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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_{12}U), but not Poly(I:C): Differential Recognition of Synthetic dsRNA Molecules[1]

Brian B. Gowen,[2]* Min-Hui Wong,* Kie-Hoon Jung,* Andrew B. Sanders,* William M. Mitchell,† Lena Alexopoulou,‡ Richard A. Flavell,§ and Robert W. Sidwell*

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_{12}U), 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_{12}U) 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_{12}U), 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)[3] 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_{12}U) 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 $\sim 2.7 \times 10^5$ Da (14). Poly(I:C_{12}U) was derived by investigators at Johns Hopkins University as a nontoxic analog with similar IFN

*Institute for Antiviral Research, Utah State University, Logan, Utah 84341; †Vanderbilt University Medical Center, Nashville, TN 37232; ‡Centre d'Immunologie de Marseille-Luminy, Centre National de la Recherche Scientifique-Institut National de la Santé et de la Recherche Médicale, Marseille, France; and §Section of Immunobiology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510

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[2] Address correspondence and reprint requests to Dr. Brian B. Gowen, Institute for Antiviral Research, Utah State University, 5600 Old Main, Logan, UT 84322. E-mail address: bgowen@cc.usu.edu

[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* protozoan Ag; RIG-I, retinoic acid-induced protein-I; TRIF, Toll/IL-1R domain-containing adaptor; $CCID_{50}$, 50% cell culture infectious dose.

Table I. *Effect of poly(I:C_{12}U) treatment on PTV infection and disease outcome in 3- to 4-wk-old mice*

Strain	Treatment[a]	Alive/Total	Day of Death[b] Mean ± SD	Day of Death[b] Range	Log-Rank Probability > χ^2	ALT[c,d] ± SD	Liver Score[c,e] ± SD
C57BL/6	Poly(I:C_{12}U), 10 μg	10/10***			<0.0001	19 ± 17**	0.2 ± 0.3*
	Poly(I:C_{12}U), 1 μg	5/10**	5.0 ± 1.7	4–8	0.0022	906 ± 909	1.2 ± 1.1
	Poly(I:C_{12}U), 0.1 μg	0/10	4.4 ± 0.5	4–5	0.3246	1565 ± 872	1.8 ± 1.3
	Ribavirin, 75 mg/kg	10/10***			<0.0001	14 ± 6**	0.0 ± 0.0*
	Sterile saline	0/22	4.9 ± 1.4	4–8		1528 ± 692	1.2 ± 1.1

[a] Singe-dose poly(I:C_{12}U) 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.
[b] Mean and range day of death of mice dying before day 21.
[c] Determined on day 3 of infection; five to six mice per group.
[d] Measured in international units per liter.
[e] Score of 0 (normal liver) to 4 (maximal discoloration).
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with saline-treated control.

induction capacities as the parent compound (15). As an inducer of IFN, poly(I:C_{12}U) has potent antiviral and immunomodulatory properties. This synthetic, dsRNA polymer consists of one strand of polyriboinosine (poly I) hybridized to a complementary strand of polyribocytosine containing a uridine residue statistically at every 13th monomer (poly C_{12}U) 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:C_{12}U) that presents an initial site for endoribonucleolytic enzyme-catalyzed hydrolysis. The lack of poly(I:C_{12}U) 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:C_{12}U).

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:C_{12}U), trade name, Ampligen, was provided by HEMISPHERx Biopharma at a concentration of 2.4 mg/ml. Poly(I:C) was obtained from Amersham Biosciences. Both were prepared for injection in sterile saline. Materials to generate cationic liposome-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.

Evaluation of dsRNAs in TLR3$^{-/-}$ *and wild-type mice infected with PTV*

PTV, Adames 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-MK_2 cells (American Type Culture Collection). Weanling 3- to 4-wk-old *TLR3*$^{-/-}$ and C57BL/6 mice were inoculated by s.c. injection with 1.3×10^4 50% cell culture infectious doses ($CCID_{50}$) 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:C_{12}U). 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:C_{12}U) or saline 24 h postinfectious challenge. Day 1 samples were also collected from untreated animals to provide a comparison point early during the course of infection. Virus titers were assayed using an infectious cell culture assay as described previously (7). Briefly, a specific volume of liver homogenate or serum was serially diluted and added to triplicate wells of LLC-MK_2 cell monolayers in 96-well microplates. The viral cytopathic effect was determined 6–7 days postvirus exposure, and the 50% end points were calculated as described previously (24).

