Immunoadjuvant effects of polyadenylic:polyuridylic acids through TLR3 and TLR7

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Abstract

Double-stranded RNA (dsRNA) is produced upon viral infection and can activate innate immunity. Polyinosinic:polycytidylic acids [poly(I:C)] is a synthetic mimic of dsRNA and functions through an endosomal receptor, Toll-like receptor (TLR) 3 or cytosolic receptors. Another type of dsRNA, polyadenylic:polyuridylic acids [poly(A:U)], can also act as an immune adjuvant, but it remains unclear how it exhibits its adjuvant effects. Here, we have characterized the adjuvant effects of poly(A:U). Poly(A:U) could induce both IFN-α and IL-12p40 from murine bone marrow dendritic cells (DCs). Poly(A:U)-induced IFN-α production depended on a DC subset, plasmacytoid dendritic cell (pDC), and required TLR7. IL-12p40 was also produced by poly(A:U)-stimulated pDC in a TLR7-dependent manner. In addition to pDC, conventional dendritic cell (cDC) also produced IL-12p40 in response to poly(A:U). This IL-12p40 induction resulted from two cDC subsets, CD24high cDC and CD11bhigh cDC in a TLR3- and TLR7-dependent manner, respectively. In vivo injection of poly(A:U) with antigen led to clonal expansion of and IFN-γ production from antigen-specific CD8⁺ T cells. Consistent with the in vitro findings, TLR3 and TLR7 were required for the clonal T-cell expansion. Notably, TLR3, rather than TLR7, was critical for generating IFN-γ-producing CD8⁺ T cells. CD8⁺ T-cell responses induced by poly(A:U) were independent of type I IFN signaling. Our results demonstrate that poly(A:U) functions as an in vivo immunoadjuvant mainly through TLR3 and TLR7.

Introduction

Nucleic acids can activate innate immunity and function as potent immune adjuvants through pattern recognition receptors including Toll-like receptors (TLRs) or RIG-I (retinoic acid-inducible gene-I)-like receptors (1, 2). Single-stranded RNA (ssRNA) is recognized by TLR7 in mice and TLR7 and TLR8 in humans (3, 4). DNA with unmethylated CpG motifs is sensed by TLR9 (5). Nucleic acids can be manipulated with little contamination of other ingredients and are now considered to be promisingly applicable to the treatment for allergy or cancer (6–8).

Double-stranded RNA (dsRNA) is produced in virally infected cells and sensed by pattern recognition receptors. Polyinosinic:polycytidylic acids [poly(I:C)] has been widely used as a synthetic dsRNA which works as a potent immune adjuvant. Poly(I:C) is recognized not only by an endosomal receptor TLR3 (9) but also by cytosolic receptors including RNA helicases such as RIG-I or melanoma differentiation-associated gene 5 (MDA5) (10–12). Gene-targeting experiments have revealed the roles of those receptors in poly(I:C)-induced in vivo responses (11). TLR3 is essential for IL-12p40 production, whereas MDA5 is critical for IFN-α induction. Although poly(I:C) can bind to RIG-I in vitro (10), RIG-I is dispensable for poly(I:C)-induced type I IFN production in vivo (11). Another type of dsRNA, polyadenylic:polyuridylic acids [poly(A:U)], is also utilized as an immune modulator (13, 14). When injected with protein or viral antigens into mice, poly(A:U) can promote T-helper 1 generation and antibody production (13). In vivo target delivery of a tumor-associated epitope to antigen-presenting cells in combination with poly(A:U) leads to regression of tumor growth and anti-tumor immune effects against antigen-bearing tumor cells, indicating poly(A:U) as one of promising immunotherapeutic approaches (14). Poly(A:U) has also been used with moderate success for treating breast cancers with minimum...
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side effects (15–17). However, it remains unclear how poly(A:U) activates innate immune cells. Here, we have analyzed the molecular mechanisms on the adjuvant effects of poly(A:U). Analysis on in vitro bone marrow (BM) dendritic cells (DCs) revealed that poly(A:U) functioned as TLR3 and TLR7 agonists, depending on DC subsets. Furthermore, poly(A:U) could augment in vivo antigen-specific CD8+ T-cell responses, in which both TLR3 and TLR7 are involved.

