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Length of dsRNA (poly I:C) drives distinct innate immune responses, depending on the cell type

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ABSTRACT

Poly I:C, a synthetic dsRNA analogue, has been used extensively for decades to study innate responses in vivo and in different cell types. We have found substantial variability while using poly I:C from different sources. In this study we found that poly I:C from 2 commercial sources induced sharply opposite responses in myeloid and fibroblasts, depending on the length of the poly I:C. Although short poly I:C (~1–1.5 kb) induced greater amounts of TNF-α, IL-8, and IFN-β and a stronger antiviral response in myeloid cells, it was a poor inducer in fibroblasts. By contrast, long poly I:C (>5 kb) preferentially elicited higher cytokine and antiviral responses in fibroblasts and showed diminished responses in myeloid cells. Poly I:C activated NF-κB and STAT-1 signaling in a length- and cell-type–dependent fashion. Mechanistically, short poly I:C was better internalized in the myeloid cells and long poly I:C in the fibroblasts. Finally, long poly I:C required SR-A, whereas short poly I:C required RIG-I and Raftlin. We provide evidence that the length of dsRNA drives distinct innate responses in different cell lineages. These findings may augment in selecting the appropriate poly I:C type to design cell-type–specific potent adjuvants for vaccines against infectious diseases or cancers. *J. Leukoc. Biol.* 94: 1025–1036; 2013.

Abbreviations: ATTC=American Type Culture Collection; CHIR=Canadian Institute of Health Research; DC=dendritic cell; $DxSO_4$=dextran sulfate; HEL= human embryonic fibroblast; IPS-I=IFN-beta promoter stimulator-I; IκBα= inhibitor of nuclear factor of kappa light chain gene enhancer in B-cells alpha; IRF-3/7=interferon regulatory factor 3/7; KC=keratinocyte-derived cytokine; MARCO=macrophage receptor with collagenase structure; MDA-5=melanoma differentiation-associated gene-5; MEF=mouse embryonic fibroblast; MFI=mean fluorescence intensity; poly I:C=polyinosinic: polycytidylic acid; PVDF=polyvinylidene difluoride; RIG-I=retinoic acid inducible gene I; ROI=region of interest; RPMI=Rose Park Memorial Institute; SR-$A^{-/-}$=scavenger receptor class A deficient; SCARA=scavenger receptor class A; TPH-1=human acute monocytic leukemia cells; VSV-GFP= vesicular stomatitis virus-green fluorescent protein

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Poly I:C, a synthetic analogue of viral dsRNA, has long been known as a strong inducer of innate immune responses against infectious diseases and cancers. It was initially identified as an antiviral agent that activates various immune cells by recognizing TLR3 in the endosome [1]. It also induces an antiviral state by recognizing the cytoplasmic dsRNA sensors MDA-5 [2, 3] and RIG-I [4]. Upon binding to poly I:C, TLR3 signaling through the adaptors TIRF and MDA-5 via IPS1 leads to the activation of NF-κB and IRF-3 and-7, with subsequent induction of type 1 IFNs, proinflammatory cytokines, and chemokines [5]. We and others have shown that poly I:C treatment protects mice against viral infections [6, 7] and activates NK cells directly [8] or through accessory cells [9] to kill tumor cells.

In addition to innate immunity, poly I:C plays an important role in shaping adaptive immune responses. It augments maturation of DCs to competent APCs, a prerequisite for the generation of adaptive immunity and also a major function of adjuvant. Further evidence has shown that poly I:C potently activates and boosts memory CD8 [10] and CD4 T cells [11, 12] through the activation of DCs that produce an array of cytokines, including IL-12 and type 1 IFNs [7, 13]. Therefore, in recent years, poly I:C has been preferentially used as one of the most potent adjuvants for vaccines [11, 14, 15].

Poly I:C is a large dsRNA-like complex consisting of synthetic polymers, and the different preparations vary in the distribution of strand lengths and biological functions [16, 17]. However, there is no evidence yet to show whether poly I:C from different sources, or even from the same supplier but from different batches, varies in functionality. In our work, we have noted that poly I:C from the same supplier

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but from different batches yields sharply contrasting innate responses. An initial study indicated that the structure of dsRNA determines its antiviral effects through IFN-β production [18]. More recent studies have shown that the length of dsRNA is important in the differential ability of MDA-5 and RIG-I to induce antiviral IFNs in fibroblasts [2, 4, 19]. In another study, extracellular, viral, long dsRNA (~3 kb) has been found to elicit an antiviral state in MEFs, independent of IFN-β production through IRF-3 or MDA-5 [20]. In the context of DC maturation, poly I:C from different sources has differential effects on the various cytokine profiles [13]. It has been shown that in epithelial and fibroblasts, extracellular dsRNA is transported to the endosome or cytosol through SA-R [21, 22] or the transmembrane receptor CD14 [23]. Another study showed that the endoplasmic lipid raft protein Raftlin is indispensable for uptake and delivery of extracellular poly I:C to the cytoplasm in human myeloid DCs and epithelial cells [24]. However, the transport of poly I:C in myeloid cells, as well as the length dependency in nonmyeloid cells, remains largely unknown.

Despite several studies conducted on poly I:C in the context of innate and adaptive immunity or adjuvant effects, some fundamental questions as to whether poly I:C from different suppliers vary in length and induces differential functional response in different cell types remain to be resolved. In this study, poly I:Cs from 2 sources varied in length and therefore induced differential cytokine and antiviral responses in myeloid and nonmyeloid cells. Further, the entry of poly I:C into different cell types depended on the length of the poly I:C, which may be associated with SR-A- or Raftlin-mediated endocytosis.

MATERIALS AND METHODS

Cells, animals, and reagents

The murine macrophage cell line RAW264.7 (ATCC, Manassas, VA, USA) was maintained in RPMI supplemented with 10% FBS, L-glutamine, penicillin-streptomycin, and β-mercaptoethanol. MEFs, prepared in our laboratory from 13.5-day-old embryos from WT C57BL/6 mice, as well as TLR3$^{-/-}$, MDA5$^{-/-}$, and RIG-I$^{-/-}$ MEFs (generously provided by Drs. Michael Gale, Jr., University of Washington, Seattle, USA, and John Hiscott, McGill University, Quebec, Canada, to our colleague and group member Dr. Karen Mossman) were maintained in α-MEM supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin. Human PBMCs were isolated from fresh blood of healthy individuals by the Ficoll-Hypaque density gradient protocol (GE Healthcare, Piscataway, NJ, USA) and were cultured in complete RPMI medium. Vero cells, mouse lung fibroblasts (L929), and HELs (ATCC) were maintained in complete α-MEM. poly I:Cs (Sodium salt) were purchased from Sigma (Burlington, ON, Canada) and GE Healthcare (Quebec, Canada). Phospho-IκB-α, phosphor-STAT1 and β-actin antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). $DxSO_4$ was obtained from Sigma-Aldrich. For mouse anti-SR-A antibody [25], the hybridoma clone 2F8 was grown and maintained as described elsewhere [26]. Purified anti-mouse CD14 (4CI) monoclonal blocking antibody was purchased from BD Biosciences (Mississauga, ON, Canada). A breeding pair of SR-A$^{-/-}$ mice [27, 28] was the kind gift of Dr. Siamon Gordon (Sir William Dunn School of Pathology, University of Oxford, Oxford, UK, to D. B.), and WT C57BL/6 control mice were purchased from Charles River (Montreal, QC, Canada). The VSV-GFP was from Dr. Brian Lichty (McMaster Immunology Research Centre, Hamilton, ON, Canada).

Treatment of cells with poly I:Cs

For cytokine responses, 2×10^5 cells/well were cultured in 96-well plates and treated with poly I:C in serum-free medium at a concentration of 10 μg/ml or 10 nM/ml, to ensure equal amounts of molecules per poly I:C length, unless otherwise indicated. For viral challenge, cells were cultured in 12-well ($1–2\times10^5$ cells/well) or 24-well ($0.5–1\times10^5$ cells/well) plates and pretreated with poly I:C (10 μg/ml) in 2-fold serial dilutions, as noted in the figure legends.

