Toll-Like Receptor 3 Signal in Dendritic Cells Benefits Cancer Immunotherapy

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Pattern recognition receptors (PRRs) play a crucial role in the innate immune system and contribute to host defense against microbial infection. PRR-mediated antimicrobial signals provide robust type-I IFN/cytokine production and trigger inflammation, thereby affecting tumor progression and autoimmune diseases. Accumulating evidence demonstrates that among the PRRs, only the signaling pathway of endosomal toll-like receptor 3 (TLR3) induces no systemic inflammation and mediates cross-priming of antigen-specific CD8$^+$ T cells by dendritic cells. Treatment with a newly developed TLR3-specific ligand, ARNAX, along with tumor-associated antigens (TAAs), induces tumor-specific cytotoxic T lymphocytes, modulates the tumor microenvironment to establish Th1-type antitumor immunity, and leads to tumor regression without inflammation in mouse tumor models. Combination therapy using ARNAX/TAA and PD-1/PD-L1 blockade potently enhances antitumor response and overcomes anti-PD-1/PD-L1 resistance. In this review, we will discuss the TLR3-mediated signaling in antitumor immunity and its application to cancer immunotherapy.

Keywords: adjuvant, cancer immunotherapy, checkpoint inhibitors, cross-priming, dendritic cells, double-stranded RNA, innate immunity, toll-like receptor 3

INTRODUCTION

The innate immune system senses pathogen- and host-derived nucleic acids to maintain host homeostasis (1). Nucleic-acid-sensing innate immune receptors can be classified into two groups: (1) direct antiviral receptors that induce robust type-I IFN/cytokine production (2–4) and (2) dendritic cell (DC)-priming receptors that induce adaptive immunity (5, 6). The former consists of endosomal toll-like receptor (TLR) 7, 8, and 9, cytoplasmic RNA sensors retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated protein 5 (MDA5) and DNA sensors cGAS and STING, whose activation is closely associated with systemic IFN/cytokinemia and autoimmune diseases resulting from the recognition of endogenous RNA/DNA (7–9). The resultant IFN/cytokines induce inflammation and trans-activate antigen (Ag)-presenting DCs. On the other hand, TLR3 belongs to the latter group due to its restricted expression in myeloid DCs and usage of the signaling adaptor protein toll-IL-1 receptor-containing adaptor molecule-1 (TICAM-1) (also named TRIF) (10). The TLR3–TICAM-1 pathway predominantly works in professional Ag-presenting DCs to cross-prime CD8$^+$ T cells as well as to induce production of Th1-type cytokines/chemokines (11, 12). However, polyinosinic:polycytidylic acid [poly(I:C)] that activates both TLR3 and MDA5 has been used as a TLR3 ligand. Studies with a recently developed TLR3-specific agonist, ARNAX, demonstrate that TLR3–TICAM-1 signaling primarily induces DC-priming without systemic cytokine production (13–15). The results suggest that TLR3-specific signal is non-inflammatory and RNA-driven inflammation is rooted in the systemic cytoplasmic pathway (**Table 1**). Hence, in the context of

DC-priming, targeting endosomal TLR3 is a promising strategy for induction of antitumor immunity.

Toll-like receptor 3 is expressed on endosomal membranes in myeloid DCs, as well as on both cell and endosomal membranes in macrophages, fibroblasts, and some kinds of epithelial cells (16). Professional Ag-presenting DCs, including mouse CD8α+ and CD103+ DCs as well as human CD141+ DCs, highly express TLR3 (17, 18). TLR3 recognizes virus-derived double-stranded RNA (dsRNA) and virus- or host-derived single-stranded RNA having incomplete stem structures (19). Upon ligand recognition within endosomes, TLR3 oligomerizes and recruits the adaptor molecule TICAM-1, which activates the transcription factors NF-κB, IRF3, and AP-1, leading to the production of cytokines (IL-6, TNF-α, IL-12) and type-I IFN (especially IFN-β) (20, 21). TBK-1 is critical for IRF3 activation in the TICAM-1 signalosome, which resides in perinuclear regions as speckles following the dissociation from endosomal TLR3 (22). TICAM-1 is expressed in various cells and tissues at low levels, and TICAM-1 activation is tightly regulated by the conformational context of protein–protein associations (23). Spatiotemporal regulation of the TLR3–TICAM-1 pathway might be important for triggering non-inflammation and Th1-type adaptive immune responses.

