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IN VITRO ANTIVIRAL ACTIVITY OF POLY-Γ-GLUTAMATE AGAINST NEWCASTLE DISEASE VIRUS INFECTION ON MURINE MACROPHAGE CELLS

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ABSTRACT

The High-molecular-weight Poly-γ-glutamate (HM-γ-PGA) is a promising natural and edible polymer with diverse biological functions. Among the applications of HM-γ-PGA, its capacity to promote immune-stimulating effect via Toll-like receptor-4 (TLR4) signaling solidifies its significance as a potential natural antiviral agent. In the pre-treatment assay, HM-γ-PGA can protect murine macrophage cells (RAW 264.7) against Newcastle Disease (ND) infection. Such protection can be explained by the induction of antiviral state of HM-γ-PGA in RAW 264.7 cells via TLR4-mediated IRF-3, IFN-B, and IFN-related gene induction as shown in time-dependent changes in mRNA expression confirmed by polymerase chain reaction (PCR). Moreover, since mRNA expression does not necessarily correlate with the secreted protein levels, pro-inflammatory cytokine secretion was also measured by murine Enzyme-Linked Immunosorbent Assay (ELISA). Therefore, our findings suggest that HM-γ–PGA can be a potential antiviral substance that can inhibit NDV infection through its stimulation of antiviral state on RAW 264.7 cells. These results have been consistent with the previous studies showing that this natural polymer can protect RAW 264.7 and mice against influenza infection. However, it should be noted that although murine macrophage cells are susceptible to NDV, they are not the natural host cells of the virus, thus further in vitro and in vivo studies using chicken and chicken-derived cells are needed to fully assess the efficacy and applicability of HM-γ–PGA in the poultry industry.

Key words: Poly-γ-glutamate, Newcastle disease, antiviral effect

1 BACKGROUND

Newcastle disease virus (NDV) is a member of the *Paramyxoviridae* family under the genus *Avulavirus* (Mayo 2002a, 2002b) and currently designated as avian paramyxovirus virus serotype 1 (APMV-1) (Alexander 2000). According to the Office International des Epizooties (OIE) in 2009, NDV strains can be classified into five pathotypes according to the clinical signs shown by the affected chickens, namely viscerotropic velogenic (high mortality and hemorrhagic intestinal lesions), neurotropic velogenic (high mortality, respiratory and nervous signs), mesogenic (low mortality, respiratory signs with occasional nervous signs), lentogenic (subclinical or mild infection), and asymptomatic enteric (subclinical enteric infection).

Included in the List A of sufficiently serious diseases of poultry (Alexander 2000), ND remains prevalent worldwide, though a number of live and inactivated NDV vaccines are available to control the disease (Seal *et. al*. 2000; Lee *et. al*. 2008). However, the currently available commercial vaccines have their limitations and one of them is the absence of

genetic markers for serological differentiation between vaccinated and naturally infected birds. There are also reports suggesting that the types of NDV strains that have been identified circulating in poultry already showed major antigenic drift. Thus, there is a need for better NDV vaccines which can solve such problems, wherein viral vector vaccines prove to be a good alternative (Kumar *et. al*. 2011), as exemplified by the first licensed commercial recombinant vaccine, a recombinant Newcastle disease virus vaccine, using fowl pox virus as the vector to express immunogenic proteins from the Newcastle disease virus (Yamanouchi *et. al*. 1998). However, recombinant vaccines are not widely used by the poultry industry due to their inability for use in population-based mass application procedures and high cost (Seal *et. al*. 2000).

Currently available vaccines cannot provide adequate immunity in poultry even with the use of multiple vaccinations and live vaccines do not completely prevent infection or virus shedding (Alexander 2001; Kumar *et. al*. 2011), thus natural substances with the ability to inhibit or prevent NDV infections might provide much needed additional protection against the viral infection. One of the natural substances which is widely studied for its various biological functions and applications is the high-molecular-weight poly-γ-glutamate (HM-γ-PGA) (>3000 kDa), a natural, edible, and biodegradable polymer derived from *Bacillus subtilis* subsp. *chungkookjang* (Bae *et. al*. 2010; Sung *et. al*. 2005; Poo *et. al*. 2010). Recently, Moon *et al*. (2012) demonstrated the antiviral function of HM-γ-PGA against influenza virus through stimulation of type I interferon (IFN) and Mx1 proteins both *in vitro* and *in vivo*. Additionally, several studies have shown that HM-γ-PGA is functionally better than low molecular weight Poly-γ-glutamate (LM-γ-PGA) (10-1,000 kDa) when it comes to anti-tumor activity, immune stimulation and when used as an adjuvant (Lee *et. al*. 2009; Yoshikawa *et. al*. 2008).

