Immunomodulatory effects of poly(I:C)-stimulated exosomes derived from chicken macrophages

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ABSTRACT Exosomes are small membrane vesicles that contain proteins and nucleic acids derived from secretory cells and mediate intracellular communication. Immune cell-derived exosomes regulate immune responses and gene expression of recipient cells. Macrophages recognize viral dsRNA via Toll-like receptor 3, thereby inducing the activation of transcription factors such as interferon regulatory factor 3 and nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**). In this study, we aimed to identify the immunomodulatory functions of exosomes derived from chicken macrophages (**HD11**) stimulated with polyinosinic-polycytidylic acid (poly[I:C]); exosomes were then delivered into HD11 cells and CU91 chicken T cells. Exosomes purified from poly(I:C)-activated macrophages stimulated the expression of type I interferons, proinflammatory cytokines, anti-inflammatory cytokines, and chemokines in HD11 and CU91 cells. Moreover, poly(I:C)-stimulated exosomes induced the NF-κB signaling pathway by phosphorylating TAK1 and NF-κB1. Therefore, we suggest that after the activation of Toll-like receptor 3 ligands following infection with dsRNA virus, chicken macrophages regulate the immune response of naive macrophages and T cells through the NF-κB signaling pathway. Furthermore, poly(I:C)-activated exosomes can be potentially utilized as immunostimulators.

Key words: exosomes, TLR3, poly(I:C), NF-κB signaling pathway, chicken, macrophage

INTRODUCTION

Macrophages play important roles in the immune system, such as in phagocytosis, antigen presentation, and cytokine production (Aderem et al., 1999; Koh et al., 2011). Macrophages initiate an inflammatory response by recognizing pathogen-associated molecular patterns (**PAMPs**) originating from pathogenic bacteria and viruses using Toll-like receptors (**TLRs**) and activate TLR signaling pathways (Beutler, 2004; Reimer et al., 2008; Takeda et al., 2004). Polyinosinic-polycytidylic acid (**poly[I:C]**), a viral-like synthetic dsRNA, is recognized by Toll-like receptor 3 (**TLR3**) and melanoma differentiation-associated protein 5 (**MDA-5**), which activates transcription factors such as interferon regulating factor and nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**) by stimulating the expression of type I interferons (**IFNs**) and

inflammatory cytokines (Kawai et al., 2010). Moreover, antigen-presenting cells (**APCs**) and macrophages communicate with T cells via direct cell-cell contact and soluble factors (Veerman et al., 2019). For example, macrophages activate T cells using 3 signals: 1) antigen-specific interaction by presenting foreign antigens to major histocompatibility complexes (**MHCs**) I and II, which are recognized by T cell receptors; 2) co-stimulatory signals such as the CD40 of APCs and CD40L of T cells, and 3) a cytokine signal (Guerriero, 2019; Kambayashi et al., 2014). Activated helper T cells differentiate into various subtypes including T_H1, T_H2, T_H17, and memory helper T cells, depending on APC-secreted cytokines (Pennock et al., 2013). In addition to cytokines, exosomes secreted from immune cells can also transfer information by delivering various molecules of donor cells (Veerman et al., 2019).

Exosomes, small membrane vesicles with a diameter of 40 to 100 nm, are released by most eukaryotic cells and are present in most biological fluids such as urine, blood, saliva, and cerebrospinal fluid (Lu et al., 2018; Mathivanan et al., 2010). Exosomes are released by the fusion of multivesicular bodies containing intraluminal vesicles with the plasma membrane (Lu et al., 2018; Van der Pol et al., 2012). Exosomes contain lipid rafts

(sphingolipids, cholesterol, ceramide, and flotillins), proteins (tetraspanins, immune-regulatory molecules, enzymes, MVB-synthesizing proteins, signaling proteins, chaperones, and cytoskeletal proteins), and nucleic acids (DNA, mRNAs, and miRNAs) (Gross et al., 2012; Mathivanan et al., 2010; Sato-Kuwabara et al., 2015). Exosomes transfer genetic material to recipient cells, which are protected from degradation, and have high biocompatibility and long circulation times; thus, exosomes show great potential as therapeutic tools (Andaloussi et al., 2013; Zhu et al., 2018).

