TLR3 Is Essential for the Induction of Protective Immunity against Punta Toro Virus Infection by the Double-Stranded RNA (dsRNA), Poly(I:C 12U), but not Poly(I:C): Differential Recognition of Synthetic dsRNA Molecules


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TLR3 Is Essential for the Induction of Protective Immunity against Punta Toro Virus Infection by the Double-Stranded RNA (dsRNA), Poly(I:C_{12U}), but not Poly(I:C): Differential Recognition of Synthetic dsRNA Molecules


In the wake of RNA virus infections, dsRNA intermediates are often generated. These viral pathogen-associated molecular patterns can be sensed by a growing number of host cell cytosolic proteins and TLR3, which contribute to the induction of antiviral defenses. Recent evidence indicates that melanoma differentiation-associated gene-5 is the prominent host component mediating IFN production after exposure to the dsRNA analog, poly(I:C). We have previously reported that Punta Toro virus (PTV) infection in mice is exquisitely sensitive to treatment with poly(I:C_{12U}), a dsRNA analog that has a superior safety profile while maintaining the beneficial activity of the parental poly(I:C) in the induction of innate immune responses. The precise host factor(s) mediating protective immunity following its administration remain to be elucidated. To assess the role of TLR3 in this process, mice lacking the receptor were used to investigate the induction of protective immunity, type I IFNs, and IL-6 following treatment. Unlike wild-type mice, those lacking TLR3 were not protected against PTV infection following poly(I:C_{12U}) therapy and failed to produce IFN-α, IFN-β, and IL-6. In contrast, poly(I:C) treatment significantly protected TLR3^{−/−} mice from lethal challenge despite some deficiencies in cytokine induction. There was no indication that the lack of protection was due to the fact that TLR3-deficient mice had a reduced capacity to fight infection because they were not found to be more susceptible to PTV. We conclude that TLR3 is essential to the induction of antiviral activity elicited by poly(I:C_{12U}), which does not appear to be recognized by the cytosolic sensor of poly(I:C), melanoma differentiation-associated gene-5. The Journal of Immunology, 2007, 178: 5200–5208.

Punta Toro virus (PTV) is phylogenetically closely related to Rift Valley fever and sandfly fever viruses, the only members of the Phlebovirus genus of the Bunyaviridae family of viruses associated with significant human morbidity and mortality (1). PTV is endemic in rural areas of Panama with seroconversion rates of up to 35% previously documented (2). Unlike with the highly pathogenic phleboviruses, human infection with PTV produces disease generally limited to a mild febrile illness. Infection models in small rodents have been described that produce acute disease with hepatic involvement similar to that observed in cases of Rift Valley fever in humans and domesticated ungulates. Several groups have described the susceptibility of hamsters to severe disease induced by PTV infection (2, 3). Pifat and Smith initially described the mouse model of phleboviral disease and assessed the susceptibility of various strains of mice to PTV infection (4). The availability of these rodent models makes PTV a viable alternative to the use of Rift Valley fever virus for antiviral studies because the latter is highly restricted and requires high-level containment facilities. To that end, numerous evaluations of promising antivirals have been conducted using the PTV models of acute phlebovirus-induced disease (5–12). Moreover, several large studies have involved the evaluation of immune modulators and have demonstrated that the PTV is acutely sensitive to IFN inducers (5, 10). The importance of type I IFN is borne out in the mouse PTV infection model. Treatment with neutralizing Abs to IFN-α/IFN-β completely abolishes resistance to infection reported in adult mice (4). Potent type I IFN-inducers in the form of dsRNA poly(I:C) and poly(I:C_{12U}) have consistently proven to be highly effective in protecting mice from lethal PTV challenge.

Poly(I:C) was originally identified by investigators at Merck as an IFN inducer before the cloning of the human IFNs (13). A variety of synthetic and natural dsRNAs were effective inducers of IFN in tissue culture and rodents with poly(I:C) being the most potent. The elements required for the induction of IFN in vivo is a stable double-stranded polynucleotide at physiological temperatures with a ribose backbone and a minimum molecular mass of ~2.7 × 10^{5} Da (14). Poly(I:C_{12U}) was derived by investigators at Johns Hopkins University as a nontoxic analog with similar IFN

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3 Abbreviations used in this paper: PTV, Punta Toro virus; ALT, alanine aminotransferase; CLDC, cationic liposome-DNA complex; mda-5, melanoma differentiation-associated gene-5; rEA, recombinant Eimeria protosozan Ag; RIG-I, retinoic acid-induced protein-I; TRIF, Toll/IL-1R domain-containing adaptor; CCID_{50}, 50% cell culture infectious dose.