In vivo cytokine analysis

Eight-week-old mice were injected by i.p. or i.v. routes with varying amounts of poly(I:C_{12}U) or poly(I:C), and serum was collected at the indicated times for the analysis of type I IFN levels and IL-6 release. IFN-β and 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:C_{12}U), elicits protective immunity to PTV infection in 3- to 4-wk-old mice lacking of TLR3*

Strain	Treatment[a]	Alive/Total	Day of Death[b] Mean ± SD	Day of Death[b] Range	Log-Rank Probability > χ^2
TLR3$^{-/-}$	Poly(I:C_{12}U), 10 μg	0/9	4.1 ± 0.3	4–5	0.6775
	CLDC, 1 μg	5/8**	3.7 ± 0.6	3–4	0.0163
	Ribavirin, 75 mg/kg/day	6/8**	6.0 ± 2.8	4–8	0.0003
	Sterile saline	0/9	4.2 ± 1.0	3–6	
Wild type	Poly(I:C_{12}U), 10 μg	10/10***			<0.0001
	CLDC, 1 μg	10/10***			<0.0001
	Ribavirin, 75 mg/kg	10/10***			<0.0001
	Sterile saline	1/11	4.5 ± 0.7	4–6	

[a] Single-dose poly(I:C_{12}U), CLDC 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.
[b] 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.

Results

Determination of optimal poly(I:C_{12}U) dosing for the treatment of PTV infection in C57BL/6 mice

Poly(I:C_{12}U) is an experimental drug that has previously been shown to induce remarkable protection in C57BL/6 weanling mice challenged with PTV and limit liver dysfunction and disease associated with infection (10). Because the aforementioned studies were reported over a decade ago, we first evaluated several quantities of poly(I:C_{12}U) in 3- to 4-wk-old mice to determine the optimal dose that would elicit complete protection against PTV challenge. As shown in Table I, a single injection of 10 μg of poly(I:C_{12}U), administered 24 h postinfection with a highly lethal PTV inoculum, provided full protection as all animals survived and presented with minimal to no liver disease. No protection was afforded by the lowest dose of poly(I:C_{12}U) as all animals in the treatment group died. The observed protection seen with the 10-μg dose of poly(I:C_{12}U) was comparable to that of the positive control drug, ribavirin, included to ascertain the treatability of the infectious virus dose inoculated (Table I). Moreover, the lowest effective dose of 10 μg was similar to that previously reported (10) and therefore was selected for further investigation in mice lacking TLR3.

TLR3-deficient mice fail to develop protective immunity to PTV infection following treatment with poly(I:C_{12}U)

To test the hypothesis that TLR3 plays a role in the induction of antiviral defenses against PTV by poly(I:C_{12}U), 3- to 4-wk-old *TLR3*$^{-/-}$ and wild-type mice were treated 24 h postinfectious challenge. There were no survivors in the group of *TLR3*$^{-/-}$ mice treated with poly(I:C_{12}U) (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:C_{12}U) 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:C_{12}U) 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

Table III. *TLR11 agonist, rEA, but not mismatched dsRNA poly(I:C_{12}U), protects 4-wk-old TLR3-deficient mice from lethal PTV disease*

