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Cytokine Storm Mitigation

Ming Wang
China Agricultural University

Prepared by:
College of Veterinary Medicine, China Agricultural University, Beijing 100094, China

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IMPORTANT INFORMATIVE STATEMENTS

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Final Contract Report

Contract Title: **Cytokine Storm Mitigation**

Contract No. W7702-165789/001/EDM

Reported by: Prof. Ming Wang

Contractor: Prof. Ming Wang, College of Veterinary Medicine, China Agricultural University, Beijing 100094, China

Date: April 9, 2017

Executive Summary

This contract was awarded to Professor Ming Wang, China Agricultural University on March 2, 2016. The primary objective of this contract work was to evaluate the approach to drug combination therapy of cytokine storm mitigating drugs with antiviral drugs. Two relevant viral infection models (highly pathogenic influenza virus and Dengue fever virus infections in mice) were used in the contract work. Catalytic DNA (DNAzymes) and siRNAs targeting Bcl-2 and c-jun genes were combined with liposome-encapsulated, polyinosinic-polycytidylic acid and poly-L-lysine double-stranded RNA (LE Poly ICLC) in the testing of their efficacy on cytokine storm and animal survival.

Background

Newly emerging and re-emerging viral threats have continued to challenge medical and public health systems and incur economic costs to both individuals and countries. The influenza virus is a main cause of those threats and is responsible for millions of severe cases and 250,000–500,000 deaths each year. These infections in humans are accompanied by an aggressive pro-inflammatory response and insufficient control of an anti-inflammatory response, a combination of events called ‘cytokine storm'. Currently, Relenza (zanamivir) and Tamiflu (oseltamivir) known as neuraminidase inhibitors have been used to prevent and reduce symptoms of flu. Although these drugs showed clinical effectiveness in combating the virus [1], they suffered a number of drawbacks including neurological toxicity and emerging drug-resistant strains [2]. Therefore, it is imperative that new classes of anti-influenza agents are developed to control influenza epidemics.

Nucleic acid molecules are emerging as a potent force in both further characterizing important molecular pathways and have a potential to be developed as a new class of anti-flu drugs. The ability to selectively attenuate the expression of specifically targeted genes represents an appealing method of therapy as well as a means of dissecting molecular function. As such, strategies to specifically knockdown gene expression have received considerable attention in translational medicine. Previous work has shown that antiviral drugs developed at Defence Research and Development Canada, Suffield Research Centre (DRDC SRC) including liposome-encapsulated, polyinosinic-polycytidylic acid and poly-L-lysine double-stranded RNA (LE Poly ICLC) [3] and antisense oligonucleotides are effective in prevention and treatment of influenza H5N1 and H1N1 infections in mice [4]. In order to develop effective prophylactic and therapeutic drugs against the virus infections, it is important to mitigate the cytokine storm and associated lung damage using novel drugs which suppress or down-regulate inflammation. DNAzymes are promising drugs which have been shown to be effective in silencing the master genes which regulate inflammation [5]. Treatment of influenza A virus infection with DNAzymes has shown some promise in effective in reducing inflammation and enhance therapeutic outcome in experimental animals [6, 7]. Combination of pre- and post-treatment with DNAzymes in experimental animals will likely enhance the effectiveness of LE Poly ICLC to protect against pandemic strains of

influenza A viruses.

Small interfering RNA (siRNA), a 21-23nt double-stranded RNA responsible for post-transcriptional gene silencing in a sequence-specific manner, has attracted great interests as promising class of genomic drugs for cancers and viral infections. Despite high silencing efficiency and on-target specificity, the clinical translation of siRNA has been hindered by its inherent features: poor intracellular delivery, limited blood stability, unpredictable immune responses and unwanted off-targeting effects. To overcome these hindrances, researchers have made various advances to modify siRNA itself and to improve its delivery. The second generation siRNA features chemically and structurally modified siRNAs to solve their intrinsic problems. As for DNAzymes, great efforts have also been made to improve delivery efficiency, such as, various formulations including siRNA conjugates, polymerized siRNA, and nucleic acid-based nanoparticles.

The development of gene-based drugs against viral diseases can benefit both public health and military sectors. These drugs can play in an important role in E&E viral disease management, as they provide effective broad-spectrum and specific antiviral prophylaxis and therapy to these diseases in the absence of commercial vaccines. When fully developed, they may represent promising life-saving medicines, and demonstrate the benefits of advances in biotechnology. Therefore, based our previous work and collaboration history with DRDC, we were awarded this Contract in 2016.