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induction capacities as the parent compound (15). As an inducer of IFN, poly(I:C12U) has potent antiviral and immunomodulatory properties. This synthetic, dsRNA polymer consists of a single strand of polyriboinosine containing a uridine residue statistically at every 13th monomer (poly C12U) in a RNA polymeric linkage. The introduction of uridine provides a site in which the hydrogen bonds involved in chain association with inosine are not available. This specific configuration provides a thermodynamically unstable locus in poly(I:C12U) that presents an initial site for endoribonucleolytic enzyme-catalyzed hydrolysis. The lack of poly(I:C12U) toxicity as compared with its parent dsRNA, poly(I:C), has been linked to this single modification.

There is accumulating evidence that two pathways are involved in activation events resulting from exposure to dsRNA, a replication intermediate of many RNA viruses (16). In addition to the TLR3 response pathway (17), a TLR3-independent pathway mediated by RNA helicase cytoplasmic sensors that contain caspase-recruiting domains has been uncovered recently (18, 19). Signaling by these dsRNA sensors occurs through distinct pathways that converge to share various kinases and transcriptional factors that regulate the production of IFN-β, a critical factor in regulating antiviral immunity (20). Due to its endosomal restriction (21), TLR3 is likely involved in the recognition of dsRNA that is internalized via the phagocytic process of virally infected cells. The cytosolic RNA helicase dsRNA detectors, retinoic acid-induced protein-I (RIG-I) and melanoma differentiation-associated gene-5 (mda-5), can sense viral infection within the cell. Recent evidence suggests that mda-5 plays a dominant role over TLR3 and RIG-I in the type I IFN response to poly(I:C) (22, 23). In this study, we present results demonstrating the essential role of TLR3 in the induction of protective immunity by the mismatched dsRNA, poly(I:C12U).

Materials and Methods

Mice

TLR3−/− mice were derived and backcrossed onto a C57BL/6 background at Yale University (17). A breeding colony was established and housed in the animal facility at Utah State University under specific pathogen-free conditions. C57BL/6 mice (wild-type) were obtained from The Jackson Laboratory. Carefully age- and gender-matched mice were used in all experiments. All animal procedures used in these studies complied with guidelines set by the U.S. Department of Agriculture and Utah State University Animal Care and Use Committee.

Test materials

Poly(I:C12U), trade name, Ampligen, was provided by HEMISPHERx Biopharma at a concentration of 2.4 mg/ml. Poly(I:C) was obtained from Amershams Biosciences. Both were prepared for injection in sterile saline. Materials to generate cationic lipidosome-DNA complexes (CLDC) were provided by Juvaris BioTherapeutics. Liposomes, DNA, and the preparation of CLDC for injection have been described previously (12). Recombinant Eimeria protozoan Ag (rEA) was provided by Barros Research Institute. Ribavirin was supplied by ICN Pharmaceuticals.

Effect of poly(I:C12U) treatment on PTV infection and disease outcome in 3- to 4-wk-old mice

Table I. Effect of poly(I:C12U) treatment on PTV infection and disease outcome in 3- to 4-wk-old mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatmenta</th>
<th>Alive/Total</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>Log-Rank Probability &gt; χ²</th>
<th>ALT−d ± SD</th>
<th>Liver Score−e ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Poly(I:C12U), 10 µg</td>
<td>10/10***</td>
<td>&lt;0.0001</td>
<td>19 ± 17**</td>
<td>0.2 ± 0.3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly(I:C12U), 1 µg</td>
<td>5/10**</td>
<td>5.0 ± 1.7</td>
<td>4–8</td>
<td>0.0022</td>
<td>906 ± 909</td>
<td>1.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Poly(I:C12U), 0.1 µg</td>
<td>0/10</td>
<td>4.4 ± 0.5</td>
<td>4–5</td>
<td>0.3246</td>
<td>1565 ± 872</td>
<td>1.8 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Ribavirin, 75 mg/kg</td>
<td>10/10***</td>
<td>4.9 ± 1.4</td>
<td>4–8</td>
<td>&lt;0.0001</td>
<td>14 ± 6**</td>
<td>0.0 ± 0.0*</td>
<td></td>
</tr>
<tr>
<td>Sterile saline</td>
<td>2/22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1528 ± 692</td>
<td>1.2 ± 1.1</td>
</tr>
</tbody>
</table>

