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A Toll-like receptor 3 (TLR3) agonist ARNAX for therapeutic immunotherapy

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SUMMARY

Vaccine immunotherapy consisting of tumor antigens combined with an immune-enhancing adjuvant fosters cytotoxic T cell (CTL) proliferation. Clinically, polyI:C has been used as an adjuvant to enhance cancer vaccine protocols. However, according to its long history, polyI:C promotes inflammation that causes cytokine toxicity. Although checkpoint inhibitor immunotherapy has improved the prognoses of patients with progressive cancer, over 75% of patients continue to experience resistance to antibody (Ab) against anti-programmed cell death-protein 1 (PD-1) or its ligand, PD-L1 therapy. In most cases, patients suffer from adverse events resulting from inflammation during anti-PD-1/L1 Ab therapy, which is a serious obstacle to patients' quality of life. We have studied the functional properties of double-stranded (ds)RNA and polyI:C, and developed a nucleic acid adjuvant that barely induces a significant increase in the level of serum inflammatory cytokines in mouse models. This adjuvant, termed ARNAX, consists of DNA-capped dsRNA that specifies the endosomal target for Toll-like receptor 3 (TLR3) in dendritic cells (DCs). We expect that this adjuvant is safe for administration in elderly patients with cancer receiving immunotherapy. Here, we summarize the properties of ARNAX for immunotherapy in mice. We suggest that DC-priming is essential to induce anti-tumor immunity; neither exogenous inflammation nor the administration of tumor antigens is always a prerequisite for DC-mediated CTL proliferation. If our mouse data can be extrapolated to humans, ARNAX and the liberated endogenous tumor antigens may facilitate effect of current therapies on patients with therapy-resistant tumors.

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1. Introduction

Recent findings have confirmed that the activation of innate immunity bestows a trigger of the acquired immune response [1]. A typical outcome of innate immune activation is to alarm the host cells to the presence of microbial patterns in infections. This is a rapid response mediated by systemic type I interferon (IFN) and cytokines produced by the affected cells [2]. The innate immune system is conserved across

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vertebrates, including jawless vertebrates [3]. According to comparative studies on the various innate immune systems among vertebrates, similar receptors facilitate the recognition of various microbial patterns [3]. These receptors are termed pattern-recognition receptors (PRRs), of which the Toll-like receptors (TLRs) represent a family of PRRs conserved among vertebrates.

The TLRs appear to have evolved in parallel with the development of the acquired immune system in vertebrates [4]. During a viral infection in mammals, infected cells produce viral double-stranded (ds)RNA, which is followed by rapid cytokine production. The synthetic dsRNA mimic, polyI:C, also induces a cytokine response by injection, suggesting that dsRNA is an innate pattern molecule [5]. The dsRNA sensors are distributed in the cell membrane and cytoplasm (Fig. 1), and their orchestration results in the activation of a cytokine network [6] followed by lymphocyte activation. Infected cells are typically damaged and die after issuing a warning via cytokines, whereas bystander cells survive to receive the products of the dead cells. The surviving cells responding to acute phase response appears to trigger delayed lymphocyte activation and memory formation secondary to dsRNA sensing in bystander cells.

Cytoplasmic RNA sensors are ubiquitously expressed in mammals with cell-specific variation to detect viral RNA [6], whereas TLR3 is the only membrane-associated receptor for dsRNA that can recognize dsRNA outside the cell membrane. TLR3 is primarily expressed in myeloid and neuroectodermal cells, and myeloid TLR3 preferentially acts as a receptor for the extrinsic recognition of viral dsRNA [5]. Dendritic cells (DCs) consist of a number of subsets, and those expressing TLR3 facilitate the cross-presentation of exogenous antigens [7]. However, it remains unknown whether robust cytokine production is absolutely required for DC-priming and the acquired response (including cross-priming of T cells) as described in previous reviews [6].

Viral RNA possesses several structural motifs for the activation of various RNA sensors in the innate immune system [6]. TLR3 agonist including synthetic dsRNA similarly functions without the sign of infections. Viral infections and a polyI:C injection simultaneously activate systemic dsRNA sensors in vivo [5]. To identify the function of each RNA sensor associated with DC-priming and its immune-enhancing effects [7], we need to establish specific agonists for each type of dsRNA receptors is required. Thus, we created a TLR3-specific agonist based on the theoretical strategy of structural biology and performed a functional identification of the immune response using mouse models [8,9]. The results were essentially reproduced in human peripheral blood mononuclear cells.

Here, we summarize the results of TLR3-specific stimulation based on the in vivo findings in mouse models. The TLR3-specific ligand contributes to DC-priming and cytotoxic T cell (CTL) proliferation without inducing inflammatory cytokinemia to facilitate vaccine immunotherapy. Robust cytokine production accompanying a viral infection is not essential to evoke a DC-mediated acquired immune response. This synthetic TLR3 agonist was termed, ARNAX.

2. RNA sensors in nucleic acid sensing

The dsRNA sensors reside in the endosomal membrane or cytoplasm and signal the presence of viral infections in both mouse and human cells [6]. Viral RNA has a signature recognized by host RNA sensors, which typically induces the production of IFN and other pro-inflammatory cytokines. RNA sensors are classified into two families based on their structural motifs, leucine-rich repeat (LRR) and DExD/H box helicase. Most of the RNA sensors converge on several adaptors, including TICAM-1 (TRIF), MAVS, and MyD88 (Fig. 1). These adaptors activate transcription factors, such as NF-κB, activator protein-1 (AP-1) and interferon-regulatory factors (IRF) [2,10]. In particular, the activation of IRF3 or 7 activation preferentially induces a type I IFN response in certain cell types, leading to amplified activation of the interferon-α/β receptor (IFNAR) pathway [11,12]. Therefore, RNA harboring virus-specific signatures can be recognized as non-self RNA [13]. The non-self signatures are referred to chemically-synthesis of RNA adjuvants. Although the host cells occasionally harbors incompletely paired RNA (e.g., miRNA or cytosolic non-coding RNA), they minimally activate RNA sensors in the same cell, which suggests that they behave as self-RNA.