Methods

Mice
C57BL/6J mice were purchased from CLEA Japan. IFN-α/βR-deficient mice were purchased from B&K universal (Hull, UK). TLR3, TLR7 and TLR3/TLR7 double-deficient mice with C57BL/6 background were established and maintained as described previously (18, 19). Mice were maintained under the specific pathogen-free conditions in the animal facility of the RIKEN Research Center for Allergy and Immunology.

Reagents
Poly(I:C) was purchased from Amersham Bioscience (Piscataway, NJ, USA). Polyadenylic acid (polyA) and polyuridylic acid (polyU) were purchased from Sigma (St Louis, MO, USA). Antibodies against CD11c (HL3), CD8α (53-6.7), CD62L (MEL-14) and IFN-γ (XMG1.2) were purchased from XMG1.2. Antibodies against CD11c (HL3), CD8α (53-6.7), CD62L (MEL-14) and IFN-γ (XMG1.2) were purchased from BD Pharmingen (San Jose, CA, USA). Antibodies against B220 (RA3-6B2), CD24 (M1/69) and CD11b (M1/70) were purchased from Biolegend (San Diego, CA, USA). Anti-mPDCA-1 antibody was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Poly(A:U) was generated by annealing polyA and polyU. Briefly, the same amounts of each RNA were mixed in the presence of annealing buffer (20 mM Tris–HCl, 10 mM MgCl2 and 50 mM NaCl) and incubated at 100°C for 5 min. Then, RNAs were subsequently placed at room temperature for >15 min and used as poly(A:U). For RNase treatment, dsRNAs were incubated for 5 h with 1 mg ml−1 of RNaseA (Roche, Mannheim, Germany).

Generation of BM DCs
BM cells were cultured in the presence of 100 ng ml−1 of human recombinant Fms-like tyrosine kinase 3 ligand (Flt3L) (PeproTeck, London, UK) for 7–8 days and used as Flt3L-induced BM DCs as described previously (20). To prepare granulocyte macrophage colony-stimulating factor (GM-CSF)-induced BM DCs, BM cells were cultured in the presence of 10 ng ml−1 of mouse recombinant GM-CSF (R&D, Minneapolis, MN, USA) for 6 days (21). DC subset sorting
Flt3L-induced BM DCs were stained with FITC–anti-CD11c, PE–anti-mPDCA-1 and APC–anti-B220. PDCA-1+ B220+ CD11c+ cells were sorted as plasmacytoid dendritic cell (pDC). Flt3L-induced BM DCs were also stained with FITC–anti-CD24, PE–anti-CD11b, PE–Cy7–anti-CD11c and APC–anti-B220. Among B220+ CD11c+ cells, two types of conventional dendritic cells (cDCs), CD24highCD11blow (CD24high) and CD24lowCD11bhigh (CD11bhigh) cDCs, were sorted. Sorting was performed with FACS Vantage or BD Aria (BD Biosciences, San Jose, CA, USA).

Measurement of cytokine production
DCs were plated in 96-well flat-bottom plates (1.0 × 105 cells per well) and stimulated with indicated concentrations of various stimuli. In certain experiments, polyU was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and utilized. After 24 h of DC stimulation, culture supernatants were collected and amounts of cytokines in the supernatants were measured with ELISA. IFN-α and IFN-β ELISA kits were purchased from PBL (Piscataway, NJ, USA). IL-12p40 ELISA kit was purchased from TECHNE Corporation (Minneapolis, MN, USA).

Analysis on antigen-specific CD8+ T-cell responses
One hundred and fifty milligrams of poly(A:U) or poly(I:C) mixed with 1 mg of ovalbumin (OVA) (Worthington Biochemical Corporation, Lakewood, NJ, USA) per head was intraperitoneally injected into the mice. One week after injection, spleenocytes were harvested and stained with CyChrome–anti-CD8α (BD Biosciences) and PE–OVA-H-2Kb tetramer (MBL, Nagoya, Japan) for detecting antigen-specific CD8+ T-cell populations. For intracellular cytokine detection, spleenocytes were cultured with or without 1 µg ml−1 of a dominant CD8+ T-cell epitope of OVA peptide (257–264), SIINFEKL, in the presence of 1 µl ml−1 GolgiPlug (BD Biosciences) for 6 h. Then, cells were stained with APC–anti-CD8α and PE–Cy7–anti-CD62L and fixed with 4% PFA. Fixed cells were permeabilized according to the manufacturer’s instructions and further stained with FITC–anti-IFN-γ. Stained spleenocytes were analyzed with FACSCalibur (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Results