Major achievements

1. Completed the testing of Dz-Bcl2 and Tamiflu combinations in H5N1 infection mouse model.
2. Completed anti-inflammatory drug testing in Dengue virus infection model.
3. Completed testing of siRNAs targeting c-jun in combination of LE-PolyICLC in mice;
4. Completed testing of siRNAs targeting Bcl-2 in combination of LE-PolyICLC in mice

Technical and Scientific Summary

1. Efficacy of combination of anti-bcl2 DNAzyme and Tamiflu in H5N1 infection mouse model

In previous work, we demonstrated that suppression of c-jun by Dz13 could improve the survival of the mice infected with H5N1 influenza virus [8]. In this work, we tested the combinational effect of anti-bcl-2 DNAzyme (DzBcl) and Tamiflu on highly pathogenic H5N1 influenza infection in mice. Experimentation with animals was governed by the Regulations of Experimental Animals of Beijing Authority and approved by the Animal Ethics Committee of the China Agriculture University.

Mice were separated into 4 groups, and 10 mice in each group. On day 0, mice were anesthetized with Zoletil (Virbac, Carros, France) (16.7mg/kg) and infected intranasally with 2 LD_{50} virus mixed with DzBcl (2.60 mg/kg). Tamiflu was administered intranasally (i.n.) to the respective groups in a volume of 50 μl on days 1, 4 and 8 post infection. Control group was given same volume of phosphate buffered saline (PBS) instead. Mice in each group were examined for survival up to 12 days. As shown in Figure 1, the combined use of the DNAzyme and Tamiflu could effectively improve the mouse survival (60% at the end of the testing), while both the DNAzyme alone and Tamiflu alone provided only partial protection.

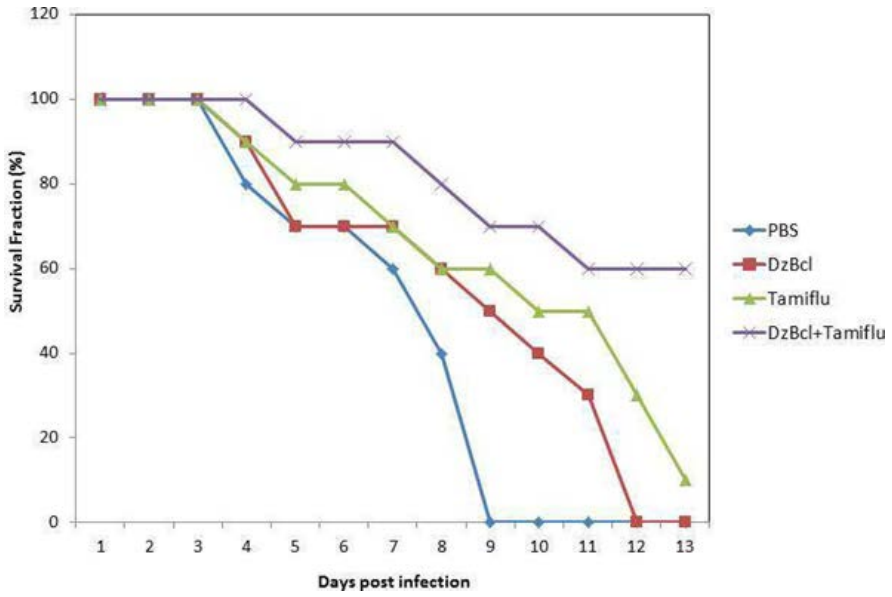

Figure 1. Combinational use of anti-Bcl-2 DNAzyme and Tamiflu in H5N1 mouse infection model.

2. Efficacy evaluation of anti-inflammatory drugs in Dengue virus infection model

We evaluated the potential of LE-PolyICLC against the Dengue 2 virus in a lethal Balb/c mouse model established using NGC strain [9]. The protective efficiency of LE-PolyICLC by i.v. route was compared with s.c. and i.p. routes initially, and PolyICLC as the control drug. Results indicated that LE-PolyICLC induced varying levels of protection by the different administration routes. The two groups treated with LE-PolyICLC and PolyICLC by i.v. route presented 100% survival rate, while 57.14% of the mice in 0.9% NaCl-treated group survived the viral challenge. Survival rate of the group treated with LE-PolyICLC by s.c. route was 85.71%, which was the same as i.p. route (Table 1).

Table 1. The protective efficiency of LE-PolyICLC against dengue 2 virus infection in mice.

Group	No. of survivors[a] /total No.	% Survival
LE-PolyICLC (ip) [b,d]	6/7	85.71
LE-PolyICLC (sc) [b,d]	6/7	85.71
LE-PolyICLC (iv) [b,d]	7/7	100
PolyICLC (iv) [b,d]	7/7	100
0.9% NaCl (iv) [c,d]	4/7	57.14
Untreated control [c,e]	7/7	100

Further analysis of clinical signs of infection showed that LE-PolyICLC treated by the intravenous (i.v.) route was more efficacious than the other routes (Figure 2).