In this study, the antiviral effect of HM-γ-PGA was evaluated on murine macrophage cell line (RAW 264.7). Based on the results, this study has shown that HM-γ-PGA protects murine macrophage cells from NDV infection through Type I interferon induction.

2 MATERIALS AND METHODS

2.1 Preparation of HM-γ–PGA

By the method of Kim *et al*., (2007) poly-γ-glutamate (γ–PGA) produced from *Bacillus subtilis* subsp. *chungkookjang*, was prepared and provided by BioLeaders Corporation (Daejeon, Korea) in 0.85% sterile NaCl solution. Briefly, the culture broth of *B. subtilis* subsp. *chungkookjang* was collected and mixed with 3 times volume of ethanol. The precipitate was lyophilized and reconstituted in 10mM Tris–HCl buffer (pH 7.5), treated with proteinase K, and dialyzed in distilled water. Next, the γ–PGA was purified by anion–exchange chromatography, and dialyzed using Sep–Pak Plus Waters Accell Plus QMA cartridge (Millipore, USA) equilibrated with distilled water. Next, the cartridge column charged with γ–PGA was stepwise developed with NaCl solutions from 0.1 to 1.0M. By estimating the concentration of glutamate in hydrolyzed γ–PGA using an amino acid analyzer, the content of γ–PGA was calculated by the following formula; content of γ–PGA (%)=(amount of glutamate/amount of sample)×(A/B)×100. A=129 (molecular mass of γ–glutamyl residue in γ-PGA); B=147 (molecular mass of glutamate).

The number and weight-average molecular masses (M*n* and M*w*, respectively) along with the polydispersity (M*w*/M*n*) of γ–PGA molecules were measured by gel permeation chromatography using a GMPWXL column (Viscotek, USA) and a LR125 Laser Refractometer (Viscotek, USA). Polyacrylamide standards (American Polymer Standard, USA) were used to construct a calibration curve and polydispersity of high molecular weight γ–PGA was measured. The content of high molecular mass γ–PGA was increased to > 99%, and polydispersity was decreased after anion–exchange chromatography. To thoroughly get

solubilized gamma–PGA, the pH was adjusted to 7.0 by adding 5N NaOH solution to the acid form PGA (Lee *et. al*. 2009). Only purified HM-γ-PGA was used in this study.

2.2 Cell Culture and Virus

RAW 264.7 cells (ATCC TIB–71) were maintained in Dulbecco's Modified Eagles Minimum essential medium (DMEM, Invitrogen, USA) containing 10% fetal bovine serum (FBS)(Gibco, USA) and 1% antibiotic/anti-mycotic (Gibco, USA) at 37 °C with 5% CO_2 until use.
The NDV–GFP (green fluorescence protein) was kindly provided by Dr. J. Jung of University of Southern California. The virus was amplified in 9- to 10-day-old embryonated eggs. Collected allantoic fluid was ultracentrifuged at 20,000 × g for 2.5 hours at 8°C in 20% sucrose solution for viral concentration. Viral pellet was resuspended in PBS and 500 ul aliquots were kept at -70°C until use.

2.3 Antiviral Assay

To evaluate the anti-viral effect of HM-γ-PGA against NDV infection, the inhibition of virus replication was examined by the method of Moon *et. al*., (2012) with some modifications. RAW 264.7 cells were cultured with a suitable number (8×10^5 /well) onto 12–well plates (Nunc, Denmark) for 12 hours. Then, the medium was substituted to DMEM (for w/o treatment and virus only groups) and DMEM with 1mg/ml γ–PGA. DMEM with 1000 units of recombinant mouse interferon-β (Sigma, USA) served as the positive control. After 12 hours of incubation, cells were infected with 1 multiplicity of infection (MOI) of NDV–GFP virus. The green fluorescence protein expression was observed at 200 × magnification 12 hours post- infection.

Cell viability was carried out via trypan blue exclusion test as described elsewhere. Briefly, RAW 264.7 cells were treated with either 1mg/ml of γ–PGA or DMEM only for 12 hours. After incubation, the cells were infected with NDV–GFP virus and the clarified cells from each group were mixed with 0.4% trypan blue stain (Invitrogen, USA) at 1:1 ratio. After staining, 10 µl of the mixture was applied to a haemocytometer wherein to get the percentage of viable cells, the total number of viable/live cells per ml of aliquot was divided by the total number of cells/ml of aliquot multiplied by 100. Cell counting was done thrice.

