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The efficacy of poly-ICLC against Ebola-Zaire virus (EBOV) infection in mice and cynomolgus monkeys

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ABSTRACT

The potential protection of poly-ICLC (Hiltonol®) a double stranded RNA (dsRNA) against EBOV infection was assessed with prophylactic and therapeutic administration to wild type and TLR3-negative mice, and in non-human primates (NHPs) by measuring EBOL serum titers, survival extension, and serum liver and kidney function markers. Various doses of aqueous and liposomal poly-ICLC monotherapy provided robust protection in otherwise lethal murine EBOV challenge models, when treatment is started on the day 0 or one day after virus challenge. There was no advantage of liposomal vs. the aqueous poly-ICLC form. Protection appeared to be independent of TLR-3. NHPs treated with poly-ICLC and challenged with EBOV survived longer but eventually succumbed to Ebola infection. Nevertheless, the liver and kidney serum markers were markedly reduced in the infected and treated NHPs. In the two longest surviving poly-ICLC- treated NHPs, the day 10 serum EBOV titer was reduced 2.1 and 30 fold respectively.

1. Introduction

EBOV infection has been shown to block host cell response to interferon (IFN), to inhibit double-stranded RNA (dsRNA) mediated induction of antiviral gene expression and to block INF-α/β production in infected cells (Basler et al., 2003). VP35 protein an essential component of EBOV RNA replication machinery functions as an IFN antagonist (Basler et al., 2000). In addition to the evasion of the interferon IFN system the IFN regulatory factor (Basler et al., 2003), the dsRNA-dependent protein kinase (PKR) and 2′5′-oligoadenynalate synthetase (2–5 OAS) (Harcourt et al., 1998) are also inhibited, which are the mediators of the innate immune antiviral state. EBOV inhibits the activity of natural killer (NK) cells (Warfield et al., 2004), the macrophages (Gupta et al., 2001); as well as the dendritic cells (Geisbert et al., 2003), major histocompatibility I (MHC I) antigens (Harcourt et al., 1999), various cytokines (Wauquier et al., 2010) and other elements of adaptive immunity (Bosio et al., 2003; Rao et al., 2002; Harcourt et al., 1998). The mechanism of this inhibition is unclear, but has been partly linked to the Ebola envelope glycoprotein (GP) (Takada and Kawaoka, 2001). Harcourt and colleagues in 1998 have described not only the failure of EBOV to activate dsRNA mediated innate immunity, but also its inhibition of the induction of this immunity by exogenous dsRNA (Harcourt et al., 1998). The inhibition eventually causes the failure of the host defenses to mount an immune response and protection, resulting an infection with a high mortality rate. Significantly, the same authors also found that this inhibition of dsRNA-activated innate immunity did not begin until more than 24 h after infection suggesting, that there is a significant peri-exposure window within which exogenous activation of innate immunity could provide protection. This prediction is consistent with the preclinical findings presented below showing protection by poly-ICLC when given one or two days post-viral challenge with EBOV. The dual role of poly-ICLC as an antiviral agent (Wong et al., 1995, 1999, 2005; Levy and Salazar, 1992; Kende, 1992; Stephens et al., 1977b; Kende et al., 1987), and as an immune adjuvant Caskey et al. (2011), Stephens et al., 1977a, Fujimoto et al. (2004) is consistent with its function as a viral-mimic in stimulating an immediate defense against viral attack, while at the same time facilitating the development of long-term immunity, by improved efficiency of antigen processing and T-cell activation (Fujimoto et al., 2004.). Poly-ICLC also provides robust protection in an otherwise lethal murine model of SARS, even though viral titers are not significantly suppressed (Kumaki et al., 2016).

2. Materials and methods

Ethics Statement. Opinions, interpretations, conclusions, and

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recommendations are those of the author and are not necessarily endorsed by the U.S. Army. Research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

2.1. Mice

Pathogen free, female, 22–25 gr 8–10 weeks old BALB/c mice were obtained from the National Cancer Institute Frederick Research and Development Facility at Frederick, MD. The mice were housed in full compliance with the Guide for Care and Use of Laboratory Animals, with food and water ad libitum. The pathogen free, female, 22–25 gr 8–10 weeks old $TLR^{-/-}$ and the B6129 mice were obtained from Jackson Laboratory, Bar Harbor, Main.