**Singledose poly(I:C12U) and saline treatments administered i.p. 24 h postvirus challenge. Ribavirin given i.p. twice per day for 5 days beginning 4 h previrus challenge.**

*Mean and range day of death of mice dying before day 21.

† Determined on day 3 of infection; five to six mice per group.

‡ Measured in international units per liter.

§ Score of 0 (normal liver) to 4 (maximal discoloration).

* p < 0.05; ** p < 0.01; *** p < 0.001 compared with saline-treated control.

Evaluation of dsRNAs in TLR3−/− and wild-type mice infected with PTV

PTV, Adams strain, was obtained from Dr. D. Pifat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). Virus stocks were prepared following four passages of the original virus stock through LLC-MK2 cells (American Type Culture Collection). Weaning 3- to 4-wk-old TLR3+/− and C57BL/6 mice were inoculated by s.c. injection with 1.3 × 105 50% cell culture infectious doses (CCID50) of PTV. Single doses of dsRNAs or other immunostimulatory materials were administered i.p. 4 h pre- or 24 h postinfectious challenge, as indicated in the table footnotes. A ribavirin treatment group was also included in several experiments for comparison. The mice in each group were observed for death out to 21 days. When possible, additional mice (n = 5) were included and sacrificed on day 3 of infection for virus titer determination and liver disease analysis. Livers were scored on a scale of 0–4 for hepatic icterus, with 0 being normal and 4 being maximal yellow discoloration. Serum alanine aminotransferase (ALT) activity was determined using the ALT (SGPT) Reagent Set purchased from Pointe Scientific.

A temporal study was conducted to compare systemic and liver virus loads, hepatic discoloration, and ALT levels in infected TLR3−/− and wild-type mice treated with poly(I:C12U). Groups of 8-wk-old mice (n = 5) were sacrificed for sample collection on days 2, 3, 4, or 5 of infection following therapeutic intervention with poly(I:C12U) or saline. Serum was collected at the indicated times for the analysis of type I IFN levels and IL-6 release. IFN-α levels were measured using ELISA reagents from PBL as specified by the manufacturer. IL-6 was detected using the IL-6 Ready-SET-Go ELISA kit from eBioscience.

Statistical analysis

Log-rank analysis was used to evaluate differences in survival data. The Fisher’s exact test (two-tailed) was used for evaluating differences in total survivors. The Mann-Whitney U test (two-tailed) was performed to analyze the differences in mean day to death, virus titers, serum ALT, and cytokine levels. Wilcoxon ranked sum analysis was used for mean liver score comparisons.
Table II. CLDC, but not mismatched dsRNA poly(I:C12U), elicits protective immunity to PTV infection in 3- to 4-wk-old mice lacking of TLR3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Alive/Total</th>
<th>Day of Deatha</th>
<th>Log-Rank Probability &gt; χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR3−/−</td>
<td>Poly(I:C12U), 10 μg</td>
<td>0/9</td>
<td>4.1 ± 0.3</td>
<td>0.6775</td>
</tr>
<tr>
<td></td>
<td>CLDC, 1 μg</td>
<td>5/8**</td>
<td>3.7 ± 0.6</td>
<td>0.0163</td>
</tr>
<tr>
<td></td>
<td>Ribavirin, 75 mg/kg/day</td>
<td>6/8**</td>
<td>6.0 ± 2.8</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Sterile saline</td>
<td>0/9</td>
<td>4.2 ± 1.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Wild type</td>
<td>Poly(I:C12U), 10 μg</td>
<td>10/10***</td>
<td>4.5 ± 0.7</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>CLDC, 1 μg</td>
<td>10/10***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ribavirin, 75 mg/kg</td>
<td>10/10***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sterile saline</td>
<td>1/11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Single-dose poly(I:C12U), CLDC and saline treatments administered i.p. 24 h post virus challenge. Ribavirin given i.p. (twice per day) for 5 days beginning 4 h previrus challenge.

