or cIAP2 can potentiate the deleterious effect of dsRNA. Two non-mutually exclusive models can be proposed to explain the sensitizing effect of cIAP invalidation. In the first one, cIAP elimination by SMs allows the formation of the ripoptosome which can bind to TRIF following TLR3 stimulation by dsRNA treatment, and favouring induction of apoptosis. In the second one, TLR3 ligation allows the recruitment of the adapter TRIF that functions as a platform to recruit signaling molecules such as RIP1, TRAF2, cIAPs, and TRADD for activation of the NF- κ B pathway. In absence of cIAP,

ubiquitination of RIP1 is defective which favours (or stabilizes) its association with caspase-8 and induces apoptosis (Figure 1b). This second model is supported by the fact that TLR3-induced apoptosis can occur without a prior invalidation of cIAPs in some tumor cells, and that ripoptosome formation may not be a prerequisite for caspase-8 activation.

d.2. The downstream antiapoptotic FLIP-dependent checkpoint

Two FLIP isoforms exist in the cell: FLIP_s (short form) et FLIP_L (long form). FLIP_L is similar to caspase-8 but lacks the catalytic site. FLIP_s contains the two DED and is structurally related to the FLIP inhibitor from viruses. FLIP_s and FLIP_L bind FADD and block caspase-8-mediated apoptosis in response to death receptor ligation [71, 72]. FLIP represents one of the most important anti-apoptotic proteins whose expression is tightly regulated by the NF- κ B pathway for blocking TNF-mediated caspase-8-dependent apoptosis [73]. Moreover, heterodimers FLIP-caspase-8 assemble preferentially in the cell because of a greater affinity and/or stability than caspase-8 homodimers [74].

Like classical death receptors of the TNFR family, TLR3-induced caspase-8-mediated apoptosis is negatively regulated by FLIP. Indeed, FLIP invalidation by specific shRNA potentiates TLR3-dependent caspase-8 activation and apoptosis in different tumor cell lines ([54] and unpublished data). At the contrary, FLIP_L overexpression blocks the apoptotic effect of dsRNA poly(I:C) treatment [54, 55]. In contrast to TNF-RI pathway, for which the role and the regulation of FLIP have been extensively studied, TLR3-mediated FLIP regulation as well as the mechanism of FLIP-dependent blockade of pro-apoptotic activation of caspase-8 are not clear and require further investigations. However, we can hypothesize (from death receptor signalling literature) that FLIP inhibit TLR3-induced apoptosis through associating with caspase-8 to form FLIP-caspase-8 heterodimers, and hence, preventing the formation of apoptotic caspase-8 homodimers (Figure 1b)

Although both IAPs- and FLIP-dependent checkpoints are likely to protect cells from TLR3-triggered apoptosis, it remains unknown to which extent they each contribute to the resistance of normal cells and of different tumor cells. For example, it would be interesting to determine whether the higher sensitivity to TLR3-induced apoptosis of metastatic head and neck cancer cells relative to primary tumors [75] could be explained by differences in the efficacy of either or both of those two molecular barriers.

3.2.3. TLR3 and necroptosis

It is important to note that although FLIP_L prevents apoptotic activation of caspase-8, FLIP_L-caspase-8 heterodimers are proteolytically active, which is not true for FLIP_s-caspase-8 heterodimers [74, 76, 77]. This non-apoptotic protease activity of FLIP_L-caspase-8 heterodimers is required to protect from lethality of mouse embryos during development, indicating that caspase-8 plays a survival role [78]. Indeed, caspase-8 knock-out is lethal at around embryonic day 10.5 due to alteration in the development of yolk sac vasculature [79, 80]. A molecular mechanism of caspase-8-induced survival has been recently highlighted, and indicates that FLIP_L-caspase-8 heterodimers confers protection from necroptosis [78, 81],

a form of programmed necrotic cell death, which is regulated by RIP1 and RIP3 [82, 83]. To prevent necroptosis, caspase-8 protease activity is required to cleave and inactivate RIP1 and RIP3, but also CYLD, a deubiquitinating enzyme that removes Lys 63-linked polyubiquitin chains on RIP1 and regulates the interaction between RIP1 and caspase-8 [66, 84]. Necroptosis can be triggered by death receptor ligation in condition of caspase-8 inhibition, and is inhibited by necrostatin-1, a specific inhibitor of RIP1 kinase activity [85]. Necroptotic cell death is currently under intensive investigations (for review see [86-88])