Strain	Treatment[a]	Alive/Total	Day of Death[b] Mean ± SD	Day of Death[b] Range	Log-Rank Probability > χ^2	ALT[c,d] ± SD	Liver Score[c,e] ± SD
TLR3$^{-/-}$	Poly(I:C_{12}U), 10 μg	0/10	4.1 ± 0.6	3–5	0.4861	2700 ± 1576	3.2 ± 0.4
	rEA, 1 μg	10/10***			<0.0001	155 ± 77**	3.3 ± 0.3
	Sterile saline	1/10	4.1 ± 0.6	3–5		3837 ± 234	3.5 ± 0.0
Wild type	Poly(I:C_{12}U), 10 μg	10/10***			<0.0001	3 ± 6**	0.6 ± 0.2**
	rEA, 1 μg	10/10***			<0.0001	93 ± 56**	3.3 ± 0.3
	Sterile saline	1/20	4.8 ± 1.1	3–7		3650 ± 823	3.2 ± 0.3

[a] Single-dose poly(I:C_{12}U), 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).
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with respective saline-treated controls.

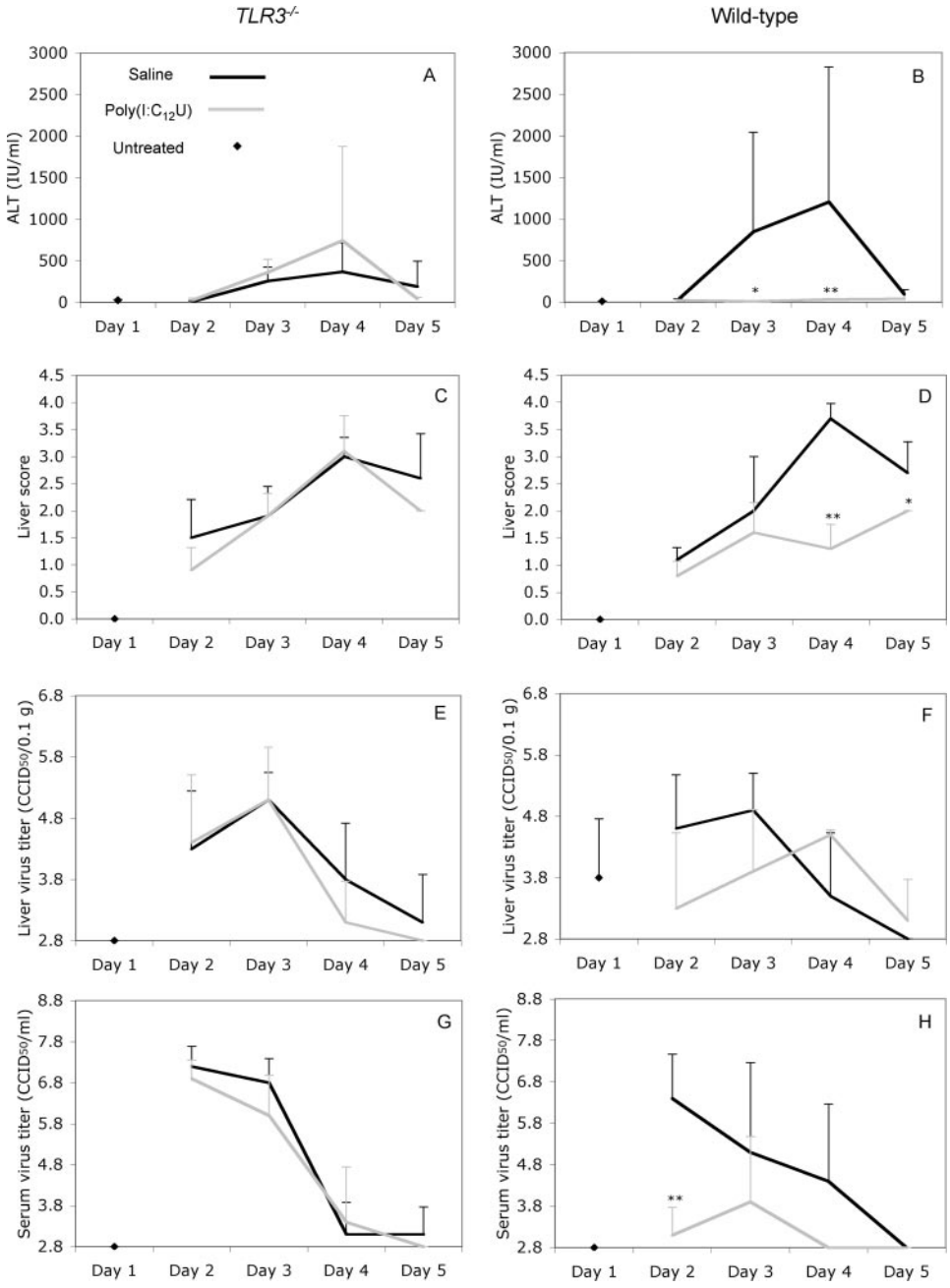

FIGURE 1. Poly(I:C_{12}U) treatment limits liver disease and systemic virus burden in wild-type but not *TLR3*$^{-/-}$ mice. Groups of 8-wk-old *TLR3*$^{-/-}$ (*A*, *C*, *E*, and *G*) and wild-type (*B*, *D*, *F*, and *H*) mice were challenged with PTV and treated i.p. with 10 μg of poly(I:C_{12}U) or 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.

serum ALT levels. As expected, treatment of wild-type mice with poly(I:C_{12}U) and rEA elicited 100% protection against the lethal challenge inoculum (Table III). Interestingly, poly(I:C_{12}U), 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:C_{12}U)

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:C_{12}U) 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 mice and peaked on day 4 before returning to normal levels in the majority of mice by day 5. There were no differences in ALT levels between poly(I:C_{12}U)-treated and saline-treated *TLR3*$^{-/-}$ mice, whereas levels remained near baseline in the wild-type mice that received poly(I:C_{12}U) therapy (Fig. 1, *A* and *B*). Interestingly, despite the large variation seen on days 3 and 4 of infection, wild-type saline-treated mice presented with mean ALT levels three times greater than their TLR3-deficient counterparts. In the case of liver damage assessed by gross visual examination, disease, as reflected by discoloration, was first noted on day 2 and peaked on day 4 in saline-treated mice (Fig. 1, *C* and *D*).