b Mean and range day of death of mice dying before day 21.

c *** p < 0.001 compared with respective saline-treated controls.

d alive/total alive.

e Probability

f Log-Rank

TLR3−/− mice treated with poly(I:C12U) (Table II). In contrast, five of eight mice stimulated with CLDC, which likely act primarily via TLR9 recognition of CpG motifs present in the plasmid DNA backbone (25), survived the infection. In the wild-type mice, both the poly(I: C12U) and CLDC protected 100% of the mice (Table II), verifying that the immunomodulatory drug preparations were highly active. Ribavirin treatment was also included as an additional positive control because it routinely protects ≥90% of wild-type mice from lethal PTV challenge. Notably, ribavirin only protected 75% (six of eight) of the TLR3−/− mice from death in this experiment, whereas complete protection was observed in wild-type animals (Table II). This may have been due to the slightly smaller size of the TLR3−/− mice used (~3 wk of age) compared with the wild-type mice (~3–4 wk of age). Alternatively, the TLR3 deletion may reduce the capacity of these mice to limit the infection and combat the disease. Notwithstanding, both CLDCs and ribavirin significantly improved survival outcome.

In a similar experiment, mice were treated 4 h before virus challenge, and five extra mice per group were included for sacrifice on day 3 of infection to assess differences in liver disease as a consequence of PTV infection. In addition, more rigorous interstrain age matching of the mice (all ~4 wk of age) was implemented. As shown in Table III, poly(I:C12U) failed again to protect TLR3−/− mice from a highly lethal dose of virus and was ineffective at limiting liver disease as reflected by elevated levels of serum ALT and high liver scores. Conversely, rEA, the positive control immune modulator that acts through TLR11 in mice (11), was highly effective at protecting mice from death and significantly reducing liver scores.

Table III. TLR11 agonist, rEA, but not mismatched dsRNA poly(I:C12U), protects 4-wk-old TLR3-deficient mice from lethal PTV disease

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Alive/Total</th>
<th>Day of Deatha</th>
<th>Log-Rank Probability &gt; χ²</th>
<th>ALT−d ± SD</th>
<th>Liver Score−e ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR3−/−</td>
<td>Poly(I:C12U), 10 μg</td>
<td>0/10</td>
<td>4.1 ± 0.6</td>
<td>0.4861</td>
<td>2700 ± 1576</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>rEA, 1 μg</td>
<td>10/10***</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>155 ± 77**</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Sterile saline</td>
<td>1/10</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>3837 ± 234</td>
<td>3.5 ± 0.0</td>
</tr>
<tr>
<td>Wild type</td>
<td>Poly(I:C12U), 10 μg</td>
<td>10/10***</td>
<td>4.1 ± 0.6</td>
<td>&lt;0.0001</td>
<td>3 ± 6**</td>
<td>0.6 ± 0.2**</td>
</tr>
<tr>
<td>rEA, 1 μg</td>
<td>10/10***</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>93 ± 56**</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Sterile saline</td>
<td>1/20</td>
<td></td>
<td>4.8 ± 1.1</td>
<td>3–7</td>
<td>3650 ± 822</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

a Single-dose poly(I:C12U), rEA, and saline treatments administered i.p. 4 h previrus challenge.

b Mean and range day of death of mice dying before day 21.

c Determined on day 3 of infection; four to five mice per group.

d Measured in international units per liter.

e Score of 0 (normal liver) to 4 (maximal discoloration).

f * p < 0.05; **, p < 0.01; ***, p < 0.001 compared with respective saline-treated controls.
As expected, treatment of wild-type mice with poly(I:C12U) and rEA elicited 100% protection against the lethal challenge inoculum (Table III). Interestingly, poly(I:C12U), known to induce type I IFN (10), dramatically abrogated hepatic icterus, whereas rEA, which has not been shown to induce type I IFN (11, 26), did not reduce mean liver scores in either mouse strain. There were no significant differences when comparing the TLR3−/− and wild-type saline-treated placebo and rEA treatment groups, suggesting that both strains were equally susceptible to PTV infection and responded similarly to rEA.