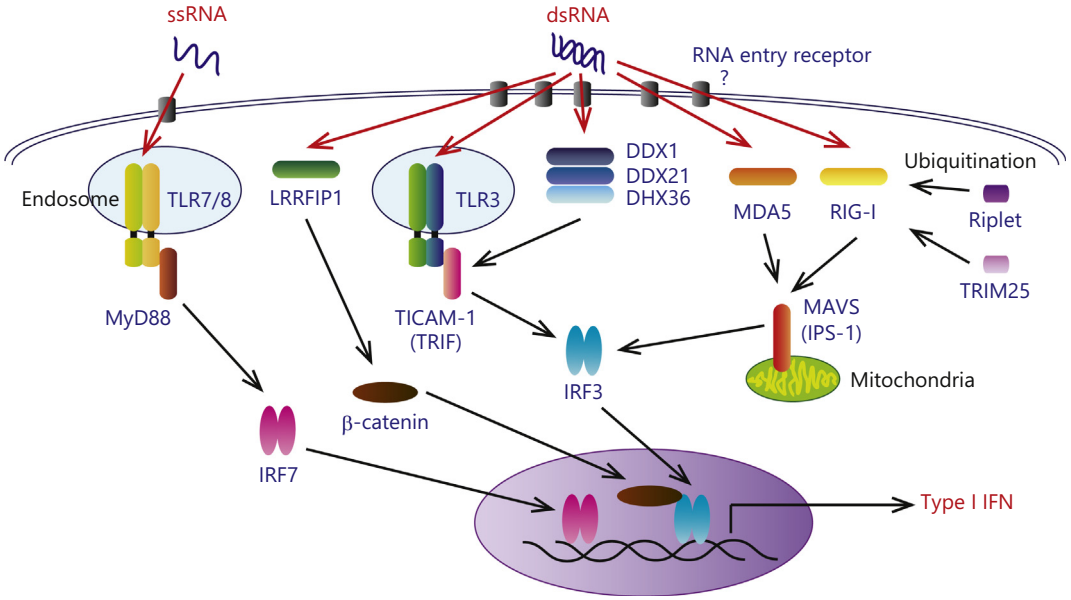

Fig. 1. RNA-dependent IFN-inducing pathways. The signal responses are depicted with reference to polyI:C stimulation, except for TLR7, which recognizes single-stranded RNA. There are multiple dsRNA sensors (including sentinels [23]) in the cytoplasm of various cell-types, and they principally use MAVS or TICAM-1 as the adaptor (in some cases β-catenin involved). PolyI:C activates all of these sensors without transfection. However, cytoplasmic RNA sensors minimally activate IFN signaling in response to external dsRNA of viral origin (i.e., blunt-paired), suggesting that the cytoplasmic sensors essentially function to alarm the presence of viral RNA in the same cell (i.e., viral infection). TLR3 exclusively responds to extrinsic dsRNA to recruit TICAM-1 as the adaptor in myeloid cells. Even without transfection, TLR3 responds to polyI:C and 'structured' RNA [19], but does not usually respond to extrinsic blunt-paired dsRNA. Some unidentified RNA-uptake machinery helps external dsRNA to activate myeloid cells. In plasmacytoid DCs, TLR7 uses the MyD88 adaptor to activate IRF7 and produce a large amount of IFN-α. Additionally, RIG-I/MDA5 are representative viral RNA sensors that recognize viral RNA patterns with the aid of Riplet or Trim25 [23].

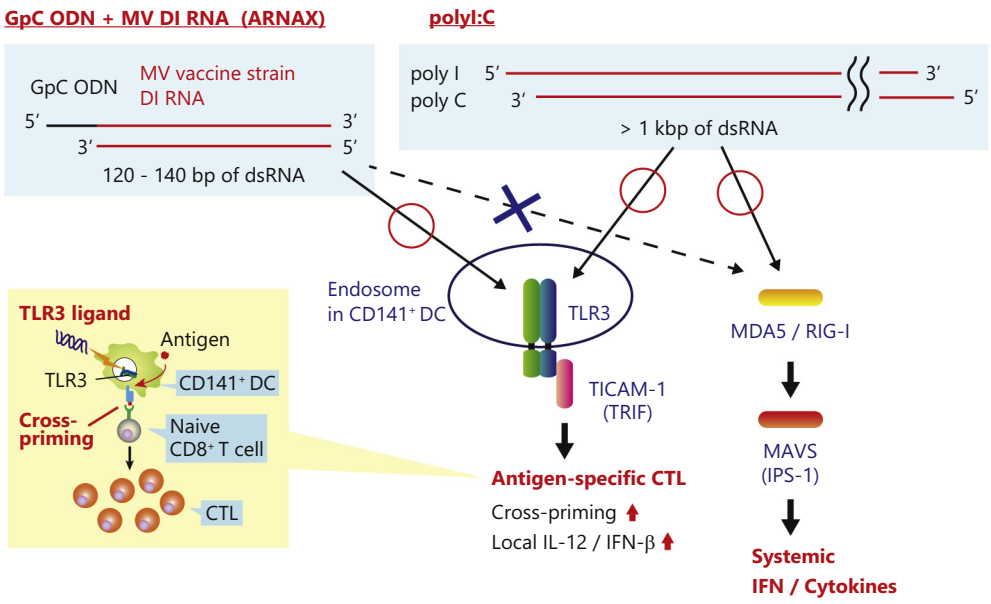

Fig. 2. Structure and function of ARNAX. (Left panel) ARNAX is a synthetic DNA/RNA hybrid molecule. The structure of ARNAX is shown in the upper inset. ARNAX is a TLR3-specifc agonist that sufficiently primes antigen-presenting DCs [29,30]. DCs cross-prime exogenous antigens in response to ARNAX to induce the proliferation of antigen-specific CTLs. ARNAX also induces the class-switching of B cells to predominantly produce IgA [44]. Both DC-priming functions contribute to vaccine and tumor immunity. Moreover, ARNAX barely induces an inflammatory cytokine response, but sufficiently activates the immune system. (Right panel) PolyI:C acts on both TLR3 present in the endosomes of DCs or macrophages, as well as MDA5 in the cytoplasm. In general, systemic inflammation and cytokinemia are observed in viral infections due to the ubiquitous distribution of MDA5 in whole body cells. Other cytoplasmic dsRNA sensors/sentinels may foster IFN/cytokinemia. Therefore, the major outcome of viral infections is an inflammatory response. PolyI:C is a viral dsRNA-mimic that induces similar systemic response via i.p. or i.v. administration. However, ARNAX does not induce inflammation by any route of administration in mice since there is no activation of the MAVS pathway.