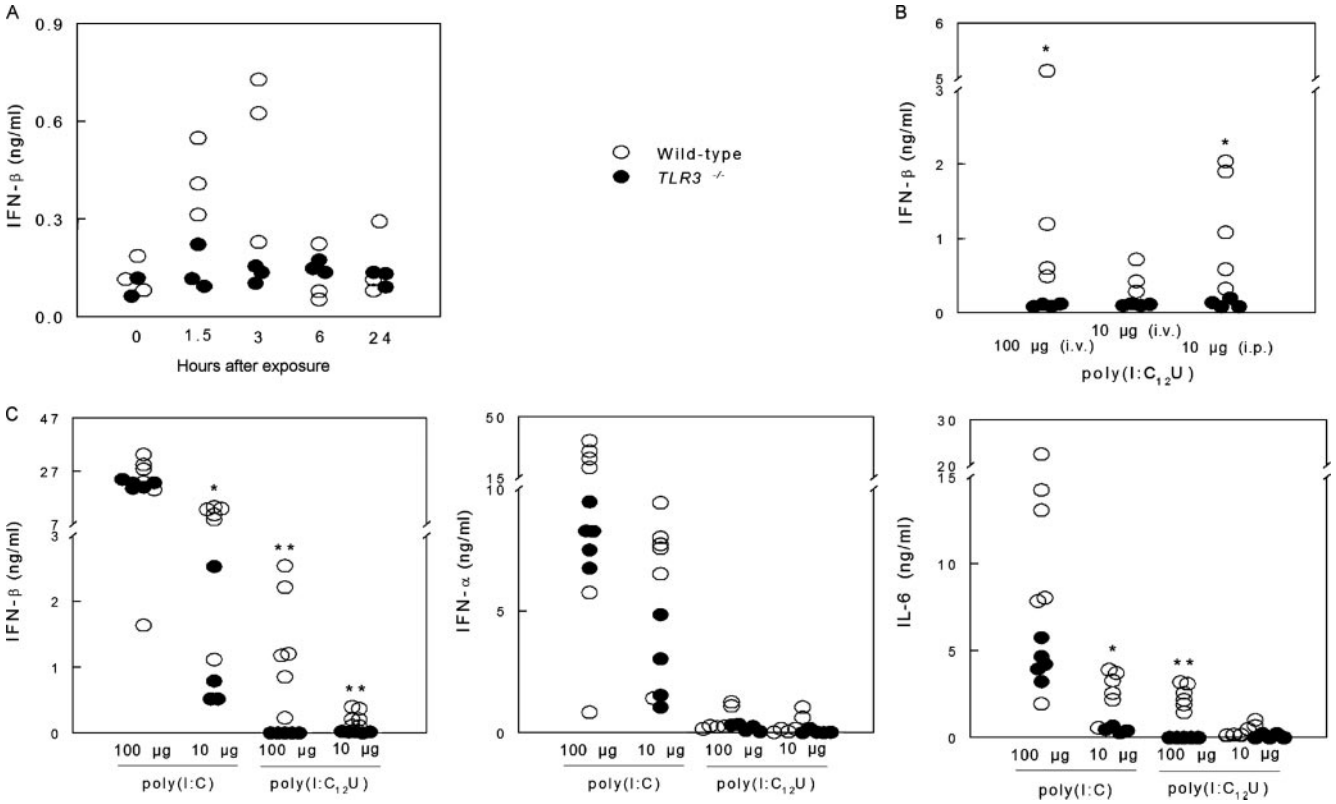

FIGURE 2. Type I IFN and IL-6 induction in uninfected 8-wk-old *TLR3*$^{-/-}$ and wild-type mice following exposure to synthetic dsRNAs. Groups (n = 3) of *TLR3*$^{-/-}$ and wild-type mice were injected i.p. with 10 μg of poly(I:C$_{12}$U), and systemic IFN-β levels were determined for serum samples collected at the indicated times postexposure (*A*). Groups of three to five mice were injected either i.v. or i.p. with 100 or 10 μg of poly(I:C$_{12}$U), 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:C$_{12}$U) 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.

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:C$_{12}$U). 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 $\pm$ 0.3 and 3.4 $\pm$ 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:C$_{12}$U) treatment.

The control of liver and systemic viral burden during the course of infection following poly(I:C$_{12}$U) 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:C$_{12}$U)-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 day 1 in several infected, untreated wild-type animals included as controls (Fig. 1, *E* and *F*). Although not detectable on day 1 of the infection, serum virus spiked dramatically by day 2, with the exception of the poly(I:C$_{12}$U)-treated wild-type mice, which were able to control the infection to barely detectable virus levels with a >3 $\log_{10}$ reduction observed (Fig. 1, *G* and *H*). As seen with the control of liver disease (Fig. 1, *B* and *D*), the beneficial effect of poly(I:C$_{12}$U) therapy observed in the wild-type animals was lost in the TLR-deficient mice. With respect to peak liver and serum viral loads, no remarkable differences were seen between saline-treated wild-type and *TLR3*$^{-/-}$ mice (Fig. 1, *E–H*). However, mean serum virus titers in *TLR3*$^{-/-}$ mice dropped precipitously by >3 $\log_{10}$ after day 3, while a more gradual decrease was observed in the wild-type mice. The lack of significant differences in viral burden between the saline-treated groups of mice are consistent with previous work that suggests that detrimental inflammatory responses mediated by TLR3 may contribute to the more severe liver disease profile seen with the wild-type animals (27).

TLR3-deficient mice fail to produce type I IFNs and IL-6 in response to poly(I:C$_{12}$U)

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:C$_{12}$U) 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. 2*A*). 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. 2*A*). 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:C$_{12}$U) likely factors in their failure to overcome PTV infection despite treatment proven effective in wild-type mice. The data suggest that the low