**TLR3-deficient mice fail to reduce disease severity and viral load in response to poly(I:C12U)**

We have recently shown that PTV infection can be lethal in older C57BL/6 mice (27). Mortality, however, can be significantly reduced by limiting the handling of 8-wk-old animals following PTV challenge (B. B. Gowen, unpublished data). Thus, to facilitate sample collection during peak infection times, we used older animals to evaluate virologic, clinical, and pathologic disease parameters temporally during the course of infection to further investigate the contribution of TLR3 to the protective effect of poly(I:C12U) immunotherapy. As seen in Fig. 1, A and B, remarkable levels of ALT were not present until day 3 of infection in the TLR3−/− and wild-type saline-treated placebo and rEA treatment groups, suggesting that both strains were equally susceptible to PTV infection and responded similarly to rEA.

![FIGURE 1. Poly(I:C12U) treatment limits liver disease and systemic virus burden in wild-type but not TLR3−/− mice. Groups of 8-wk-old TLR3−/− (A, C, E, and G) and wild-type (B, D, F, and H) mice were challenged with PTV and treated i.p. with 10 μg of poly(I:C12U) or saline 24 h after infection. Mean serum ALT levels (A and B), liver scores (C and D), liver virus titers (E and F), and serum virus titers (G and H) for samples collected on the indicated days postvirus inoculation. The data points represent the means and SDs of five animals per group and are representative of two similar experiments. *p < 0.05, and **p < 0.01, compared with saline-treated controls.](http://www.jimmunol.org/)

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In concordance with the liver dysfunction indicated by the ALT values, a significant reduction in hepatic icterus compared with the saline control treatment on days 4 and 5 was only demonstrated in the wild-type mice treated with poly(I:C12U). Again, the suggestion of greater liver disease in the wild-type mice was observed as they had higher day 4 mean liver scores compared with the TLR3−/− mice (3.7 ± 0.3 and 3.4 ± 0.4, respectively). Consistent with the lack of protection seen in the previous challenge studies (Tables II and III), the data indicate that TLR3 plays a vital role in limiting disease severity associated with PTV infection following poly(I:C12U) treatment.

The control of liver and systemic viral burden during the course of infection following poly(I:C12U) or saline treatment was also examined. Unexpectedly, we did not find any appreciable differences in liver viral loads, in part, due to the high degree of variability seen with the wild-type mice (Fig. 1, E and F). The mean titers were lower on days 2 and 3 in the poly(I:C12U)-treated wild-type mice but not statistically significant as demonstrated with serum ALT levels and liver scores. Notably, in contrast to their TLR3−/− counterparts, virus was unexpectedly detected as early as 100 μg of poly(I:C12U), and systemic levels of IFN-β were determined after 3 h of exposure (B). Groups of four to six mice were treated with 100- or 10-μg quantities of poly(I:C12U) or poly(I:C), and serum IFN-β, IFN-α, and IL-6 levels were assessed following a 3-h exposure period (C). Each data point represents the level of cytokine for a single mouse. *, p < 0.05, and **, p < 0.01, compared with TLR3−/− mice.

TLR3-deficient mice fail to produce type I IFNs and IL-6 in response to poly(I:C12U)

The dsRNA, poly(I:C), is a potent inducer of IFN-β, a critical factor in the establishment of host antiviral defenses. To examine whether lack of functional TLR3 alters the IFN-β response profile to mismatched dsRNA, groups of wild-type and TLR3−/− mice were treated with the 10-μg poly(I:C12U) dose used in the PTV challenge experiments, and systemic IFN-β release was determined at various time points. Following a 1.5-h exposure period, an increase in IFN-β levels was observed in wild-type mice compared with the TLR3−/− mice (Fig. 2A). At the 3-h time point, mean IFN-β levels peaked in the wild-type mice while remaining at basal levels in the TLR3−/− mice. By 6 h, IFN-β levels had returned to baseline in the wild-type mice (Fig. 2A). There was no appreciable increase of IFN-β detected at any of the time points evaluated for the TLR3−/− mice. The inability of TLR3-deficient animals to mount an IFN-β response to poly(I:C12U) likely factors in their failure to overcome PTV infection despite treatment proven effective in wild-type mice. The data suggest that the low...
to moderate levels of IFN-β induced by the 10-µg i.p. dose of poly(I:C12U) are sufficient to provide adequate protection against PTV challenge in wild-type mice.

