ANTICANCER RESEARCH *35*: 4385-4392 (2015)

Review

Functional Alteration of Tumor-infiltrating Myeloid Cells in RNA Adjuvant Therapy

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Abstract. *Macrophages, as well as dendritic cells (DCs), are derived from myeloid progenitor cells. Recent evidence suggests that tumor-infiltrating macrophages differ in many aspects from conventional tissue macrophages, including nature, function and markers. Tumors usually contain various myeloid lineage cells in their non-parenchymal environment. In immunotherapy for cancer, tumor cells and non-parenchymal cells are exposed to tumor-associated antigens (TAA) and tumor-cell-derived nucleic acids. In addition, a dsRNA mimic, polyinosinic:polycytidylic acid (polyI:C), exhibits strong adjuvant activity, which acts both on the immune system and tumor constituents. Herein we discuss the RNA recognition system and unique cellular output in tumor-associated myeloid cells in response to immunotherapy. We especially focus on the mechanism by which RNA adjuvant alters the tumor-supportive nature of tumor-infiltrated myeloid cells to those with tumoricidal activity. We discuss how RNA administration makes tumor cells collapse and its significance of evoking cell death signals in tumor cells and macrophages. This knowledge will be applicable to the development of an alternative immunotherapy for cancer.*

Life has developed a system that performs the translation from the messenger RNA (mRNA) to proteins to realize genetic information. mRNA is spliced, edited and matured out of the nucleus to be translated into a protein in the ribosome, the process of which is essentially conserved across yeast and humans. Expression of the basic activity of translation is supported by RNA (ribosomal (rRNA), transfer (tRNA)) and enzymatically complemented by proteins in the ribosome (Figure 1). In addition, there exist a number of RNA activities in cells (Figure 1): anti-sense RNA activity, ribozyme activity remaining in the ribosome, microRNA (miRNA), RNA interference (RNAi) qualified by small-interfering RNA (siRNA), transcriptional regulation and a large amount of non-coding RNA in the nucleus and circular RNA in cytoplasm whose functions remain largely unknown. These RNAs function in both normal and tumor cells, and closely participate in biological phenomena observed in tumorigenesis.

Macrophages and dendritic cells (DCs) are major players of innate immunity. Innate immunity basically regards the microorganisms as non-self to be eliminated. We refer microbe-specific molecules to as pattern molecules by which humans conduct pattern recognition in the innate immune system (1). The pattern recognition function is carried with pattern recognition receptors (PRR) incidental to such macrophage systems. Microbial RNA is an important pattern molecule that can be recognized by PRRs as non-self. The final response of pattern recognition in human macrophages and DCs is characterized by cytokine/interferon induction and immune-cell activation. Humans can overcome most infections because of the natural presence of the innate immune response against all microbes (1).

Pattern recognition is practically enforced by PRRs in and outside the cytoplasm. Myeloid cells, characteristically, possess a sophisticated extra-cytoplasmic recognition system for microbial RNA (Figure 2). Since this short review cannot cover all types of pattern recognition, we summarize recent findings on the tumor microenvironment and systemic immune response to RNA identified in myeloid cells.

Immune Responses Induced by Extra-cellular RNA

Self RNAs in nucleus and cytoplasm do not start the immune cell response. On the other hand, it has been reported that

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Key Words: Tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), dendritic cells (DCs), Poly(I:C), Toll-like receptor 3, TICAM-1, antitumor immunotherapy, review.

nuclear reprogramming occurs in fibroblasts in response to RNA, which is exogenously added to the cells (2, 3). Extracellular RNA modulates the nuclear transcriptional function through a receptor that recognizes RNA (4). Activation of the transposon can, also, occur with RNA. These findings suggest that RNA serves as an inter-cellular mediator distinct from the known cell-reserving functions (Figure 1). This response against exogenous RNA is mainly triggered by an endosomal membrane-associated RNA sensor, Toll-like receptor 3 (TLR3), rather than cytoplasmic sensors. TLR3 is highly expressed in macrophages and some of the DC subsets (4). The discovery in our laboratory about the TLR3 signal using human TLR3 antibody facilitated the elucidation of multifarious TLR3 functions (5, 6).