2.2. Poly-ICLC

Poly-ICLC (Hiltonol) was produced by Oncovir Inc., Washington D.C. It was prepared in a concentration of 2 mg/ml poly-IC, 1.5 mg polylysine, 5 mg carboxymethyl cellulose in 0.9% sodium chloride sterile solution, and adjusted to pH 7.6–7.8 with sodium hydroxide. The solution was kept refrigerated until its use. Poly-ICLC doses were injected by the subcutaneous (s.c.) route at 20 mcg/mouse or at 250 mcg/kg in NHPs.

2.3. Liposome preparation

Liposomal poly-ICLC was prepared according the published method of Wong et al. (1999). Poly-ICLC was diluted in 0.9% sodium chloride made from RNA-ase free water (Sigma, St Louis, Mo). All phospholipids and cholesterol used for encapsulation of the poly-ICLC were purchased from Avanti Polar Lipids (Alabaster, AL). Cationic lipids were prepared using phosphatidylcholine, cholesterol and stearylamine at a molar ration of 7:2:1 and were prepared by the freeze-drying method.

2.4. Ebola virus challenge of mice

Adult BALB/c mice are uniformly susceptible to serially passed mouse-adapted Ebola Zaire strain of virus (MA-EBOV). On day 0 the mice were challenged intraperitoneally (i.p.) with 1000 LD_{50}, or subcutaneous (s.c.) route with 300 LD_{50} of MA-EBOV. This a 100% lethal dose with a mean time to death (MTD) of 6–7 days. The mice were observed twice daily for three weeks for clinical signs of the disease (sluggish movement or no movement, hunched back). `The mice will reach the end-point either by death (those that during the evening/night), death by Euthanasia or by survival. Mice with clinical signs of the infection (sluggishness or no movement, can not get up, hunched back) will be euthanized by CO_2 inhalation in a CO_2 chamber.

2.5. EBOV challenge of NHPs

On day 0, the NHPs were challenged intramuscularly (i.m.) with 1000 plaque forming units (PFU) of EBOV. This is a 100% lethal dose in cynomolgus monkeys, with a historical mean time of death (MTD) of 6.1 days. The study end-point is survival after 30 days or euthanasia of the moribund animals. Criteria of euthanasia: labored or agonal breathing, lies down and will not get up when approached, severe no flexor response, bleeding. Euthanasia will be performed by exsanguinations following deep anesthesia with 9 mg/kg Telazol administered on i.m. route.

2.6. Statistical evaluation of treatment efficacy

The efficacy of the treatment groups vs the untreated virus control was determined by two tailed Fisher exact test. A sample size of 8 animals per group per time point detected a minimum confidence level assuming a standard deviation of between animals at 0.3 log 10.

2.7. Immunohistochemistry of cynomolgus tissues

The immunoperoxidase assay used is a published method of Geisbert et al., 2003. EBOV GP and VP40 antigens were detected in tissues cut at 5–6 μm on a rotary microtome, mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and immunohistochemically stained for detection of the viral antigen by an immunoperoxidase method according to kit procedures (Envision System, DAKO Co., Carpenteria, CA).

2.8. EBOV titration

Serial dilutions of EDTA NHP plasma was assayed by counting plaques on Vero cells maintained as monolayers in 6-well plates under agarose, as previously described by Jahrling (1999).

