Polynosinic Acid Is a Ligand for Toll-like Receptor 3*

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Innate immune responses are critical in controlling viral infections. Viral proteins and nucleic acids have been shown to be recognized by pattern recognition receptors of the Toll-like receptor (TLR) family, triggering downstream signaling cascades that lead to cellular activation and cytokine production. Viral DNA is sensed by TLR9, and TLRs 3, 7, and 8 have been implicated in innate responses to RNA viruses by virtue of their ability to sense double-stranded (ds) RNA (TLR3) or single-stranded RNA (murine TLR7 and human TLR8). Viral and synthetic dsRNAs have also been shown to be a potent adjuvant, promoting enhanced adaptive immune responses, and this property is also dependent on their recognition by TLR3. It has recently been shown that mRNA that is largely single-stranded is a ligand for TLR3. Here we have investigated the ability of single-stranded homopolymeric nucleic acids to induce innate responses by murine immune cells. We show for the first time that polynosinic acid (poly(I)) activates B lymphocytes, dendritic cells, and macrophages and that these responses are dependent on the expression of both TLR3 and the adaptor molecule, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF). We therefore conclude that TLR3 is able to sense both single-stranded RNA and dsRNA.

Recognition of microbial pathogens is an important prerequisite for the activation of innate and adaptive immune mechanisms responsible for their elimination. The Toll-like receptors (TLRs) are a key element in this recognition, capable of detecting microbial components of both bacterial and viral origin (1). The importance of these molecules for immunity to viruses is illustrated by the fact that viruses have evolved genes that act as negative regulators of the TLR signaling pathway (2). TLRs have been implicated in the recognition of viral envelope proteins (TLR2 and TLR4) (3, 4), DNA (TLR9), dsRNA (TLR3), and ssRNA (TLR7/8). Microbial DNA containing unmethylated CpG motifs (CpG DNA) is detected by TLR9, and this TLR has been implicated in innate immunity to several viruses (5, 6). It has long been appreciated that type 1 interferons are induced by viral infection and play an important role in anti-viral responses. Double-stranded RNA and its synthetic analogue polynosinic-polycytidylic acid (poly(I:C)) are potent stimulators of type 1 IFN production (7, 8). Double-stranded RNA can be detected by TLR3 (9) and by intracellular pattern recognition receptors belonging to the retinoic acid-inducible protein (RIG)-like helicase family, and these molecules have been found to be important for effective viral immunity (6, 10, 11). TLRs 7 and 8 have been implicated in responses to single-stranded RNA viruses. Purified influenza genomic RNA and the synthetic homopolymer polyuridylic acid (poly(U)) (but not poly(A), poly(C), poly(G), or poly(I)) were shown to elicit IFN-α when transfected into plasmacytoid dendritic cells (DCs) from wild type but not TLR7-deficient mice (12), and GU-rich oligonucleotides derived from an human immunodeficiency virus RNA sequence elicited TLR8-dependent innate responses when transfected into human DC (13).

The signaling pathways activated by ligation of most TLRs utilize the adaptor molecule MyD88. However two TLRs, TLR3 and TLR4, signal through a pathway that involves an alternative adaptor molecule, TRIF (14, 15). Signaling through TLR3 exclusively involves TRIF, whereas TLR4 utilizes both MyD88 and TRIF to activate distinctive signaling cascades (16). The TLRs also differ in their subcellular localization. Most are expressed at the cell surface, but those involved in innate responses to viruses by sensing viral nucleic acids (TLRs 3, 7, 8, and 9) are localized to intracellular compartments including endosomes (1, 17, 26). It has been argued that this distribution maximizes their opportunity to intercept viral ligands while removing them from contact with cellular nucleic acids. This distribution also explains both the sensitivity of responses mediated by these TLRs to agents that prevent endosomal acidification (12, 18) and the apparent need to transfct ssRNA ligands for them to effectively induce TLR7/8-mediated responses. In contrast to ssRNA, poly(I:C) and CpG DNA can induce responses when added extracellularly; it is not clear whether this reflects some expression of TLR3 and TLR9 at the cell surface or the existence of a mechanism responsible for shuffling their respective ligands to intracellularly located TLRs.

In addition to poly(I:C) other nucleic acids including mRNAs of microbial and mammalian origin (19) and siRNA (20) have...
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also been shown to be TLR3 ligands, possibly by virtue of possession of short double-stranded sections in their secondary structure. To date, ssRNAs have only been shown to be ligands for TLRs 7 (mouse) and 8 (human). In this report we show for the first time that extracellularly presented poly(I) induces innate responses by B cells, DC, and macrophages. Our data demonstrate that these responses are dependent on TLR3, showing that this TLR can sense ssRNA as well as dsRNA.