Since TLR3 behaves like a death receptor and activates caspase-8 through the recruitment of RIP1, it is reasonable to assume that TLR3 could also induce necroptotic cell death in condition of caspase-8 inhibition. Indeed, it has been reported that Poly(I:C)-induced TLR3 activation can trigger necroptosis in presence of the pan-caspases inhibitor Z-VAD [54], this cell death is inhibited by necrostatin-1 treatment. FLIP isoforms play differential roles in this type of cell death, FLIP_L preventing both apoptosis and necroptosis while FLIP_S is an inhibitor of only apoptosis [54]. However, TLR3-mediated necroptosis seems to be cell specific and probably depends on the expression of RIP3 [54, 55], which is also true for other inducers of necroptosis. Nevertheless, these data indicate that TLR3 activation can trigger the formation of a “necroptosome” containing at least RIP1 and RIP3 which could have relevant function in virus-infected cell death and in immune responses. Further studies are required to address the role of necroptosis in virus-induced diseases and in TLR3-mediated tumor growth inhibition.

3.3. dsRNA in clinical trials

It is known for long time that in human and primate, Poly(I:C) has a short half-life (~6min) because of rapid hydrolysis by RNase from serum, and its capacity to induce IFN production is weak compare to what is observed in mouse models [89, 90]. Moreover, no GMP preparation are currently available and poly(I:C) has too much toxicity by causing fever, renal failure, coagulopathies and hypersensitivity reactions [90], indicating that Poly(I:C) can't be used in clinic. However, two type of Poly(I:C) analogues are currently evaluated in several clinical trials: Poly-ICLC that correspond to Poly(I:C) complexed with polylysine and carboxymethylcellulose, and Poly(I:C₁₂U) or Ampligen (Hemispherx Biopharma of Philadelphia) which is a Poly(I:C) modified by introduction of unpaired bases (uracil). Poly-ICLC is 4- to 10-fold more resistant to hydrolysis than Poly(I:C), with a longer half-life in serums of primate, and a great inducer of IFN [91]. Poly(I:C₁₂U) is a GMP-grade molecule that, in contrast to Poly-ICLC, undergoes accelerated hydrolysis because of regular regions of mismatching. However, Poly(I:C₁₂U) maintains pharmacological activity [92]. Poly-ICLC remains toxic – notably with doses greater than 12 mg/m² – whereas Poly(I:C₁₂U) showed no evidence of dose-limiting organ toxicity. Indeed, Poly(I:C₁₂U) has been previously tested in the treatment for chronic fatigue syndrome and AIDS without apparent toxicity [92]. Moreover, Poly(I:C₁₂U) is shown to specifically target TLR3 [93].

Owing to its strong capacity to activate the adaptive immunity notably through its action on dendritic cells, dsRNA ligands are currently tested in several clinical trials mainly as