ARNAX SIGNALING PATHWAY

ARNAX is a synthetic DNA–dsRNA hybrid molecule consisting of 140 bp of measles virus vaccine strain-derived dsRNA with a 5′ GpC-type phosphorothioated oligodeoxynucleotides (ODNs) cap (**Figure 1A**) (13, 24). DNA–RNA conjunction sites and dsRNA regions are relatively resistant to nucleases (25) and measles virus-derived dsRNA fails to induce RNA interference against host cell-derived RNAs, suggesting a stable and safer structure. The GpC ODN cap guides dsRNA to TLR3-positive cells for endocytosis, where the dsRNA activates TLR3 (26). dsRNA with a length of >90 bp is required for sufficient activation and signal transduction of the TLR3–TICAM-1 pathway (18). These ARNAX structural motifs do not stimulate cytoplasmic RNA sensors, RIG-I and MDA5, or DNA sensors: they are ubiquitously expressed all over the body.

The viral dsRNA analogue poly(I:C)—a well-known ligand for TLR3—additionally activates the cytoplasmic dsRNA sensor MDA5 (27, 28), resulting in systemic and robust production of type-I IFNs/cytokines that causes undesirable inflammation. In contrast to poly(I:C), ARNAX induces marginal inflammatory cytokine/IFN-β production in a TLR3–TICAM-1-dependent fashion, demonstrating that the TLR3–TICAM-1 pathway contributes to minimal and local cytokine release to effectively prime DCs. Furthermore, the Th1-type cytokine IL-12 is substantially induced by ARNAX, as per poly(I:C), in a TLR3-dependent manner upon subcutaneous injection in mice (29). Non-inflammatory features of ARNAX and its Th1-skewing profile can be attributed to the restricted expression profile and early endosome localization of TLR3 as well as the TICAM-1 signalosome composition.

ARNAX activates professional Ag-presenting DCs to cross-prime CD8+ T cells. The TLR3–TICAM-1–IRF3–IFN-β signaling axis in DCs is indispensable for ARNAX-induced Ag-specific CD8+ T-cell priming (**Figure 1A**) (15). MAVS (signaling adaptor of RIG-I-like receptors), MyD88 (adaptor of all TLRs except for TLR3), and STING (adaptor of DNA sensors) do not contribute to ARNAX-induced cross-priming of CD8+ T cells (30). Importantly, DC-mediated local, but not systemic, IFN-β production is sufficient for CD8+ T-cell cross-priming (15, 31, 32), although the molecular determinants that regulate cross-priming downstream of the IFN-α/β receptor remains undefined. Thus, ARNAX is a non-inflammatory DC-priming adjuvant that specifically targets the TLR3–TICAM-1 pathway.

TABLE 1 | Nucleic-acid-sensing innate immune receptors.