We established a specific monoclonal Ab against human TLR3 [14,15]. TLR3 is typically localized in the endosome of myeloid cells, including DCs [14], where TLR3 encounters dsRNA to induce TLR3 signaling. PolyI:C, a viral dsRNA-mimic, enters endosomes whereas blunt-paired dsRNA rarely enters cells (Fig. 2). This finding suggests that a modification of synthetic dsRNA is required for endosomal uptake [16]. When a virus (e.g., measles) infects DCs, the infected DCs undergo apoptosis to disrupt viral spreading. The damaged DCs stop the immune function as well. Secondary immunosuppression frequently accompanies such dysfunction of DCs [8,17]. A structure-function analysis of TLR3 agonists indicates that endosomal TLR3 recognizes >120 bp blunt base-paired dsRNA or structured RNA [18,19] that contains long dsRNA portions with mismatched regions. TLR3 is expressed on the cell surface of some fibroblasts and epithelial cells [14,20], which are activated by the extrinsic addition of these dsRNA. Moreover, there is no sequence-specific activation of TLR3 in response to dsRNA. Although TLR3 minimally responds to approximately 40 bp dsRNA by a reporter assay [18,19], a length >120 bp is required for TLR3 clustering to sufficiently amplify the signal [19]. Thus, >120 bp length of dsRNA is a non-self pattern to yield high-affinity coupling with TLR3 to dsRNA. The TLR3 response occurs exclusively in the endosome in DCs. Thus, TLR3 ligands for DC-targeting also require additional structures which facilitate endosomal trafficking (Figs. 1 and 2).

In some cases, these RNA patterns are released from infected cells in the form of native dsRNA or vesicles (e.g., exosomes) even when the cells are damaged; however, the majority of these particles are rarely taken up into other cells (Fig. 1). Hence, how the activation of TLR3 occurs in infected hosts through exogenous dsRNA remains to be investigated.

In the cytoplasm, DExD/H box helicases recognize dsRNA-containing motifs [21–23]. Retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are representative cytoplasmic RNA sensors (Fig. 1). Additionally, other DExD/H box helicases may be engaged in RNA-sensing in a cell-type-specific manner [22,23] as depicted in Fig. 1. Their functions are typically modulated by accessory molecules and/or sentinels in the cytoplasm [23]. However, why so many molecules join the modulation of helicases remains unknown. RIG-I recognizes the structure of short dsRNA capped with 5′-di/triphosphate, which is a hallmark of viral replication [24]. MDA5 recognizes long dsRNA (>1 kbp) without bulges [21,25]. Both negative- and positive-stranded RNA viruses and some DNA viruses produce one of these virus-specific patterns in infected cells. These viral RNAs are also non-self patterns in the cytoplasm.

Since RIG-I and MDA5 are ubiquitously expressed in human whole-body cells, infected cells evoke the production of IFNs and other cytokines in response to intrinsically-produced viral pattern RNAs in the same cytoplasm [26]. Type I and III IFNs and other pro-inflammatory cytokines released from infected cells prohibit the systemic spread of viruses through the activation of the IFNAR pathway in other cells [11,27]. The IFN pathway enhances evoking the acquired immune response. Thus, infected cells accomplish systemic host cell protection against viruses, despite their own destruction.

In summary, dsRNA >1 kb in length or capped with 5′-di- or triphosphate represent non-self RNA based on the ability to up-regulate cytokine production in the same cell cytoplasm. RNA having complete or incomplete stem structures over 120 bp and ability to enter endosome behave as a non-self RNA. The two PRR responses to dsRNA participate in DC-priming: one inducing systemic robust cytokines followed by secondary DC-priming and the other (so far only TLR3) inducing local minute cytokines and direct DC-priming. The receptor responsible for dsRNA trafficking from the cell-surface to the TLR3 endosomes remains unknown [28].

3. Functional properties of the TLR3-specific adjuvant ARNAX

Appropriate structures of patterns in viral dsRNA products for cytoplasmic sensors other than RIG-I, MDA5 and TLR3 have not been determined yet. It is known that polyI:C activates the other cytoplasmic sensors of DExD/H box helicases and LRR proteins, which suggests that they bind and recognize the long RNA duplex in the cytoplasm [29]. A short RNA duplex with no phosphate modification at the 5′ end does not induce a cytokine response in human and mouse cells to

date. Thus, short fragments of viral dsRNA may not activate these undefined cytoplasmic sensors.

TLR3 recognizes >40 bp RNA duplex irrespective of the 5′ modification. Using a reporter gene assay, the degree of TLR3 activation has been found to depend on the length of the dsRNA; the longer in the dsRNA length, the better activates TLR3 by dsRNA in human HEK293 cells [16]. There is no sequence dependency for TLR3 activation, which enables us to select a safe (no RNA interference) sequence for humans [29,30]. However, synthetic dsRNA, which have an OH in their 5′ ends, hardly attains endosomal TLR3 [30]. The point is what is the best option to guide >120 bp dsRNA to the endosome circumventing activation of cytoplasmic sensors.