Lastly, following the instructions of the manufacturer, the GFP expression levels of media treated, 1 mg/ml HM-γ-PGA and IFN-β treated cells 12 h before NDV-GFP infection was measured 24 hours post infection (hpi) using Glomax multi-detection system (Promega, USA) (Puig-Saus 2011). Briefly, the cells from each treatment group as described above were collected separately and centrifuged at 1200 rpm for 3 min. The resulting cell pellet was diluted in PBS and transferred to 96-well black plate for GFP detection

2.4 NDV-GFP mRNA expression on RAW264.7 cells

By the method of Shin *et. al*. (2010) with some modifications, total mRNA from RAW 264.7 cells was extracted and amplified in order to estimate the NDV-GFP mRNA expression. Briefly, cells (8×10^5 /well) were cultured on 12–well plates (Nunc, Denmark) for 12 hours. Then, the medium was replaced with DMEM (for w/o treatment and Virus only groups) and DMEM with 1mg/ml HM-γ–PGA. After 12 hours of incubation, cells were infected with 1 MOI of NDV–GFP virus. Cells were collected at 0, 6, 12, and 24 hours post-infection. Total mRNA was extracted from RAW 264.7 cells using the RNeasy Mini Kit (Qiagen, USA). Reverse transcription of the total mRNA was carried out using M-MLV Reverse Trancriptase (Enzynomics, Korea), oligo (dT) 16-primers and dNTP (0.5 µM). M-MLV Reverse Trancriptase was incubated at 72°C for 5 min and 37°C for 60 min and terminally inactivated by heating at 72°C for 15 minutes. The polymerase chain reactions (PCR) using specific primers for Matrix gene of APMV-1 (Wise *et. al.* 2004) and murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene (internal control) (Reed *et. al*. 2009) was carried out using emerald PCR master mix (Takara, USA) with 1 pM of each primer set

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(Table 1). The cDNA was amplified for 30 cycles with optimized annealing temperature for each primer set. Final extension was performed at 72°C for 5 min. Equal amounts of PCR products were run on 1.5% ethidium bromide impregnated agarose gels and visualized using GelDoc Imaging System (Bio-Rad, USA). On the other hand, Relative Band Intensity (RBI) of the Matrix gene and GAPDH mRNA expression was determined using GelDoc Imaging System Band Quantification Software (Bio-Rad, USA).

Table 1: Primer sets used to quantify viral mRNA expression

Genes	Primers	
	Forward	Reverse
APMV-1 M Gene	5'-AGTGATGTGCTCGGACCTTC-3'	5'-CCTGAGGAGAGGCATTTGCTA-3'
GAPDH	5'-TGACCACAGTCCATGCCATC-3'	5'-GACGGACACATTGGGGGTAG-3'

2.5 Detection of Pro-inflammatory cytokines induced by HM-γ-PGA on RAW 264.7 cells by (ELISA)

By the method of Wadsworth and Koop (1999) with some modifications, the pro-inflammatory inducing effect of HM-γ–PGA on RAW 264.7 cells was examined using commercial ELISA kits for murine Interleukin-6 (IL-6), Interleukin-12 (IL-12), Tumor Necrosis Factor –Alpha (TNF-ɑ) (BD Bioscience, USA)(von Maltzan & Pruett 2011) and IFN–β (PBL Interferon Source, USA) (Moon *et. al*. 2012). Briefly, a suitable number (2×10^6/well) of RAW 264.7 cells was seeded to each well of a 6–well TC plate (Nunc, Denmark) and cultured. Twelve hours later, the culture medium was removed and the cell monolayer was washed once with PBS. The cells were then treated with 100ng/ml of lipopolysaccharide (LPS) derived from *E. coli* 0111:B4 (Sigma–Aldrich, USA) or 1mg/ml of HM-γ–PGA in DMEM with 1% FBS and incubated at 37°C with 5% CO_2. Wells treated with DMEM with 1% FBS only served as negative control. Supernatant from each treatment group was harvested at 0, 6, 12, 24, and 36 hours post-treatment and clarified by centrifugation at 2500 × g for 10 minutes at 4 °C. Clarified supernatant was dispensed into the murine IFN–β ELISA plate for the measurement of secreted murine IFN–β, while 10-fold diluted supernatant was dispensed into the mouse TNF–ɑ, IL-6 and IL-12 capture antibody coated ELISA plate. The test was performed in triplicate.