The ability of poly(A:U) to induce IFN-α
First, we have investigated the effects of poly(A:U) on Flt3L-induced BM DCs, which include both pDC and cDC. The DC could not produce IL-12p40 or IFN-α in response to polyA or polyU alone, but could secrete both cytokines when stimulated with poly(A:U) (Fig. 1A). IFN-α production was seen at 1.8 µg ml−1 and reached a plateau at >16.7 µg ml−1 of poly(A:U) (Fig. 1B). IL-12p40 production was also observed at 1.8 µg ml−1 and increased up to 50 µg ml−1 in a dose-dependent manner (Fig. 1B). Induction of these cytokines was abolished when poly(A:U) was treated with RNase, verifying that the effects are dependent on RNAs (Fig. 1C). Gel electrophoresis analysis revealed that this poly(A:U) consisted of 80–350 bp (data not shown). We have also synthesized polyAs and polyUs, which include 10, 30 or 50 nucleotides. Poly(A:U) derived from these synthesized ssRNAs showed similar effects, but consistently induced less amounts of cytokines (data not shown). Therefore, poly(A:U) from Sigma was used in further experiments.

PolyU is a component of poly(A:U) and can stimulate pDC to produce type I IFN through TLR7 when conjugated with cationic lipids (4). We have then analyzed whether poly(A:U) can activate pDC to produce IFN-α through TLR7 (Fig. 2).
pDCs can be identified as mPDCA-1+B220+CD11c+ cells (22). Wild-type pDC could produce IFN-α in response to poly(A:U), whereas TLR7-deficient pDC did not produce IFN-α in response to poly(A:U), although the mutant pDC responded to a TLR9 agonist, CpG DNA. All stimuli listed in Fig. 2, including CpG DNA (D19) and poly(A:U), failed to induce IFN-α from B220+CD11c+ DCs (20, data not shown).

Another dsRNA, poly(I:C), failed to induce IFN-α production from Flt3L-induced BM DC (Fig. 1B). However, poly(I:C) can activate another type of BM DC, GM-CSF-induced BM DC, to produce IFN-α in a MDA5-dependent manner (11). Poly(A:U) could not induce IFN-α production from GM-CSF-induced BM DC (data not shown). Thus, unlike poly(I:C), poly(A:U) can activate TLR7 signaling, but fails to activate the MDA5-mediated pathway.

The ability of poly(A:U) to induce IL-12p40

We have next analyzed how poly(A:U) induces IL-12p40 production. Flt3L-induced cDC can be further divided into CD24high and CD11bhigh cDCs, which correspond to splenic...
CD11bhigh cDC and CD24high cDC responded to poly(A:U) and this ability was abolished in TLR7-deficient CD24 high cDC. Thus, TLR3 and TLR7 are differentially expressed in CD24 high cDC, but neither in pDC nor in CD11b high cDC. In contrast, TLR7 expression is very low in CD11b high cDC, but not CD24 high cDC, produced IL-12p40 (Fig. 3C). Consistent with the TLR7 expression pattern, y(A:U), cDC produced more amounts of IL-12p40 than pDC (24, 25). Thus, TLR3 and TLR7 are differentially expressed among DC subsets. 

Based on this knowledge, we have purified three DC subsets from Flt3L-induced BM DC using cell sorter (Fig. 3A) and measured the amounts of IL-12p40 from those DC subsets (Fig. 3). Poly(A:U)-stimulated pDC produced IL-12p40 and this ability was abolished in TLR7-deficient pDC, suggesting that poly(A:U)-induced IL-12p40 production from pDC was dependent on TLR7 (Fig. 3B). In response to poly(A:U), cDC produced more amounts of IL-12p40 than pDC (Fig. 3C). Consistent with the TLR7 expression pattern, CD11b high cDC, but not CD24 high cDC, produced IL-12p40 in response to a TLR7 agonist, R848 (18; Fig. 3C). IL-12p40 induction from poly(A:U)- and R848-stimulated CD11b high cDC was abolished in TLR7 deficiency. Meanwhile, CD24 high cDC could produce IL-12p40 not only by poly(A:U) but also by poly(I:C). This induction was retained in TLR7-deficient, but abolished in TLR3-deficient CD24 high cDC. Thus, CD11b high cDC and CD24 high cDC responded to poly(A:U) by producing IL-12p40 in a TLR7- and TLR3-dependent manner, respectively (Fig. 3C).