B/C-type oligodeoxynucleotide (ODN) competes with polyI:C for cellular uptake [16,29], suggesting the presence of a common nucleic acid receptor involved in their internalization. Although CpG DNA induces a cytokine and IFN response via TLR9, GpC DNA does not. After several chemical synthesis trials of TLR3 ligands that modify the 5′ end with GpC DNA, we found that capping the dsRNA with 25 bp phosphorothioated-GpC ODN allowed dsRNA to enter the endosomes [29,30] (Fig. 2). The GpC-ODN-capped dsRNA activates TLR3 at the endosome without activating TLR9 or RIG-I/MDA5 [30].

The DNA-dsRNA compound, ARNAX, can activate DCs via exogenous addition to the cells [29,31]. Therefore, the dsRNA portion can be delivered to the endosomes of DCs by DNA-capping; >120 bp dsRNA sufficiently activates the TLR3/TICAM-1 pathway [18,29]. In addition, dsRNA recognition by TLR3 is not affected by a modification of the GpC cap in ARNAX, which suggests that TLR3 indistinguishably recognizes DNA-capped dsRNA. Neither the cytoplasmic sensors nor DNA sensors involve the immune response and inflammation in the ARNAX-mediated in vivo events based on gene-disrupted mouse studies. In addition, in both DCs and HEK293 cells, ARNAX specifically activates human TLR3 [30]. Using ARNAX as a probe, we can define the function of TLR3 in human DCs. In situations during which high levels of TLR3 are expressed, the TLR3/TICAM-1 pathway is involved in the maturation of antigen-presenting DCs [30–32]. The results are consistent with previous reports on the functional definition of RIG-I/MDA5 [33,34] and DC-priming by TLR3 [14,32].

According to previous reports, the mitochondrial antiviral signaling protein (MAVS) pathway is ubiquitously expressed in whole-body cells and typically participates in cytokinemia in mice [33,34]. The effect of systemic polyI:C administration in humans was described approximately 50 years ago [36], and human clinical trials involving polyI:C were dropped down as designated 'untrelable'. Therefore, non-self RNA patterns are also functionally categorized into two entities: (1) those inducing systemic cytokine production; and (2) those facilitating acquired immune activation. Published in vivo studies have been interpreted to indicate that the RIG-I/MDA5/MAVS pathway mainly participates in systemic cytokine production [33,34], whereas TLR3/TICAM-1 promotes acquired immune-activation [14,29,35]. Indeed, activation of the MAVS pathway induces type I IFN to secondarily participate in DC priming. Thus, the initial trigger of DC-priming is not RNA sensor stimulation but type I IFN in the MAVS pathway.

The final design of the TLR3-specific ligand is presented in Fig. 2. While this molecule does not activate the MAVS pathway following extrinsic addition, it induces cross-presentation in DCs (Fig. 2). Therefore, TLR3 activates a T cell response via cross-priming without the help of RIG-I/MDA5 or robust cytokine production [30,31]. Thus, ARNAX enables DC-priming for T cell activation without inducing inflammation [36,37]. It is important to note that all previous adjuvants induced cytokine toxicity (i.e., 'inflammatory' adjuvants) [38] whereas ARNAX only targets DCs for immune activation (i.e., DC-priming adjuvant) [36,37].

4. ARNAX enhances the antitumor effect by inducing a PD-1/L1 blockade

TLR3-specific activation is applicable to targeted therapy to facilitate DC-priming and T cell activation in mouse models [30,31,39]. In the absence of inflammation, mice reject implant tumors by inducing tumor-associated antigen (TAA)-specific CTLs following treatment with ARNAX combined with the target antigen [31,39]. Several host factors participate in the prognosis of tumor-bearing mice. The important factor responsible for the efficacy of ARNAX is dependent on the tumor environment. Thus, if tumors leak their antigens, the adjuvant could cooperate in evoking a specific immune response towards endogenous antigens.

Anti-cancer vaccine immunotherapy may offer cancer patients a higher quality of life and a safer treatment option with reduced burden following surgery, at a lower cost. However, the only inflammatory adjuvants (e.g., Alum and oil) [38] that are currently approved for immunotherapy enhance the inflammatory side-effects in cancer patients [40]. Moreover, there are no approved RNA adjuvants due to the associated side-effects, which disturb comfortable care for the elderly.

Although PD-1/L1 antibody (Ab) therapy exerts an excellent therapeutic effect, the efficacy on solid tumors remains low, at approximately 20% [40]. It has been established that TAAs are present in the majority of spontaneous cancers and induce an anti-tumor CTL response, which can be reinvigorated by checkpoint inhibitors [40]. Thus, cancer vaccines could be administered if an appropriate adjuvant for DC-priming and CTL proliferation is developed.

In mouse models, combination therapy with a TAA/adjuvant and PD-1/L1 Ab is an appropriate choice for cancer immunotherapy, since TAAs can be provided for the proliferation of specific CTLs [31,41]. However, the side effects caused by inflammation and autoimmunity associated with immunotherapy need to be resolved [40,41]. In human studies, CD141+ and CD1c + DCs are activated in response to TLR3 ligand [42]. In clinical tests, the administration of polyI:C as an adjuvant and PD-1/L1 Ab treatment appears effective [41] but induces additive inflammatory side-effects. Thus, the rate of remission is expected to be much higher by combining ARNAX with PD-1/L1 Ab, compared with PD-1/L1 Ab monotherapy [41]. Since each tumor has a unique spectrum of TAAs [43], endogenous TAA and ARNAX may enhance the effects of PD-1/L1 Ab therapy with a low side-effect profile [36,37].

5. DC-priming therapy with ARNAX

Tumors typically express TAAs, and in rare cases, adjuvant-only therapy induces tumor-specific CTLs to shrink tumors in both mice and humans. This indicates that endogenous TAAs serve as antigens to specifically direct the adjuvant activity. Therefore, TAAs are indispensable for ARNAX treatment, and DC-priming occurs independent of the robust cytokine-inducing ability of the adjuvants [31,39]. This finding is further confirmed when comparing the effects of ARNAX with polyI:C (Fig. 2). In both cases, the TAAs and DC TLR3 pathway are essential for the induction of tumor-specific CTLs (Fig. 3). In addition, the level of TLR3 expression is elevated in response to IFN in CD103+ DC/CD8α + DCs (mouse) and CD141+ DC/CD1c + DCs (humans) [32,42], which are representative of antigen-presenting DCs [43]. DC-priming culminates in TAA and TLR3 agonist [41,43].