TLR3 conveys a signal by the adapter molecule named Toll-IL-1R homology domain-containing adaptor molecule 1, (TICAM-1) (7). The N-terminus of toll-like receptor adaptor TICAM-1 is connected to tumor necrosis factor receptor-associated factor (TRAF) proteins and nucleosome assembly protein 1 (NAP1) to recruit an interferon regulatory factor (IRF)3-activating tank-binding kinase 1 (TBK1) (8), whereas the C-terminus of TICAM-1 binds the second adapter receptor-interacting protein 1 (RIP1) to pass the signal for activation of the RIP3 pathway (9) (Figure 2). These pathways are functionally expressed in a cell type-specific fashion: NAP1 involves survival signal, while RIP1 exerts a cell death signal in a case-sensitive manner. In the case of myeloid cells, IRF3 is a major transcription factor for type I interferon (IFN) induction. Upon IRF3 activation, DCs capture the exogenous antigens and induce cross-presentation, as well as promote natural killer (NK) cell activation *via* a collateral pathway with up-regulation of IRF3-dependent NK cell activating molecule (INAM) (10, 11). That is, RNA response of myeloid cells is critically responsible for not only acute IFN/cytokine response but also for activation of cellular immunity involving lymphocytes (Figure 2).

The RIP1/RIP3 pathway is part of the RNA response of myeloid cells (12). Myeloid cells activate a transcription factor (NF)-κB by the TICAM-1-RIP1 axis, when they detect the RNA with TLR3, directing to the survival. However, cells fall to programmed cell death when the RIP3 pathway is dominant. If caspase 8 works in macrophages, cells undergo apoptosis, and if caspase 8 does not work (which situation occurs in cells where gene expressions are disrupted secondary to accidents such as virus infection or carcinogenesis), cells go necroptosis (Figure 3). RIP3 activation encourages the fragmentation of mitochondria by dynamin-related protein 1 (DRP1) under the involvement of mixed lineage kinase domain-like (MLKL) and phosphoglycerate mutase family member 5 (PGAM5), as well as reactive oxygen species (ROS) (Figure 3). Inflammation prompted by mitochondria occurs separately from the activation of inflammasome and is involved in initiation of necroptois (13, 14). The RIP3 output occurs fundamentally based on infection and malignant transformation of host cells, according to the case and situation (12). TLR3 is usually up-regulated in response to inflammation stimuli in myeloid cells and keeps a standing-by state for RNA recognition that contributes to the homeostasis of the host cell network (4).

RNA Sensors in Macrophages and Dendritic Cells

RNA recognition involves multiple RNA sensors to elicit different responses to the RNA (15, 16). Generally, RNA viruses penetrated into the cells replicate the plus-strand RNA from the genomic RNA and, then, the positive-strand RNA is translated into protein as mRNA. This means that RNA virus infection, such as hepatitis C virus (HCV), requires a step -not existing in humans- that makes the RNA from RNA. For completing this step, viruses bring unique RNA polymerases to the host cell. Host cells are continuing to be homeostatic (or less likely to be cancerous) without misreading of DNA, which is a result of elaboration by the proofreading mechanism for transcription of DNA, but RNA barely has a mechanism to check the proof because of its disposable nature. Furthermore, RNA polymerase of the virus is always misreading when the RNA exceeds 1,000 bases. It is inevitable that the viral RNA continues mutations in human cells (evolution in the viral side). Innate immunity provides the cytoplasm RNA helicase of retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) to detect viral replication events in the cell (16). Viral mRNA is not capped with m7guanosine nucleotides (m7GTP) because it is not produced by DNA-dependent RNA polymerase (Pol II) of self-cell origin. Viral mRNA is usually liberated as a 5'-triphosphate intermediate. This uncapped mRNA is translated into protein by the Cap-independent initiation factor (eIF) complex. At the same time, RIG-I identifies this 5'-triphosphated RNA as non-self by discrimination of the structural differences of RNA (16). MDA5 identifies a long dsRNA as non-self within the cytoplasm (16). These sensors constitute the RNA recognition system in the cytoplasm, thus opposing to the extra-cytoplasmic RNA recognition by TLR3. Therefore, myeloid cells, that admit the RNA phagocytosis, correspond to the system with different RNA recognition machinery in the cytoplasm and cell-surface (Figure 2). RIG-I/MDA5 can transmit signals by the common adapter mitochondrial antiviral-signaling protein (MAVS) (16). On the other hand, TLR3 captures RNA released from other cells in the myeloid cells (Figure 1). RNA should be a ligand for TLR3 as long as it possesses a stem-structure that resists to RNase (17) or a form of exosomes (18, 19). In any case, TLR3 delivers the signal through the adaptor TICAM-1 (7). TICAM-1 and MAVS