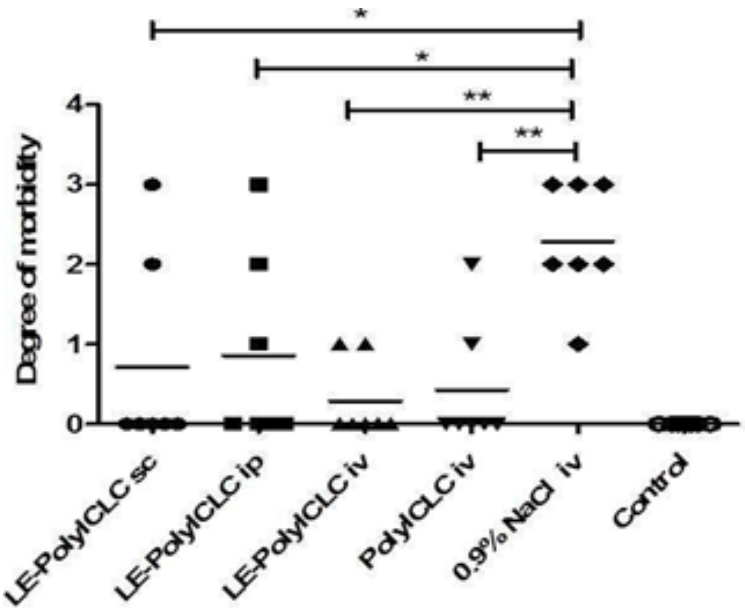

Figure 2. Morbidity of Balb/c mice treated with drugs after being challenged with DNV-2 strain New Guinea C (NGC). The semi-quantitative analysis of severity of morbidity in each group after virus challenge was performed using a scale ranging from 0 to 3 (0 = none, 1 = mild paralysis in one hind leg or alteration of the spinal column with a small hump, 2 = severe paralysis in one hind leg and alteration of the spinal column with a small hump or severe paralysis in both hind legs, 3 = two severe hind leg paralysis and deformed spinal column or death).

To determine whether LE-PolyICLC could influence virus RNA replication and viral antigen expression, the weight loss of DENV2-infected mice (Fig. 3*A*), morbidity degrees (Fig. 3*B*), viral load in the brain by reverse transcription polymerase chain reaction (RT-PCR) (Fig. 3*C*) and viral protein production by Western blotting (Fig. 3*D*) were measured. The data suggested that the i.v. administration of LE-PolyICLC could inhibit virus replication, leading to a reduction in brain viral titers and viral antigen expression of infected mice.