| Receptor	Ligand	Signaling adaptor	Localization	Cell	Function	Reference
TLR3	Viral dsRNA, virus/host structured ssRNA, Poly(I:C), ARNAX	TICAM-1 (localization: cytoplasm)	Endosome	Myeloid DC, macrophage, fibroblast, epithelial cell	Antiviral, NK activation, CTL induction, IgA production	(5, 6, 10, 11, 13)
TLR7	Virus/host ssRNA, imidazoquinoline compound	MyD88 (localization: cytoplasm)	Endosome	Plasmacytoid DC, B cell	Antiviral (type-I IFN), Ab production	(2, 7)
TLR8	Virus/host ssRNA, imidazoquinoline compound	MyD88 (localization: cytoplasm)	Endosome	Myeloid DC, monocyte, neutrophil	Antiviral, inflammatory cytokine production	(2, 7)
TLR9	CpG DNA, chromatin/DNA complex	MyD88 (localization: cytoplasm)	Endosome	Plasmacytoid DC, B cell	Antiviral (type-I IFN), Ab production	(2, 7)
RIG-I	Viral 5′ppp-dsRNA	MAVS (localization: mitochondrion)	Cytoplasm	Ubiquitous	Antiviral (type-I IFN) inflammatory cytokine production	(2, 3, 8)
MDA5	Viral long dsRNA, Poly(I:C)	MAVS (localization: mitochondrion)	Cytoplasm	Ubiquitous	Antiviral (type-I IFN), inflammatory cytokine production, NK activation	(2, 3, 8)
cGAS	dsDNA	STING (localization: endoplasmic reticulum)	Cytoplasm	Ubiquitous	Antiviral (type-I IFN), inflammatory cytokine production, CTL induction	(4, 9)
STING	Cyclic dinucleotide		Endoplasmic reticulum	Ubiquitous	Antiviral (type-I IFN), inflammatory cytokine production, CTL induction	(4, 9)
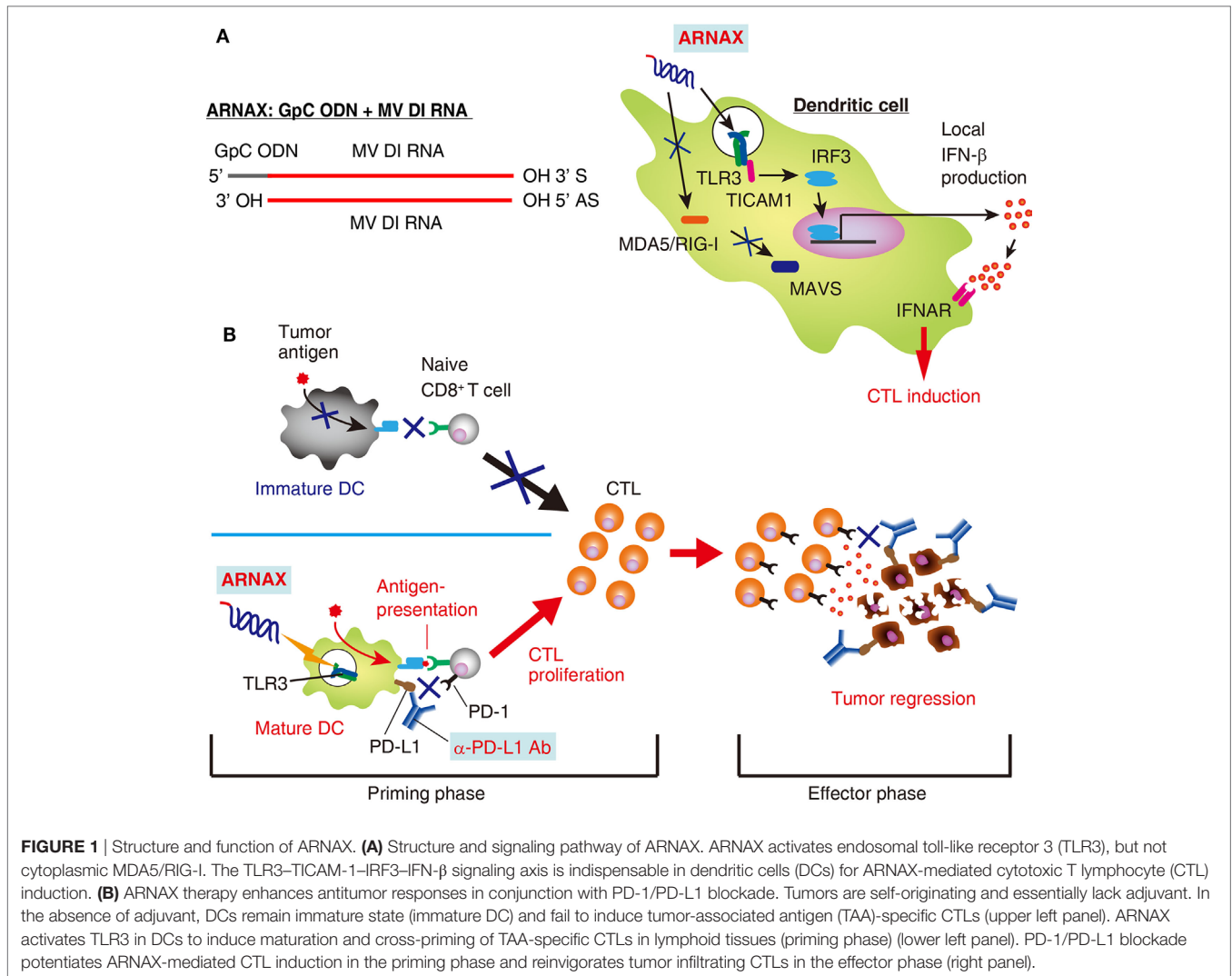
FIGURE 1 | Structure and function of ARNAX. **(A)** Structure and signaling pathway of ARNAX. ARNAX activates endosomal toll-like receptor 3 (TLR3), but not cytoplasmic MDA5/RIG-I. The TLR3–TICAM-1–IRF3–IFN-β signaling axis is indispensable in dendritic cells (DCs) for ARNAX-mediated cytotoxic T lymphocyte (CTL) induction. **(B)** ARNAX therapy enhances antitumor responses in conjunction with PD-1/PD-L1 blockade. Tumors are self-originating and essentially lack adjuvant. In the absence of adjuvant, DCs remain immature state (immature DC) and fail to induce tumor-associated antigen (TAA)-specific CTLs (upper left panel). ARNAX activates TLR3 in DCs to induce maturation and cross-priming of TAA-specific CTLs in lymphoid tissues (priming phase) (lower left panel). PD-1/PD-L1 blockade potentiates ARNAX-mediated CTL induction in the priming phase and reinvigorates tumor infiltrating CTLs in the effector phase (right panel).