TLR3 signals also promote the class-switching of B cells via DC and CD4 T cell activation to elevate IgA production [44,45]. Since antigen-presenting DCs express high levels of TLR3, ARNAX can selectively prime DCs for IgA production, thereby minimizing systemic cytokine induction [46].

Therefore, it is important to understand how the TAAs are increasingly released as an effective form from tumors. When tumors die via necrotic stress, they may secret various types of proteins [47,48], which may represent a source of TAAs. Moreover, whole-genome sequencing analyses suggest that the quantity, quality, and immunogenicity of mutant antigens differ between individual cancers [41]. If a method for an increase of free endogenous TAAs is established, the step of exon-sequencing for the detection of TAAs can be skipped. TAA liberation may be enhanced by radiation or chemotherapy [48,49].

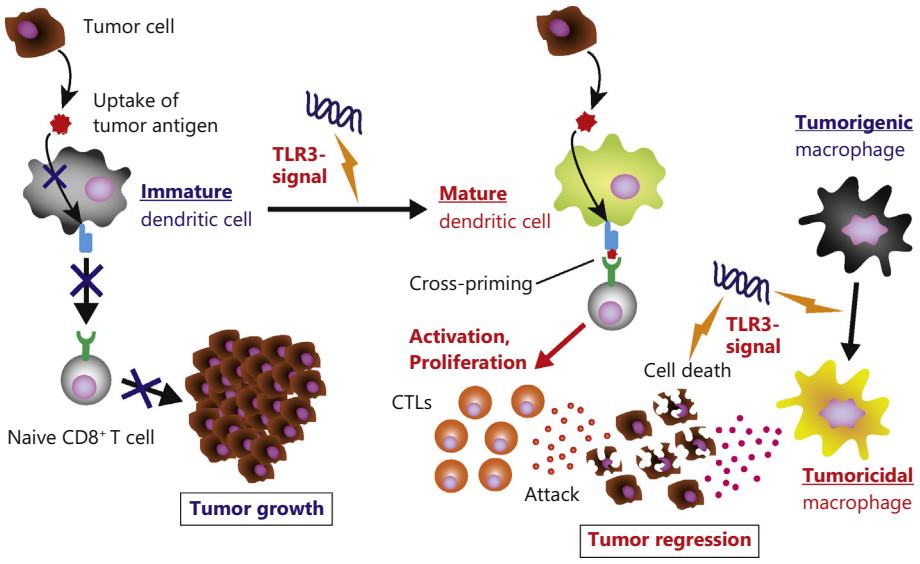

Fig. 3. Antitumor immunity induced by ARNAX. (Left panel) DC-priming by ARNAX. RNA-induced signaling by ARNAX facilitates DC maturation as observed by DC-priming and antigen-specific T cell proliferation. ARNAX sufficiently promotes CTL proliferation without the help of the MAVS pathway to induce tumor regression in mouse models. (Right panel) TLR3-signaling also converts the macrophage function from tumor-promoting to tumoricidal in the microenvironment. This TLR3 function also contributes to TLR3 adjuvant-mediated tumor regression.

Combination therapies involving ARNAX and radiation/chemotherapy are worth attempting without identification of TAAs.

Immuno-resistance is caused by both CTL-dependent and -independent axes [48–51]. Furthermore, distinct factors are involved in CTL dysfunction, which can be multifarious. At any tumor site, possible causes range from a lack of antigenicity, reduced potential of antigen processing, failure of peptide cleavage, peptides unsuitable to MHC class I (MIC), incompatibility of the peptide with the MIC complex, matching failure with T cell receptors, or a low density of MIC/peptides. For CTLs, additional causes include the functional failure of CTLs, insufficient CTL proliferation, loss of CTL tumor-entering ability, and tumor-killing failure.

PD-1Ab only ameliorates CTL proliferation and tumor-killing efficacy. Therefore, resistance to PD-1/L1 Ab treatment is one hallmark of CTL dysfunction in a tumor. Moreover, in the absence of CTL proliferation, no therapeutic efficacy is evoked with PD-1/L1 Ab. One of the measures to improve post-operative cancer outcomes with PD-1/L1 resistance is to combine PD-1/L1 Ab therapy with CTL-induced vaccine therapy [41]. Since cancer-specific antigens differ between individual cases [43], if the antigens from cancer cells are successfully liberated by radiation pretreatment, only an appropriate adjuvant can break PD-1/L1 resistance. This is true in mouse tumor-implant models susceptible to CTL and CTL-dependent tumoricidal axes [50–52].

6. Modification of the tumor microenvironment by adjuvant

Tumor consists of parenchymal and non-parenchymal cells. The latter contains myeloid cell moieties having TLR3, and takes part in regulation of tumor growth [50–52]. On the other hand, tumor cells frequently express TLR3 [53], and secret inflammatory cytokines. However, polyI:C barely induces tumor cell progression or invasion. In CT26 (colon cancer)-bearing Balb/c mice, TLR3 stimulation rather leads to cell damage via the RIP3 pathway of TLR3/TICAM-1 in the tumor cells [53]. Lewis lung carcinoma (LLC) tumor in mice is acutely rejected into hemorrhgic necrosis by polyI:C treatment [54]. This rapid tumor shrinkage is triggered by non-parenchymal macrophages responded to polyI:C. LLC tumor contains tumor-infiltrating M2 macrophages, which become an additional antitumor effector through polyI:C response [54]. Hence, TLR3 agonist works in other cells than DCs in tumor-bearing mice.