**Poly(A:U) functions through TLR3 and TLR7 as an immune adjuvant in vivo**

We have further analyzed if and how poly(A:U) has adjuvant effects in vivo. For this purpose, we have examined antigen-specific CD8+ T-cell responses (25). Mice were first immunized with OVA in the absence or presence of dsRNAs. After immunization, antigen-specific CD8+ T cells in the spleen were monitored by their reactivity to OVA-H-2Kb tetramer (Fig. 4). Injection of OVA alone into wild-type mice did not induce expansion of OVA-specific CD8+ T-cell populations. However, simultaneous injection of OVA and poly(A:U) significantly increased the percentage of antigen-specific CD8+ T cells (Fig. 4A and B), indicating that poly(A:U) can function as an immune adjuvant in vivo. Antigen-specific T cells were increased also in the mice injected with OVA and poly(I:C) (Fig. 4A and B).

Based on in vitro findings, we have next examined the mutant mice lacking either of or both TLR3 and TLR7 (Fig. 4). Compared with wild-type mice, both TLR3- or TLR7-deficient mice showed decreased expansion of antigen-specific CD8+ T cells in response to OVA and poly(A:U). Significant increase was still detected in these mutants, but was abolished in TLR3/TLR7 double-deficient mice, indicating that poly(A:U) exhibits the in vivo adjuvant effects mainly through TLR3 and TLR7. When poly(I:C) was injected with antigen, TLR3-deficient mice showed slightly reduced levels of clonal T-cell expansion compared with wild-type mice. However, TLR7 deficiency did not affect the clonal expansion and TLR3/TLR7 double-deficient mice still exhibited significant increase of clonal T-cell populations. The results suggest that TLR3, but not TLR7, is involved in the in vivo adjuvant effects provoked by poly(I:C).

Next, we have analyzed IFN-γ production from antigen-specific CD8+ T cells (Fig. 5). For this purpose, splenocytes from immunized mice were cultured in vitro with or without SIINFEKL and the percentages of IFN-γ-producing cells out of CD8+CD62L+ T cells were calculated. CD8+CD62L+ T cells are major IFN-γ producers (26) and percentages of these cells among CD8+ T cells were not significantly different among wild-type and mutant mice analyzed: wild-type mice 11.9 ± 4.5% (n = 7), TLR3-deficient mice 10.4 ± 5.2% (n = 7), TLR7-deficient mice 15.4 ± 5.4% (n = 7) and TLR3/TLR7 double-deficient mice 14.6% and 24.1% (n = 2). In wild-type mice immunized with antigen and poly(A:U), small, but significant population of CD8+CD62L+ T cells produced IFN-γ in response to SIINFEKL. The percentages of the IFN-γ-producing cells from TLR7-deficient mice were comparable to those from wild-type mice. However, TLR3 as well as TLR3/TLR7 double-deficient mice lacked such responses. Thus, both TLR3 and TLR7 are necessary for clonal expansion, while TLR3 is mainly involved in inducing IFN-γ production from T cells upon immunization with poly(A:U). Poly(I:C) could also enhance numbers of IFN-γ-producing T cells in wild-type mice. The increase was not significantly decreased in TLR3- or TLR7-deficient mice. These results suggest that poly(I:C) mainly functions as an immune adjuvant through the TLR3/TLR7-independent pathway.

**Type I IFNs in poly(A:U)-induced CD8+ T-cell responses**

Type I IFNs can promote cross-priming of CD8+ T cells and play critical roles in virus-induced CD8+ T-cell responses (27). Poly(A:U) could activate pDC to produce IFN-α in a TLR7-dependent manner (Fig. 2), but induced <0.1 ng ml−1
of IFN-α from wild-type CD24<sup>high</sup> and CD11b<sup>high</sup> cDCs. These cDCs also produced <0.1 ng ml<sup>-1</sup> of IFN-β in response to poly(A:U), although wild-type pDC stimulated with poly(A:U) could produce 11.3 ± 0.4 ng ml<sup>-1</sup> of IFN-β. Furthermore, we have also measured serum cytokine levels in wild-type mice after injection of poly(A:U) and OVA. Serum levels of both IFN-α and IFN-β remained <0.1 ng ml<sup>-1</sup> at 1, 3, 6 and 9 h after the injection, although serum IL-12p40 levels were prominently increased (n = 4, 7.8 ± 2.1, 10.2 ± 3.1 and 5.2 ± 3.1 ng ml<sup>-1</sup> at 3, 6 and 9 h, respectively). These results suggest the possibility that type I IFNs play minor roles in poly(A:U)-induced CD8<sup>+</sup> T-cell responses. However, it is still possible that local production or small amounts of type I IFNs can be involved in the responses. In order to
further assess the involvement of type I IFNs, we have investigated the responses of IFN-α/βR-deficient mice to poly(A:U) (Figs 4 and 5). In IFN-α/βR-deficient mice, antigen-specific CD8\(^+\) T-cell populations were expanded (Fig. 4) and IFN-γ-producing cells from IFN-α/βR-deficient mice were also generated (Fig. 5). Although these numbers are slightly less than those from wild-type mice, \(P\) values between wild-type and IFN-α/βR-deficient mice were 0.095 and 0.408 in Figs 4(B) and 5(B), respectively, indicating that the differences are not significant statistically. Thus, poly(A:U) can promote CD8\(^+\) T-cell responses in a type I IFN-independent manner.