INDUCTION OF ANTITUMOR IMMUNITY

The presence of functional tumor-specific cytotoxic T lymphocytes (CTLs) in the tumor microenvironment is mandatory for tumor regression (33). ARNAX can induce tumor-specific CTLs by activating Ag-presenting DCs. In mouse implant tumor models EG7 and MO5 (OVA expressing T lymphoma and melanoma, respectively), injection of ARNAX along with OVA regresses tumor growth (15). OVA-specific CD8+ T cells proliferate in both lymphoid tissues and within tumors. Furthermore, CD8α+ DCs and CD8+ T cells increasingly infiltrate into tumor. Numerous genes associated with antitumor immunity are significantly upregulated in whole EG7 tumors during the ARNAX + OVA therapy (15). Chemokine genes responsible for recruiting DCs and T cells (*Ccl4*, *Ccl5*, *Ccl27*) (34), NK/T-cell function-related genes (*Gzmb*, *Prf1*, *Fasl*), cell adhesion-related genes, and cytokine receptor genes such as *IL2rb* and *IL12rb1* are also upregulated. ARNAX and TAA therapy thus fosters Th1-type antitumor immunity in these tumor models. Hence, vaccine immunotherapy with TLR3 adjuvant enables to establish antitumor immunity against certain tumor types.

Notably, ARNAX monotherapy induces tumor growth retardation (15). It is likely that DCs internalize tumor cell debris, which contains TAAs, and cross-prime CD8+ T cells *via* TLR3-mediated activation of DCs (35). Alternatively, TLR3 signaling may facilitate the infiltration of preexisting tumor-reactive CTLs into tumor sites by inducing chemokine production. Mouse CD8α+ DCs and human CD141+ DCs express the C-type lectin-like receptor CLEC9A on their cell surface, which is involved in phagocytosis of dead cell debris that contains TAAs (36, 37). The quality of TAAs and their efficient delivery to DCs are important factors influencing the validation of vaccine immunotherapy with TLR3 adjuvant.

MODULATION OF THE TUMOR MICROENVIRONMENT

The tumor microenvironment strongly affects tumor progression and antitumor immunity (38–40). Tumor-associated macrophages (TAMs), granulocytic or monocytic myeloid-derived suppressor cells (G- or M-MDSCs), and regulatory T cells are

major constituents of the immunosuppressive tumor microenvironment (41, 42). The extent and composition of immune cell infiltration within tumors considerably differ among tumor types (42, 43). Accumulating evidence suggests that modulation of the tumor microenvironment from immunosuppressive to immunosupportive is a crucial factor for the success of cancer immunotherapy (44–46). It has emerged from several studies that TLR activation changes the properties of the tumor microenvironment (47–49). Among the TLR ligands, TLR2 ligand enhances the survival of M-MDSCs and their differentiation into macrophages, which augments the immunosuppressive activity of M-MDSCs toward CD8+ T cells through iNOS expression from macrophages (49). In contrast, TLR3 ligand converts TAMs from an M2- to an M1-like phenotype (47). When activated with poly(I:C), TAMs robustly produced TNF-α in 3LL (mouse lung carcinoma cell line) tumor in mice, resulting in tumor cell death and growth suppression. The TLR3–TICAM-1 pathway is critical for poly(I:C)-induced tumor regression via stromal macrophages in the 3LL tumor mouse model. In addition, G-MDSCs act as effector but not suppressor cells upon activation with TLR3 ligand in the EL4 tumor model (48). G-MDSCs produce reactive oxygen species through the TLR3–TICAM-1 pathway, leading to tumor growth inhibition (48). Furthermore, the proportion of G-MDSCs in EG7 tumors is greatly decreased by ARNAX + TAA therapy (15), which makes CTL unexhausted. Thus, TLR3 signaling functionally ameliorates the tumor microenvironment to potentiate antitumor immunity.