Antiviral assay

Cells (0.5×10^5 cells/well) were seeded in a 24-well plate and treated with serial dilutions of microgram per nanomolar concentrations of poly I:C for the periods as indicated in the figure legends. In some cases, cell-free supernatants from poly I:C-stimulated cells (PBMCs, RAW264.7 cells, or splenocytes) were serially diluted and added to Vero cells or MEFs for the specified time as mentioned in the figure legend. Cells were subsequently infected with VSV-GFP at 0.1 PFU/cell for 1 h in serum-free medium. The viral inoculum was then replaced with 2× F11 MEM containing 1% methylcellulose. The intensity of GFP fluorescence was measured 24 h later with a Typhoon Trio fluorescence imager and quantified by ImageQuant TL software (both from GE Healthcare). A dose–response curve was generated for each poly I:C type by GraphPad Prism software (La Jolla, CA, USA).

Cytokine and chemokine measurements by ELISA

Poly I:C-treated, cell-free supernatants were harvested and assayed for TNF-α, human IL-8/mouse KC, and IFN-β, with commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. NO production from poly I:C-treated (48 h), cell-free supernatants was measured by a Griess nitrite assay, as per standard procedures.

Immunoblot analysis

RAW264.7 cells were treated with 2 different poly I:Cs for 2 h. Whole-cell protein lysates were isolated in lysis buffer, and 30 μg of protein per sample was run through 10% SDS-PAGE and electroblotted onto a PVDF membrane. The membrane was incubated with phospho-IκB-α and phosphor-STAT1 (both from Cell Signaling, Inc.) antibodies and then with the secondary antibody. It was developed by ECL reagent and photographed. The membrane was then stripped and reprobed with anti-β-actin antibody (Cell Signaling, Inc.), as a loading control.

Poly I:C labeling, confocal imaging, and quantification of images

Two types of poly I:Cs were labeled with Alexa Fluor 594-conjugated dye (BD Biosciences) and a Ulysis nucleic acid labeling kit (Millipore/Invitrogen, Billerica, MA, USA) according to the manufacturers' instructions The labeled poly I:Cs were then purified using Micro Bio-Spin RNAse-free columns packed with a special grade of Bio-Gel P-30 polyacrylamide gel matrix (Bio-Rad, Hercules, CA). The cells were then treated with Alexa Fluor 594-labeled poly I:C for 1–2 h, followed by 4% paraformaldehyde fixation for 20 min. After washes in PBS, the cells were stained for nuclei with SYTO 21 (Invitrogen) and covered with Vectashield mounting medium (Vector Laboratories, Burlington, ON, Canada) under a glass coverslip. Images were acquired with an LSM510 confocal microscope (Zeiss, Jena, Germany) and the data were analyzed with LSM510 image software (Zeiss). ImageJ v1.43m [29, 30] was used to define the cell ROI with a binary mask based on Alexa Fluor-594 staining. Briefly, the masks were defined by background subtraction, Gaussian blur, and autothresholding, according to an algorithm published by Jiang and Li [31], to define the cell ROI. In RAW264.7 binary masks, the

nucleus was added manually. An image calculator was used to extract cell and noncell (background) ROIs from the original images. MFI was calculated for each image's cell and background ROI and was plotted with Prism v4.01 (GraphPad Prism).

Raftlin siRNA transfection of RAW264.7 cells

Murine Raftlin siRNA duplexes (sc-152681 and sc-152682) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and RAW264.7 cells were transfected according to the manufacturer's protocol. Briefly, RAW264.7 cells (1×10^5 cells/well), cultured in serum- and antibiotic-free medium on coverslips inserted into 12-well plates, were transfected with 20 pmol of siRNA with Lipofectamine 2000 (Life Technologies, Gaithersburg, MD, USA). After 48 h, the cells were washed once and then incubated 1–2 h with Alexa Fluor 594-labeled poly I:C-S (short) or poly I:C-L (long), washed, fixed, and processed for confocal imaging, as described in the prior section. For cytokine analysis, transfected cells were stimulated with poly I:C-S and poly I:C-L for 24 h and cell-free supernatants were analyzed by ELISA. Raftlin knockdown in RAW264.7 cells was evaluated by extracting RNA with the RNeasy mini kit (Qiagen, Toronto, ON, Canada), cDNA was synthesized, and semiquantitative RT-PCR was performed with the following primers: mouse Raftlin, (forward) 5′-CAC GAG GTT AGC CTC TCT GC-3′, (reverse) 5′-TCT GGG ATG AGC TTC TGG TC-3′; mouse Raftlin-2, (forward) 5′-GGA GGA ACC TCA GCA TGA AAG-3′, (reverse) 5′-CTT TAG GTG TCT CCC AGC AC-3′; mouse 18SrRNA, (forward) 5′-GTG CAT GGC CGT TCT TAG TT-3′, (reverse) 5′-TGC CAG AGT CTC GTT CGT TAT-3′.

Statistical Analysis

All statistical analyses were performed with GraphPad Prism software (La Jolla, CA, USA). Comparisons between 2 groups were performed by *t* test and between 3 or more groups by 1-way ANOVA. Data are expressed as the mean ± SEM, unless otherwise noted. $P < 0.05$ was considered to show statistical significance.

RESULTS

Poly I:Cs from 2 different sources elicited substantial distinct activation of myeloid cells

Poly I:C has been widely used for decades to study innate immune responses against infections and cancers. Our lab has been routinely used poly I:C from different suppliers to study antiviral innate immunity. To our great surprise, we have observed substantial variation in results in poly I:C from different suppliers and even in different batches from the same supplier. This prompted us to investigate the underlying reasons for the variation. To achieve our goal, we stimulated human and murine primary or transformed myeloid cells with poly I:C from 2 commercial sources (termed poly I:C-A and -B), and cytokines and chemokines were assessed. Stimulation of murine and human myeloid cells with poly I:C-A and -B exerted sharply opposite cytokine and chemokine responses. In contrast to poly I:C-A, stimulation of murine splenocytes and human PBMCs with poly I:C-B induced significantly greater amounts of TNF-α (**Fig. 1A** and **B**) and IL-8/KC (Fig. 1C and D) production. We then treated murine (RAW264.7) and human (THP-1) macrophage cell lines with the 2 poly I:Cs. These cells, similar to primary myeloid cells, produced significantly higher amounts of TNF-α with poly I:C-A when compared with poly I:C-B (Fig. 1E and F). In a separate set of experiments, human PBMCs and mouse splenocytes were stimulated with these 2 poly I:Cs for 48 and 72 h, and IFN-γ was measured. Poly I:C-A induced significantly greater quantities of IFN-γ than did poly I:C-B (Supplemental Fig. S1).

Difference in length of poly I:C from 2 sources resulted in differential innate responses

We have observed differential cytokine responses with 2 poly I:Cs in the same myeloid cells. A recent report also has delineated differential IFN responses with various sizes of viral dsRNA in fibroblasts [20]. The results led us to hypothesize that the variable cytokine responses in myeloid cells with 2 poly I:Cs is due to differences in the poly I:Cs' lengths. To this end, we analyzed 2 poly I:Cs in 1% agarose gel, to compare their molecular sizes. The agarose gel data clearly showed that the 2 poly I:Cs were very different in molecular size. Most of poly I:C-A had a length of about 0.5–1.5 kb, whereas poly I:C-B was ~2.0–8.0 kb (**Fig. 2A**). As mentioned in Methods, according to their lengths, we now refer to poly I:C-A as poly I:C-S and poly I:C-B as poly I:C-L, unless otherwise indicated. To further ascertain that the differential innate responses are attributable to the length and not the source and manufacturing conditions of poly I:C, we shortened poly I:C-L by RNAse III enzymatic digestion to a length (~1.5 kb) similar to that of poly I:C-S, as verified in 1% agarose gel (Fig. 2B). We then stimulated human and murine myeloid cells with digested poly I:C-L, along with normal poly I:C-L and -S. Stimulation of human PBMCs with the digested poly I:C-L resulted in sharply opposite effects, releasing significantly greater amounts of TNF-α (Fig. 2C) and IL-8 (Fig. 2D), compared with the undigested poly I:C-L, but identical to the response to the poly I:C-S. Similar levels of TNF-α were also detected in the murine myeloid cells (Fig. 2E).