3. Results

3.1. Two or single treatments of Ebola-infected BALB/c mice

Mice (n = 7–10/group) were treated s.c. twice or once with 20 μg of aqueous or liposomal poly-ICLC prior to infection on day 0 with 300 LD_{50} of MA-EBOV. Two treatments with aqueous or liposomal poly-ICLC on day −7 and −5 had no effect on the course of the infection (Table 1). Two treatments on day −4 and −2 was ineffective with the aqueous compound, while the liposomal form of the antiviral yielded 40% survivors, p value of 0.1300, which was not significantly different from the aqueous treatment, since survival less than 50% is not statistically significantly different due to the low number of mice. Two treatments on days 0 and + 2 yielded 80% and 100% survivors with the aqueous and the liposomal poly-ICLC, respectively ($p = 0.0025$ and < 0.0001). A single dose of aqueous poly-ICLC on day −1 or 0 protected 40% and 60% of the mice ($p = 0.1734$ and 0.0271), while the liposomal poly-ICLC on these schedules protected 56% and 80%, respectively ($p = 0.0261$ and 0.0021), indicating no advantage of either modality. A single aqueous poly-ICLC dose on day + 1 or +2 protected 83% and 25% of the Ebola-infected mice ($p = 0.0034$ and 0.3660), while on the same schedule (Table 1) the liposomal compound protected 75% and 25% of the infected mice, respectively ($p = 0.0075$ and 0.3660).

Five day treatment cycles were also evaluated with aqueous and liposomal poly-ICLC. When a treatment cycle started on day 1, and was repeated every other day for 5 doses, 70% of the mice remained alive with both formulations ($p = 0.0031$). When the cycle started on day 0, 90% of the mice were protected ($p = 0.0002$), whereas a cycle beginning on day +1 yielded 100% protection with both formulations ($p = < 0.0001$). This benefit was lost when start of the treatment cycle was delayed until day +2, only the aqueous compound yielded 40% survivors ($p = 0.043$).

3.2. Prophylactic and therapeutic treatment efficacy with diminished number of doses

The above findings were re-confirmed with a repeat experiment (Fig. 1). When the treatment was started on day 0, five (Group 1), four (Group 2) or three (Group 3) doses of 20 μg/mice aqueous poly-ICLC (dark-shaded bars) protected 100%, 100%, and 80% of the mice against lethal EBOV challenge ($p = 0.001$ or lower). Liposomal poly-ICLC (light-shaded bars) was less consistently effective. The difference

Table 1
Prophylactic and therapeutic efficacy of aqueous or liposomal Poly-ICLC in MA-EBOV[a] infected BALB/c mice.

Treatment days	Aqueous poly-ICLC			Liposomal poly-ICLC[b]		
	% Survived	Numbers Survived	P values vs placebo[c]	% Survived	Numbers Survived	P values vs placebo[c]
−7, −5	0	0/7	1.0000	0	0/10	`1.000
−4, −2	0	0/7	1.0000	40	4/10	0.1500
−1	40	4/10	0.1734	56	4/7	0.0261
0	60	6/10	0.0271	80	8/10	0.0021
0, +2	80	8/10	0.0025	100	8/8	< 0.0001
+1	83	6/7	0.0034	75	6/8	0.0075
+2	25	2/8	0.3650	25	2/8	0.3650
0, +2 ml PBS[d]	0	0/8	–	–	–	–
−1, 0, +2, +4, +6	70	7/10	0.0031	70	7/10	0.0031
0, +2, +4, +6, +8	90	9/10	0.0002	90	9/10	0.0002
+1, +2, +4, +6, +8	100	10/10	< 0.0001	100	10/10	< 0.0001
+2, +3, +4, +6, +8	40	4/10	0.0433	0	0/10	1.0000
1,0, +2, +4, +6	0	0/10	–	–	–	–

[a] Virus: 300 LD_{50} MA-EBOV, s.c. route on day 0.
[b] 0.8 mg/kg (~20 μg) polyICLC, s.c. route on days as indicated in Table.
[c] Fisher's 2-sided exact test.
[d] Phosphate Buffer Solution pH-7.2.