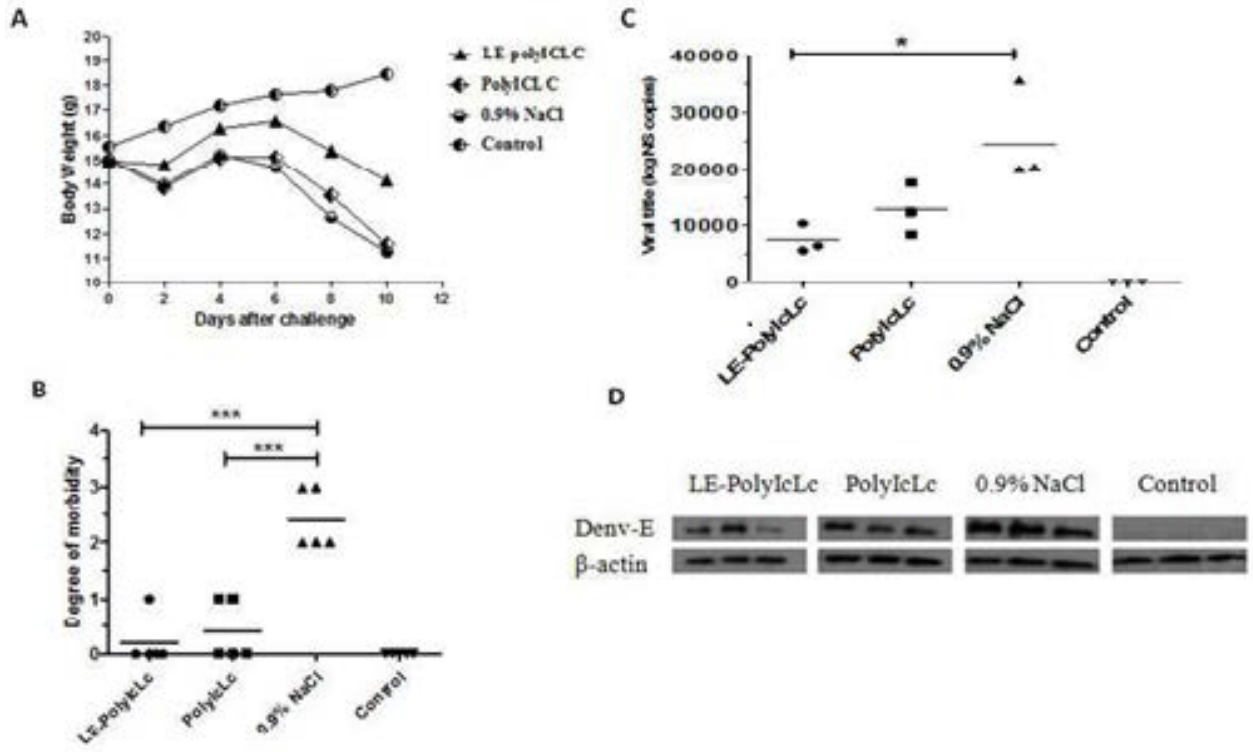

Figure 3. Effects of LE-PolyICLC for inhibiting virus replication in mice. The LE-PolyICLC was treated by the intravenous (i.v.) route at the same dose and time point. Three mice in each group were euthanized on day 10 post infection (p.i.), the body weights were recorded daily (A). The degree of morbidity was performed using a scale ranging from 0 to 3 (B).the viral titers in brains were determined by real-time quantitative RT-PCR (C). Brain tissues were collected randomly from three mice of each group on day 10 p.i., viral E protein expression was analyzed by Western blotting (D). Asterisks indicate statistically significant differences (*p<0.05, **p< 0.001,***p< 0.0001).

To investigate the influence of LE-PolyICLC on the expression of cytokines induced by Dengue 2 virus, TNF-α, IFN-β, IFN-γ, and IL-6 levels were assessed by real-time quantitative RT-PCR method on days 10 dpi. The mRNA levels in spleen of TNF-α, IFN-β, IFN-γ, and IL-6 in the LE-PolyICLC-treated group were significantly increased compared with the PolyICLC or 0.9% NaCl-treated groups at 10 dpi. ($p< 0.0001$) (Fig.4*A*). The liver mRNA levels of these cytokine were lower than spleen, but were not significant for IL-6 (Fig. 4*B*). TNF-α, IFN-β, IFN-γ, IL-6, and IL-12 p40 cytokine production in the serum from these groups were also determined *via* ELISA. IFN-γ and TNF-α levels in the LE-PolyICLC-treated group were significantly increased compared with the PolyICLC or 0.9% NaCl-treated groups at 10 dpi ($p < 0.001$) (Fig. 4*C* and 4*D*). IFN-β and IL-12P70 levels

were also increased, albeit not significantly (Fig. 4*E–G*), and yet compared with 0.9% NaCl-treated groups at 10 dpi, the IL-6 levels in the LE-PolyICLC- and PolyICLC-treated groups were significantly decreased ($p < 0.05$; Fig. 3*F*).