Poly I:C-mediated innate responses were cell-type dependent and were linked to differential type 1 IFN expression

We then sought to evaluate whether poly I:C from the same source but different in length would induce differential innate responses depending on cell type. Type 1 IFNs are the major innate antiviral agents produced by poly I:C stimulation of myeloid cells and fibroblasts. To this end, we performed a standard VSV-GFP antiviral IFN bioassay by treating murine macrophages (RAW264.7) and fibroblasts (WT C57BL/6 MEF) with poly I:C-S, poly I:C-L, and digested-poly I:C-L followed by infection with VSV-GFP. Although stimulations of RAW264.7 cells with poly I:C-S and digested-poly I:C-L elicited robust protection against VSV replication, poly I:C-L displayed significantly diminished inhibition of VSV replication (**Fig. 3A**). In contrast, poly I:C-L showed complete prevention of VSV replication in WT MEFs, whereas poly I:C-S and digested-poly I:C-L failed to limit VSV replication (Fig. 3B). This phenomenon was further confirmed in HEL cells in a similar experimental protocol (Fig. 3C). We then assessed type 1 IFN (IFN-β) production by RAW264.7 cells and MEFs after stimulation with different

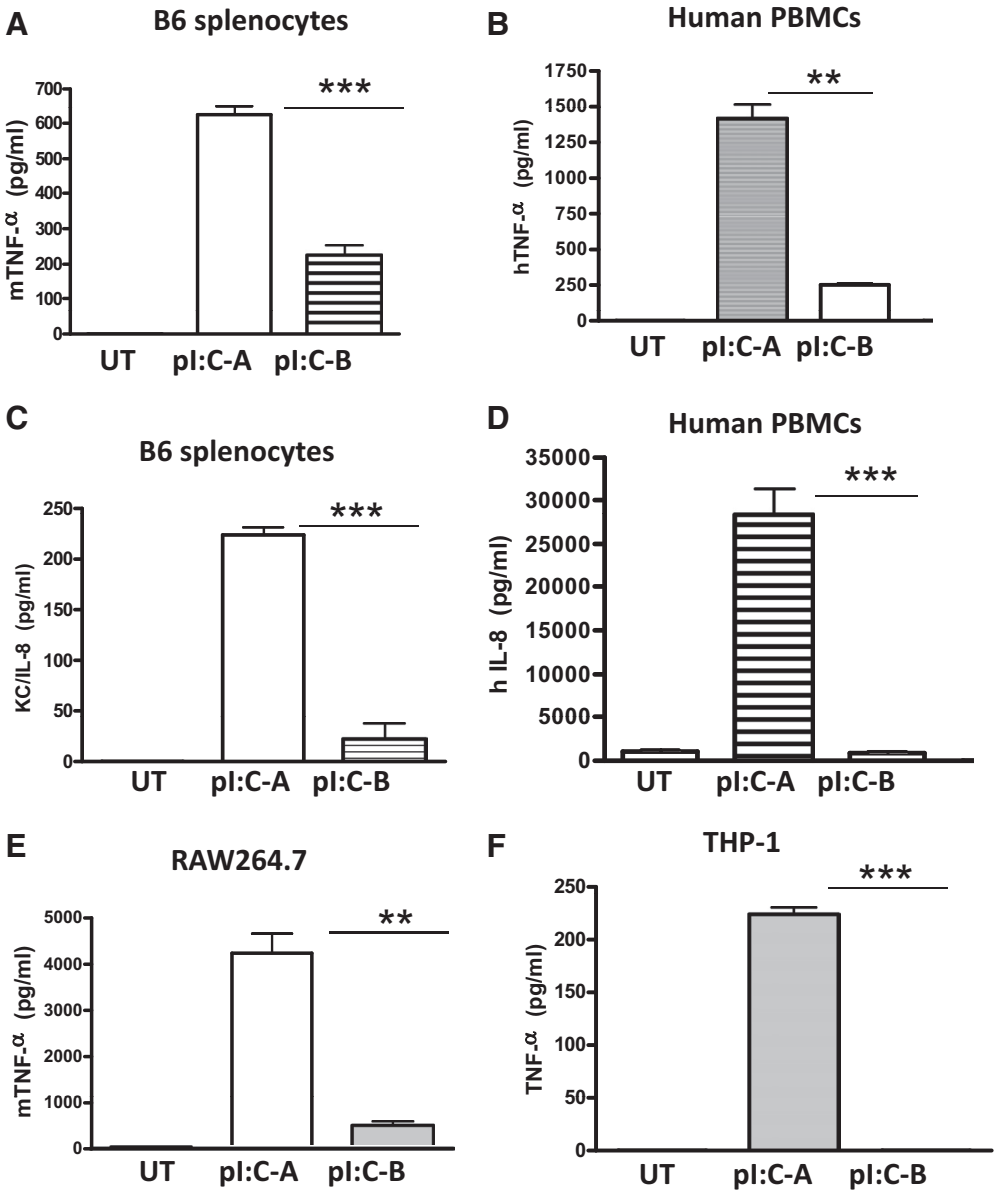

Figure 1. Cytokine and chemokine responses with different sources of poly I:Cs in human and murine myeloid cells. (A, C) C57BL/6 mouse (naïve) splenocytes and (B, D) human PBMCs from healthy individuals were isolated and cultured ex vivo in the absence or presence of poly I:Cs from 2 different sources for 24 h. Cell-free supernatants were assessed for TNF-α or IL-8/KC by ELISA. (E) RAW264.7 cells and (F) human macrophages (THP-1) were stimulated with 2 different poly I:Cs, and cell-free supernatants were measured for TNF-α by ELISA. Data are expressed as the mean ± SEM of results of 5 experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

lengths of poly I:C. As measured by ELISA, poly I:C-S induced greater amounts of IFN-β in RAW264.7 cells than did poly I:C-L (Fig. 3D). In WT MEFs, we observed sharply opposite trends; poly I:C-L produced a significantly higher amount of IFN-β than did poly I:C-S (Fig. 3E). NO is another known potent antiviral agent that is generated mainly by macrophages by TLR stimulation. We demonstrate that poly I:C-S-treated, in contrast to poly I:C-L-treated, RAW264.7 cells released significantly higher amounts of NO (Fig. 3F). Poly I:C stimulation resulted in NF-κB, IRF-3, and STAT-1 transcriptional activation through dsRNA receptor dimerization with subsequent induction of innate cytokines, including type 1 IFNs. We observed poly I:C length-dependent differential cytokine and antiviral responses in myeloid cells, and we therefore chose to determine whether the length of poly I:C influences NF-κB and STAT-1 signaling. Poly I:C-L and -S stimulated RAW264.7 cell lysates examination by Western immunoblot revealed greater activation of IκB-α and STAT1 signals with poly I:C-S than with the poly I:C-L (Fig. 3G).

The poly I:C length-dependent cytokine responses were not due to LPS contamination or dose kinetics

Poly I:C-L and -S vary widely in molecular size (base pairs). Therefore the question arises as to whether higher doses could compensate for the discrepancy. RAW264.7 cells were stimulated with very low to higher doses (0.1–50 μg/ml) of both poly I:C-L and -S, and the TNF-α was measured. Although the production of TNF-α was elevated according to the increased doses of both poly I:Cs, the significant differences in TNF-α levels between poly I:C-L and -S remained consistent from lower to higher doses (**Fig. 4A**). The time kinetics of both poly I:C treatments showed the highest