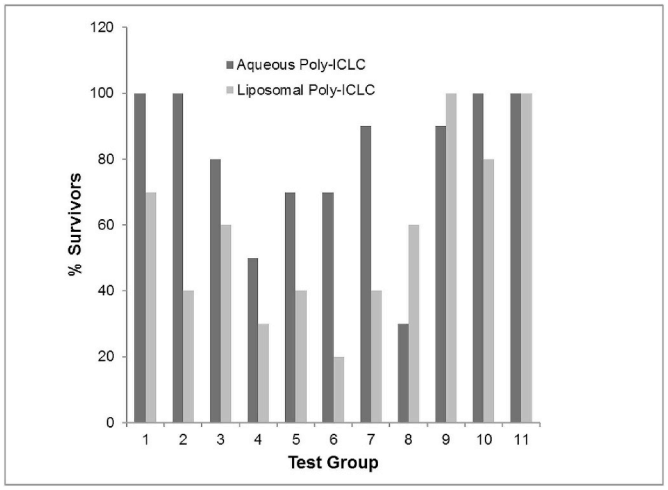

Fig. 1. Poly-ICLC efficacy administered with decreasing frequency against MA-EBOV. Challenge in BALB/c mice 10 mice/group were challenged i.p. route on day 0 with 1000 LD_{50} ME-EBOV. **Virus control:** PBS pH-72. **No survivals**, Groups **1–8**: 20 μg/mouse **Group treatment days of Groups 1–8**: Group **1** on days: 0, 2, 4, 6, 8; Group **2** on days: 0, 2, 4, 6 Group **3** on days: 0, 2, 4; Group **4** on days: 0, 2; Group **5** on days: 1, 2, 4, 6, 8; Group **6** on days: 1, 2, 4, 6; Group **7** on days: 1, 2, 4; Group **8** on days: 1, 2; Group **9** on days: 0, 2, 4, 6, 8 (dose: 40 μg/mouse); Group **10** on days: 0, 2, 4, 6, 8 (dose 20 μg/mouse); Group **11** on days: 0, 2, 4, 6, 8 (dose: 10 μg/mouse).

among the survivors was significantly different in favor of the four doses of the aqueous compound (Group 2 dark-shaded bar) vs. the liposomal poly-ICLC (Group 2 light-shaded bar), with a p value of 0.011. Two doses of aqueous poly-ICLC administered on Day 0 and 2 is not a treatment option Group 4 dark bar, yielding only 50% protection while the liposomal compound protected only 30% of the mice (Group 4 light-shaded bar).

Therapeutic administration of poly-ICLC starting on day +1 post-infection with diminished number of doses revealed no difference between therapeutic efficacy of five, four or three doses (Fig. 1). Five (Group 5), four (Group 6) or three (Group 7) doses of the aqueous poly-ICLC (dark shaded bars), administered on days +1, +2, +4, +6, +8, or +1, +2, +4, +6, or +1, +2, +4 protected 70% (p = 0.003), 70% (p = 0.003), and 90% (p= < 0.0001) of the infected mice, respectively. Two doses (Group 8) of the aqueous compound administered on days +1 and 2 protected only 30% of the treated animals (p = 0.211). Five (Group 5), four (Group 6) and three (Group 7) doses of the liposomal poly-ICLC (light shaded bars) protected 40%, 20% and 40% of the infected treated mice, which was not significant due to the respective p values of 0.087, 0.474 and 0.087. Two doses (Group 8, light bar) of the liposomal poly-ICLC yielded 60% long-term survivors (p = 0.011). The treatment efficacy of the aqueous and liposomal poly-ICLC was not statistically different, although the treatment efficacy obtained with three doses of the aqueous compound (Group 7) has a borderline significance vs the liposomal poly-ICLC (p value of 0.057).

Using five doses of aqueous and liposomal poly-ICLC, (dark or light shaded bars respectively) the prophylactic efficacy of varying amounts of poly-ICLC (40 μg Group 9; 20 μg Group 10; 10 μg Group 11) was assessed against 1000 LD_{50} of MA-EBOL challenge. The treatment started simultaneously with the viral challenge and the respective aqueous or liposomal poly-ICLC doses were repeated every other day until day +8 (Fig. 1 Groups 9, 10, 11). The number of long-term survivors with both treatment modalities was 100%, except those with the 40 μg aqueous compound: 90% (Group 9) and with 20 μg liposomal poly-ICLC (Group 10) treatment: 80% of the mice survived (p= < 0.0001). Thus all doses were highly significant, with no statistical difference between the groups. The poly-ICLC treated survivors of this study segment were back-challenged with 1000 LD_{50} of MA-EBOV i.p. route at 8 weeks. All of them were immune, and resisted the re-challenge with no additional treatment.