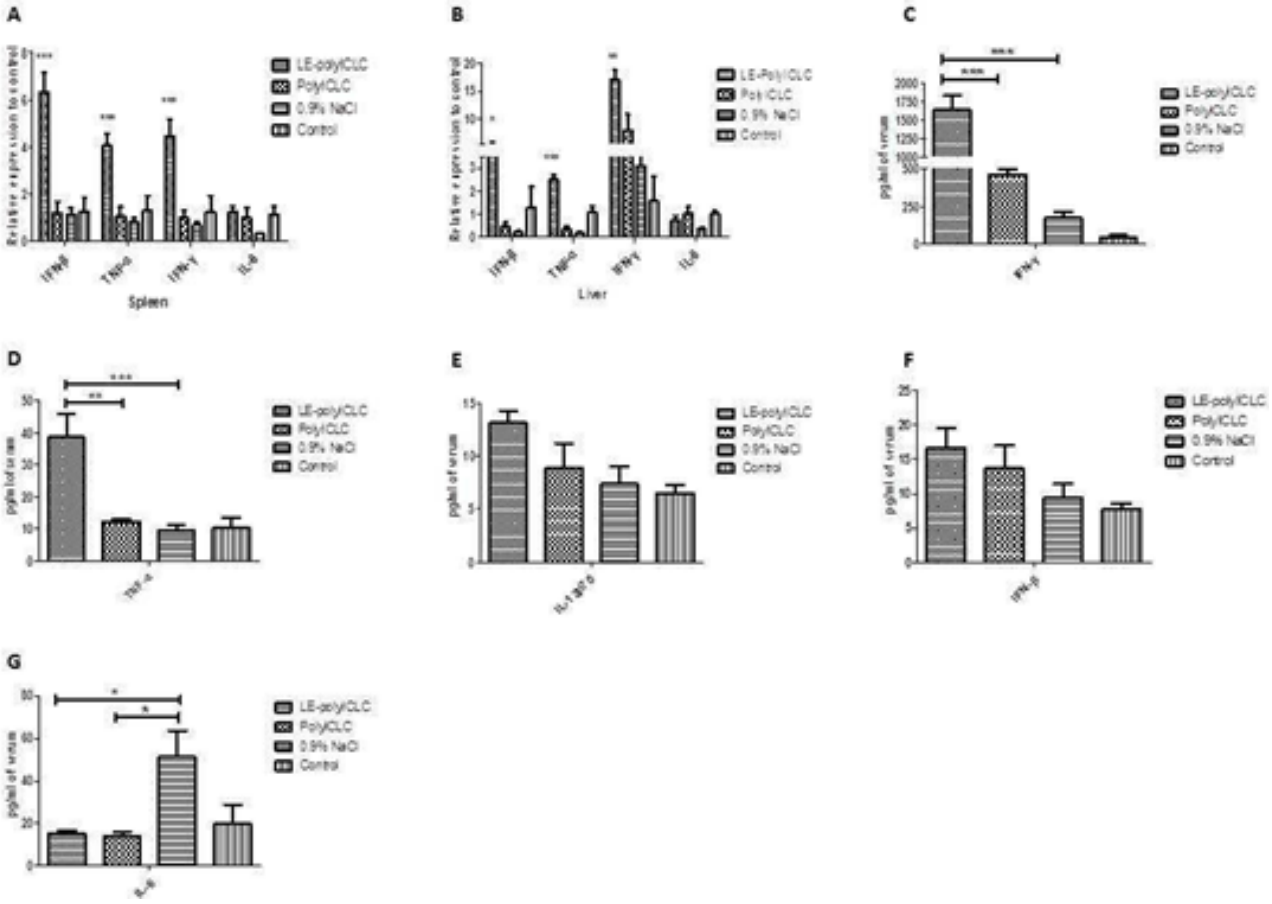

Figure 4. Effect of LE-PolyICLC on cytokine expression. At day 10 p.i., mRNA levels of cytokines in the liver and spleen were measured by real-time quantitative RT-PCR (A.B). Protein levels of cytokines in the serum were measured by ELISA (C.D.E.F.G). Data were presented as mean ± SD of three representative independent.

To examine the effect of LE-PolyICLC on the humoral immune responses in mice, on day 10 after virus infection, the serum samples were collected and the titers of total antibodies against DENV2 were detected by ELISA. The titers of antibodies in mice administrated with LE-PolyICLC were higher than PolyICLC or 0.9% NaCl-treated groups, although not statistically significant (Fig. 5). Nonetheless, these data indicated that LE-PolyICLC could enhance antibody response.

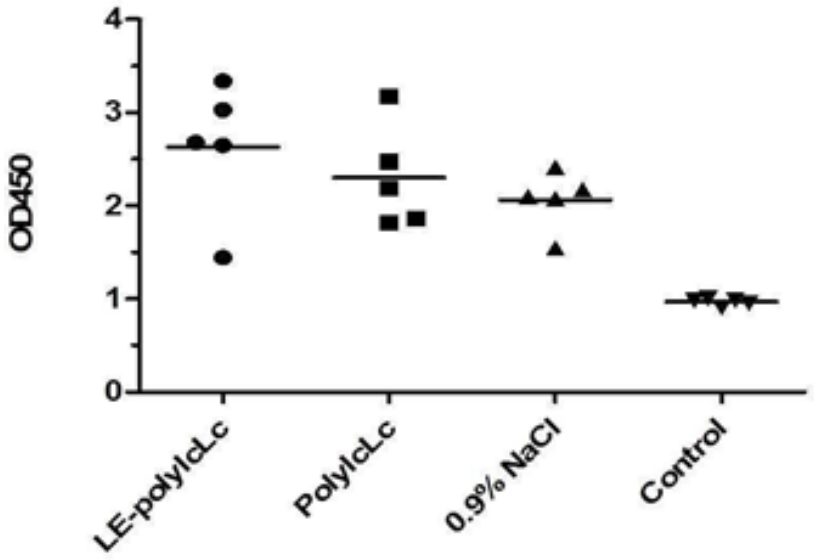

Figure 5. E protein-specific total antibody level. Sera were collected 10 days after virus infection. Mean value ± S.E.M of OD450 in each group was presented (n=5).

To determine whether the severity of histopathological lesion was improved by LE-PolyICLC treatment, the histopathological lesion in brains of the mice at day 10 were examined. As shown in Figure 6, treatment of the mice with LE-PolyICLC reduced the severity of pathological lesion on brain (Fig 6).