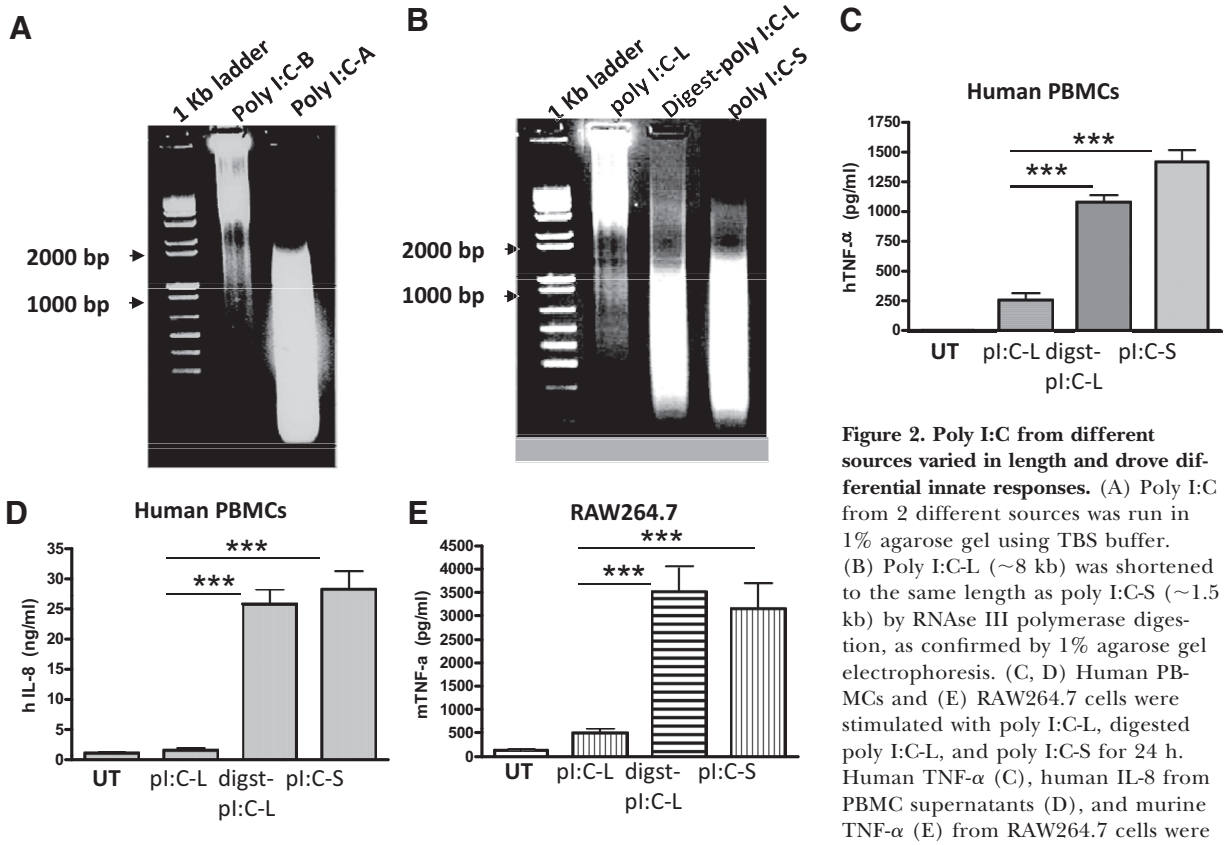

Figure 2. Poly I:C from different sources varied in length and drove differential innate responses. (A) Poly I:C from 2 different sources was run in 1% agarose gel using TBS buffer. (B) Poly I:C-L (~8 kb) was shortened to the same length as poly I:C-S (~1.5 kb) by RNAse III polymerase digestion, as confirmed by 1% agarose gel electrophoresis. (C, D) Human PBMCs and (E) RAW264.7 cells were stimulated with poly I:C-L, digested poly I:C-L, and poly I:C-S for 24 h. Human TNF-α (C), human IL-8 from PBMC supernatants (D), and murine TNF-α (E) from RAW264.7 cells were measured by ELISA. Data are expressed as the mean ± SEM of results of 3 experiments. ***$P < 0.001$.

TNF-α production at 12 h of poly I:C stimulation and then a decline at 24 h, with consistent differences between poly I:C-L and -S (Fig. 4B).

To validate whether the striking differences in cytokine responses between poly I:C-L and -S preparations are masked or influenced by LPS contamination, we completely digested both poly I:C-L and -S with RNAse III enzyme for 30 min (no band detected in agarose gel) and partially digested (15 min) poly I:C-L to achieve a length equivalent to that of poly I:C-S. The digested poly I:C preparations were cleaned and purified on an RNase-free Micro-Bio spin column (Bio-Rad). As a positive control, LPS was digested similarly. RNAse III enzyme potently cleaves or digests long dsRNA, but is unable to digest LPS. RAW264.7 cells were treated with all the poly I:C and LPS preparations, and TNF-α levels were measured. As depicted in Fig. 4C, completely digested poly I:C-S or -L failed to induce any detectable TNF-α production; however, partially digested poly I:C-L induced significantly greater amounts of TNF-α than the intact poly I:C-L, but similar to that induced by the intact poly I:C-S. This experiment provides strong evidence to rule out any possibility of LPS contamination.

Entry of poly I:C into cells was both length and cell-type dependent

It is apparent that only the presence of or binding preference to dsRNA receptors does not determine the poly I:C response; instead, some other factors or molecules could be involved in the process. We asked whether the entry of poly I:C-L and -S into cells would also be different. Poly I:C-L and -S were labeled with fluorescence-conjugated (Alexa Fluor 594; BD Biosciences) RNA labeling dye and column purified. The RAW264.7 cells and WT MEFs were treated for the specified time and examined by confocal microscopy. Poly I:C-S displayed higher levels of internalization in the RAW264.7 cells (**Fig. 5A**), and poly I:C-L in the MEFs (Fig. 5C). Similarly, we conducted an experiment with human fibroblasts (BJ and HEL cell lines) and THP-1 macrophage-like cells, using poly I:C-L and -S; the results were similar to those obtained with murine cells (data not shown). Figure 5B and D displays the quantification of the staining intensity of poly I:C-L and -S in RAW264.7 cells and WT MEFs, respectively, as measured by a standard NIH ImageJ method.

Poly I:C-L was dependent on SR-A and poly I:C-S on Raftlin

One study has demonstrated that poly I:C is recognized and internalized by SR-A in human bronchial epithelial cells (BEAS-2B) [21]. Because SR-As have been shown to be essential for extracellular viral dsRNA entry and type 1 IFN response in MEFs [22], we sought to ascertain whether this would be true for poly I:Cs of various lengths both in fibroblasts and myeloid cells. To this end, splenocytes isolated