Several recent reports have demonstrated that mice lacking TLR3 or its Toll/IL-1R domain-containing adaptor, TRIF, have no deficits in their ability to respond to the related dsRNA, poly(I:C), yet poly(I:C) elicits higher levels of this type I IFN at both the high and low doses in the wild-type mice compared with the TLR3<sup>−/−</sup> mice (Fig. 2C). Only two of the six wild-type animals mounted an appreciable IFN-α response to poly(I:C12U). In contrast, a robust IL-6 response was observed in all of the wild-type animals dosed with 100 µg of poly(I:C12U), but only partial, low-level induction was seen with the 10-µg amount. As with the type I IFNs, the TLR3<sup>−/−</sup> mice failed to respond to either poly(I:C12U) dosing (Fig. 2C). The IL-6 release following exposure to poly(I:C) was extremely variable in most of the wild-type mice. As seen with IFN-α, a defect in IL-6 release in TLR3<sup>−/−</sup> mice was apparent at both the 100- and 10-µg doses (Fig. 2C). These data are consistent with IL-6 deficiencies previously documented in TRIF-deficient mice (23). Taken together, the type I IFN and IL-6 cytokine data suggest that poly(I:C12U) is predominantly recognized by TLR3.

### Table IV. Poly(I:C) protects mice from lethal PTV infection in 3- to 4-wk-old TLR3-deficient mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Alive/Total</th>
<th>Day of Death&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Log-Rank Probability &gt; χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Poly(I:C12U), 100 µg</td>
<td>1/8</td>
<td>Mean ± SD: 4–7</td>
<td>0.0907</td>
</tr>
<tr>
<td></td>
<td>Poly(I:C), 100 µg</td>
<td>5/8**</td>
<td>4.7 ± 1.2</td>
<td>0.0027</td>
</tr>
<tr>
<td></td>
<td>Poly(I:C12U), 10 µg</td>
<td>1/10</td>
<td>4.6 ± 1.0</td>
<td>0.2940</td>
</tr>
<tr>
<td></td>
<td>Poly(I:C), 10 µg</td>
<td>8/10***</td>
<td>6.0 ± 1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sterile saline</td>
<td>0/10</td>
<td>4.4 ± 1.4</td>
<td>3–8</td>
<td></td>
</tr>
</tbody>
</table>

* Wild type: Poly(I:C12U), 100 µg 10/10***, Poly(I:C), 100 µg 10/10***, Poly(I:C12U), 10 µg 10/10***, Poly(I:C), 10 µg 10/10***, Sterile saline 0/15 4.5 ± 0.7 4–6
* Mean and range day of death of mice dying before day 21.

* p < 0.05; ** p < 0.01; *** p < 0.001 compared with respective saline-treated controls.

Based on the cytokine profiling data, we predicted that poly(I:C) treatment would effectively protect TLR3-deficient mice from a lethal inoculum of PTV. As shown in Table IV, 63 and 80% of TLR3<sup>−/−</sup> mice treated with 100 and 10 µg of poly(I:C), respectively, survived a highly fatal challenge dose of virus. Before significant protection was not afforded by poly(I:C12U), even at the 10-fold excess protective dose of 100 µg. Wild-type animals were completely protected, irrespective of dsRNA or administered dose (Table IV). The slight defects in type I IFN and IL-6 induction in TLR3<sup>−/−</sup> mice treated with poly(I:C) may have contributed to the slightly lower yet highly significant protection induced in these animals as opposed to the 100% protection observed with the wild-type mice.