EXPERIMENTAL PROCEDURES

Animals—BALB/c mice, mice deficient in TRIF or TLR3 (on a mixed 129/C57Bl background) and their littermate controls, and mice deficient in MyD88 or TLR4 (C57Bl/6 background) were maintained in our animal facilities. C57Bl/6 mice were obtained from Banting and Kingman. Founder animals for the knock-out strains were generously given to us by Prof. S. Akira. Unless otherwise indicated, mice were used at >8 weeks of age. All animal studies were carried out in accordance with UK Home Office regulations for animal care and use.

Antibodies and Reagents—We purchased lipopolysaccharide (LPS) from Escherichia coli 055 B5, poly(I), poly(C), poly(G), poly(U), poly(I:C), dextran sulfate, and chloroquine from Sigma, peptidoglycan (Staphylococcus aureus), PGN from Fluka, and CpG containing oligodeoxynucleotide 1826 (CpG DNA) from MWG. Antibodies to CD86 (a gift from Dr. D. Flaherty), CD40 (a gift from Dr. J. Andersen), and major histocompatibility complex II were coupled with fluorescein isothiocyanate or biotin by standard methods.

Preparation of B Lymphocytes and Analysis of Responses—B lymphocytes were purified from the spleens of wild type or deficient mice by treatment with anti-Thy1 antibody and complement as described (21). Unless otherwise indicated in the text, experiments were performed on a pool of cells generated from at least two mice.

For analysis of modulation of co-stimulatory molecules, B cells were cultured with various stimuli at 1–2 × 10⁶ cells/ml in RPMI/10% fetal calf serum for 24 or 48 h before being stained with appropriate antibodies. Expression of co-stimulatory molecules was analyzed by flow cytometry using a FACScan cytometer. Proliferation was assessed by culturing purified B cells (2 × 10⁶/well) with or without stimuli for 48 h, pulsed with [³H]thymidine (0.5 μCi/well) for the last 6 h of culture, and measuring uptake by liquid scintillation spectroscopy. In some experiments supernatants of stimulated B cells were collected for analysis of interferon production.

Preparation of Dendritic Cells and Macrophages and Analysis of Responses—Dendritic cells were derived by culturing murine bone marrow cells at 5 × 10⁵/ml in RPMI/10% fetal calf serum in the presence of 20% v/v of supernatant from J558 cells transfected with the mGM-CSF gene (a gift from Dr. B. Stockinger). After 7 days non-adherent cells were collected, washed, and cultured at 2 × 10⁶/ml in 24-, 48-, or 96-well plates with indicated stimuli for 24 h. After incubation, supernatants were collected and stored at −20 °C for cytokine analysis. Cells were detached by a brief treatment with phosphate-buffered saline/2 mM EDTA, washed, stained with various antibodies, and analyzed by flow cytometry.

Macrophages were derived by culturing bone marrow cells at 5 × 10⁶ cells/ml in Dulbecco’s modified Eagle’s medium/10% fetal calf serum supplemented with 20% v/v of macrophage colony-stimulating factor (M-CSF) containing supernatant from LADMAC cells (a gift from Dr. S. Hou). After 6 days macrophages were detached using phosphate-buffered saline/EDTA, harvested, washed, and recultured with stimuli as described. Secretion of cytokines and up-regulation of co-stimulatory molecules were analyzed as described.

Culture of Bronchiolar Epithelial Cells—Cells of the BEAS-2B line, obtained from the European Collection of Cell Cultures, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v fetal calf serum. For experiments, cells were harvested from culture flasks using trypsin/EDTA (Sigma), washed, plated in 24 plates at 5 × 10⁴/well, and cultured overnight before the addition of stimuli as described. After 20 h cell-free supernatants were collected and stored at −20 °C until used.

Use of Transfected HEK 293 Cells—HEK 293 cells stably transfected with huTLRs, kind gifts from Dr. K. Fitzgerald (HEK-TLR3 and 4 cells) and Dr. S. Paludan (HEK-TLR8 cells), were seeded into 96-well plates (2 × 10⁴ cells/well) and transfected the next day with expression vectors for a κB-luciferase reporter gene and Renilla luciferase internal control as described previously (22). On the following day cells were stimulated as described and reporter gene activity assessed 6 h later. Data are expressed as mean fold induction ± S.D. relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Cytokine/Chemokine Analysis—Production of IL-12, TNFα, and IP-10 was assessed using enzyme-linked immunosorbent assays according to the manufacturer’s instructions. Kits for TNF and IL-12 were from Caltag Medsysystems and that for IP-10 was from R&D. Type 1 interferon was detected using a bioassay as previously described (23). IFNα was detected using an ELISA kit from PBL Laboratories according to the manufacturer’s instructions.