2.6 Stimulation of an antiviral state by HM-γ–PGA treatment

To confirm the up-regulation in mRNA expression level of antiviral related genes on RAW 264.7 cells, the method of Moon *et. al*. (2012) was used with some modifications. Briefly, the RAW 264.7 cells were seeded to each well of a 6–well TC plate (Nunc, Denmark) and cultured for 12 hours. The cells were treated with 1mg/ml of HM-γ–PGA in DMEM with 1% FBS. Separately, the cells were treated 100 ng/ml LPS in DMEM as the positive control and DMEM with 1% FBS only as the negative control. The treated cells were harvested at 0, 3, 6, 12, and 24 hours post-treatment. Afterward, preparation of total RNA and RT–PCR were performed using same method above. The specific primers for PCR are listed in Table 2 (Moon *et. al*. 2012; Wilden *et. al*. 2009; and Reed *et. al*. 2009).

Table 2. Primer sets used to quantify antiviral gene mRNA expression.

Genes	Primers	
	Forward	Reverse
GAPDH	5'-TGACCACAGTCCATGCCATC-3'	5'-GACGGACACATTGGGGGTAG-3'
Interefron Beta (IFN-β)	5'-TCCAAGAAAGGACGAACATTCG-3'	5'-TGCGGACATCTCCCACGTCAA-3'
Myxovirus resistance 1 (Mx1)	5'-GATCCGACTTCACTTCCAGATGG-3'	5'-CATCTCAGTGGTAGTCAACCC-3'
Interferon Regulatory Factor 3 (IRF-3)	5'-GTGCCTCTCCTGACACCAAT-3'	5'-CCAAGATCAGGCCATCAAAT-3'
Interferon Regulatory Factor 7 (IRF-7)	5'-AAGCTGGAGCCATGGGTATG-3'	5'-GACCCAGGTCCATGAGGAAG-3'
Interferon–stimulated gene 15 (ISG15)	5'-CAATGGCCTGGGACCTAAA-3'	5'-CTTCTTCAGTTCTGACACCGTCAT-3'
Guanylate–binding protein 1 (GBP1)	5'-AAAAACTTCGGGGACAGCTT-3'	5'-CTGAGTCACCTCATAAGCCAAA-3'

2.7 Statistical analysis

Differences between groups were analyzed by Student's t–test. P values less than 0.05 were regarded as significant and those less than 0.01 were regarded as highly significant.

3 RESULTS

3.1 Inhibition of virus replication by HM-γ–PGA on RAW264.7 cells

Previous studies revealed that HM-γ-PGA has beneficial functions to immune responses (Kim *et. al*. 2007; Lee *et. al*. 2009; Poo *et. al.* 2010). Recent studies also confirmed the role of γ–PGA in inducing cytokines involved in antiviral states in cells, leading to inhibition of virus replication (Moon *et. al*. 2012).

HM-γ–PGA treated RAW 264.7 cells showed markedly reduced virus replication while the untreated group demonstrated high level of GFP expression (Fig. 1A). Based on this observation, the researcher measured the GFP expression of the infected cells to compare the result of observation with a numerical value (Fig. 1C). HM-γ–PGA treated cells showed more than two-fold reduction of GFP expression compared to virus alone.

Likewise, after virus infection, HM-γ–PGA treated cells showed less than 10% cell death while non-treated cells showed more than 50% cell death within 30 hours post-infection (Fig. 1D). Nonetheless, cells treated with natural substance alone showed less than 5% cytotoxicity compared to the media treated group, thus showing that HM-γ–PGA has negligible toxicity. Additionally, with the failure of the NDV-GFP virus to bud successfully from RAW 264.7 cells, we opted to measure the mRNA expression of the Matrix gene of the virus via RT-PCR to estimate virus replication (Fig. 1B). As expected, the M gene expression of the HM-γ–PGA treated cells is relatively lower than the virus alone from 6 to 24 hours post-infection. The non-treated group also showed continuous expression of the M gene up to 24 hours post-infection while HM-γ–PGA treated cells demonstrated non-increasing pattern beginning 12 hours.

3.2 Induction of antiviral genes and pro-inflammatory cytokines by HM-γ–PGA in RAW 264.7 cells

To determine the mechanism by which HM-γ–PGA induces antiviral state in RAW 264.7 cells, the antiviral related gene expression and secreted pro-inflammatory cytokines from the murine macrophage cells were confirmed after HM-γ–PGA stimulation. RAW 264.7 cells were treated with 1mg/ml of HM-γ–PGA and compared with 100ng/ml of LPS treated cells.
HM-γ–PGA treated RAW 264.7 cells showed increased mRNA expression of transcriptional factors IRF-3, IRF-7 and IFN-stimulated genes such as Mx1 and GBP1 almost comparable with the level induced by LPS beginning 3 hours post-treatment (Fig. 2) However, IFN-β as well as ISG-15 mRNA expression levels was not as high as compared to LPS treated cells, though still evidently higher in contrast to the negative control. Since mRNA expression does not necessarily correlate with the secreted protein levels, pro-inflammatory cytokine secretion was also measured after HM-γ–PGA stimulation via murine cytokine ELISA kits (Fig. 3). The present research showed that HM-γ–PGA can induce cytokine secretion in RAW 264.7 cells comparable with the LPS treated cells.