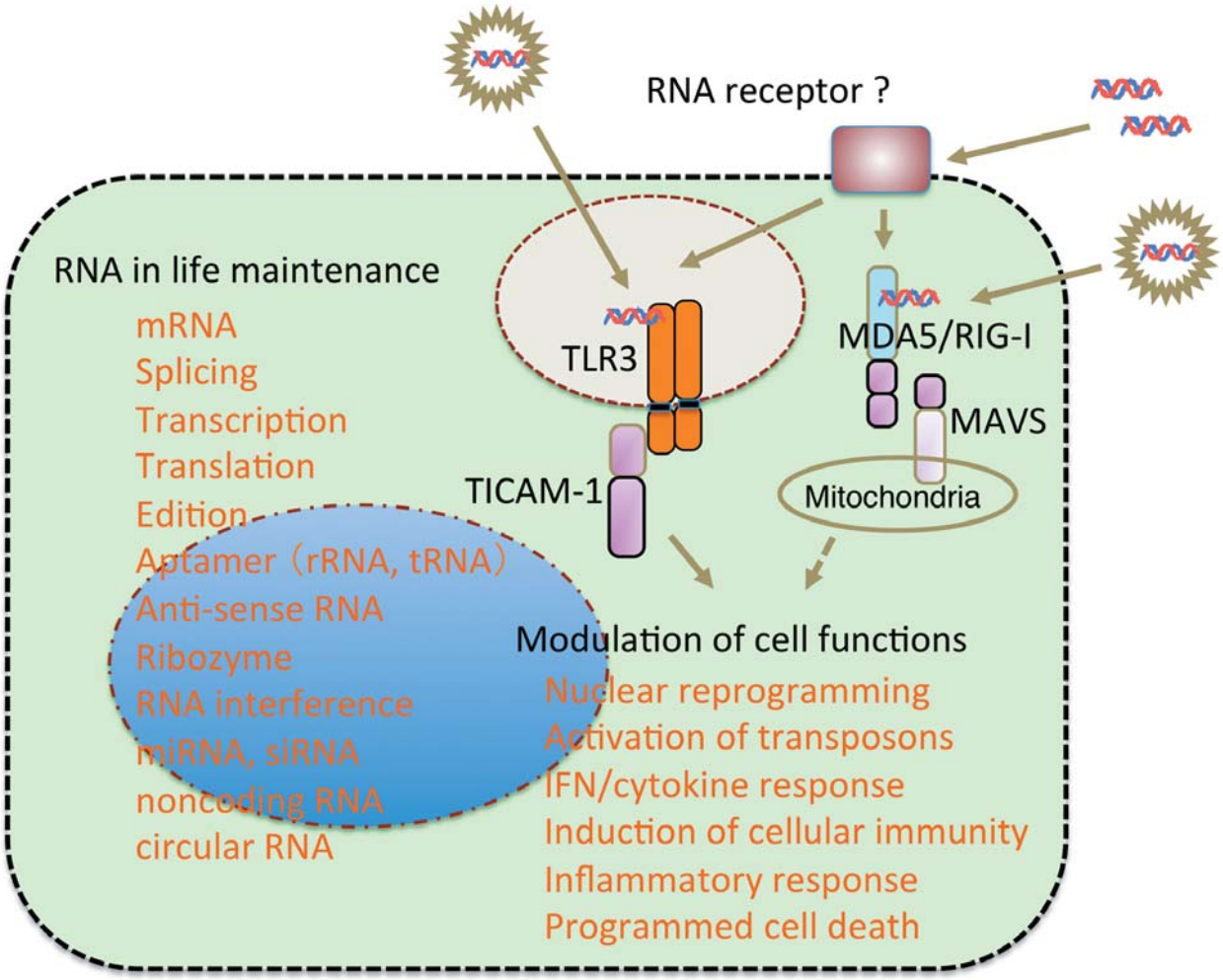

Figure 1. *RNA sensors for modulation of cellular functions. RNA, essentially, plays functions for life support in the nucleus and cytoplasm (left). On the other hand, exogenous RNA taken-up into cells induces signal response capable of inducing epigenetic conversion in myeloid cells (right). The molecular mechanism for capturing RNA in endosomes into the cytoplasm is not yet clear. Out of the RNA sensors described herein, we illustrated the sensors whose function is known. TICAM-1 and MAVS are adapter molecule (see text).*