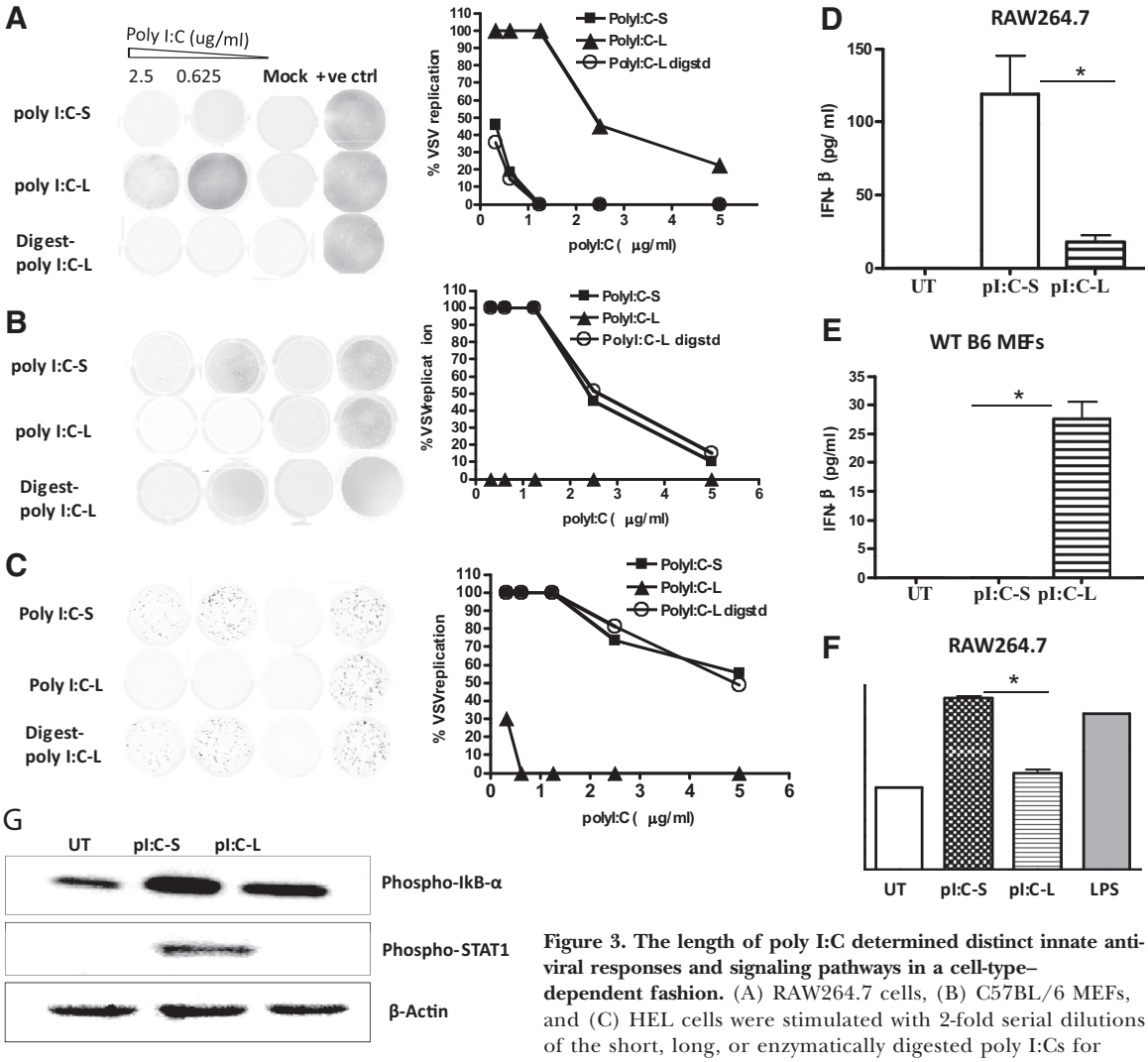

Figure 3. The length of poly I:C determined distinct innate antiviral responses and signaling pathways in a cell-type–dependent fashion. (A) RAW264.7 cells, (B) C57BL/6 MEFs, and (C) HEL cells were stimulated with 2-fold serial dilutions of the short, long, or enzymatically digested poly I:Cs for 16–20 h and =then challenged with VSV-GFP for 1 h followed by Typhoon scanning to measure viral replication. Representative cell wells are depicted from at least 3 individual experiments. (A, C, right panels) poly I:C dose responses on viral replication. Data are expressed as the mean ± SEM of results of 3 experiments. (D) RAW264.7 and (E) C57BL/6 MEF cells were stimulated with poly I:C-S and -L for 16 h, and cell-free supernatants were measured for IFN-β production by ELISA. (F) RAW264.7 cells were stimulated with poly I:C-S and -L for 48 h, and cell-free supernatants were measured for NO production by Griess assay. (G) The RAW264.7 cells were stimulated with poly I:C-S or -L for 2 h, cell lysates were prepared in lysis buffer and the proteins run on 10% SDS-polyacrylamide gels, and immunoblot analysis was performed with anti-phospho-IκB-α and anti-phospho-STAT1 antibodies. (A–C) Data are expressed as the mean ± SEM of results of 5 experiments. (D) Representative of 3 separate experiments. *$P <$ 0.05.

from WT mice were either pretreated with the SR-A inhibitor $DxSO_4$ or an anti-SR-A antibody. The cells from SR-$A^{-/-}$ mice were stimulated with the 2 different poly I:Cs, and the cytokines were measured. Blocking of SR-A in WT splenocytes with anti-SR-A antibody or an SR-A inhibitor ($DxSO_4$) significantly reduced TNF-α secretion by poly I:C-L compared with that in IgG-treated cells but with no apparent effect on poly I:C-S-mediated TNF-α response (**Fig. 6A**). Furthermore, splenocytes from SR-A-deficient mice displayed levels of TNF-α similar to those in WT mice after poly I:C-S stimulation (Fig. 6B). However, SR-$A^{-/-}$ splenocytes produced slightly less, although not significantly, TNF-α than those of WT mice (Fig. 6B). We then assessed type 1 IFN responses with the different poly I:Cs in murine lung fibroblasts (L929) and RAW264.7 cells. The cells were pretreated with anti-SR-A antibody or $DxSO_4$ followed by poly I:C stimulation and then challenged with VSV-GFP. Blocking of SR-A in murine fibroblasts (L929) resulted in significantly less inhibition of VSV replication with poly I:C-L treatment; however, it had no influence on the poly I:C-S-mediated antiviral response (Fig. 6C and Supplemental Fig. S2). A similar antiviral assay in RAW264.7 cells revealed no inhibition of viral replication with anti-SRA antibody pretreatments followed by poly I:C-S or -L stimulation, but the

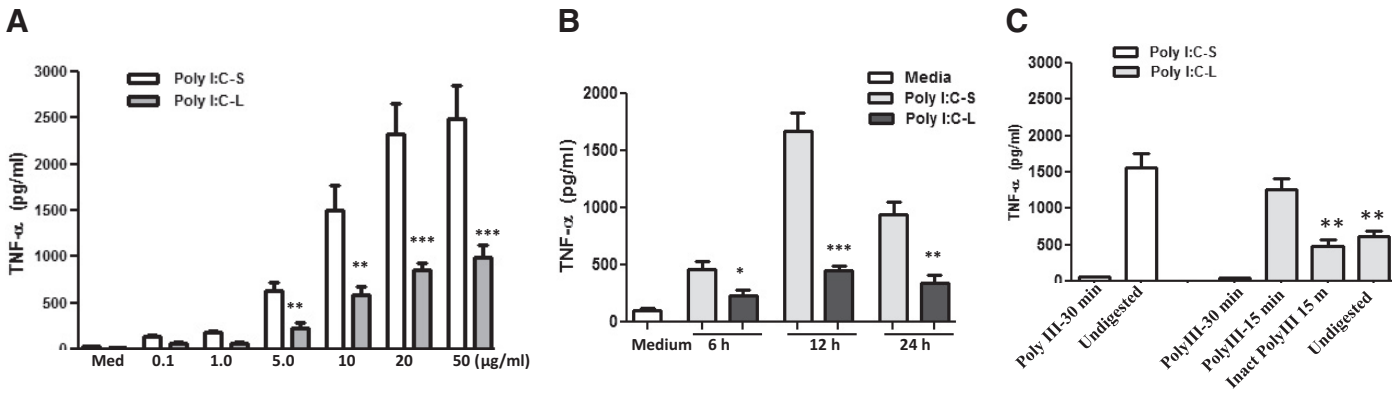

Figure 4. The poly I:C length-dependent cytokine responses were not due to LPS contamination or dose kinetics. (A) RAW264.7 cells were stimulated with the various doses of poly I:C-S and -L indicated, and the cell-free supernatants were assayed for TNF-α by ELISA. (B) RAW264.7 cells were stimulated with poly I:C-S and -L for 6, 12, and 24 h, and cell-free supernatants were measured for TNF-α production by ELISA. (C) Poly I:C-S was digested by active RNAse III enzyme for 30 min, and poly I:C-L was digested by both active and heat inactivated (90°C for 30 min) RNAse III for 15 or 30 min and then purified on a Micro-Bio spin column. RAW264.7 cells were then stimulated for 16 h with the poly I:C preparations, and the cell-free supernatants were assayed for TNF-α production by ELISA. Data are expressed as the mean ± SEM of results of 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

SR-A inhibitor ($DxSO_4$) interfered with viral replication (Fig. 6D and Supplemental Fig. S2).

To delineate the requirement of the known dsRNA receptors, including TLR-3, MDA-5, and RIG-I for poly I:C S or -L, MEFs from the respective receptor knockout mice were assessed for anti-viral IFN by using the gold-standard VSV-GFP method. Poly I:C-L showed TLR-3- and RIG-I-receptor–independent anti-viral innate responses in MEFs (**Fig. 7A**). Poly I:C-S, on the other hand, was absolutely dependent on RIG-I, but only partially on TLR-3, for an antiviral response (Fig. 7A).