RESULTS

Both viral RNA and synthetic single-stranded poly(U), (but not poly(I), poly(A), or poly(C)), have been shown to induce innate responses when transfected into plasmacytoid DC but were without effect when presented extracellularly in the absence of the transfection reagent (12). We assessed the effects of homopolymeric nucleic acids on B cells by culturing them with poly(I), poly (U), or known ligands for TLRs 3 and 4 and analyzing the expression of co-stimulatory molecules. As expected, poly(I:C) and LPS induced marked up-regulation of major histocompatibility complex II, CD86, and CD40. Culture with poly(I) also induced up-regulation of these molecules, but poly(U) was ineffective (Fig. 1A). Culture with single-stranded poly(I) also induced the proliferation of murine B cells as did poly(I:C) and LPS, but poly(U) failed to induce this response (Fig. 1B). These data suggest that, unlike plasmacytoid DC, murine B cells are able to sense extracellular ssRNA and that the molecule or molecules responsible have a different specificity from TLR7 that senses ssRNA transfected intracellularly.

To examine whether this difference was related to cell type we examined the responses of bone marrow-derived dendritic
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The sensing of transfected ssRNA by TLR7 in murine plasmacytoid DC.

It is well established that polyanions, including poly(I), are ligands for class A scavenger receptors (SR-A) (34). Although SR-A ligation has not been shown to lead to innate responses it does trigger receptor-mediated endocytosis. It was therefore possible that responses to poly(I) were dependent on SR-A. To assess this we investigated the effects of other known SR-A ligands on B cell and BMDC responses. We found that neither B cells nor BMDC were activated by either poly(G) or dextran sulfate (data not shown), indicating that responses to poly(I) are not directly mediated by SR-A.

Given the roles of TLR3, TLR7, and TLR9 in sensing microbial nucleic acids, it seemed likely that a TLR family member was responsible for mediating the observed responses to poly(I). To investigate this we examined the responses of B cells and DC from animals deficient in MyD88, the adaptor molecule used by all TLRs except TLR3. We also investigated the responses of cells from TLR4-deficient mice to assess the possible role of TLR4 and to exclude the possibility that the responses seen resulted from endotoxin contamination of the RNA preparations used. The data show that induction of up-regulation of co-stimulatory molecules and proliferation induced by LPS were abolished in MyD88−/− B cells (Fig. 3, A–C). In contrast, responses to poly(I:C) and poly(I) were MyD88-independent. Neither poly(U) nor poly(C) induced these responses. As anticipated, B cells from TLR4−/− mice failed to respond to LPS. However, these cells did respond effectively to stimulation with both poly(I:C) and poly(I).

The responses of BMDC from MyD88−/− and TLR4-deficient mice were also analyzed (Fig. 3D). BMDC lacking TLR4 failed to respond to LPS but responded as effectively as wild type BMDC to both poly(I:C) and poly(I). Deficiency of MyD88 did not affect the ability of LPS, poly(I:C), or poly(I) to induce the up-regulation of CD86 by BMDC, showing that LPS responses of B cells and BMDC are differentially dependent on this adaptor. These data effectively exclude the participation of TLR4 and the MyD88 adaptor molecule in responses to both poly(I:C) and poly(I). They also show that the responses induced by poly(I) are not the result of contamination of the nucleotide preparations with bacterial endotoxin.

The data therefore appeared to suggest that, like poly(I:C), poly(I) could be a ligand for TLR3. To assess this possibility we...
examined the ability of bronchiolar epithelial cells (BEAS-2B) that preferentially respond to TLR3 ligation to secrete the chemokine IP-10. Culture of BEAS-2B cells with poly(I:C) or poly(I) induced IP-10 secretion, but stimulation with LPS and peptidoglycan (a TLR2 ligand) was ineffective (Fig. 4A). Ligands for murine TLRs 7 and 9 also failed to induce this response (data not shown). These results were consistent with the idea that poly(I) could be a ligand for TLR3 and further excluded any involvement of possible LPS contamination. A characteristic of TLR3 responses is that they are sensitive to inhibition by agents that disrupt endosomal acidification. We therefore examined the effects of chloroquine on B cell responses to nucleic acids and LPS. B cell proliferation induced by CpG DNA was highly sensitive to this agent, and responses to both poly(I:C) and poly(I) were significantly inhibited although LPS responses were unaffected (Fig. 4B).