In most of the mouse tumor-implant models, ARNAX/TAA combination therapy results in tumor shrinkage and prolonged survival [31,37,39]. However, in other models (e.g., Lewis Lung Carcinoma [LLC]-ovalbumin [OVA]), tumor remission rarely occurs when the polyI:C/TAA therapy is employed [51,53]. While these are PD-L1-resistant tumors, the resistance to antigen/adjuvant vaccine therapy is not explained. Another cause may be factors associated with the tumor microenvironment and cells/macrophages infiltrating into the tumor, which make the environment immune-resistant [52,53,55]. In general, CTL-dependent and -independent tumor regression coincides with tumor cell proliferation; the background of tumor regression depends on their balance, as demonstrated by the immunoediting theory [56]. Some adjuvants may act as modifiers of the tumor microenvironment to improve intratumoral immune cell function [54,57].

We identified a TLR3 agonist that functions in both immune cells and the microenvironment in tumors (Fig. 3). DCs and macrophages present in tumors express high levels of TLR3 in tumor sites [54,57]. Tumor shrinkage appears to be associated with an improved tumor microenvironment following ARNAX treatment [39]. In addition, LLC-OVA becomes sensitive to radiation after treatment with TLR3 agonists [49]. The microenvironmental modulation of immune-resistant tumors by treatment with a TLR3 agonist appears crucial for prognosis, but the exact mechanism remains poorly understood.

In contrast to ARNAX, polyI:C activates TLR3 and MDA5 and works on both tumor and immune cells [55,57]; polyI:C makes the environmental situation more complicated than ARNAX. Additionally, PolyI:C may affect both DC-priming and the inflammatory status of the macrophages that have infiltrated into tumors, which can both negatively and positively affect the tumors [54,58]. The possible events that can occur in response to dsRNA in macrophages and tumor cells are depicted in Fig. 3. The tumor factors that are released are presumed to be damage-associated molecular patterns (DAMPs) and exosomes [58,59] in addition to TAAs. The diverse functions of DAMP/exosomes are associated with the reconstruction of the tumor microenvironment [60], as well as TAA-specific CTL induction and tumor regression. In addition, it has been shown that tumor macrophages elevate the level of TLR3 present in the endosomes [54,61]. Further evidence is required to prove whether ARNAX is superior to other inflammatory adjuvants in making the tumor microenvironment immune-sensitive. The answer lies, in part, in the macrophage response to ARNAX in the tumor

microenvironment. This last issue should be experimentally addressed in mouse tumor models and future clinical tests.

7. Conclusion

Multiple adjuvants have been developed for effective vaccination. These adjuvants enhance immune activation but always induce inflammation. We have shown that inflammation occurs independent of immune-enhancement following ARNAX vaccination. This TLR3-specific adjuvant predicts that the infection-DC maturation axis is independent of the in vivo infection-cytokinemia axis when based on the function of ARNAX. In mouse tumor-implant models, ARNAX targets both host and tumor cells, thereby enhancing the anti-tumor response. Most inflammatory adjuvants also act on tumors, and tend to trigger tumor progression and invasion. Thus, the selection of non-inflammatory adjuvants may be critical in the search to improve the tumor microenvironment.