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

Strain	Treatment[a]	Alive/Total	Day of Death[b] Mean ± SD	Day of Death[b] Range	Log-Rank Probability > χ^2
TLR3$^{-/-}$	Poly(I:C$_{12}$U), 100 μg	1/8	5.3 ± 1.1	4–7	0.0907
	Poly(I:C), 100 μg	5/8**	4.7 ± 1.2	4–6	0.0027
	Poly(I:C$_{12}$U), 10 μg	1/10	4.6 ± 1.0	3–6	0.2940
	Poly(I:C), 10 μg	8/10***	6.0 ± 1.4	5–7	<0.0001
	Sterile saline	0/10	4.4 ± 1.4	3–8	
Wild type	Poly(I:C$_{12}$U), 100 μg	10/10***			<0.0001
	Poly(I:C), 100 μg	10/10***			<0.0001
	Poly(I:C$_{12}$U), 10 μg	10/10***			<0.0001
	Poly(I:C), 10 μg	10/10***			<0.0001
	Sterile saline	0/15	4.5 ± 0.7	4–6	

[a] Single-dose poly(I:C$_{12}$U), poly(I:C), or saline treatments administered i.p. 4 h before virus challenge.
[b] 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.

to moderate levels of IFN-β induced by the 10-μg i.p. dose of poly(I:C$_{12}$U) 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) (22, 23). In those studies, mice were injected i.v. with 100- to 200-μg quantities of poly(I:C), compared with the experiments described above where 10-μg doses of poly(I:C$_{12}$U) were administered by i.p. injection. To assess whether the lack of IFN-β induction by poly(I:C$_{12}$U) was a consequence of the lower quantity and route of delivery, we injected mice i.v. with 100- and 10-μg amounts and measured systemic IFN-β 3 h postexposure. As shown in Fig. 2*B*, only basal levels of IFN-β were detected in *TLR3*$^{-/-}$ mice, irrespective of quantity and route of administration. Considering the results from previous studies and the structural similarities between poly(I:C) and poly(I:C$_{12}$U), the complete lack of responsiveness by the TLR3-deficient mice to i.v. administration of 10-fold excess poly(I:C$_{12}$U) was surprising. A modest dose-dependent response was evident in the wild-type mice treated i.v. with poly(I:C$_{12}$U); however, the 10-μg i.p. dose elicited nearly equivalent amounts of IFN-β to that of the 100-μg i.v. dose (Fig. 2*B*).

Because i.v. injection of poly(I:C$_{12}$U) did not appear to augment the amounts of IFN-β released compared with i.p. dosing, we next evaluated high-dose (100 μg) i.p. administration of poly(I:C$_{12}$U) in parallel with poly(I:C). As expected, the 100-μg dose of poly(I:C) induced a profound amount of IFN-β in both wild-type and *TLR3*$^{-/-}$ mice (Fig. 2*C*). Interestingly, the 10-μg amount of poly(I:C) stimulated the release of significantly more IFN-β in the wild-type animals compared with the TLR3-deficient mice, resolving a defect in this capacity at the lower treatment dose. Consistent with previous experiments, only basal levels of IFN-β were observed in TLR3-deficient mice treated with poly(I:C$_{12}$U), even at the highest dose (100 μg) (Fig. 2*C*). Although a highly significant increase in the systemic levels of IFN-β were seen in the wild-type mice treated with poly(I:C$_{12}$U), the detected amounts were considerably lower than those seen in the mice treated with 10 μg of poly(I:C). These data suggest that poly(I:C) is much more potent than poly(I:C$_{12}$U) in the induction of IFN-β and that, when given at lower doses, the involvement of TLR3 in the systemic response to poly(I:C) can be resolved.

In addition to the analysis of IFN-β following exposure to synthetic dsRNAs, the levels of IFN-α and IL-6 were also determined. In the case of IFN-α, with the exception of one or two mice, poly(I:C) elicited higher levels of this type I IFN at both the high and low doses in the wild-type mice compared with the *TLR3*$^{-/-}$ mice (Fig. 2*C*). Only two of the six wild-type animals mounted an appreciable IFN-α response to poly(I:C$_{12}$U). In contrast, a robust IL-6 response was observed in all of the wild-type animals dosed with 100 μg of poly(I:C$_{12}$U), but only partial, low-level induction was seen with the 10-μg amount. As with the type I IFNs, the *TLR3*$^{-/-}$ mice failed to respond to either poly(I:C$_{12}$U) dosing (Fig. 2*C*). The IL-6 release following exposure to poly(I:C) was very remarkable in most of the wild-type mice. As seen with IFN-α, a defect in IL-6 release in *TLR3*$^{-/-}$ mice was apparent at both the 100- and 10-μg doses (Fig. 2*C*). 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:C$_{12}$U) is predominantly recognized by TLR3.