4 DISCUSSION

The capacity of NDV to cause a highly contagious infection resulting to high mortality and reduced farm efficiency remains to threaten the global poultry industry. Currently available vaccines cannot provide adequate immunity in poultry even with the use of multiple vaccinations (Alexander 2001) hence natural substances which have antiviral activity are of

great importance. One of the natural substances which is widely studied for its various biological functions and applications is the HM-γ–PGA, a natural, edible, and biodegradable polymer derived from *Bacillus subtilis* subsp. *chungkookjang* (Bae *et. al*. 2010; Sung *et. al*. 2005; Poo *et. al*. 2010). HM-γ–PGA is secreted from γ-PGA synthetase ABC complex on the wall of *Bacillus subtillis* subsp. *chungkookjang* (Sung *et. al*. 2005). This naturally secreted HM-γ–PGA is a safe and edible polymer which contains negligible toxins which do not interfere with its beneficial effects/applications such as satisfactory adjuvant function, antitumor effect, innate immunity inducible role (Kim *et. al*. 2007; Lee *et. al*. 2009). Among its biological functions, its capacity to initiate immune responses in mice via TLR4 signaling just like LPS makes HM-γ–PGA a potent immunomodulator with a big potential as a therapeutic agent (Poo *et. al*. 2010). Briefly, TLRs are considered to be a major component of the pattern recognition system which detects invading pathogens by recognizing pathogen associated molecular patterns (PAMPs). And one of the well studied TLRs is TLR4. Mouse or mammalian TLR4 signaling pathway (Fig. 9) starts by the transfer of LBP of the detected LPS to the CD14 on the surface of inflammatory cells. This reaction eventually leads to the transfer of LPS to TLR-4 via MD-2 (Palson-McDermott & O'Neill 2004). Successful activation of TLR4 initiates intracellular activation of MyD88-dependent (MyD88/TIRAP) and MyD88-independent (TRAM/TRIF) pathways. MyD88-dependent pathway utilizes MyD88 and TIRAP to successfully export NF-Kβ to the nucleus to initiate transcription of pro-inflammatory cytokine genes, while MyD88-independent pathway makes use of TRIF and TRAM to effectively activate transcription factor IRF-3 and subsequent production of IFN-B (Yamamoto *et. al.* 2003). Though, pro-inflammatory cytokines such as TNF-A, IL-6 and IL-12 are important for a successful inflammatory response, induction of type I interferon is much more considered to be indispensible for anti-viral resistance. Nonetheless, TLR4 is considered to be unique among the TLR family in that LPS recognition results in activation of both MyD88-dependent and the TRAM/TRIF-dependent signaling pathways (Keestra *et. al*. 2008).

In this study, HM-γ–PGA has been tested through several experiments to evaluate its application to control Newcastle disease virus replication on murine macrophage cells (RAW 264.7). For the pre-treatment assay, HM-γ–PGA treatment was sufficiently able to block viral replication on NDV-GFP infected RAW 264.7 cells as shown by lower NDV M gene and GFP expressions and higher cell survivability post-infection (Fig. 1). Based on these findings, γ–PGA has been hypothesized to be involved with the antiviral state in cells via induction of type I interferons. Previous study showed that the expression of antiviral effector molecules protein kinase R (PKR), 2′-5′-ologoadenylate synthetase (OAS) and myxovirus resistance protein (Mx), as induced by Type 1 interferons, correlate with the susceptibility to NDV infection both in primary macrophages and macrophage-derived tumor cells (e.g. RAW cells) as these molecules inhibit critical steps during RNA translation and/or assembly of virus particles in viral replication (Wilden *et. al*. 2009)

Likewise, the same paper also demonstrated that RAW cells have delayed IFN secretion after NDV infection thus making them susceptible to the said virus (Wilden *et. al.* 2009). However, as compared to chicken cells, mammalian cells have innate resistance with the V protein of NDV which can block production of IFN (Park *et. al*. 2003). On the other hand, Moon *et. al*. (2012) showed that HM-γ–PGA can induce significant amount of IFN-β in RAW 264.7 cells comparable with LPS induced IFN-β secretion as measured by murine cytokine ELISA. With this in mind, the researchers hypothesized that the early induction of type-I interferon can also protect RAW264.7 cells against NDV infection. In the present study, HM-γ–PGA treated Raw 264.7 cells showed increased mRNA expression of transcriptional factors IRF-3, IRF-7 and IFN-stimulated genes such as Mx1 and GBP1 almost comparable with the level induced by LPS beginning 3 hours post treatment. However, IFN-B as well as ISG-15 mRNA expression levels was not as high as compared to LPS treated cells, though still evidently higher in contrast to the negative control. Such observations are