share downstream signal molecules for IRF3 activation, the IFN-inducing signal; however, each of them has a specific output independent of each other.

Macrophages and DCs are "activated" through MAVS or TICAM-1 signal when they are exposed to RNA. Myeloid lineage cells deploy a variety of innate immune responses by RNA recognition, including cell death, release of cytokines and IFN, cell migration by chemokines, opsonization and liberation of damage-associated molecular patterns (DAMP) (20). DCs are referred to the TLR3 response as maturation, which becomes capable of conferring antigen-presenting and NK activation ability. Expression of TLR3 is limited to myeloid, fibroblast and endothelial cells in the stromal environment, while the MAVS pathway is expressed all over the body to serve as a source of mass cytokine/IFN induction (21).

Modulation of Tumor Microenvironment by RNA

Tumor-infiltrating macrophages differ in many aspects, including the origin from the normal organ-resident macrophages. Macrophages basically are derived from hematopoietic stem cells, which are originated from yolk sac according to ontogeny, and move from yolk sac to fetal liver and then the bone marrow (22). Thus, tissue macrophages are derived from the stem cells of the yolk sac, to back up the organ formation. Similarly, progenitor-type DCs in the skin (named Langerhans cells) are derived from the stem cells of fetal liver in the embryo. Macrophages are believed to be essential to the functional completion with the development of organ and tissue organization, as well as network formation of immune surveillance. They