We have also analyzed the involvement of type I IFNs in CD8\(^+\) T-cell responses provoked by poly(I:C), which can increase serum type I IFN levels (11). When injected with poly(I:C) and OVA, IFN-α/βR-deficient mice showed reduced responses in both clonal CD8\(^+\) T-cell expansion and IFN-γ-producing T-cell generation, compared with wild-type mice. The results suggest that type I IFNs contribute to the generation of optimal CD8\(^+\) T-cell responses induced by poly(I:C).

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**Fig. 4.** dsRNA-induced clonal expansion of antigen-specific CD8\(^+\) T cells. Wild-type, TLR3, TLR7, TLR3/TLR7 double-deficient and IFN-α/βR-deficient mice were immunized with PBS alone, 1 mg OVA alone, 150 μg poly(A:U) + 1 mg OVA or 150 μg poly(I:C) + 1 mg OVA. Seven days after immunization, splenocytes were harvested and percentages of tetramer-positive T cells in total CD8\(^+\) T cells were determined. Data shown in (A) are representative of 2–11 mice per each group from three independent experiments (B). Bars indicate averages of the percentages. The statistical significance of the obtained values was evaluated by Student’s t-test.
Discussion

In order to study how poly(A:U) functions as an immune adjuvant, we have also examined the responses of splenic DC subsets to poly(A:U), but the effects were not so prominent and too variable to evaluate. Therefore, we have focused to analyze in vitro BM DCs. Notably, responses to various stimuli are quite relevant between in vitro BM DCs and splenic DCs (23, 28). We have found that poly(A:U) could activate Flt3L-induced BM DC to produce IFN-α and IL-12p40. The DC produced IL-12p40, but not IFN-α, in response to poly(I:C). This indicates that this IFN-α-inducing ability is specific to poly(A:U). Flt3L-induced BM DC consists of pDC, CD24high cDC and CD11bhigh cDC. Among these subsets, poly(A:U) could induce IFN-α production from pDC, which expresses TLR7 and TLR9. It can be assumed that TLR7-induced effects can be ascribed to a ssRNA, polyU, because polyU, but not other ssRNAs including polyA, polyI and polyC, can trigger TLR7 signaling (4). As expected, poly(A:U)-induced IFN-α production was abolished in TLR7-deficient pDC. A naked ssRNA is sensitive to nucleases and polyU requires certain types of cationic lipids to activate DCs. In this study, we have analyzed the effects of poly(A:U) and poly(I:C) without any cationic lipids. In the case of poly(A:U), dsRNA formation should stabilize polyU and facilitate cellular uptake of polyU. Once incorporated, in the acidic endosome, dsRNA likely dissociates to ssRNAs. Then, polyU becomes accessible to TLR7. It is also possible that polyU annealed with polyA can function as a TLR7 agonist in the endosome. In any cases, the present results suggest that dsRNA formation can take the place of lipids and that lipids are not mandatory for polyU to function through TLR7.
Although poly(I:C) cannot induce IFN-α from Flt3L-induced BM DC, it can induce IFN-α from GM-CSF-induced BM DC. This poly(I:C) effect is dependent on MDA5 (11). Poly(A:U) failed to induce IFN-α from GM-CSF-induced BM DC, indicating that poly(A:U) has a poor ability to function as a MDA5 stimulator. Thus, poly(A:U) and poly(I:C) have their own functions as immune adjuvants.