3.3. Evaluation of poly-ICLC efficacy in TLR-3 negative and TLR-3 positive mice

Aqueous poly-ICLC of 20 μg was administered by the s.c. route on days 0, +2, +4, +6, and +8. Treatment was started simultaneously with i.p. injection of the MA-EBOV challenge. As shown in Table 2, TLR-3 positive and negative mice treated with poly-ICLC survived the viral challenge. All placebo-treated TLR-3 negative and 80% of the TLR-3 positive control mice died of Ebola infection.

Based on the dose and frequency of administration studies in mice, two efficacy studies were conducted in cynomolgus NHPs.

3.4. NHP study 1

Eight NHPs were infected by the i.m. route on day 0 with 1000 PFU of EBOV, and were then treated with 250 μg/kg of poly-ICLC s.c. on days 0, +2, +4, +6, +8 + 10, depending on the survival time. This dose level was estimated to be equivalent to the 20 μg dose used in the murine studies. One infected NHP was treated with saline as placebo.

Table 2
Survival of TLR negative and positive mice infected with Ebola virus[a] with/without poly-ICLC treatment.

Group#	Mice Strain[b]	Treatment s.c. route	Schedule (days)	% Survivors vs[c] control	p values vs the control
1	B6129	20 μg Poly-ICLC	0, +2, +4, +6, +8	100	< 0.0001
2	B6129	0.2 ml PBS[d]	0, +2, +4, +6, +8	0	
3	TLR3$^{-/-}$	20 μg Poly-ICLC	0, +2, +4, +6, +8	100	< 0.0001
4	TLR3$^{-/-}$	0.2 ml PBS	0, +2, +4, +6, +8	0	
5	BALB/c	20 μg Poly-ICLC	0, +2, +4, +6, +8	100	0.0007
6	BALB/c	0.2 ml PBS	0, +2, +4, +6, +8	20	

[a] 1,000 LD_{50} MA-EBOV i.p. route on day 0.
[b] Female, 10 weeks old mice.
[c] Fisher's 2-sided exact test.
[d] Phosphate Buffer Solution pH-7.2.

Table 3
EBOV serum titers, and EBOV GP and VP40 antigens in EBOV infected poly-ICLC treated cynomolgous monkeys i.

Monkey ID #	poly-ICLC 250 μg/kg days s.c.	Euthanasia on Day[b]	Virus Titer on Day 6[c]	Fold decrease in the titer vs control	GP and VP40 EBOV antigen[d]
C04293[a]	0,2,4,6	7	5.7×10^6	None	EBOV Positive
C04097[a]	0,2,4,6	7	4.6×10^6	None	EBOV Positive
0403069[a]	0,2,4,6,8	8	2.7×10^6	None	EBOV Positive
C04215[a]	0,2,4,6,8	8	3.9×10^6	None	EBOV Positive
C050117[a]	0,2,4,6,8	9	3.2×10^6	None	EBOV Positive
C04023[a]	0,2,4,6,8	9	1.2×10^6	None	EBOV Positive
C04047[a]	0,2,4,6,8,10	10	8.0×10^5	2.1	EBOV Positive
0403047[a]	0,2,4,6,8,10	10	6.0×10^4	31.0	EBOV Positive
0402025[a]	Saline 0,2,4 s.c.on day 0,2,4	6	1.9×10^6	–	EBOV Positive

[a] 1000 PFU MA-EBOV on day 0 by i.m. route.
[b] P = < 0.0001 vs MTD of historical controls.
[c] Titer/0.1 ml serum.
[d] At the time of the respective euthanasia Immunohistochemistry of tonsils, liver, spleen, inguinal, mesenteric, mandibular, mediastinal and axillary lymph nodes are positive for EBOV antigens.