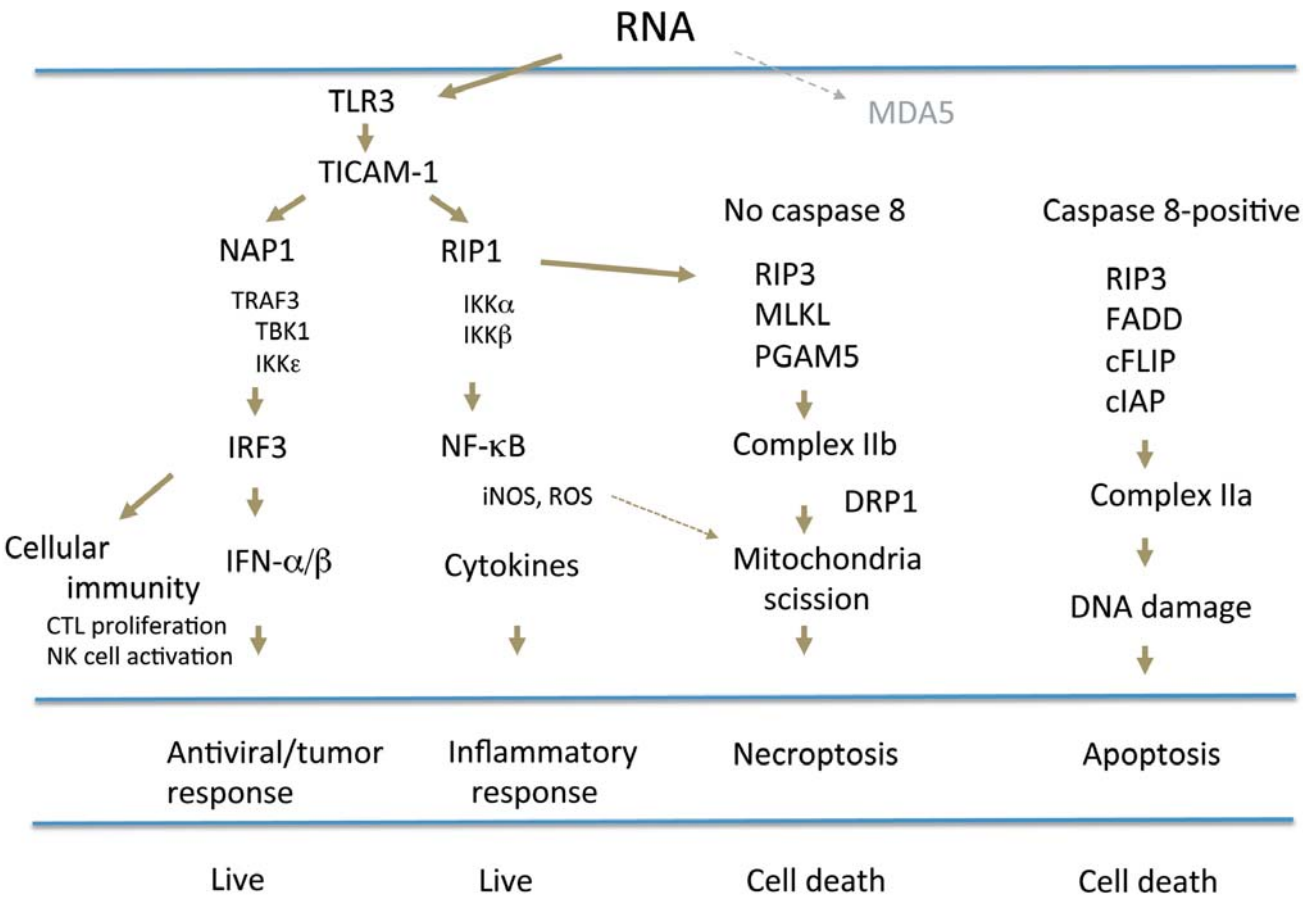

Figure 2. *Innate immune response induced by macrophage TLR3. The figure shows the TLR3 response induced by RNA that was incorporated into intracellular endosomes. Although many responses occur depending on cell types and cellular environment, all macrophage species can be activated by RNA. For survival signals, see literature (8, 12). The cell death signal is summarized in detail in Figure 3. Recently, the RNA/TLR3-mediated cell death signal system has been elucidated.*

fundamentally support the formulation of the organs they reside in (Figure 4).

In this context, it is natural that macrophages infiltrate the tumor in large quantities and help tumor progression. Tumor is established by mimicking the process of organogenesis, which the macrophages in tumor should support. However, the tumor is generated through epigenetic process after the organ is constituted. Hence, tumor-infiltrating macrophages cannot be originated from yolk sac but are derived from the monocytic lineage of the bone marrow (23). For this reason, tumor-infiltrating myeloid cells harbor a unique pattern recognition system, which is different in the properties from tissue macrophages and DCs and their response to RNA also deviate greatly from normal resident cells. Polyinosinic:polycytidylic acid (PolyI:C) injection into tumor (3LL/LLC lung carcinoma)-bearing mice induces hemorrhagic necrosis in the tumor by stimulation of tumor-associated macrophages (TAM). TAM release large amounts of tumor necrosis factor (TNF)-α, thereby inducing hemorrhagic necrosis of the tumor mass, which they should protect (Figure 4). This surprising tumor damage cannot occur with tissue macrophages. Under this scenario, tumor-infiltrating macrophages can be rebelled from tumor to support tumor destruction by TLR3 response of RNA (24). This is significant as a therapeutic strategy aiming at macrophage-targeting tumor regression by RNA.

Tumor-bearing patients induce an abnormality in the hematopoietic system of the bone marrow: undifferentiated myeloid cell populations, other than monocytes, are pooled, such as in the spleen. Notably, these abnormal myeloid cells are Gr-1-positive and referred to as myeloid-derived suppressor cells (MDSCs), while TAM form a Gr-1 (Ly6G/C)-negative population. MDSCs accumulate in tumor and help tumor growth by suppressing the immune system (Figure 4). MDSCs