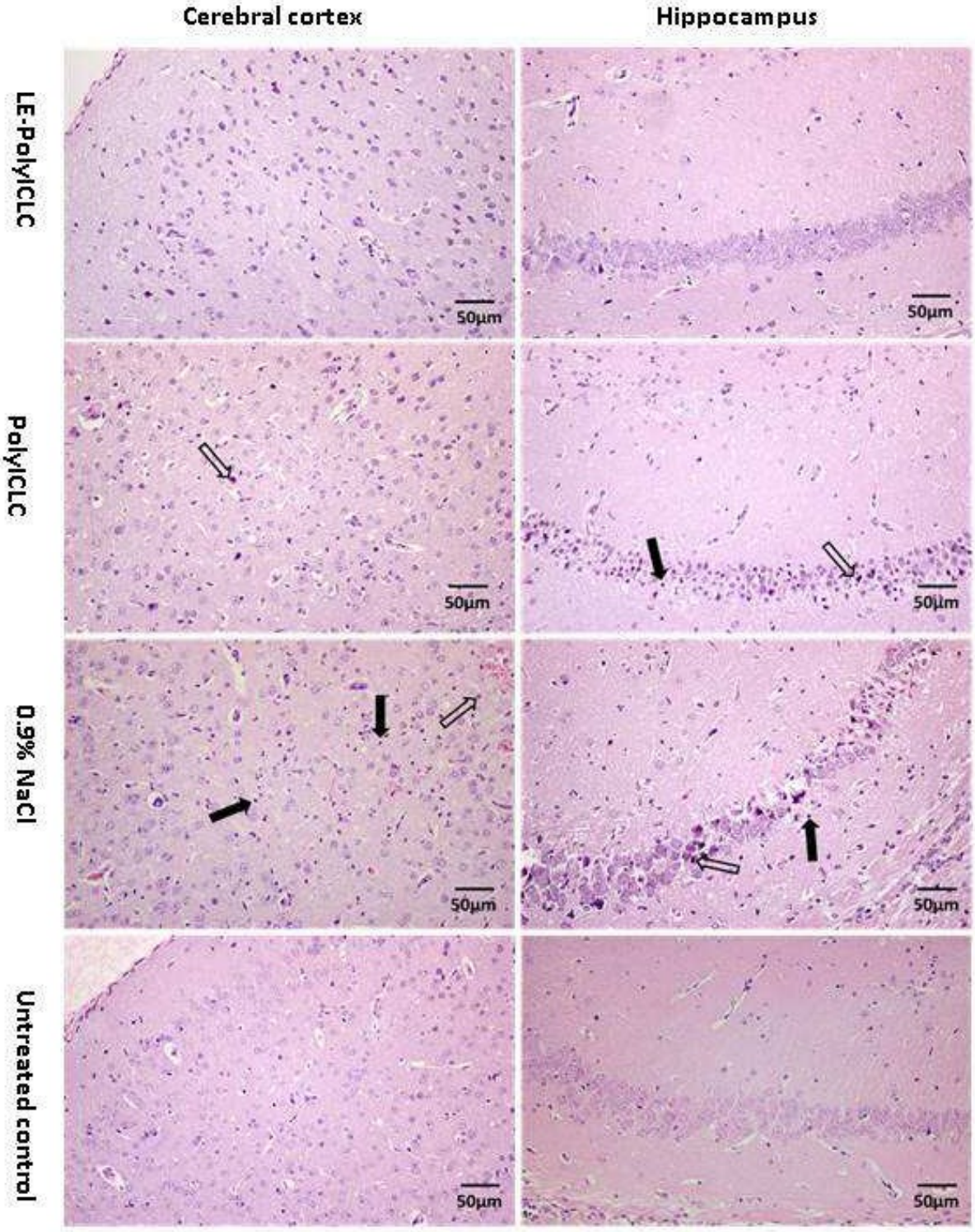

Figure 6. The histopathology of the brain after DENV2 virus infection on day 10. Representative brain sections from each group were subjected to H & E staining on day 10 post-infection. Solid arrows indicate microglia or inflammatory cellular infiltration; and open arrows denote neuronal necrosis.

3. Combinational testing of small interference RNAs (siRNAs) targeting c-jun and LE-PolyICLC in mice

Pandemic influenza is known to induce a severe over-reactive immune response in the body characterized by inflammation in the lungs, and subsequent damage to alveoli and lung tissues. The inflammation caused by viral infection is under strict control at various levels, among which transcriptional regulation of cytokines is very important in the host-virus interactions. c-jun is one of the most important transcriptional factors that participate in nearly all the biological processes. We previously showed that targeting c-jun by DNAzymes could significantly reduce H5N1 influenza virus-induced animal death [8]. In the present work, we synthesized the siRNAs targeting c-jun and tested the efficacy of combinational use of c-jun-targeted siRNA and LE-PolyICLC in H5N1 influenza virus infection model. The detailed schedule of the testing is shown in Figure 7.

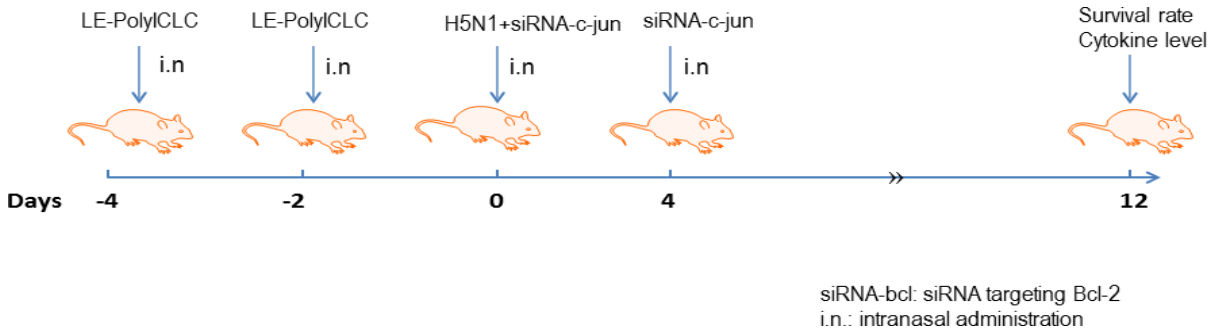

Figure 7. Scheme of combinational testing of anti-Bcl-2 siRNA and LE-PolyICLC in H5N1 mouse infection model.