Both the control and the experimental NHPs succumbed to infection (Table 3), but the distribution pattern of the time to death by euthanasia was significantly changed ($p < 0.001$) when compared with the 6.1 days median survival in 15 historical controls. As shown on Table 3, the death distribution due to euthanasia in the poly-ICLC treated group ranged from 7 to 10 days (on days 7, 7, 8, 8, 9, 9, 10, and 10 respectively) vs. 6 days of the control and a MTD of 6.1 days among the historical controls. The untreated infected cynomolgus monkey was euthanized earliest at day 6 (Table 3). In the two longest surviving NHPs, the Ebola virus titer at day 6 was reduced 2.1 - and 31-fold, respectively, versus the viral titer of the untreated control NHP prior the death (Table 3). Histopathology on the respective day of death for all animals was positive for EBOV GP and VP 40 antigens in the tonsils, liver, spleen, inguinal, mandibular, mediastinal mesenteric and axillary lymph nodes.

In the serum, on day 6, the liver enzymes, alanine aminotransferase (ALT) alkaline phosphatase (ALP), aspartate aminotransferase (AST), as well as blood urea nitrogen (BUN) and kidney creatinine levels remained normal or only modestly elevated in seven of the poly-ICLC-treated NHPs and only in one poly-ICLC-treated NHP which died on day 7 were the enzyme values elevated as contrasted to the untreated virus control monkey (Table 4). The mean values of the eight poly-ICLC-treated NHPs were lower as compared to the untreated, infected NHP: the creatinine level was more than 3 fold, the BUN level more than three-fold, the ALP level one third, the ALT level one third, and the AST level was 50% lower. Thus, lower or near normal levels of liver and kidney function markers were detected in those NHPs that survived longer due the poly-ICLC treatment.

3.5. NHP study 2

Higher doses of 1.5 mg/kg poly-ICLC were administered s.c. to four NHPs on days 0, +2, +4, +6 and/or day +8 (Table 5). They were challenged with 1000 PFU of EBOV i.m. route on day 0. The distribution pattern of the time to death by euthanasia (two deaths on day 7, and one death on each of day 8, and 10) was significantly altered by the poly-ICLC treatment ($p < 0.017$) relative to historical 6.1 days MTD of the control, but eventually all NHPs succumbed to EBOV infection. All NHPs were EBOV antigen-positive in the tonsils, spleen, liver, inguinal, mediastinal, mandibular, mesenteric and axillary lymph nodes of the time of death by euthanasia (Table 5).

4. Discussion

These studies have confirmed the robust anti-viral protection provided by a range of doses of aqueous and liposomal poly-ICLC in murine Ebola virus challenge models, when treatment is started on the day of the virus challenge or one day after virus challenge. The data also indicated that 3–5 relatively low doses of 10–20 μg at 2-day intervals appeared to be optimal even when the treatment started 1 day post-infection. There was no clear advantage of using liposomal poly-ICLC vs the aqueous poly-ICLC. It is also possible that to measure treatment efficacy, group sizes larger than 10 mice per group could be required to measure a statistically significant difference at less than 50% survival. This suggests that the initial doses of poly-ICLC may be inducing gene-expression for various dsRNA dependent systems, and additional dose (s) could maintain the activity of those systems. These include the innate OAS pathway, which activates RNase L system to degrade viral RNAs and thereby block viral infection (Silverman, 2007), and the melanoma differentiation–associated gene 5 (MDA-5). MDA-5 is an intracellular pattern recognition receptor that acts as sensors for dsRNA viral replication within the cytoplasm of human cells (Kato et al., 2008). In another study we observed induction of elevated 2–5 OAS by poly-ICLC in the serum of humans and mice, and high levels were maintained for several days. This suggests the involvement of 2–5 OAS in the induction of the antiviral state. Poly-ICLC-treated mice that

Table 4
Renal and hepatic function chemistries 6 days following poly-ICLC treatment of Ebola virus-infected NHPs on day 6.