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References

[1] R. Medzhitov, P. Preston-Hurlburt, C.A. Janeway Jr., A human homologue of the Drosophila Toll protein signals activation of adaptive immunity, Nature 388 (1997) 394–397.
[2] K. Honda, H. Yanai, A. Takaoka, T. Taniguchi, Regulation of the type I IFN induction: a current view, Int. Immunol. 17 (2005) 1367–1378.
[3] T. Seya, M. Matsumoto, T. Ebihara, H. Oshiumi, Functional evolution of the TICAM-1 pathway for extrinsic RNA sensing, Immunol. Rev. 227 (2009) 44–53.
[4] H. Oshiumi, T. Tsujita, K. Shida, M. Matsumoto, K. Ikeo, T. Seya, Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, Fugu rubripes, genome, Immunogenetics. 54 (2003) 791–800.
[5] M. Matsumoto, T. Seya, TLR3: interferon induction by double-stranded RNA including poly(I:C), Adv. Drug Deliv. Rev. 60 (2008) 805–812.
[6] V.A. Rathinam, K.A. Fitzgerald, Cytosolic surveillance and antiviral immunity, Curr. Opin. Virol. 1 (2011) 455–462.
[7] C. Dresch, Y. Leverrier, J. Marvel, K. Shortman, Development of antigen cross-presentation capacity in dendritic cells, Trends Immunol. 33 (2012) 381–388.
[8] M. Shingai, N. Inoue, T. Okuno, M. Okabe, T. Akazawa, Y. Miyamoto, M. Ayata, K. Honda, M. Kurita-Taniguchi, M. Matsumoto, H. Ogura, T. Taniguchi, T. Seya, Wild-type measles virus infection in human CD46/CD150-transgenic mice: CD11c-positive dendritic cells establish systemic viral infection, J. Immunol. 175 (2005) 3252–3261.
[9] H. Takaki, H. Oshiumi, M. Shingai, M. Matsumoto, T. Seya, Development of mouse models for analysis of human virus infections, Microbiol. Immunol. 61 (2017) 107–113.
[10] H. Oshiumi, M. Matsumoto, K. Funami, T. Akazawa, T. Seya, TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction, Nat. Immunol. 4 (2003) 161–167.
[11] A. Pantel, A. Teixeira, E. Haddad, E.G. Wood, R.M. Steinman, M.P. Longhi, Direct type I IFN but not MDA5/TLR3 activation of dendritic cells is required for maturation and metabolic shift to glycolysis after poly IC stimulation, PLoS Biol. 12 (2014), e1001759.
[12] T. Taniguchi, A. Takaoka, The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors, Curr. Opin. Immunol. 14 (2002) 111–116.
[13] M. Schlee, G. Hartmann, Discriminating self from non-self in nucleic acid sensing, Nat. Rev. Immunol. 16 (2016) 566–580.
[14] M. Matsumoto, K. Funami, M. Tanabe, H. Oshiumi, M. Shingai, Y. Seto, A. Yamamoto, T. Seya, Subcellular localization of Toll-like receptor 3 in human dendritic cells, J. Immunol. 171 (2003) 3154–3162.
[15] M. Matsumoto, S. Kikkawa, M. Kohase, K. Miyake, T. Seya, Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling, Biochem. Biophys. Res. Commun. 293 (2002) 1364–1369.
[16] S. Okahira, F. Nishikawa, S. Nishikawa, T. Akazawa, T. Seya, M. Matsumoto, Interferon-beta induction through toll-like receptor 3 depends on double-stranded RNA structure, DNA Cell Biol. 24 (2005) 614–623.
[17] N. Murabayashi, M. Kurita-Taniguchi, M. Ayata, M. Matsumoto, H. Ogura, T. Seya, Susceptibility of human dendritic cells (DCs) to measles virus (MV) depends on their activation stages in conjunction with the level of CDw150: role of Toll stimulators in DC maturation and MV amplification, Microbes Infect. 4 (2002) 785–794.
[18] I. Jelinek, J.N. Leonard, G.E. Price, K.N. Brown, A. Meyer-Manlapat, P.K. Goldsmith, Y. Wang, D. Venzon, S.L. Epstein, D.M. Segal, TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection, J. Immunol. 186 (2011) 2422–2429.
[19] M. Tatematsu, F. Nishikawa, T. Seya, M. Matsumoto, Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA, Nat. Commun. 4 (2013) 1833.
[20] K. Funami, M. Matsumoto, H. Oshiumi, T. Akazawa, A. Yamamoto, T. Seya, The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling, Int. Immunol. 16 (2004) 1143–1154.
[21] M. Yoneyama, K. Onomoto, T. Fujita, Cytoplasmic recognition of RNA, Adv. Drug Deliv. Rev. 60 (2008) 841–846.
[22] Z. Zhang, T. Kim, M. Bao, V. Facchinetti, S.Y. Jung, A.A. Ghaffari, J. Qin, G. Cheng, Y.J. Liu, DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells, Immunity. 34 (2011) 866–878.
[23] H. Oshiumi, T. Kouwaki, T. Seya, Accessory factors of cytoplasmic viral RNA sensors required for antiviral innate immune response, Front. Immunol. 7 (2016) 200.
[24] V. Hornung, J. Ellegast, S. Kim, K. Brzózka, A. Jung, H. Kato, H. Poeck, S. Akira, K.K. Conzelmann, M. Schlee, S. Endres, G. Hartmann, 5′-Triphosphate RNA is the ligand for RIG-I, Science. 314 (2006) 994–997.
[25] Q. Feng, S.V. Hato, M.A. Langereis, J. Zoll, R. Virgen-Slane, A. Peisley, S. Hur, B.L. Semler, R.P. van Rij, F.J. van Kuppeveld, MDA5 detects the double-stranded RNA replicative form in picornavirus-infected cells, Cell Rep. 2 (2012) 1187–1196.
[26] M. Matsumoto, H. Oshiumi, T. Seya, Antiviral responses induced by the TLR3 pathway, Rev. Med. Virol. 21 (2011) 67–77.
[27] A. Watanabe, M. Tatematsu, K. Saeki, S. Shibata, H. Shime, A. Yoshimura, C. Obuse, T. Seya, M. Matsumoto, Raftlin is involved in the nucleocapture complex to induce poly(I:C)-mediated TLR3 activation, J. Biol. Chem. 286 (2011) 10702–10711.
[28] K. Itoh, A. Watanabe, K. Funami, T. Seya, M. Matsumoto, The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-β production, J. Immunol. 181 (2008) 5522–5529.
[29] M. Matsumoto, M. Tatematsu, F. Nishikawa, M. Azuma, N. Ishii, A. Morii-Sakai, H. Shime, T. Seya, Defined TLR3-specific adjuvant that induces NK and CTL activation without significant cytokine production in vivo, Nat. Commun. 6 (2015) 6280.
[30] T. Seya, M. Azuma, M. Matsumoto, Targeting TLR3 with no RIG-I/MDA5 activation is effective in immunotherapy for cancer, Expert Opin. Ther. Targets 17 (2013) 533–544.
[31] Y. Takeda, K. Kataoka, J. Yamagishi, S. Ogawa, T. Seya, M. Matsumoto, A TLR3-specific adjuvant relieves innate resistance to PD-L1 blockade without cytokine toxicity in tumor vaccine immunotherapy, Cell Rep. 19 (2017) 1874–1887.
[32] S.L. Jongbloed, A.J. Kassianos, K.J. McDonald, G.J. Clark, X. Ju, C.E. Angel, C.J. Chen, P.R. Dunbar, R.B. Wadley, V. Jeet, A.J. Vulink, D.N. Hart, K.J. Radford, Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens, J. Exp. Med. 207 (2010) 1247–1260.
[33] H. Kato, O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T.S. Dermody, T. Fujita, S. Akira, Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5, J. Exp. Med. 205 (2008) 1601–1610.
[34] H. Kato, O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K.-J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C.S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, S. Akira, Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses, Nature. 441 (2006) 101–105.
[35] M. Azuma, T. Ebihara, H. Oshiumi, M. Matsumoto, T. Seya, Cross-priming for antitumor CTL induced by soluble Ag + polyI:C depends on the TICAM-1 pathway in mouse CD11c(+)/CD8α(+) dendritic cells, Oncoimmunology. 1 (2012) 581–592.
[36] T. Seya, Y. Takeda, K. Takashima, S. Yoshida, M. Azuma, M. Matsumoto, Adjuvant immunotherapy for cancer: both dendritic cell-priming and check-point inhibitor blockade are required for immunotherapy, Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 94 (2018) 153–160.
[37] M. Matsumoto, Y. Takeda, M. Tatematsu, T. Seya, Toll-like receptor 3 signal in dendritic cells benefits Cancer immunotherapy, Front. Immunol. 8 (2017) 1897.
[38] C.J. Desmet, K.-J. Ishii, Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination, Nat. Rev. Immunol. 12 (2012) 479–491.
[39] Y. Takeda, S. Yoshida, K. Takashima, N. Ishii-Mugikura, H. Shime, T. Seya, M. Matsumoto, Vaccine immunotherapy with ARNAX induces tumor-specific memory T cells and durable anti-tumor immunity in mouse models, Cancer Sci. 109 (2018) 2119–2129.
[40] A. Ribas, J.D. Wolchok, Cancer immunotherapy using checkpoint blockade, Science. 359 (2018) 1350–1355.
[41] P.A. Ott, Z. Hu, D.B. Keskin, S.A. Shukla, J. Sun, D.J. Bozym, W. Zhang, A. Luoma, A. Giobbie-Hurder, L. Peter, et al., An immunogenic personal neoantigen vaccine for patients with melanoma, Nature. 547 (2017) 217–221.
[42] F.E. Pearson, K. Chang, Y. Minoda, I.M.L. Rojas, O.L. Haigh, G. Daraj, K.M. Tullett, K.J. Radford, Activation of human CD141(+) and CD1c(+) dendritic cells in vivo with combined TLR3 and TLR7/8 ligation, Immunol. Cell Biol. 96 (2018) 390–400.