siRNA-c-jun was synthesized by RiboBio and pre-mixed with LipoFect2000 transfection reagent. The LE-PolyICLC was administered intranasally (i.n.) to anaesthetized mice in a volume of 50 µl at a dose of 1 mg/kg body weight on days 2 and 4 before viral challenge. Control mice were given same volume of PBS instead. On day 0, mice were anesthetized with Zoletil (Virbac, Carros, France) (16.7mg/kg) and infected intranasally with 0.2 LD50 of H5N1 virus mixed with siRNA-c-jun (1 mg/kg) in total volume 50ul. Second administration of the siRNA was conducted on day 4. Each group of ten mice was examined survival for 12 days.

The results in Figure 8 showed that the combinational use of siRNA-c-jun and LE-PolyICLC could significantly improve the overall survival of the animals infected by H5N1 influenza virus (A), companied with suppression of the representative cytokine (TNFα) expression (B).

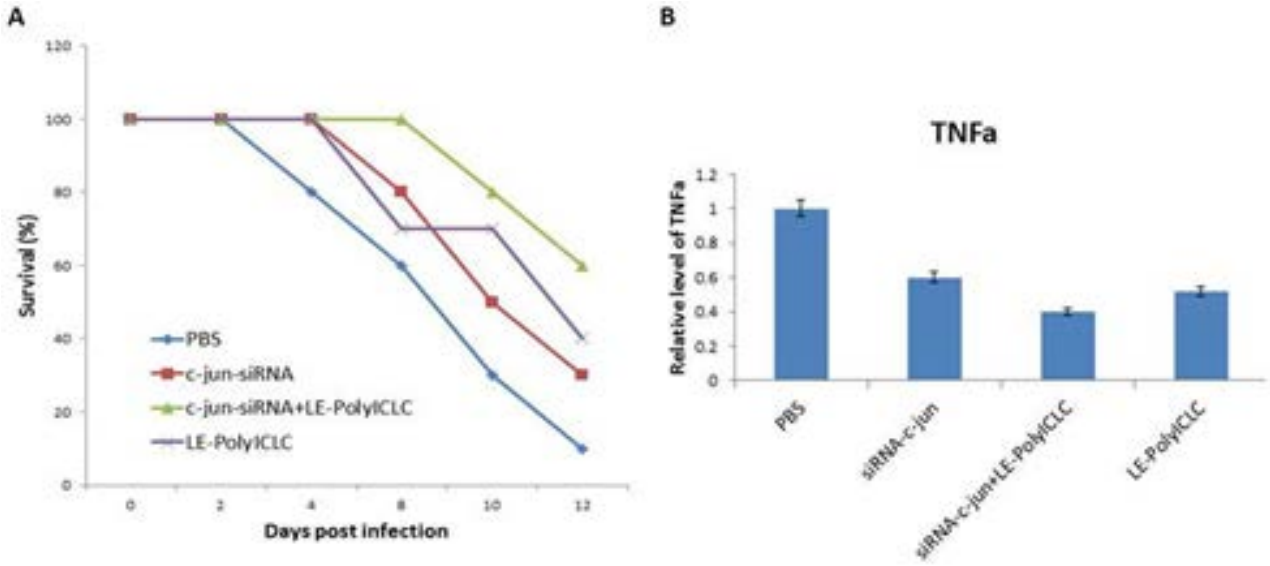

Figure 8. Effect of siRNA and LE-PolyICLC on animal survival and cytokine expression in H5N1 infection model.

4. Testing of siRNAs targeting Bcl-2 in combination of LE-PolyICLC in H5N1 virus infection model

Viruses have evolved a capacity to counter apoptosis in host cells by inducing anti-apoptotic gene expression, which facilitates the viral replication. Thus, triggering host cell apoptosis may be an alternative strategy to inhibit viral replication. To test if induction of apoptosis by targeting bcl-2 with siRNA could suppress H5N1 replication in vivo, we examined the combinational effect of siRNA-bcl and LE-PolyICLC on highly pathogenic H5N1 influenza infection in mice. The experimental procedure is shown in Figure 9.