Recent evidence has shown that CD14 in HEK293 cells [23] and the endosomal lipid raft protein Raftlin [24] in human DCs and epithelial cells act as uptake receptors, to

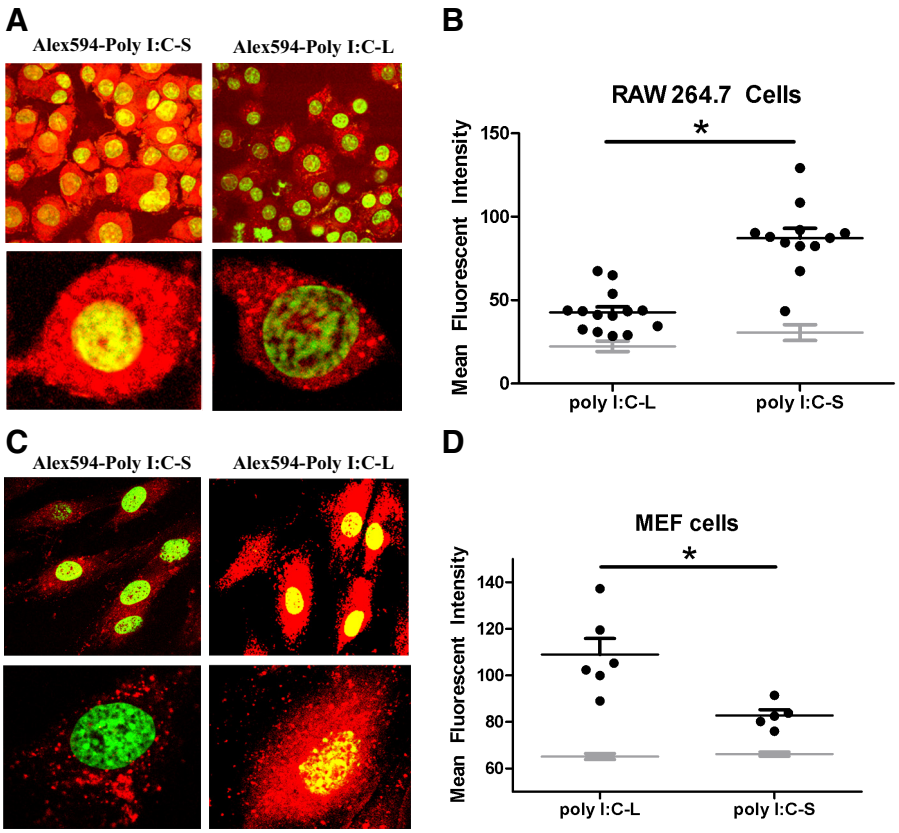

Figure 5. The length of poly I:Cs showed a distinct preference for entry, depending on cell types. (A) RAW264.7 and (C) C57BL/6 MEFs were grown on coverslips, treated with Alexa-Flour 594-labeled poly I:C-S or -L for 1 to 2 h, fixed with 4% paraformaldehyde, and the nuclei stained with SYTO 21 dye. Confocal images were acquired with an LSM510 confocal microscope and analyzed by LSM5 image software. Images represent several repeated experiments. Original magnification, ×1260 (63×20). The bottom panels show higher magnification images. (B, D) Quantification of intensity of the poly I:C staining in RAW264.7 and WT MEFs, respectively, with the standard NIH ImageJ software. Black dots indicate the number of images quantified and gray bars indicate the image background. $*P < 0.05$.

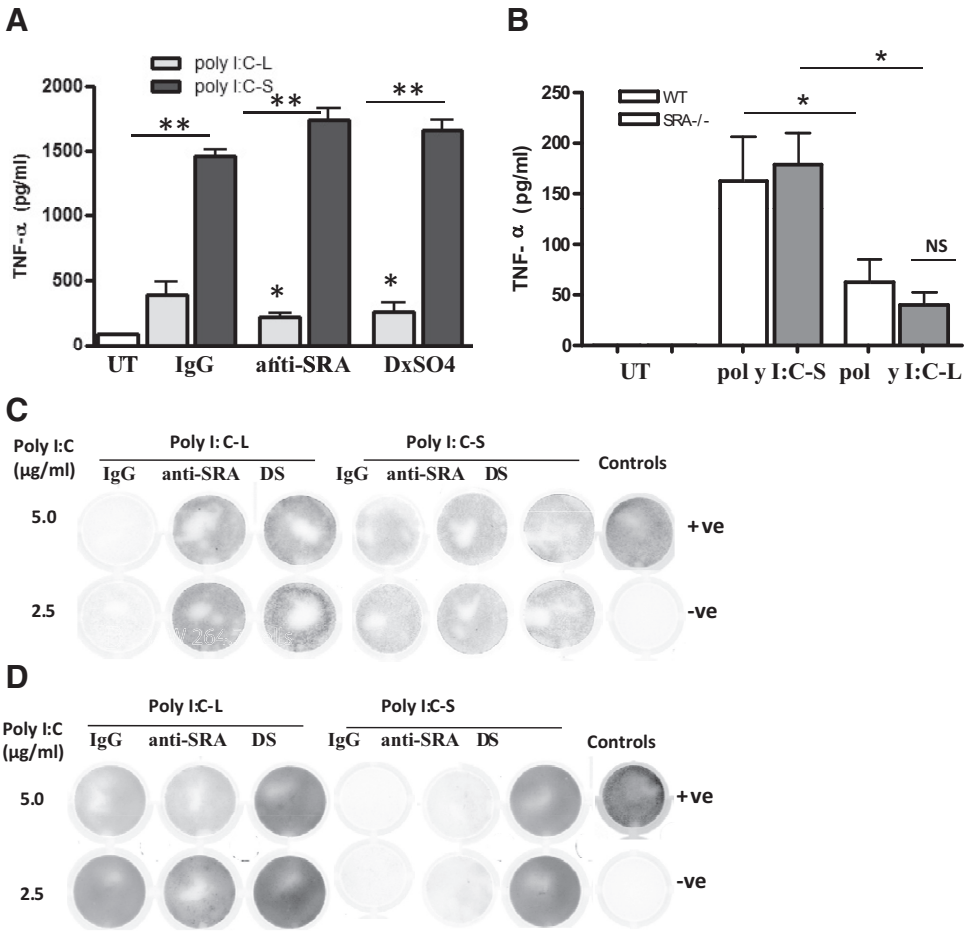

Figure 6. Poly I:C-L requires SR-A to activate splenocytes. (A) Splenocytes from WT C57BL/6 mice were pretreated with control IgG, anti-SRA antibody, or SR-A inhibitor ($DxSO_4$) followed by poly I:C stimulation for 24 h. The cell-free supernatants were analyzed by ELISA for TNF-α production. (B) Splenocytes from $SRA^{-/-}$ mice were stimulated with poly I:C-S and -L, and the supernatants were measured for TNF-α production by ELISA. Data in (A) and (B) are expressed as the mean ± SEM of results of 5 experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (C) Murine lung fibroblasts (L929) and (D) RAW264.7 cells were stimulated with serially diluted poly I:C-S and -L for 16 h. The cells were then challenged with VSV-GFP (MOI=0.1) for 24 h, and images (GFP fluorescence, indicating viral replication) were taken by Typhoon scanning of the plates. Data represent 3 independent experiments.

deliver poly I:C to the cytoplasm. To evaluate whether CD14 or Raftlin plays any role in the uptake or delivery of extracellular poly I:C-S or -L to the endosomes, particularly in myeloid cells, murine RAW264.7 cells were transfected with Raftlin siRNA to knockdown Raftlin mRNA expression (Supplemental Fig. S3), and CD14 was blocked by treating the cells with anti-mouse CD14 antibody. First, we examined the cytokine responses after 24 h stimulation with poly I:C-S or -L. Raftlin knockdown in the RAW264.7 cells significantly reduced the TNF-α production after poly I:C-S, but not -L, stimulation (Fig. 7B). However, CD14 blocking did not show any effect on cytokine responses to poly I:C-S or -L (data not shown). We then treated RAW264.7 cells with Alexa Fluor 594-labeled poly I:C-S or -L, and the levels of uptake and delivery of poly I:C into the cells were analyzed by confocal immunofluorescence. As shown in Fig. 7C, siRNA knockdown of Raftlin in the RAW264.7 cells markedly prevented the uptake of poly I:C-S but not -L, compared with the control siRNA-transfected cells. Furthermore, blocking of CD14 by anti-mouse CD14 mAb did not show any apparent effect on uptake, irrespective of the length of poly I:C.