adjuvant for antigen peptide vaccinations against various types of cancer [94]. The antigen peptide will be mainly taken up by the dendritic cells that play a major role in the innate immune response as professional Antigen-Presenting Cells (APCs). The peptide is then processed by APCs, and epitopes presented at the cell surface through MHC class I molecules for antigen cross-presentation to CD8⁺ T-cells. The adjuvant (here the dsRNA ligands) plays an important role for the up-regulation of co-stimulatory molecules by dendritic cells and their maturation, leading to direct activation of CD8⁺ and CD4⁺ T-cells through MHC class II and I molecules respectively, and indirectly to NK cells. Several phase 0/I/II clinical trials - <http://www.clinicaltrials.gov/> - are in progress in which Poly-ICLC is used in ~ 80% of the studies as adjuvant for antigen-peptide vaccination such as TARP for prostate cancer, MUC1 for triple negative breast cancer or prostate cancer, or NY-ESO1 for ovarian cancer or melanoma. Poly-ICLC is also used in combination with radiotherapy for low-grade recurrent B and T cell lymphoma, and for brain and central nervous system tumors in phase I and II clinical phase. Interestingly, a phase II clinical trial from the North American Brain Tumor Consortium for 30 patients with newly diagnosed supratentorial glioblastoma showed that treatment with radiotherapy in combination with Poly-ICLC followed by Poly-ICLC as a single agent was relatively well-tolerated and enhanced the survival of patients compared to historical studies using radiotherapy alone [95]. Then, the New Approaches to Brain Tumor Therapy (NABTT) consortium assigned 365 patients with newly diagnosed glioblastoma in phase II clinical trial for testing novel agents in combination with radiation + temozolomide and compared the results with the data from the European Organization for Research and Treatment of Cancer (EORTC) phase II study [96, 97]. It is interesting to note that patients treated with radiation + temozolomide + Poly-ICLC had significantly longer survival than patients treated with only radiation + temozolomide between 2000 and 2002 [96]. However, these encouraging data have to be interpreted with circumspection because of the changing patterns of care. Concerning Ampligen product, only two clinical trials (phase I/II) are in progress (recruiting status) in which the dsRNA Ampligen is used as adjuvant for oxidized tumor cell lysate vaccination for patients with ovarian, fallopian tube, or primary peritoneal cancer, or as adjuvant for HER2 protein vaccination for patients with HER2-positive breast cancers.

4. Integrated view of the activities of TLR3 ligand in cancer

TLR3 agonist would have multiple cellular targets that could all contribute to the efficacy of their use in cancer.

As described above, targeting TLR3 expressed by tumor cells could trigger apoptosis and/or block cell cycle progression. It can also elicit the secretion of chemokines, which may recruit immune effectors at the site of the tumor and thereby enhance anticancer immune responses [36], or reduce the sensitivity of cancer cells to immuno-chemotherapy [98]. Furthermore, many of the cytokines secreted by TLR3-stimulated cancer cells, and particularly the type I IFNs will enhance the intratumoral innate immune responses, while the upregulation of CD54 on cancer cells may also enhance the cytotoxic activity T cells, as it has been observed *in vitro* [37].

Among the human immune cells, TLR3 is mostly expressed by myeloid Dendritic Cells (mDC) [99], which represent the major Antigen-Presenting Cells, and by macrophages. However, TLR3 is only one of the dsRNA receptor present in mDC, altogether with RIG-I, MDA-5 and the two helicases complexes DDX1-DDX21-DHX36 and DHX9. In the presence of Poly(I:C), human mDC undergo phenotypic maturation and produce high amounts of IL-12 p70 [100, 101]. Moreover, TLR3 activation had been shown to enhance the antigen cross-presentation capability of mouse CD8⁺ DC [102]. Recently, a subset of human mDC expressing BDCA3⁺ was found to internalize material from dead cells *in vitro*, and to cross-present exogenous antigens to CD8⁺ T cells upon treatment with poly(I:C) [103].

Activation of human T cells by Poly(I:C) is generally regarded as an indirect consequence of TLR3 DC stimulation. However, TLR3 can also be expressed by T cells, at least on gamma/delta T cells, and acts as co-stimulatory receptor to enhance proliferation and/or cytokine production of T-cell receptor-stimulated T lymphocytes [104]. The clinical grade poly(I:C)-analogue (Ampligen) was reported to promote optimal human DC maturation and Th1-type T cell responses *in vitro* [105].