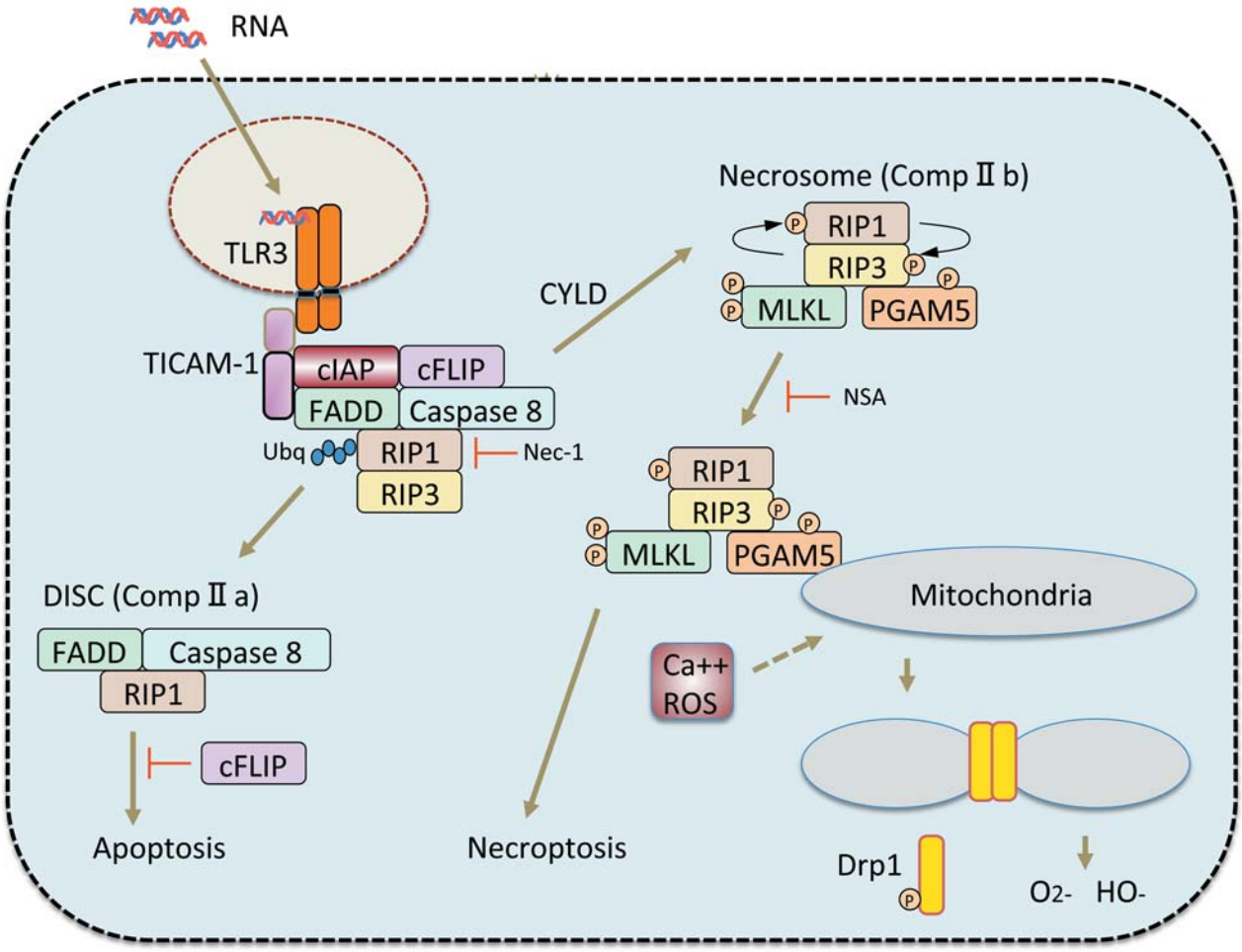

Figure 3. *Cell death pathway in macrophages. When RNA having an RNase-resistant stem structure is incorporated into endosomes, it induces a survival signal, which evokes activation of the immune system (elimination of tumors or microorganisms). In macrophages and tumor cells, it is likely that schematic reactions, such as the figure proceeds. Whatever the choice, apoptosis or necroptosis, it is made by the presence or absence of the function of caspase 8. The latter case involves the scission of mitochondria.*