DISCUSSION

Three major fundamental findings generated by the current study were previously unknown. First, commercially available poly I:C from different sources induces distinct innate immune responses. These differential responses were found to be due to the wide variation in molecular sizes (length). Secondly, different cell types, myeloid and fibroblasts, for instance, recognize and respond to these poly I:Cs differentially, depending on their lengths. Poly I:C-S displayed a stronger response in myeloid cells; by contrast, poly I:C-L showed better response in fibroblasts. Thirdly, the level of entry of poly I:C into the cells, which is thought to be an important mechanistic determinant linked to innate responses, was also found to be length dependent. Poly I:C-S exhibited better entry into the macrophages which is mediated by Raftlin, whereas poly I:C-L gained better entry into fibroblasts, where SR-As plausibly play an important role. Poly I:C has been widely used to study innate immunity and as a vaccine adjuvant, and it is therefore important to understand the detail biology of the commercially available poly I:Cs and their immunomodulatory capacity in the context of targeting cell types.

Although poly I:C has been appreciated as a potent inducer of innate immune responses against viral infections and cancers, it has never been revealed whether poly I:Cs from different commercial sources could be different in functionality, depending on cell type. We have noticed a wide variability in results while using poly I:Cs from different commercial suppliers. This provoked us to investigate

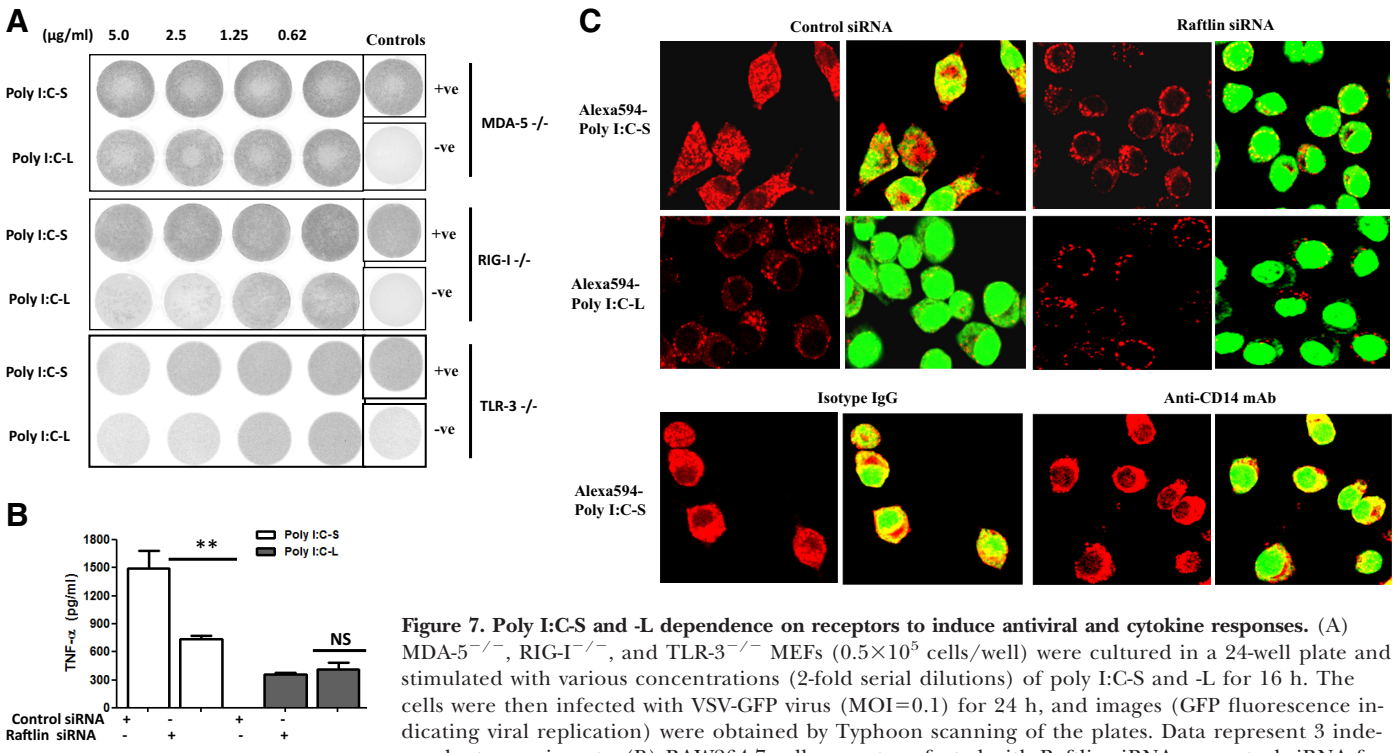

Figure 7. Poly I:C-S and -L dependence on receptors to induce antiviral and cytokine responses. (A) MDA-5$^{-/-}$, RIG-I$^{-/-}$, and TLR-3$^{-/-}$ MEFs (0.5×10^5 cells/well) were cultured in a 24-well plate and stimulated with various concentrations (2-fold serial dilutions) of poly I:C-S and -L for 16 h. The cells were then infected with VSV-GFP virus (MOI=0.1) for 24 h, and images (GFP fluorescence indicating viral replication) were obtained by Typhoon scanning of the plates. Data represent 3 independent experiments. (B) RAW264.7 cells were transfected with Raftlin siRNA or control siRNA for 48 h and then stimulated with poly I:C-L and -S (10 μg/ml) for 16 h. The cell free supernatants were assayed for TNF-α production by ELISA. Data are expressed as the mean ± SEM of results of 3 independent experiments. $^{**}P < 0.01$; NS, nonsignificant. (C) RAW264.7 cells were transfected with Raftlin siRNA or control siRNA and grown on coverslips for 48 h. In another set of experiments, the RAW264.7 cells were preincubated with 10 μg/ml of anti-mouse CD14 mAb (4CI) or isotype control IgG for 1 h. The cells were then treated with Alexa Fluor-594-labeled poly I:C-S and -L for 1–2 h, fixed in 4% paraformaldehyde, and the nuclei stained with SYTO-21 dye. Confocal images were acquired with an LSM510 confocal microscope and analyzed by LSM5 image software. Original magnification, was 1260 (63×20). The images represent at least 3 separate experiments.

the underlying factors associated with these variable effects with different poly I:Cs in different cell types. We first tested 2 different commercial sources of poly I:Cs in human and murine myeloid cells. To our surprise, we observed significantly contrasting innate cytokine responses with 2 sources of poly I:Cs. Of note, these differential poly I:C effects were not species specific, since comparable cytokine responses were evident both in human and murine myeloid cells. Further, these distinct poly I:C responses were not due to differences in molarity or concentration of poly I:C, as we found differential cytokine responses using equal nanomolar amounts of both poly I:Cs in the same cell type (data not shown).

We then thought that these 2 poly I:Cs might be different in length. In fact, they are very different: one was ~500 bp to 1.5 kb (poly I:C-A/poly I:C-S) and the other was >2–8 kb (poly I:C-B/poly I:C-L). We confirmed this length-dependent innate effect by enzymatically cleaving the poly I:C-L to the size of poly I:C-S, which yielded innate cytokine responses similar to those of the manufacturer-provided poly I:C-S. This result clearly indicates that the length, not the commercial source, of poly I:C determines the differential innate responses in myeloid cells. In line with our findings, a previous study showed that human DCs or macrophages stimulated with poly I:C-L (>5 kb, by tracking the source of the poly I:C) failed to induce TNF-α, IL-6, or IL-8 [32]. However, the investigators used only 1 type of poly I:C to achieve different objectives from ours. We next evaluated whether the length of poly I:C induces distinct innate antiviral responses depending on the cell type. Importantly, Poly I:C-S, but not poly I:C-L, induced stronger antiviral responses in myeloid cells. In sharp contrast, and poly I:C-L, but not poly I:C-S, induced stronger antiviral responses in fibroblasts. To our knowledge, this is the first systematic study to show that poly I:C-S induces stronger innate cytokine and antiviral responses in myeloid cells and poly I:C-L in fibroblast or epithelial cells. Mechanistically, these length-dependent innate responses seem to be associated with the distinct capabilities of different lengths of poly I:Cs, depending on the cell type.