NHP#	poly-ICLC[b] 250 μg/kg on days	Euthanasia on day	Creatinine	BUN	ALP[c]	ALT[d]	AST[e]
C04293[a,b]	0,2,4,6	7	2.0	48	1539	250	1351
C04097[a,b]	0,2,4,6	7	1.7	61	489	137	676
0403069[a,b]	0,2,4,6,8	8	0.9	14	557	39	261
C04215[a,b]	0,2,4,6,8	8	1.1	26	644	60	368
C050117[a,b]	0,2,4,6,8	9	0.8	27	358	40	373
C0404023[a,b]	0,2,4,6,8	9	1.1	28	672	69	509
C04047[a,b]	0,2,4,6,8,10	10	0.9	13	556	34	223
0403047[a,b]	0,2,4,6,8,10	10	0.8	13	490	34	188
0402025[a]	0.1 ml Saline s.c. days 0,2,4	6	4.1	109	958	119	857
Mean value of treated NHPs		–	**1.2**	**28**	**663**	**83**	**492**

[a] 1,000 PFU EBOV on day 0 by i.m. route.
[b] 250 μg/kg poly-ICLC s.c route on days 0,2,4,5,8,10.
[c] Alkaline Phosphatase.
[d] Alanine Aminotransferase.
[e] Aspartate Aminotransferase.

survived the initial Ebola challenge were also protected from back-challenge 8 weeks later (Fig. 1, Groups 9,10,11), indicating the development of protective immunity despite the antiviral action of this compound. Similarly, poly-ICLC does not interfere with the immunity provided by live virus smallpox vaccine (Dryvax) in mice and monkeys, as measured by antibody response and resistance to viral challenge (Baron et al., 2003).

The activation levels of poly-ICLC with lymphoproliferative response was used only in mice, when four adjuvants were ranked by the activation of the lymphoproliferative response. The mice were injected by i.m. route with 10 μg recombinant Rift Valley Fever virus vaccine (rRV), with/without 10 μg LTK63, or 50 μg poly-ICLC, or 25 μg CpG, or 0.2% Alhydrogel. At day 1, 2, 6 and 7 the mice were euthanized, their spleen removed, and a single -cell suspension was prepared. Triplicate or quadruplicate cultures of 5×10^5 cells were added into 96 well microplates. Into the cells 16 units of IL-2 was added, and the cultures were immediately re-stimulated in-vitro by 10 μg/well rRV, with/without 10 μg/well LTK63 or 50 μg poly-ICLC, or 25 μg CpG, or 0.2% Alhydrogel. Into the cultures alamar blue solution was added, and after incubation the fluorescence of the culture supernatant was read by a SAFIRE Fluorometer to determine the intensity-based lymphoproliferative response. According to the lymphoproliferative response the adjuvants were ranked CpG > poly-ICLC > LTK63 > Alhydrogel. The ranking was the same after one or two injections. (Kende, Unpublished Observations).

We also showed that TLR3−/− mice (JAX B6; 129S1-Tlr3mFlv/J) were totally protected by poly-ICLC from otherwise lethal challenge with 1000 LD_{50} MA-EBOV (Table 2), which suggests that the protection afforded by poly-ICLC is independent of the TLR-3 pathway. More recent studies may shed additional light on this issue. Pretreatment with poly-ICLC provides robust protection from stroke in a murine ischemia-reperfusion model (Packard et al., 2011). This protection appears to occur through activation of the MDA-5 and IFN-1 pathways, which may induce a relative anti-inflammatory state that co-opts the much more severe inflammation and tissue loss normally generated by tissue injury through TLR4 and the myeloid differentiation primary response gene (MyD88) associated pathway. As in the case of Ebola, TLR3−/− mice are also protected from ischemic injury (Gesuete et al., 2016). Whether this relative anti-inflammatory state is the mechanism by whereby poly-ICLC attenuates certain viral infections, including Ebola, remains to be confirmed.

In spite of the robust protection seen in the murine models, all of the cynomolgus monkeys treated with NHP-equivalent or higher doses of poly-ICLC (250 μg/kg-1.5 mg/kg) and challenged with EBOL eventually succumbed to Ebola infection. As early as day 7, the tonsils, spleen, liver, inguinal, mediastinal, mandibular, mesenteric and axillary lymph nodes were positive for EBOV GP and VP40 antigens in accordance with the results of Geisbert et al., 2003.