possess TLR2 and TLR3 like other myeloid cells, and well respond to their ligands. It is likely that TAMs, MDSCs and regulatory T-cells (Treg) join the stroma, namely non-parenchymal tissue, in the tumor to form a favorable microenvironment to support malignant tumor growth (Figure 4). The functions of TAMs and MDSCs hold a complexity depending on the phase and species of the tumor; however, the consensus is that they are modulated by innate immune signaling pathways, TLR3/TICAM-1 or MDA5/MAVS (24, 25). TLR3/TICAM-1 signal, basically, convert the tumor microenvironment from malignant-support to benign-support (*i.e.* anti-neoplastic) (Figure 4). This conversion may contribute to re-programming and tissue regeneration. Concerning the output of the TLR3/TICAM-1 signal in cells with malignant transformation, what is the function of re-programming-inducible genes up-regulated by TLR3, constitutes questions for future elucidation. They may contribute to tissue repair in a normal process of regeration, but also facilitate the generation of cancer stem cells in certain cancerous microenvironment. It has also been reported that a few stem cells are detected in some tumor cases while being in dormancy.

RNA Adjuvant Therapy Targeting Tumor-infiltrating Myeloid Cells

Because the targets of RNA adjuvant are dendritic cells and macrophages in our purpose for immune modulation, immunotherapy with RNA has been employed together with tumor antigens to enhance antitumor immune response (26). PolyI:C activates metallomatrix proteases and acts as a strong inducer of inducible nitric oxide synthase (iNOS) in macrophages (27). TLR3 is specifically phosphorylated at