Poly(A:U) could activate all three DC subsets to produce IL-12p40. However, underlying mechanisms are different among the DC subsets. In pDC as well as CD11bhigh cDC, IL-12p40 production by poly(A:U) was abolished in TLR7 deficiency. However, in CD24high cDC, the production was retained in TLR7 deficiency, but abolished in TLR3 deficiency. In response to poly(I:C), CD24high cDC also produced IL-12p40 in a TLR3-dependent manner. Thus, TLR3-dependent effects on CD24high cDC were common between two types of dsRNAs. It should be noted that poly(A:U) function as an agonist for both TLR3 and TLR7 and that its effect is mediated in a DC subset-dependent manner.

We have also examined critical roles of TLR3 and TLR7 in in vivo responses to poly(A:U). Injection of a protein antigen and poly(A:U) into mice induced the expansion of antigen-specific CD8+ T cells, indicating that poly(A:U) can function as an immune adjuvant through the cross-priming. The expansion decreased in TLR3- or TLR7-deficient mice and was abolished in TLR3/TLR7 double-deficient mice (Fig. 4). These results indicate that TLR3 and TLR7 play critical roles in optimizing the poly(A:U)-induced clonal T-cell expansion, which is consistent with the in vitro experiments on DC responses to poly(A:U). Poly(I:C)-induced expansion was partly dependent on TLR3 and TLR3/TLR7 double-deficient mice still responded to poly(I:C). Thus, poly(A:U) functions in a TLR-dependent manner, while poly(I:C) can exert its adjuvant effects through a TLR-independent pathway.

Poly(A:U) could also facilitate IFN-γ production from CD8+ T cells in vivo. According to the in vitro analysis, poly(A:U) could induce IL-12p40 through TLR3 and TLR7. It can be assumed that this IL-12p40 production is involved in inducing IFN-γ production from CD8+ T cells. The induction was not impaired in TLR7-deficient, but abolished in TLR3-deficient mice, indicating that TLR3 plays major roles in generating IFN-γ-producing T cells. It is unclear why TLR7 fails to compensate the function of TLR3. TLR3 and TLR7 are expressed in distinct DC subsets and a TLR3-expressing DC subset is featured by a potent ability to ingest apoptotic cells (29, 30). Importantly, TLR3-mediated signals can promote cross-priming (31). We can speculate that a TLR3-expressing DC subset should efficiently incorporate poly(A:U), which becomes accessible to the interaction with TLR3. This DC subset-specific function should contribute to the dominant involvement of TLR3 signaling in generating IFN-γ production from CD8+ T cells. In the case of poly(I:C), MDA5 signaling should apparently play a dominant role in IFN-γ induction in CD8+ T cells.

Type I IFNs are involved in CD8+ T-cell responses induced by viral infection (29). Although poly(A:U) could induce type I IFNs from pDC, poly(A:U) injection into mice did not increase serum type I IFN levels significantly. Furthermore, IFN-α/βR-deficient mice responded to poly(A:U) by expanding clonal CD8+ T cells and generating antigen-specific IFN-γ-producing CD8+ T cells. This is in contrast to severe defects in TLR3-deficient mice and indicates that poly(A:U) can activate CD8+ T-cell responses through TLR3 in a type I IFN-independent manner. It is an important issue to clarify how TLR3 signaling leads to CD8+ T-cell responses. Type I IFNs are involved in, but not mandatory for generating CD8+ T-cell responses. This is supported by the finding that TLR2 signaling, which fails to induce type I IFNs, can induce CD8+ T-cell responses (25). It is also notable that type I IFN-independent CD8+ T-cell responses were observed in poly(I:C)-injected mice (Figs 4 and 5).

In this study, through the in vitro and in vivo experiments, we have clearly demonstrated that poly(A:U) can function as a potent immune adjuvant by activating through TLR3 and TLR7.

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**Abbreviations**

BM bone marrow 
cDC conventional dendritic cell 
DC dendritic cell 
dsRNA double-stranded RNA 
Flt3L Fms-like tyrosine kinase 3 ligand 
GM-CSF granulocyte macrophage colony-stimulating factor 
MDA5 melanoma differentiation-associated gene 5 
OVA ovalbumin 
pDC plasmacytoid dendritic cell 
polyA polyadenylic acid 
poly(A:U) polyadenylic:polyuridylic acid 
poly(I:C) polyinosinic:polycytidylic acid 
polyU polyuridylic acid 
RIG-I retinoic acid inducible gene-I 
ssRNA single-stranded RNA 
TLR Toll-like receptor

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