[43] T.N. Schumacher, R.D. Schreiber, Neoantigens in cancer immunotherapy, Science. 348 (2015) 69–74.
[44] H. Takaki, S. Kure, H. Oshiumi, Y. Sakoda, T. Suzuki, A. Ainai, H. Hasegawa, M. Matsumoto, T. Seya, Toll-like receptor 3 in nasal CD103(+) dendritic cells is involved in immunoglobulin A production, Mucosal Immunol. 11 (2018) 82–96.
[45] H. Takaki, S. Ichimiya, M. Matsumoto, T. Seya, Mucosal immune response in nasal-associated lymphoid tissue upon intranasal administration by adjuvants, J. Innate. Immun. 10 (2018) 515–521.
[46] Y. Takeda, H. Takaki, A. Fukui-Miyazaki, S. Yoshida, M. Matsumoto, T. Seya, Vaccine adjuvant ARNAX promotes mucosal IgA production in influenza HA vaccination, Biochem. Biophys. Res. Commun. 506 (2018) 1019–1025.
[47] H. Kono, K.L. Rock, How dying cells alert the immune system to danger, Nat. Rev. Immunol. 8 (2008) 279–289.
[48] J.A. Joyce, D.T. Fearon, T cell exclusion, immune privilege, and the tumor microenvironment, Science. 348 (2015) 74–80.
[49] S. Yoshida, H. Shime, Y. Takeda, J.M. Nam, K. Takashima, M. Matsumoto, H. Shirato, M. Kasahara, T. Seya, Toll-like receptor 3 signal augments radiation-induced tumor growth retardation in a murine model, Cancer Sci. 109 (2018) 956–965.
[50] E. Gonzalez-Gugel, M. Saxena, N. Bhardwaj, Modulation of innate immunity in the tumor microenvironment, Cancer Immunol. Immunother. 65 (2016) 1261–1268.
[51] H. Shime, M. Matsumoto, T. Seya, Double-stranded RNA promotes CTL-independent tumor cytolysis mediated by CD11b(+)Ly6G(+) intratumor myeloid cells through the TICAM-1 signaling pathway, Cell Death Differ. 24 (2017) 385–396.
[52] A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation, Nature 454 (2008) 436–444.
[53] R. Takemura, H. Takaki, S. Okada, H. Shime, T. Akazawa, H. Oshiumi, M. Matsumoto, T. Teshima, T. Seya, PolyI:C-induced, TLR3/RIP3-dependent necroptosis backs up immune effector-mediated tumor elimination in vivo, Cancer Immunol. Res. 3 (2015) 902–914.
[54] V. Kumar, S. Patel, E. Tcyganov, D.I. Gabrilovich, The nature of myeloid-derived suppressor cells in the tumor microenvironment, Trends Immunol. 37 (2016) 208–220.
[55] G.P. Dunn, C.M. Koebel, R.D. Schreiber, Interferons, immunity and cancer immunetherapy, Nat. Rev. Immunol. 6 (2006) 836–848.
[56] H. Shime, A. Maruyama, S. Yoshida, Y. Takeda, M. Matsumoto, T. Seya, Toll-like receptor 2 ligand and interferon-γ suppress anti-tumor T cell responses by enhancing the immunosuppressive activity of monocytic myeloid-derived suppressor cells, Oncoimmunology. 7 (2017), e1373231.
[57] H. Shime, M. Matsumoto, H. Oshiumi, S. Tanaka, A. Nakane, Y. Iwakura, H. Tahara, N. Inoue, T. Seya, Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 2066–2071.
[58] S.K. Gupta, P.K. Yadav, A.K. Tiwari, R.K. Gandham, A.P. Sahoo, Poly (I:C) enhances the anti-tumor activity of canine parvovirus NS1 protein by inducing a potent anti-tumor immune response, Tumour Biol. 37 (2016) 12089–12102.
[59] A. Patidar, S. Selvaraj, A. Sarode, P. Chauhan, D. Chattopadhyay, B. Saha, DAMP-TLR-cytokine axis dictates the fate of tumor, Cytokine. 104 (2018) 114–123.
[60] T. Seya, H. Shime, M. Matsumoto, Functional alteration of tumor-infiltrating myeloid cells in RNA adjuvant therapy, Anticancer Res. 35 (2015) 4385–4392.
[61] J.M. Pitt, M. Vetizou, R. Daillere, M.P. Roberti, T. Yamazaki, B. Routy, P. Lepage, I.G. Bonece, M. Chamaillard, G. Kroemer, L. Zitvogel, Resistance mechanisms to immune-checkpoint blockage in cancer: tumor-intrinsic and –extrinsic factors, Immunity. 44 (2016) 1255–1269.