Long viral dsRNA (up to 3 kb) has been shown to elicit stronger antiviral response in fibroblasts [20]. However, the length-dependent type 1 IFN response of poly I:C in myeloid cells remains to be explored. In our study, poly I:C-S produced greater amounts of IFN-β in RAW264.7 cells and poly I:C-L in WT MEFs. These results strongly reflect the antiviral IFN bioassay data. Our data further support a very recent finding showing that long viral dsRNA elicits a greater antiviral IFN-β response in murine fibroblasts [20]. We, for the first time, showed that murine macrophages

with different lengths of poly I:C stimulation show differential antiviral responses including type 1 IFN. Type 1 IFNs are the key players in bridging innate to adaptive immunity as well as directly acting on CD8 T cells [33] by increasing survival during antigen-driven clonal expansion [34, 35] or promotes CD8 T cell cross-priming by direct stimulation of DCs [36]. Further, poly I:C boosts CD8 T cells memory responses, probably mediated by type 1 IFNs and CD4 T cells [37]. The current study thus provides important rationale in choosing the appropriate type of poly I:C, long or short, while studying cell-type–specific immunomodulation or adjuvant effects.

We further observed elevated NF-κB and STAT-1 signal activations in myeloid cells after stimulation with poly I:C-S, but not poly I:C-L. These findings also show a clear link to the poly I:C-S-mediated stronger cytokine and antiviral responses in murine macrophages contrasting to the poly I:C-L. These results, in part, corroborate those in a previous study that showed no activation of NF-κB or secretion of proinflammatory cytokines induced by poly I:C-L stimulation of DCs or macrophages, but did by stimulation of endothelial and synovial fibroblasts [32]. It remains unclear, particularly in RAW264.7 cells, what other receptors or factors besides TLRs or RLRs could contribute to these differential antiviral responses.

The poly I:C-S and -L used in this study differed widely in molecular size. Therefore, we asked whether balancing lower or higher doses, depending on the molecular size, could compromise the length-dependent cytokine responses. Our data on dose response and time kinetics (Fig. 4A and B), unequivocally demonstrate that it was the length, not doses or kinetics, of poly I:C that drove the differential cytokine responses. Because the significant differences in cytokine responses to poly I:C-S and -L remained consistent from the lowest (0.1 μg/ml) to the highest (50 μg/ml) doses. We further ruled out that the striking differences in cytokine responses to poly I:C-S and -L were not due to LPS contamination, rather associated with the poly I:C length. RNAse III enzymatic complete digestion of poly I:C-L or -S totally abolished the cytokine responses. In contrast, partial digestion of poly I:C-L, to make it equivalent to the size of poly I:C-S, enabled it to induce comparable cytokine responses but significantly higher than those induced by the intact poly I:C-L. In addition, digestion of poly I:C-L with inactivated RNAse III for 15 min did not alter the cytokine response. This clearly ruled out any chance of LPS contamination of the poly I:C preparations.

Evidence suggests that the length of dsRNA determines which cellular receptors preferentially bind to a given dsRNA molecule [38, 39]. However, the biological significance of dsRNA length is not well understood. We hypothesized that the entry of poly I:C into different cell types may depend on length. Our confocal data unequivocally show that the uptake of poly I:C-S is significantly better by myeloid cells and of poly I:C-L by fibroblasts. These findings directly correlate with the cytokine and antiviral results. Although the underlying reasons for the length-dependent poly I:C uptake in different cell types is not well understood, it is clearly not due to the differences in molarity or number of molecules contained in the poly I:C, because we used a very small (nanogram) amount of poly I:C for this uptake study.

The SR-As located on the cell surface has been identified as a strong candidate receptor to mediate internalization of dsRNA, including poly I:C [21, 22]. However, these studies did not focus on length of dsRNA or cell types. We investigated whether SR-As are implicated in mediating poly I:C uptake, depending on poly I:C size and cell type. It is well known that SR-As enter cells through an endocytic pathway in the context of dsRNA [40], and SR-A-mediated gene expression is dependent on endocytosis in macrophages [41]. A more recent study revealed that SR-As mediate the entry of extracellular viral dsRNA through clathrin-induced endocytosis in MEFs [22]. However, it remains unclear whether this same phenomenon is true for macrophages, as well as the length-dependent requirement of SR-As in fibroblasts. We provide further evidence that fibroblasts require SR-A for optimal antiviral IFN response, which is ambiguous in macrophages. The complete inhibition of viral replication by $DxSO_4$, but the lack of inhibition by anti-SR-A antibody implies that other redundant SR-As contribute to the preferential poly I:C uptake in RAW264.7 cells. There could be several explanations for these SR-A-mediated effects of different lengths of poly I:C in lung fibroblasts and macrophages. DeWitte-Or et al. [22] have shown abundant expressions of transcripts for all candidate SR-As, including SR-AI and -AII; SCARA-3, -4, and -5; and MARCO in epithelial and endothelial cells. SCARA-3, -4, and -5 and MARCO, in contrast, are more known as myeloid-expressed SRs, because they are primarily, but not exclusively, expressed in myeloid cells (unpublished data). These SR-A data show a strong correlation with the antiviral and IFN-β results that we found in MEFs and further confirm the recent findings by DeWitte-Or et al. [22]. Nevertheless, it remains interesting to dissect the detail and specific role of each candidate SR-A, including MARCO, in poly I:C-L- and -S-induced intracellular antiviral signaling pathways, in both myeloid and nonmyeloid cells.

It is evident from a number of recent studies that viral dsRNAs, based on their lengths, differentially recognize TLR3, MDA-5, or RIG-I and induce various type 1 IFNs or interferon-stimulated genes in fibroblasts [4, 20]. Therefore, the length of dsRNA seems to play a crucial role in determining the cellular receptor that preferentially binds to a given dsRNA in nonprofessional immune cells. However, this phenomenon remains elusive for professional immune (myeloid) cells. We observed comparable expressions of all these dsRNA receptors in both cell types (Supplemental Fig. S3A). We assessed the dependence on these well-known dsRNA receptors of poly I:C-S and -L to induce antiviral responses by $TLR3^{-/-}$, $MDA\text{-}5^{-/-}$, and $RIG\text{-}I^{-/-}$ in MEFs. Poly I:C-L did not require RIG-I and TLR-3 receptors to induce antiviral responses, but MDA-5 was essential. In contrast, poly I:C-S was completely dependent on RIG-I and MDA-5, but only partially on TLR3, for an effective antiviral response. Therefore, fibroblasts or epithelial cells deficient in RIG-I or

TLR3 receptor could be treated with poly I:C-L, but not poly I:C -S, regimens.

Recent evidence has shown that the endosomal lipid raft protein Raftlin plays a pivotal role in the uptake and delivery of extracellular poly I:C into the cytoplasm in human DCs and epithelial cells [24]. The CD14 receptor in human epithelial cells works as a shuttle for dsRNA into the endosomal compartment [23]. However, definitive information is not available as to what receptors in myeloid cells are necessary for poly I:C-L or -S to induce cytokine and antiviral responses. We have shown, for the first time, that Raftlin is essential for poly I:-S, but not -L, uptake and entry into myeloid cells, to induce strong cytokine responses. We found that knocking down Raftlin in RAW264.7 cells markedly diminished poly I:C-S uptake and significantly reduced TNF-α production. However, blocking CD14 by mAb to anti-mouse CD14 in RAW264.7 cells had no effect on uptake or cytokine response.

Poly I:C has been widely used as a potent inducer of innate immune responses, and the findings in this study suggest that 2 fundamental factors—the length of poly I:C and the type of targeting cells—must be taken into consideration when using poly I:C to study innate immunity in vitro or in vivo and when using poly I:C as an immunomodulator or adjuvant for vaccine regimens against viral infections or cancers.

AUTHORSHIP

M.F.M. performed the experiments, analyzed the data, and wrote the paper; A.N.A. performed the experiments and analyzed the data; M.R. performed the experiments; A.B. analyzed the confocal data; D.B. provided reagents; and A.A.A. designed the experiments and wrote the paper.

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myeloid cells · fibroblasts · cytokine · antiviral · internalization