Signaling via TLR3 has been shown to be exclusively dependent on the adaptor molecule TRIF, whereas TLR4 utilizes both MyD88 and TRIF adaptors. The involvement of TRIF in responses to poly(I) was assessed using B cells and BMDC from TRIF-deficient mice. B cells from TRIF−/− mice cultured with CpG DNA up-regulated CD86 as effectively as cells from wild type mice but showed an impaired response to LPS. As expected, TRIF-deficient B cells failed to up-regulate CD86 when stimulated with poly(I:C), and their response to poly(I) stimulation was also drastically impaired (Fig. 5A). When proliferative responses were assessed, B cells from TRIF-deficient mice showed markedly reduced responses to LPS, poly(I:C), and poly(I) whereas the response to CpG DNA was unaffected (Fig. 5B). Thus in B cells double-stranded poly(I:C) and single-stranded poly(I) both signal via a pathway that involves TRIF, but not MyD88, whereas LPS responses are dependent on both adaptors.
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When responses of BMDC were examined (Fig. 5C), cells from TLR3−/− mice showed impaired responses to LPS, poly(I:C), and poly(I) but responses to CpG DNA were equivalent to those made by BMDC from wild type animals. The data therefore show that responses to both poly(I:C) and poly(I) were exclusively dependent on the TRIF adaptor and those induced by the TLR9 ligand were exclusively dependent on MyD88. Although B cell responses to LPS required both adaptors, MyD88 was not required for up-regulation of CD86 by BMDC. These findings strongly supported a role for TLR3 in the recognition of single-stranded poly(I).

To confirm this directly, we examined the responses of HEK 293 cells transfected with vectors encoding human TLRs 3, 4, and 8 and co-transfected with an NFκB reporter construct. The data (Fig. 6A) show that HEK 293 cells transfected with huTLRs 4 and 8 responded only to their expected ligands, LPS and R848. Cells transfected with huTLR3 responded as expected to double-stranded poly(I:C) but not to TLR4 or 8 ligands. Responses to poly(I) were only seen in HEK cells transfected with huTLR3. These findings provide evidence that TLR3 is indeed involved in sensing single-stranded poly(I).

To confirm that, we examined the responses of B cells and BMDC from mice deficient in TLR3. B cells from TLR3−/− mice showed defective up-regulation of CD86 in response to poly(I:C) and poly(I) (Fig. 6B), but their responses to LPS and CpG DNA were unaffected. Similarly, TLR3−/− BMDC were unable to effectively up-regulate CD86 in response to poly(I:C) and poly(I) but responded normally to the TLR4 ligand LPS (Fig. 6C). TLR3-deficient BMDC also failed to secrete type 1 interferons when cultured with poly(I:C) or poly(I) (Fig. 6, D and E). Our data therefore provide the first evidence that single-stranded poly(I) induces innate responses in B cells, macrophages, and BMDC and that these responses are mediated by the TLR family member TLR3.

DISCUSSION

Recognition of microbial nucleic acids is an important function of the innate immune system, playing a critical role in protection against viral infection. Members of the TLR family have
been identified as key elements in the recognition of nucleic acid motifs. To date, it has been widely accepted that TLR9 is involved in sensing microbial DNA and TLR3 plays a vital role in detecting double-stranded RNA. Recently, TLRs 7 and 8 have been implicated in the intracellular recognition of synthetic and viral ssRNA. A role for TLR3 in sensing microbial mRNA has also been demonstrated. Here we show for the first time that murine B cells and BMDC can sense single-stranded polyinosinic acid using TLR3. Recognition of poly(I) triggers similar responses to those induced by the classical TLR3 agonist poly(I:C).