**Discussion**

Poly(I:C12U) is an experimental drug that has been shown to have varying degrees of antiviral activity against HIV (28, 29), hepatitis B virus (30), several flaviviruses (31, 32), and coxsackie B3 virus (33). We have also demonstrated remarkable efficacy using poly(I:C12U), as well as poly(I:C), in the mouse PTV infection model (5, 10). There are several lines of evidence that argue against the classic dsRNA cytosolic sensor, dsRNA-dependent protein kinase (PKR), as the prominent pathway for type I IFN induction and antiviral host defense (34, 35). Based on the original work describing the recognition of poly(I:C) by TLR3 (17), we sought to examine the role of TLR3 in the induction of protective immunity in mice by poly(I:C12U). However, the recent discoveries of additional cytoplasmic dsRNA sensors and the characterization of md-5 as the receptor for poly(I:C) suggested that md-5 would be the predominant mechanism for type I IFN induction following...
exposure to poly(I:C12U) (18, 19, 22, 23). Unexpectedly, we found that animals devoid of TLR3 failed to develop protective immunity against, and limit disease associated with, PTV infection following single-dose i.p. treatment with poly(I:C12U). Moreover, TLR3 deficiency resulted in unchecked viral replication and the absence of a type I IFN and IL-6 responses elicited in wild-type animals treated with poly(I:C12U).

A caveat associated with antiviral studies in mice with immunodeficiencies such as TLR3 deletion is that lack of efficacy may be due in part to disruption of the TLR3-mediated response to PTV infection independent of poly(I:C12U). To that end, it is conceivable that TLR3 depletion predisposes the mice to more severe disease and consequently a more difficult to treat infection. The results from the initial study (Table II) suggested that this may be the case because the positive control drugs ribavirin and CLDC, which normally protect 100 and >80% of challenged mice, respectively, were less effective. However, these results may have been influenced by the age of the TLR3−/− mice, which were slightly smaller and presumably a few days younger than the wild-type mice in this experiment. This theory is supported by the results from the second study where the mice were more rigorously age matched so that they would all be close to 4 wk of age. Indeed, very similar protection was seen among the two mouse strains in this experiment. This theory is supported by the results from the second study where the mice were more rigorously age matched so that they would all be close to 4 wk of age. Indeed, very similar protection was seen among the two mouse strains in this experiment. This theory is supported by the results from the second study where the mice were more rigorously age matched so that they would all be close to 4 wk of age. Indeed, very similar protection was seen among the two mouse strains in this experiment. This theory is supported by the results from the second study where the mice were more rigorously age matched so that they would all be close to 4 wk of age.
experienced in clinical trials. The results from our studies suggest that reduced toxicity may also be a consequence of mda-5-independent signaling triggered by poly(I:C12U), in contrast to the combined signaling from mda-5 and TLR3 in response to poly(I:C). Whether differences in receptor usage significantly contributes to the increased toxicity of poly(I:C) is yet to be determined.

The disassociation of toxic responses from beneficial innate immune responses has facilitated the clinical development poly(I:C12U). To this end, it has successfully completed a large double-blind, placebo-controlled, phase 3 clinical trial for the treatment of chronic fatigue syndrome under the trade name, Ampligen. The primary end point of exercise tolerance achieved statistical significance and was highly correlated with an increase in oxygen use. Moreover, poly(I:C12U) was generally well tolerated, and there was no significant difference in the number of serious adverse events in the poly(I:C12U)-treated group compared with the placebo control group. Poly(I:C12U) also has been examined extensively for its potential application as a treatment for HIV infection. A clinical trial is being conducted currently to evaluate poly(I:C12U) in combination with highly active antiretroviral therapy (HAART) regimens in a study of structured treatment interruption of HAART. Potentially, poly(I:C12U) immunotherapy may be an effective countermeasure, alone or in combination with other antivirals, against virus infections that are sensitive to type I IFN antiviral activities. Notwithstanding, there may be limited applicability due to the growing number of viruses that have evolved mechanisms for the evasion of host IFN responses (20).

It has recently been discovered that the RIG-I, initially thought to be a dsRNA sensor, directly binds to 5′-triphosphate ssRNA (43, 44). Despite the finding of potential dsRNA binding surfaces through the examination of the TLR3 ectodomain crystal structure (45, 46), evidence of direct binding is lacking. It is possible that other proteins serve to bridge dsRNA interactions with TLR3, as well as mda-5. Further investigation into the dsRNA-protein interactions that facilitate the molecular discrimination between RIG-I and mda-5 will be necessary.(ab, bc)

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Disclosures

W. M. Mitchell is an independent member of the board of directors for the public company HEMISPHEREx Biopharma, the manufacturer of Ampligen. All other authors have no financial conflict of interest.

References