Nevertheless, there were several indications that the poly-ICLC did have some activity in the NHP model:

1. There was a significant prolongation of surviva6l time or the time to death to euthanasia of NHPs in study 1 (8.5 days) and Study 2 (8.0 days), as indicated by the respective p values of the mean survival times compared to 6.1 day survival time of the 15 historical cynomolgus controls receiving an identical EBOV challenge.
2. In Study 1, poly-ICLC markedly reduced liver and kidney enzyme levels in the eight infected and treated NHPs. The enzyme levels were particularly reduced in those monkeys that lived longer relative to the untreated infected NHP.
3. In two longest surviving poly-ICLC-treated NHPs on day 6 the serum EBOV titer was reduced 2.1 - or 31-fold, while in those NHPs that died earlier, the virus titer was the same or somewhat higher than the titer in the untreated infected NHP was.

The results suggest that poly-ICLC should be administered repeatedly (up to five-times or more) to maximize host defenses in the NHP model. Future studies should also investigate lower-dose of viral challenges in NHP that may be more representative of a typical human exposure (e.g. 100 pfu to 10 pfu). If needed, reduction of the antiviral

Table 5
Treatment of Ebola virus infected NHP with poly-ICLC.

NHP#	Dose (mg/kg)	Schedule Days	Route	Challenge[a] (1000 PFU)	Day of euthanasia[b]	Immunohistochemistry[c].
22174	1.5	0,2,4,6	s.c.	Yes	7	Ebola positive
22602	1.5	0,2,4,6	s.c.	Yes	7	Ebola positive
23697	1.5	0,2,4,6	s.c.	Yes	8	Ebola positive
23685	1.5	0,2,4,6,8	s.c.	Yes	10	Ebola positive

[a] 1000 PFU of EBOV i.m. route on day 0.
[b] p = 0.017 the time of death due to euthanasia vs. the historical MTD.
[c] Immunohistochemistry of the tissues of tonsils, liver, spleen, inguinal, mesenteric, mandibular, mediastinal and axillary lymph nodes for EBOV antigens.

doses in NHPs can be accomplished by substituting carboxymethylcellulose (CMC) in poly-ICLC as proposed by a contract study at Roswell Park Memorial Institute (RPMI) because CMC is not metabolized or excreted (Bello and O'Malley, 1991). One of the CMC-free poly-ICLC compounds made at RPMI poly-ICDextran has an LD_{50} of 48 mg/kg on i.v. administration, while poly-ICLC's LD_{50} is 9 mg/kg which is 5.3 times higher (Bello and O'Malley, 1991). Consequently, poly-IC-Dextran can be administered in a dose 5.3 higher than the 1.5 mg/kg without toxicity, which is 7.95 mg/kg (5.3 × 1.5 mg/kg). Taking 2.5 mg/kg the maximum non-toxic dose of poly-ICLC in NHPs, then, even higher dose of poly-ICDextran can be used. Poly-ICDextran and poly-ICLC with the same dose were equally effective to protect mice against Rift Valley Fever virus infection (Kende Unpublished Observation), which make them promising candidates against EBOV infection in human and NHPs.

Recent studies have demonstrated robust adaptive immune protection in mice and NHPs by Ebola virus-like particle vaccine adjuvanted with poly-ICLC (Martins et al., 2014). As in the case of SARS, the potential for simultaneously providing immediate innate protection along with longer term immunity with such a poly-ICLC/vaccine combination needs to be investigated further, and it may be particularly relevant to Ebola epidemic containment. Like in many other infections, it is quite likely that the given EBOV vaccine will be the main protective armament. A report by Hensley et al. (2010) demonstrated in cynomolgus NHPs vaccine induced protective immunity against challenge with heterologous EBOV species, and shows that Ebola vaccines capable of eliciting potent cellular immunity may provide the best strategy for eliciting cross-protection against newly emerging heterologous EBOV species. Nevertheless, chemotherapy could be useful in unvaccinated populations, and/or adjunct to vaccination.

Authors' note

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.12.020.

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