Poly(I:C) treatment significantly protects TLR3$^{-/-}$ *mice from lethal PTV infection*

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*$^{-/-}$ mice treated with 100 and 10 μg of poly(I:C), respectively, survived a highly fatal challenge dose of virus. As before, significant protection was not afforded by poly(I:C$_{12}$U), 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*$^{-/-}$ 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:C$_{12}$U) 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:C$_{12}$U), 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:C$_{12}$U). However, the recent discoveries of additional cytoplasmic dsRNA sensors and the characterization of mda-5 as the receptor for poly(I:C) suggested that mda-5 would be the predominant mechanism for type I IFN induction following

exposure to poly(I:C_{12}U) (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:C_{12}U). 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:C_{12}U).

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:C_{12}U). 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 response to rEA, and similar lethality was observed with the saline placebo groups (Table III). The robust antiviral activity of rEA is believed to occur through potent induction of IL-12 and IFN-γ, despite the lack of IFN-α induction at the doses previously evaluated (11, 26). Further evidence refuting the diminished capacity of *TLR3*$^{-/-}$ mice to combat PTV infection was also seen in older mice. In the time course study conducted to further resolve differences in the ability of *TLR3*$^{-/-}$ and wild-type mice to respond to poly(I:C_{12}U), comparisons between the placebo-treated mice suggest that the *TLR3*$^{-/-}$ mice may be more resistant to disease resulting from PTV infection. Wild-type mice presented with higher levels of ALT and liver scores are reflective of greater liver disease. Moreover, two of five wild-type mice died before time of sacrifice on day 6 of infection while *TLR3*$^{-/-}$ mice appeared healthy (data not shown). Challenge studies in untreated *TLR3*$^{-/-}$ and wild-type mice corroborate these findings, indicating that *TLR3*$^{-/-}$ mice are no more susceptible to PTV infection than their wild-type counterparts (27).

Stimulation of IFN-β production via TLR3 occurs through signaling events that require the adaptor molecule, TRIF, exclusive to TLR3 and TLR4 pathways (36). Recent investigations in mice devoid of the TLR3, TRIF, or mda-5 have provided compelling evidence that the latter is the primary response pathway to type I IFN production following exposure to the dsRNA mimic, poly(I:C) (22, 23). Our data indicate that TLR3 is absolutely essential for the stimulation of antiviral activity and the induction of IFN-β by the related poly(I:C_{12}U). Unlike the parental poly(I:C) molecule, which elicits the release of large amounts of type I IFNs and IL-6 in TLR3-deficient mice through mda-5, the mismatched dsRNA configuration of poly(I:C_{12}U) does not appear to be a ligand for the cytosolic sensor. Thus, the specificity of mda-5 for poly(I:C) appears to be more restricted compared with TLR3, which recognizes both forms of synthetic dsRNAs.