in accordance with the findings of Wilden *et. al.* in 2009 that strong expression of anti-viral genes IRF-3, IRF-7, IFN-β and RIG-I can provide protection against NDV infection. Although, RIG-1 was not measured in this study, the strong expression of IRF-3 and IRF-7 alone can strongly suggest that antiviral state has been achieved in HM-γ–PGA treated RAW 264.7 cells since these transcriptional factors are indispensible for the induction of Type I IFNs (Sato *et. al.* 2000).

However, since mRNA expression does not necessarily correlate with the secreted protein levels, pro-inflammatory cytokine secretion was also measured. Using commercial murine cytokine ELISA kits for detection of IL-6, IL-12, TNF-α, and IFN-β, the present research showed that HM-γ–PGA can induce cytokine secretion in RAW 264.7 cells comparable with the LPS treated cells. Therefore, our findings suggest that HM-γ–PGA can be a significant antiviral substance which can inhibit NDV infection through its stimulation of antiviral state on RAW 264.7 cells.

In conclusion, the present study strongly demonstrates that HM-γ–PGA treatment can significantly protect murine macrophage cell line (RAW 264.7 cells) against NDV infection. Moreover, the results lead to further studies especially on the evaluation of the anti-NDV activity of HM-γ–PGA both in chicken and chicken-derived cells to fully assess the applicability of HM-γ–PGA treatment in the poultry industry.