COMBINATION THERAPY OF ARNAX WITH ANTI-PD-1/PD-L1 ANTIBODIES

Recent advances in cancer immunotherapy with checkpoint inhibitors have shown durable antitumor responses and good prognoses in patients with melanoma and non-small cell lung cancer, but only ~20% of patients with solid tumors respond to checkpoint blockade (50–54). The presence of preexisting tumor-specific CD8+ T cells and their infiltration into tumor sites are required for responsiveness to PD-1/PD-L1 blockade therapy (51). Many cancer patients, however, have limited numbers of tumor-specific CTLs, if at all, as well as/or the presence of therapy-resistant tumor microenvironments (55). The appearance and amount of mutation-associated neo-antigens in tumors correlates with sensitivity to PD-1 blockade (56–58), which is likely associated with preexisting tumor-specific CTLs (59). However, molecular determinants of tumor cells that define CTL induction by the immune system are still unclear. In this setting, vaccine immunotherapy that potently induces tumor-specific CD8+ T cells through DC-priming is a feasible approach to overcome primary resistance to PD-1/PD-L1 blockade.

In anti-PD-L1 antibody unresponsive mouse tumors, vaccine immunotherapy using ARNAX and tumor Ag decreases tumor progression irrespective of PD-L1 levels on tumor cells (15). Combination therapy with anti-PD-L1 antibody and ARNAX + TAA induces an antitumor response more effectively than anti-PD-L1 antibody monotherapy, especially in tumors

expressing high levels of PD-L1. Priming of tumor-specific CD8+ T cells in lymphoid tissues and the infiltration of CD8+ T cells into the tumor site are greatly enhanced by combination therapies. DCs and macrophages in lymphoid tissues express PD-L1 at low or intermediate levels (60, 61). It is conceivable that blockade of the PD-1/PD-L1 pathway augments TAA-specific CD8+ T-cell induction from ARNAX + TAA therapy at the priming phase, and infiltrated CTLs are reinvigorated at the effector phase (62, 63) (**Figure 1B**). Tumor-associated DCs and CD11b+ immunosuppressive myeloid cells express high levels of PD-L1 within tumors (60), which is independent of efficacy of ARNAX + TAA therapy in several mouse tumor models. Although PD-L1 levels on tumor cells and infiltrated myeloid cells are one of the predictive biomarkers for responsiveness to PD-1/PD-L1 blockade (64, 65), tumor PD-L1 level is not always applicable for a prognostic biomarker to the ARNAX therapy. Potent induction of tumor-specific CD8+ T cells with DC-priming adjuvant and PD-1/PD-L1 blockade is important for infiltration and reinvigoration of CD8+ T cells within tumors.

CONCLUSION

To overcome the unresponsiveness of tumors to anti-PD-1/PD-L1 therapy, many cancer immunotherapy approaches have been conducted. Vaccine immunotherapy with TAA and the DC-priming adjuvant ARNAX generates tumor-specific CTLs with minimal essential cytokine production, which appears to avoid exacerbating adverse effects observed in certain population of patients treated with checkpoint inhibitors, such as the onset of autoimmune diseases. Up until now, numerous trials of peptide vaccine therapies have been performed unsuccessfully (66). The main factor contributing to the ineffectiveness is thought to be the usage of inflammatory, as opposed to DC-priming, adjuvants in peptide vaccine therapies. On the other hand, several preclinical studies with vaccine immunotherapies using poly(I:C) as a DC-priming adjuvant have been approved (67–70). However, poly(I:C) triggers undesirable inflammation caused by cytokine toxicity (67). The introduction of the non-inflammatory adjuvant ARNAX to peptide vaccine immunotherapy and/or combination therapy with PD-1 blockade appears to be a promising strategy to overcome anti-PD-1 resistance. Notably, induction of tumor cell death by radiation, chemotherapy, and oncolytic viruses appear to liberate TAAs from tumors (71–74), which may cooperate with ARNAX to induce polyclonal tumor-reactive CTLs and facilitate suppression of tumor growth.

In cancer immunotherapy, acquired resistance to newly developed therapies is a subject of intense discussion. In the case of TLR3 adjuvant therapy, the acquisition of resistance to CTL activity by tumor cells should be investigated. Evaluation of the quality and quantity of TAAs in individual tumors could be a therapeutic prerequisite for TLR3 adjuvant therapy, but this prediction has not been confirmed. Further studies elucidating the mechanism of tumor evasion from CTL cytotoxicity and the development of appropriate protocols for TLR3 adjuvant therapy

would prove useful in this field to allow for complete tumor regression in cancer patients.

AUTHOR CONTRIBUTIONS

MM, YT, MT, and TS conceived and designed the experiments. YT and MT performed the experiments. MM and TS wrote the paper.

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