Exosomes from immune cells can regulate the immune response of recipient cells by transferring membrane-bound proteins such as those of the MHCs and nucleic acids such as mRNAs, miRNAs, and other non-coding RNAs (Veerman et al., 2019). For example, exosomes from macrophages infected with bacteria or parasites contain PAMPs and induce the expression of cytokines in a TLR-dependent manner (Bhatnagar et al., 2007; Cronemberger-Andrade et al., 2014).

A limited number of studies have been performed on chicken exosomes. For example, chicken dendritic cell-derived exosomes induce protective immunity against infection by *Eimeria* spp. (del Cacho et al., 2011; del Cacho et al., 2012; del Cacho et al., 2016). Moreover, in a previous study, we demonstrated that exosomes from LPS-stimulated chicken macrophages activate immune responses by increasing the expression of cytokines and chemokines through the MyD88/NF-κB signaling pathway (Hong et al., 2020a). However, studies on the functions of exosomes between macrophages and T cells or viral-like mimicry are limited. Therefore, in this study, we examined the immunomodulatory functions of exosomes from chicken macrophages stimulated with poly(I:C), a viral dsRNA-based immunostimulant, in chicken macrophages and T cell lines.

MATERIALS AND METHODS

Reagents and Antibodies

TAK1 (phosphor-Ser192) (#orb7051) and NFKB1 (phosphor-Ser933) antibodies were purchased from Biorbyt (Cambridge, UK). The Anti-NF-kappaB p105 (pS932) phospho antibody (#MBS8210747) was purchased from MyBioSource (San Diego, CA). Mouse anti-chicken glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) antibody (#AM4300), goat anti-mouse IgG horseradish peroxidase (**HRP**)-conjugated antibody (#A16078), and radioimmunoprecipitation assay (**RIPA**) lysis and extraction buffers were purchased from Thermo Fisher Scientific (Waltham, MA). Alexa Fluor 488 goat anti-rabbit IgG (H+L) secondary antibody and 4,6-diamidino-2-phenylindole (**DAPI**) were purchased from Invitrogen (#A-11008; Carlsbad, CA). Anti-rabbit IgG (H+L) HRP-conjugated antibody was purchased from Promega (#W4011; Madison, WI). The CD9 antibody was purchased from Cell Signaling (#13174; Danvers, MA).

Chicken Cell Line Culture

The chicken macrophage cell line HD11 (Klasing et al., 1987) and chicken T-cell line transformed by reticuloendotheliosis virus type T (REV-T) CU91 (Schat et al., 1992; Weinstock et al., 1989) were maintained in complete Roswell Park Memorial Institute (**RPMI**) 1640 medium (Thermo Fisher Scientific) containing 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) in a humidified incubator with 5% CO_2 at 41°C. For exosome purification, HD11 cells (1.0×10^7) were seeded in three 100-mm cell culture dishes (SPL Life Sciences, Pocheon, Korea) in complete RPMI 1640 medium. The next day, the medium was replaced with exosome-depleted fresh RPMI 1640 medium containing 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% exosome-depleted fetal bovine serum (#EXO-FBSHI-250A-1; System Bioscience, Palo Alto, CA) with or without 50 μg/mL poly(I:C) (#P1530; Sigma-Aldrich) and incubated for 12 h. The cell culture supernatant was collected for exosome purification.

Exosome Purification

A total of 30 mL of cell culture supernatant was collected to purify exosomes using the ExoQuick-TC kit (#EXOTC50A-1; System Biosciences). This supernatant was centrifuged at 3,000 g for 15 min. The supernatant was then transferred, mixed with 6 mL of ExoQuick-TC reagent by inverting, and incubated overnight at 4°C. The mixture was then centrifuged at 1,500 g for 30 min. After centrifugation, the exosomes were resuspended in 500 μL of phosphate-buffered saline (**PBS**; pH 7.4). The concentration of the purified exosomes was measured using a Pierce bicinchoninic acid (**BCA**) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For the characterization of exosomes, their particle size was measured using a nanoparticle analyzer (SZ-100; Horiba, Kyoto, Japan). Furthermore, western blotting was performed using antibodies against CD9 (an exosomal marker), according to previously described methods (Hong et al., 2020b).