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Figures

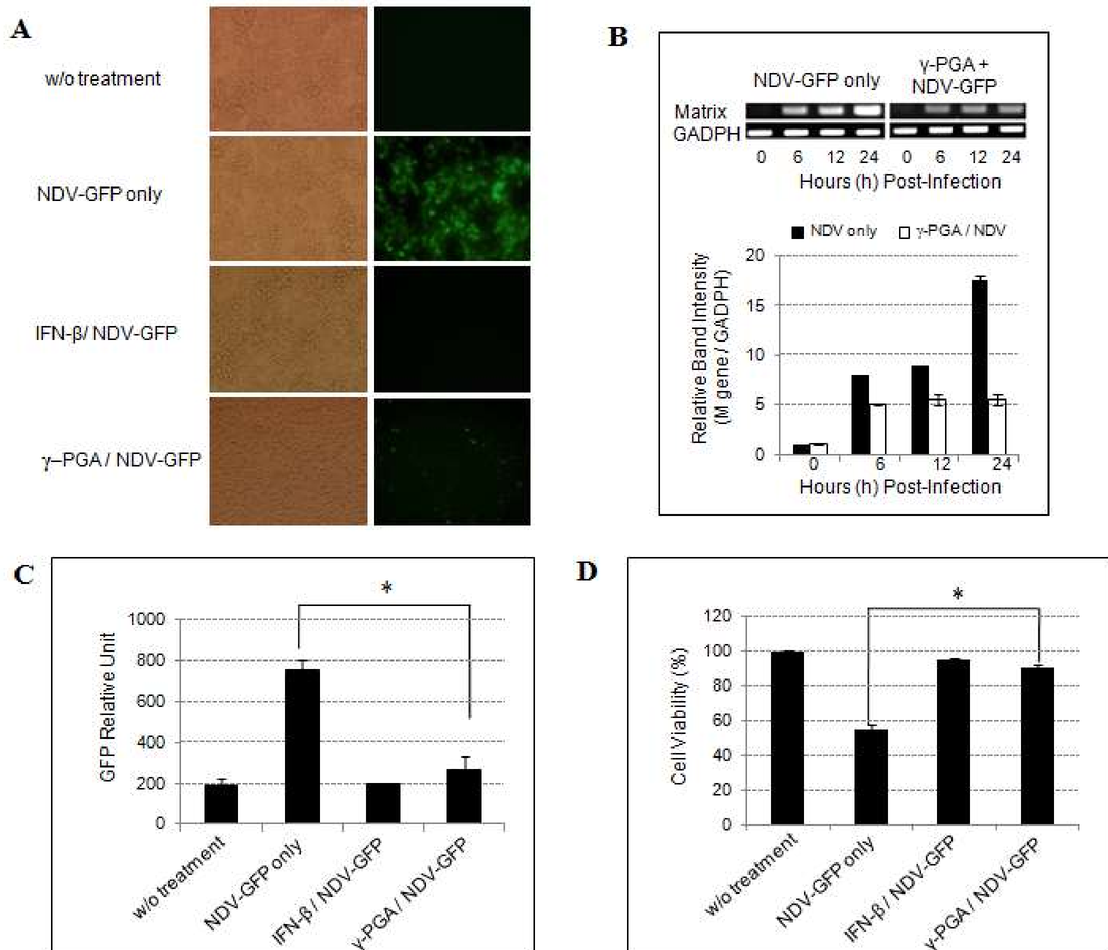

Figure 1: Antiviral function of HM-γ-PGA in murine macrophage cell line.
(A) GFP expression images of media treated, 1 mg/ml HM-γ-PGA and 1000 units/ml recombinant mouse IFN-β treated cells 12 h before NDV-gfp infection. Images taken 12 hpi (200 x magnification). (B) Viral mRNA expression level of matrix gene of NDV-gfp over time in each treatment group as confirmed by RT-PCR and their respective Relative Band Intensities (RBI) as determined (Gene/GAPDH) using GelDoc Imaging System Band Quantification Software. Error bars indicate the range of values obtained from two independent experiments.(C) GFP expression level of media treated, 1 mg/ml HM-γ-PGA and IFN-β treated cells 24 hpi was measured using Glomax multi-detection system. (D) Cell viability was determined by trypan blue exclusion test at 30 hpi. The results are presented as a percentage of the negative control (cells without treatment). Error bars on Fig. C and D indicate the range of values obtained from triplicate measurement/counting (*P < 0.05, those compared groups by student t-test are significantly different).