Moreover, Poly(I:C) was reported to induce CD4⁺ human T cells synthesis of both IL-17A and IL-21 and was able to drive the differentiation of naive T helper cells into an IL-21-producing phenotype [106]. TLR3 has also been described to directly increase IFN-gamma production by human Ag-specific CD8⁺ T cells [107]. Regarding human NK cells, in contrast to the initial description [108], their activation by dsRNA now appears to be secondary to IFN-gamma production by mDC in response to TLR3 stimulation [109, 110]. Thus, the combined activities of TLR3 on human mDC and T cells are likely to help developing Th1-polarized and strong cytotoxic T cells responses.

Indeed, syngeneic mouse tumor models have shown the importance of TLR3 expressed on non-cancer cells not only in tumor immunosurveillance but also for the control of tumor growth. Protection conferred by tumor vaccine including Poly(I:C) was mediated by primary and memory CD8⁺ T cells that has been robustly activated by antigen cross-presenting DC [111-115], and by IFN-I-activated NK cells [115]. Moreover, in a mouse model of established pulmonary metastasis, Poly(I:C) elicited a Th1-like, Th17-like, and cytotoxic immune environment following the activation of DCs and the production of IFN type [116]. Those animal models allowed also to show that combining Poly(I:C) with CD40 signaling dramatically increased the efficacy of mouse tumor vaccine [117, 118]. Such adjuvant combination was also able to convert mouse ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells [119].

TLR3 agonist might also restrain tumor-driven blood supply, as multiple human endothelial cell types express surface TLR3, and as dsRNA-induced TLR3 activation inhibits *in vitro* angiogenesis [120]. Moreover, siRNA was found to inhibit in a sequence-independent, and possibly TLR3-dependent manner, the dermal neovascularization in mice [121] and the proliferation and morphogenesis of endothelial cells in a mouse model of hepatocellular carcinoma *in vivo* [122].