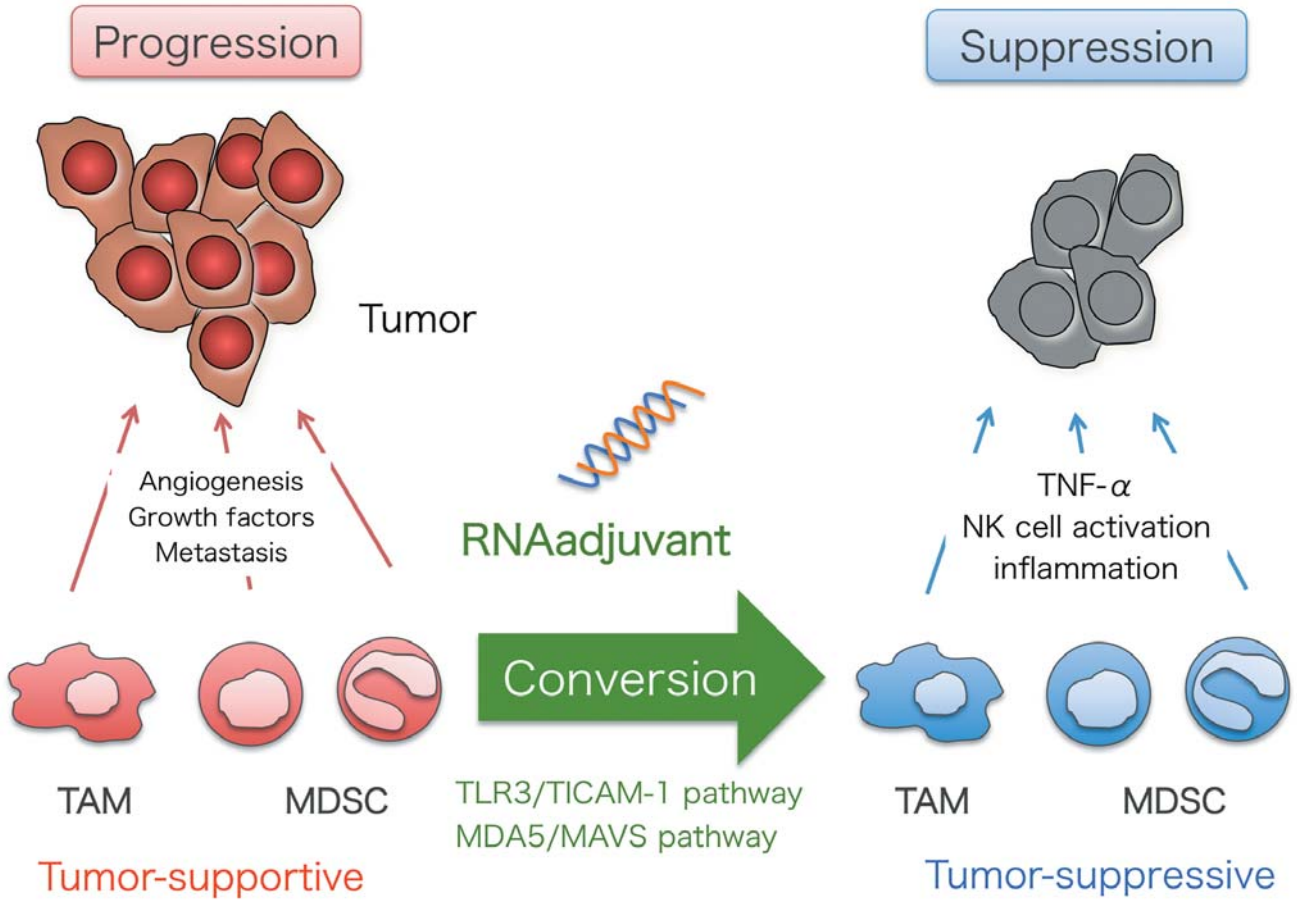

Figure 4. *Modulation of tumor-infiltrating myeloid cells by RNA. Tumor-infiltrating myeloid cells (TAMs, MDSCs) basically support tumor growth, progression and invasion. Tumor-infiltrating myeloid cells contribute to the formulation of tumor-supporting microenvironment by immune and non-immune mechanisms. RNA (in this example, polyI:C) facilitates the conversion of TAM/MDSC's tumor-supporting properties to those with anti-tumor function in the tumor. As a result, tumor regression (i.e., a "tumoricidal" microenvironment) occurs through TLR3 signals.*

tyrosine 759 and, subsequently, triggers signaling pathways to promote interferon-β (IFN-β) production (28). This action is likely to occur in myeloid cells where ROS and arginases are then up-regulated to reconstitute the tumor microenvironment.

The 1960s, immunotherapy with polyI:C (dsRNA analog) started in humans (cancer patients) and tumor regression was reported to have been observed. At that time, almost nothing about substantial function of the immune system, including lymphocytes or cytokines upon caner, was known. This became the beginning of polyI:C cancer therapy as a dream of medicine. Now, we know how the immunological concept is adapted to the knowledge of secular innate immunity. This concept is not limited to the response of the macrophage system but principally applicable to the function of dendritic cells, which activate cellular immunity by RNA (29). However, large amounts of cytokines are released in blood plasma of mice subjected to polyI:C (30). In old clinical data, >20 mg of polyI:C was injected into patients and they had untolerable side-effects, presumably due to cytokinemia (no cytokine was identified at that time).

The cytokine-inducing activity of polyI:C is dependent on the MAVS route through the RIG-I/MDA5 receptors. It was then found that the TLR3/TICAM-1 pathway is mainly participating in the triggering of cellular immunity (30). Furthermore, certain tumor types are directly damaged by polyI:C, like macrophages (31, 32). IL-12p70 induced by polyI:C under the homeostatic Batf3 function in DCs is crucial for tumor suppression (33). These findings suggest that selective activation of the TLR3/TICAM-1 pathway can provide a support for cancer immunotherapy by acting on both the immune cells and tumor cells. The only problem in polyI:C therapy is cytokine toxicity, caused by the MAVS

pathway; however, this problem will be cleared by utilizing a novel RNA adjuvant ARNAX (30). In order to realize the RNA adjuvant therapy, the macrophage response against exogenous administration of RNA adjuvant should be verified more precisely *in vivo*.

Concluding Remarks

Our study on RNA in adjuvant therapy against cancer in mice clarified that: (i) RNase-resistant structures are required for efficient therapeutic potential, (ii) accessibility of the RNA to endosomal TLR3 is critical for expression of the antitumor activity, (iii) tumor-associated myeloid cells respond to RNA with distinct manners from normal resident macrophages, (iv) dendritic cells mature in response to RNA leading to activation of the immune system; the employed pathways of TLR3 for immune activation differ from those in macrophages, (v) tumor shrinkage is an ultimate result from administration of RNA adjuvant, including polyI:C, but the mode and mechanism of tumor regression differ among the tumor species employed. Hence, our approach, application of RNA adjuvant for vaccine immunotherapy to patients with cancer, may be a potential treatment for not only immune potentiation but also induction of antitumor myeloid cells.

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Received April 3, 2015
Revised May 12, 2015
Accepted May 13, 2015