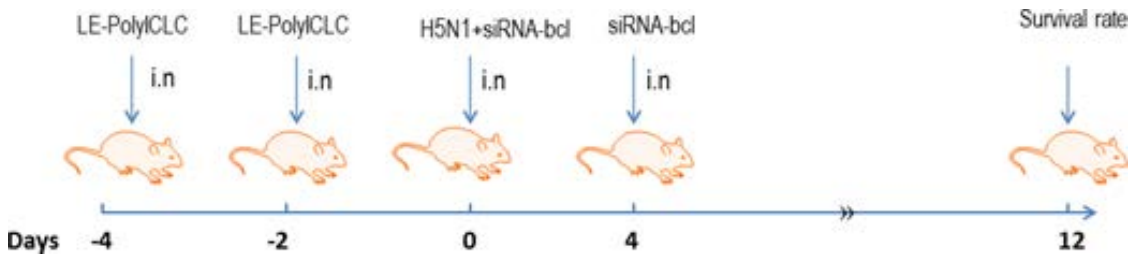

Figure 9. Scheme of combinational testing of c-jun siRNA and LE-PolyICLC in H5N1 mouse infection model. Four groups of ten mice were used in the testing. i.n. intranasal administration.

As shown in Figure 10, while the use of both siRNA-bcl and LE-PolyICLC alone could improve the animal survival, the combinational use of two exhibited better protection.

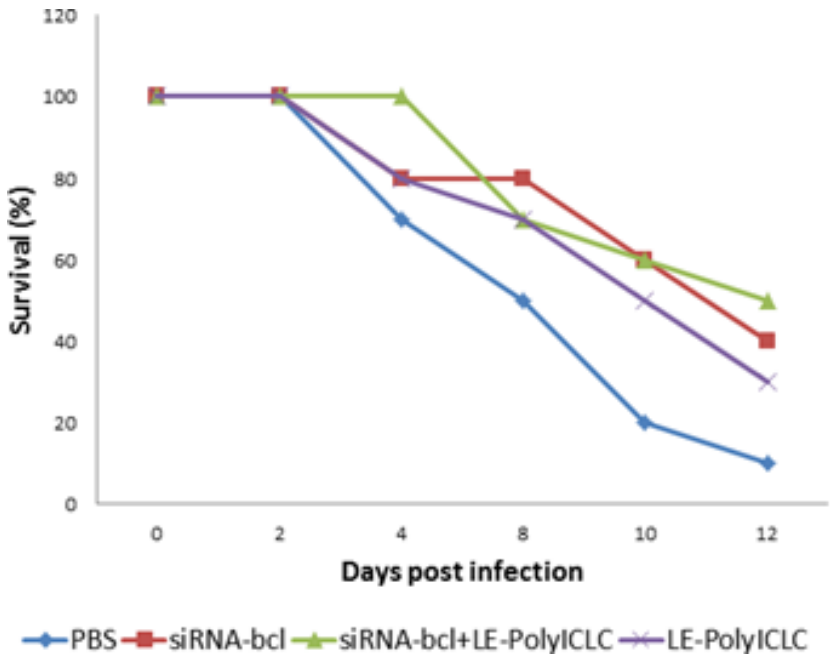

Figure 10. Effect of siRNA to Bcl-2 and LE-PolyICLC on animal survival in H5N1 mouse infection model.

Publications

1. Kawai, N., et al., *Clinical effectiveness of oseltamivir and zanamivir for treatment of influenza A virus subtype H1N1 with the H274Y mutation: a Japanese, multicenter study of the 2007-2008 and 2008-2009 influenza seasons.* Clin Infect Dis, 2009. **49**(12): p. 1828-35.
2. Tuna, N., O. Karabay, and M. Yahyaoglu, *Comparison of efficacy and safety of oseltamivir and zanamivir in pandemic influenza treatment.* Indian J Pharmacol, 2012. **44**(6): p. 780-3.
3. Wong, J.P., et al., *Liposome-mediated immunotherapy against respiratory influenza virus infection using double-stranded RNA poly ICLC.* Vaccine, 1999. **17**(13-14): p. 1788-95.
4. Wong, J.P., et al., *Activation of toll-like receptor signaling pathway for protection against influenza virus infection.* Vaccine, 2009. **27**(25-26): p. 3481-3.
5. Somasuntharam, I., et al., *Knockdown of TNF-alpha by DNAzyme gold nanoparticles as an anti-inflammatory therapy for myocardial infarction.* Biomaterials, 2016. **83**: p. 12-22.
6. Sun, L.Q. and J.P. Wong, *Frontiers in nucleic acid-based drug research and development.* Future Med Chem, 2015. **7**(13): p. 1619-21.
7. Wong, J.P., *Nucleic acid-based drugs against emerging zoonotic viruses.* Future Med Chem, 2015. **7**(13): p. 1709-19.
8. Xie, J., et al., *Regulatory roles of c-jun in H5N1 influenza virus replication and host inflammation.* Biochim Biophys Acta, 2014. **1842**(12 Pt A): p. 2479-88.
9. Hu, Y., et al., *Antiviral effects of liposome-encapsulated PolyICLC against Dengue virus in a mouse model.* Biochem Biophys Res Commun, 2016. **478**(2): p. 913-8.

Project Management

The project was performed under a close consultation with the Technical Authority at DRDC. Routine emails and tele-conferences between CAU and Technical Authority of DRDC ensured the project to be scientifically sound and technically feasible.

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1. All experimental data have been submitted to Technical Authority;
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4. The claims have been in accordance with the contract.

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