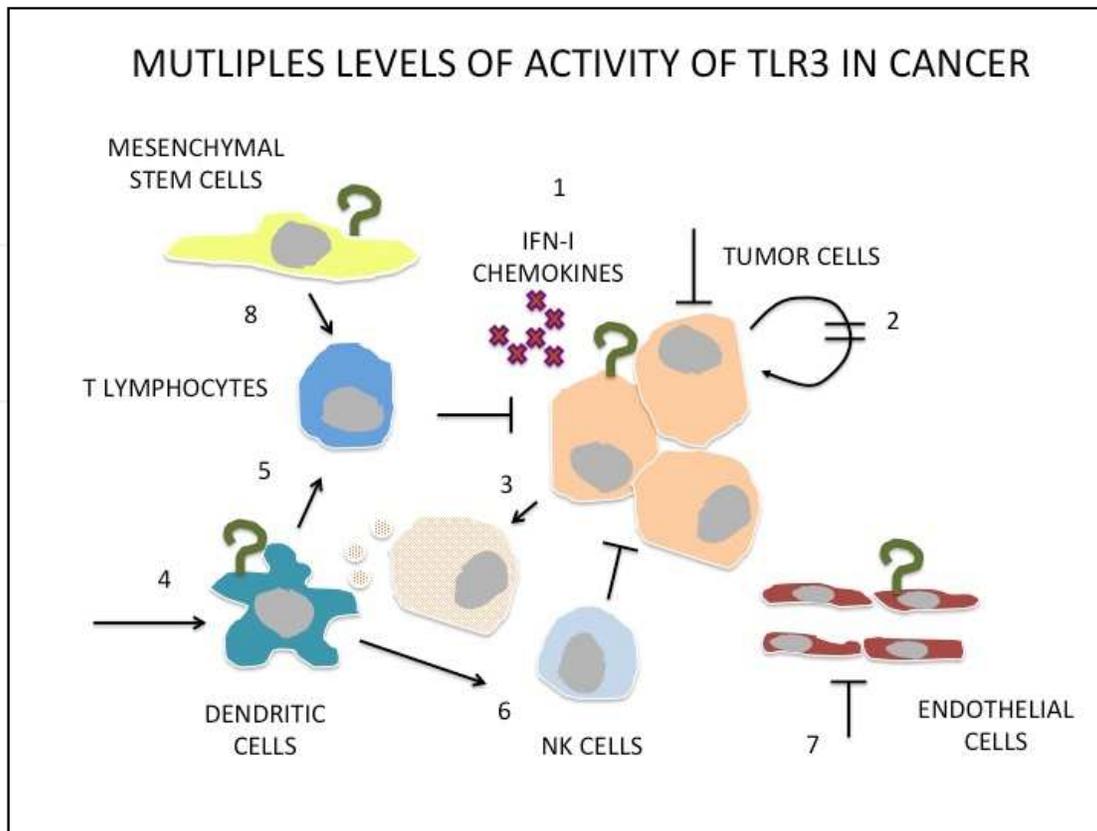


Figure 2. (1)TLR3 can stimulate cancer cells to secrete proinflammatory cytokine sand chemokines that attract and activate immune cells, respectively; (2)TLR3 can inhibit the proliferation of cancer cells; (3) TLR3 can trigger the apoptosis of cancer cells and the release of apoptotic bodies; (4)TLR3 can activate DC; (5) TLR3 can enhance the efficacy of DC to generate Th1 cells and cytotoxic T cells; (6) TLR3 can help NK cells to become cytotoxic; (7) TLR3 can inhibit tumor-driven neoangiogenesis; (8) TLR3 can switch MSC from immunosuppressive to immunosupportive phenotype.

Lastly, Toll-Like Receptor 3, which is strongly expressed by human mesenchymal stem cells (MSC), inhibits their Notch-dependent immunosuppressive effect on T cells [14]. In addition, in response to TLR3-triggering, MSC sustain and amplify the functions of neutrophils and may consequently contribute to local inflammation [123].

Many of the above-mentioned mechanisms summarized in figure 2 probably contribute to the remarkable activity of Poly(I:C) used as vaccine adjuvant in several mouse tumor models [113, 124]. Indeed, compared with other TLR agonists, DC stimulated with poly(I:C) displayed the strongest activity in stimulating proinflammatory responses and the production of tumor-specific CD8(+) T cells in several mouse tumor models [125]. Interestingly, the combination of TLR3 with TLR7 ligands increased the capacity of mouse DC to establish an in vivo anti-tumoral response [126].

5. Conclusions and prospectives

Since the first description of TLR in mouse, the members of this family of receptors have been linked to the activation of the innate immunity (Medzhitov et al., 1997). It was

therefore natural to study the adjuvancy capabilities of TLR ligands for vaccines, including anti-tumor vaccines. However, the ongoing recognition of the multiple levels of action of TLR on immune and non-immune cells indicates that a better understanding of the result of these combined activities will be required to anticipate how TLR agonist might interfere with cancer progression.

Regarding TLR3 agonists, evidences coming not only from *in vitro* experiments and from preclinical mouse models, but also from clinical data strongly suggest that they could be useful in cancer. In particular, the discovery that TLR3 behaves as a death receptor selectively in cancer cells makes it similar to TRAIL receptors that are currently targeted in phase II clinical trials. However, answering a few key questions summarized in table1 will be required in order to determine whether and how TLR3 may become a successful target in cancer.

1. Which are the molecular mechanisms that underlie the sensitivity vs. the resistance of normal and cancer cells to TLR3-triggered apoptosis?

Answer to this question should help to identify a priori tumors that would benefit from TLR3 agonist treatment

2. What is the net effect of the pro-apoptotic activity on cancer cells and the immunostimulatory effect of TLR3 ligand on tumor progression?

This important question has not been addressed yet as, in contrast with human tumors, mouse tumor appears to be rather resistant to TLR3-triggered apoptosis

3. Could TLR3 agonist synergize which (chemotherapeutic) drugs and allow increasing their efficacy while limiting their toxicity?

This question must also be addressed in a syngeneic tumor model that associates a tumor sensitive to TLR3-triggered apoptosis and a fully functional immune system

Table 1. Unsolved questions related to tlr3 and cancer

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