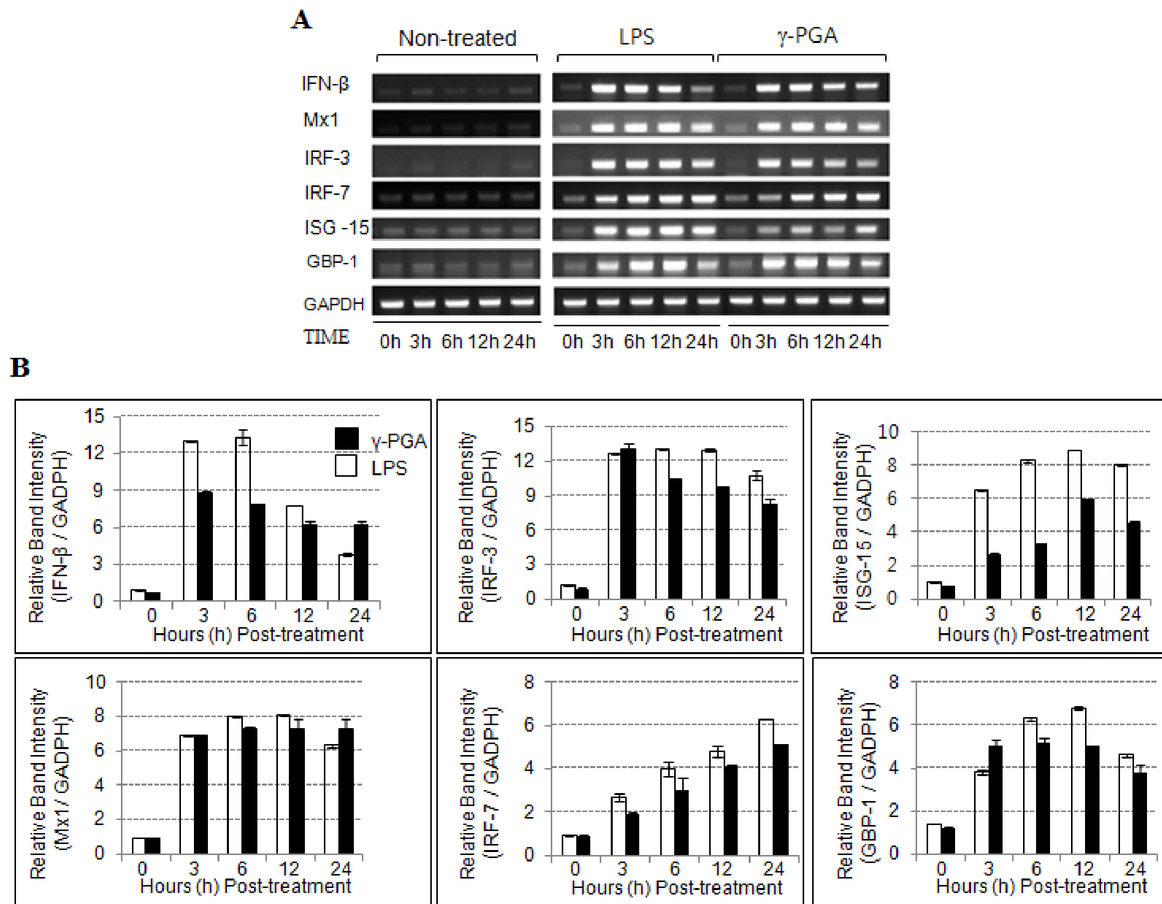

Figure 2: Induction of IFN-B, IRF-3, IRF-7 and IFN-stimulated genes by HM-γ-PGA treatment in murine macrophage cell line. (A) Cells were treated with media only, HM-γ-PGA (1 mg/ml), and 100ng/ml of LPS. The time-dependent changes in mRNA expression after treatment were confirmed by RT-PCR. All samples were normalized using GAPDH wherein equal amounts PCR products were run on 1.5% ethidium bromide impregnated agarose gels and visualized using GelDoc Imaging System. (B) RBI of the IFN-β, IRF-3, IRF-7 and IFN-related genes. Error bars indicate the range of values obtained from two independent experiments.

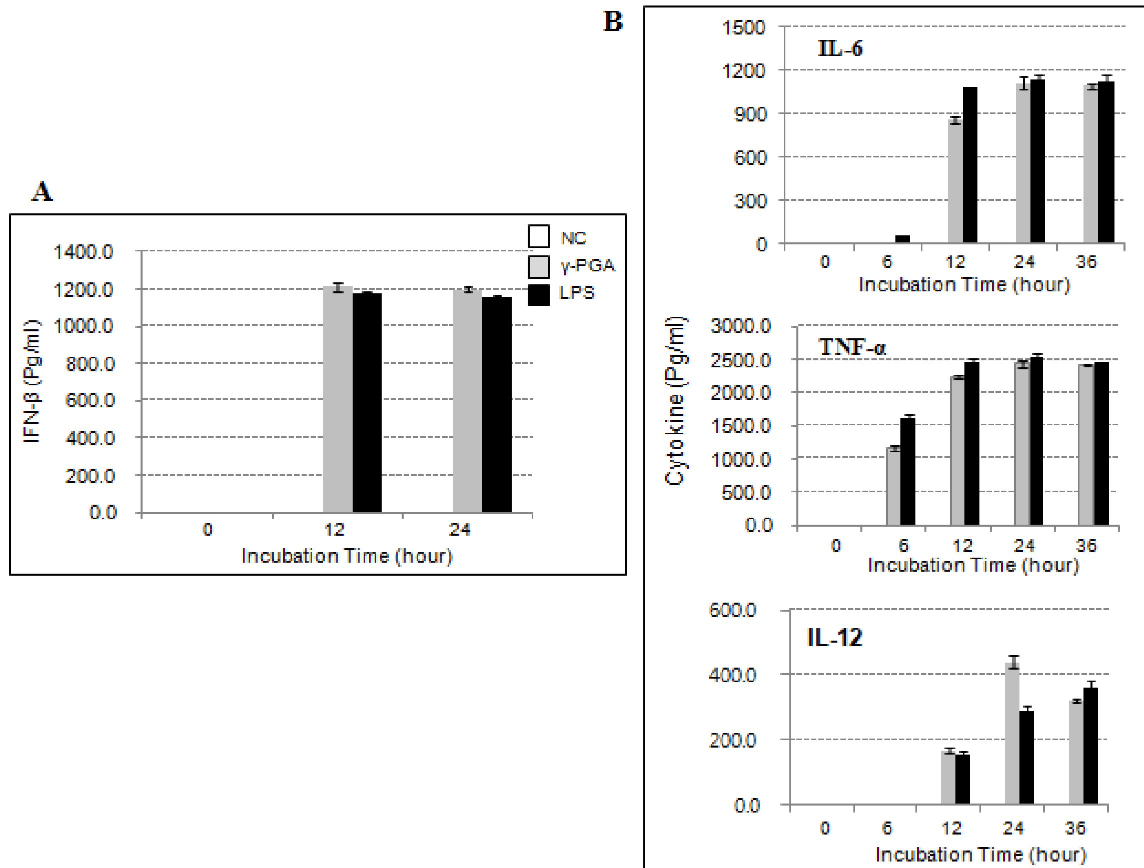

Figure 3: Ability of HM-γ-PGA to induce pro-inflammatory cytokines in murine macrophage cell line. Cells were treated with media only, HM-γ-PGA (1 mg/ml), and 100ng/ml of LPS. The time-dependent changes in IFN-β (A) and IL-6, TNF-α, and IL-12 (B) cytokine secretion after treatment were confirmed by ELISA. The data show representative means ± SD of each murine cytokine measured over time from three independent assays.