Evidence for the involvement of TLRs 7 (mice) and 8 (human) in sensing ssRNA is based on experiments in which plasmacytoid DC were transfected with viral RNAs, viral RNA sequences, or synthetic single-stranded homo- or heteropolymeric RNAs (12, 13). We set out to investigate the responses of B cells and conventional BMDC to homopolymeric RNAs presented extracellularly, the normal route by which responses to dsRNAs and CpG DNA have been examined. We found that B cells, macrophages, and BMDC responded to poly(I) but not to poly(U). This was in contrast to the TLR7- and MyD88-dependent responses of B cells and BMDC to poly(I:C).
FIGURE 6. TLR-3 is required for responses to poly(I). A, HEK293 cells transfected with the indicated TLR expression vectors and NFκB reporter were cultured with the indicated stimuli (LPS, 200 ng/ml; poly(I)/C) and responses analyzed as described. B, B cells from TLR3+/+ and TLR3−/− mice, as indicated, were cultured with medium or the indicated stimuli (concentrations as in Fig. 1 legend) for 20 h before assessing expression of CD86. Data shown are mean ± S.D. of fold increase in mean fluorescence compared with unstimulated control cells (MFIs: TLR3+/+, 15 ± 1; TRIF−/−, 14 ± 0.5) and were pooled from four experiments, each involving at least two mice of each genotype. C, BMDC generated from TLR3+/+ and TLR3−/− bone marrow were cultured with medium or the indicated stimuli (concentrations as above) for 20 h before assessing expression of CD86. Data shown are mean ± S.D. of fold increase in mean fluorescence compared with unstimulated control cells (MFIs: TLR3+/+, 76 ± 12; TRIF−/−, 65 ± 9) and were pooled from three experiments, each involving at least two mice of each genotype. D, BMDC generated from TLR3+/+ and TLR3−/− bone marrow were cultured with medium, LPS (1 μg/ml), poly(I/C), or poly(I) as indicated (μg/ml) for 20 h before assessing type 1 IFN secretion by bioassay. Data shown are the mean ± S.D. of triplicate wells and are representative of two independent experiments. E, BMDC generated from TLR3+/+ and TLR3−/− bone marrow were cultured with medium, LPS (1 μg/ml), poly(I/C), or poly(I) as indicated (μg/ml) for 20 h before assessing IFNα secretion by ELISA. Data shown are the mean ± S.D. of triplicate wells.
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Dependent responses of plasmacytoid DC that were elicited by poly(U), but not poly(I) (12, 18), suggesting that TLR7 might not be involved. This was confirmed by our finding that B cell and BMDC responses to poly(I) were not dependent on MyD88, the adaptor used by TLRs 7 and 8. Thus, if a TLR were involved in sensing poly(I) it was likely TLR3 or 4 because only these can signal independently of MyD88. Cells deficient in TLR4 were responsive to poly(I), excluding it as the responsible pattern recognition receptor. Our data showed that poly(I) responses of both B cells and BMDC were dependent on the TRIF adaptor. This, taken together with the chloroquine sensitivity of the responses and the fact that HEK cells transfected with huTLR3 (but not TLRs 4 or 8) were responsive, strongly suggested that poly(I) is sensed by TLR3. This was confirmed using TLR3-deficient B cells and BMDC.

These differences in the sensing of homopolymeric RNAs could be due to the mode of delivery or to differences related to the cell types involved. Interestingly, it has recently been demonstrated that extracellularly presented dsRNA fragments of Newcastle disease virus induced IFNα production by human plasmacytoid DC but not myeloid DC (mDC) whereas poly(I:C) demonstrated that extracellularly presented dsRNA fragments of Newcastle disease virus induced IFNα production by human plasmacytoid DC but not myeloid DC (mDC) whereas poly(I:C) demonstrated that extracellularly presented dsRNA fragments of Newcastle disease virus induced IFNα production by human plasmacytoid DC but not myeloid DC (mDC) whereas poly(I:C) was likely TLR3 or 4 because only these can signal independently of MyD88. Cells deficient in TLR4 were responsive to poly(I), excluding it as the responsible pattern recognition receptor. Our data showed that poly(I) responses of both B cells and BMDC were dependent on the TRIF adaptor. This, taken together with the chloroquine sensitivity of the responses and the fact that HEK cells transfected with huTLR3 (but not TLRs 4 or 8) were responsive, strongly suggested that poly(I) is sensed by TLR3. This was confirmed using TLR3-deficient B cells and BMDC.

In a previous report poly(I) was found to induce TNF and NO production by the RAW264.7 macrophage cell line (27). The authors concluded that these responses were mediated by SR, based on the ability of dextran sulfate to inhibit poly(I)-induced activation. It seems unlikely, however, that SRs directly mediate the responses we observed because SRs have not been shown to be linked to signaling pathways that result in cytokine production and up-regulation of co-stimulatory molecules. Furthermore, we found that SR-A ligands other than poly(I) failed to induce innate responses in B cells and BMDC.

Our findings therefore provide novel evidence that extracellular poly(I) induces innate responses by the TLR3-TRIF pathway.

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