Others have previously identified reduced responses to poly(I:C) as a result of TLR3 and TRIF deficiencies (17, 37). Consistent with such defects, we resolved an apparent deficiency in the IFN-β response of TLR3-deficient mice to the lower exposure dose of poly(I:C). The lower 10-μg doses of poly(I:C) and poly(I:C_{12}U) provided complete protection against lethal PTV challenge and is more likely physiologically relevant in the context of viral infection and potential immunotherapy. The latter is especially important considering the known toxicity of poly(I:C) discussed below. It is conceivable that, at the 100-μg dose, IFN-β release mediated by mda-5 has reached saturation, and thus, the contribution of TLR3 is masked. At the lower 10-μg dose, the defect evident in the *TLR3*$^{-/-}$ mice is brought to light. This defect was also apparent with IFN-α and IL-6 release at both the 100- and 10-μg doses. Kato et al. (23) did not report an IFN-α defect in their studies with *Trif*$^{-/-}$ mice but did observe a similar partial deficiency in IL-6 production. It is possible that the higher 200-μg dose of poly(I:C) saturated IFN-α release as was seen in our experiments with IFN-β. In the study by Gitlin et al. (22), no defect in type I IFN or IL-6 induction was observed at the 100-μg dose of poly(I:C) tested in both TLR3- and TRIF-deficient mice. Although a number of factors may have contributed to these differences, it is likely that saturation of the system with the high dose of poly(I:C) resulted and that having injected a smaller quantity such as 10 μg may have uncovered the cytokine production defects we observed. Collectively, our data suggest that, at lower exposure doses of poly(I:C), maximal cytokine induction requires a cooperative response by mda-5 and TLR3.

The ability of poly(I:C) to induce type I IFN was considerably greater than that of poly(I:C_{12}U). This more profound biological activity and induction of IFN has been documented in previous work (38, 39). Despite the above, previous studies comparing poly(I:C_{12}U) and poly(I:C) have demonstrated comparable antiviral activities against Semliki Forest virus, encephalomyocarditis virus, and PTV (10, 38, 40). In agreement with the latter, in this study, we observed similar antiviral activity elicited by both synthetic dsRNAs in wild-type mice. The results of the in vivo challenge studies indicate that the 10-μg dose of poly(I:C_{12}U) is sufficient to elicit complete protection in wild-type mice against lethal PTV infection. It is possible that poly(I:C) may offer protection at lower doses because it was able to induce greater levels of IFN. However, previous studies comparing both dsRNAs synthesized by the same source do not support this notion (40). Based on our data measuring systemic release of IFN-β, only low to moderate induction may be necessary for stimulating robust antiviral activity in the PTV infection model. Moreover, IFN-α and IL-6 levels were only appreciably higher in a few of the animals treated with the 10-μg poly(I:C_{12}U) dose. It is important to note that the cytokine data obtained does not account for the contributions of the innate immune response to the virus infection, which combined with poly(I:C_{12}U) treatment may amplify the observed type I IFN and IL-6 profiles. Also, weanling mice were used in all but the temporal viral challenge studies with the 10-μg amount, resulting in a dose of ~833 μg/kg, whereas the larger 8-wk-old mice used for cytokine induction studies received ~588 μg/kg.

The development of poly(I:C_{12}U) as a clinically useful drug has been made possible by its rapid half-life as compared with poly(I:C). Extracted from blood, poly(I:C_{12}U) is in a dsRNA conformation that allows quantitation by a solution hybridization technique using a radioactive probe under chaotropic salt conditions that inhibit RNase degradation while allowing molecular probe hybridization displacement of the homologous RNA strand (41, 42). Pharmacokinetic studies of poly(I:C_{12}U) in humans have established a half-life in blood of ~30 ± 17 min compared with over 4 h for poly(I:C). Clinical and half-life data are consistent with a model in which there is a disassociation of innate immune response gene activations by poly(I:C_{12}U) while more slowly induced toxic responses to dsRNAs are minimized. Because poly(I:C_{12}U) possesses a classical RNA structure with individual components found in in vivo nucleic acid pools, toxicity of the metabolic degradation products is not expected and has not been

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:C_{12}U), 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:C_{12}U). 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:C_{12}U) was generally well tolerated, and there was no significant difference in the number of serious adverse events in the poly(I:C_{12}U)-treated group compared with the placebo control group. Poly(I:C_{12}U) 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:C_{12}U) in combination with highly active antiretroviral therapy (HAART) regimens in a study of structured treatment interruption of HAART. Potentially, poly(I:C_{12}U) 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 poly(I:C) and poly(I:C_{12}U) by mda-5, and the lack thereof by TLR3, may provide a better understanding of the mechanisms by which the host defends itself from viruses and reveal new therapeutic strategies.

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Disclosures

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

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