Cellular Uptake of poly(I:C)-stimulated Exosomes

In order to examine intracellular internalization of poly(I:C)-stimulated exosomes (**POLY-EXOs**), purified exosomes were labeled with the DiI Stain (#D3911; Sigma-Aldrich). Briefly, 10 μg of the POLY-EXOs were diluted with 200 μL of PBS and incubated with 2 μL of a 10 μM DiI stock solution (prepared in methanol) for 2 h in the dark at 23°C (room temperature). Then, the DiI-labeled exosome solution was centrifuged at 18,000 g for 30 min. The supernatant was removed, and the pellet of DiI-labeled exosomes was then washed with 200 μL of PBS. This procedure was repeated 3 times to remove any free DiI. Then, HD11 (4.0×10^5 cells/well) and

CU91 (4.0×10^4 cells/well) cells were plated in Nunc Lab-Tek Chamber Slides (Thermo Fisher Scientific) with exosome-depleted medium and incubated with DiI-labeled POLY-EXOs for 12 h in a humidified incubator with 5% CO_2 at 41°C. The cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min and then stained with DAPI for 5 min. Finally, images were acquired using an EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific).

Quantitative Real-time PCR

HD11 cells (5.0×10^5 cells/well) and CU91 cells (1.0×10^5 cells/well) were plated in 12-well plates, treated with 50 μg/mL (final concentration) POLY-EXOs, and incubated for 0, 6, 12, 24, 36, or 48 h, in a humidified incubator with 5% CO_2 at 41°C. Then, HD11 and CU91 cells were washed with ice-cold PBS, and total RNA was extracted from the cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol. To analyze the expression levels of cytokines, primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/; Table 1), and qRT-PCR was performed using the AMPIGENE qPCR Green Mix Lo-ROX (Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's instructions, using the LightCycler 96 system (Roche, Indianapolis, IN). Chicken $\boldsymbol{GAPDH}$ was used as a control to normalize the quantity of RNA. Relative quantification of the expression levels of the target genes was performed using the $2^{-\Delta\Delta Ct}$ method after normalization with the $GAPDH$ expression levels (Livak et al., 2001). All qRT-PCRs were performed in triplicates.

Western Blotting

HD11 cells (5.0×10^6 cells/well) and CU91 cells (3.0×10^5 cells/well) were plated in 6-well plates (Thermo Fisher Scientific) containing 1.5 mL of culture medium and stimulated with 50 μg/mL POLY-EXOs for 0, 6, 12, 24, 36, and 48 h in a humidified incubator with 5% CO_2 at 41°C. After incubation, the cells were washed with ice-cold PBS, and cellular proteins were extracted using RIPA lysis and extraction buffers according to the manufacturer's protocol. The Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific) and the Halt protease inhibitor cocktail (Thermo Fisher Scientific) were added to the cell lysates. The total protein concentrations in the cells were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Next, the protein samples were mixed with 5 $\times$ sample buffer (250 mM Tris-Cl [pH 6.8], 5% β-mercaptoethanol, 10% SDS, 0.5% bromophenol blue, and 50% glycerol) and heated to 100°C for 10 min. Then, equal amounts of the protein samples (20 μg) were electrophoresed using 12% Tris-glycine SDS polyacrylamide gels; the separated proteins were transferred onto polyvinylidene difluoride membranes (GE Healthcare, Rydalmere, Australia) using the Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules, CA). The blots were first blocked with 5% skim milk (Thermo Fisher Scientific) in PBS (pH 7.4) containing 0.05% Tween-20 (Sigma-Aldrich) (PBST) and then incubated with primary antibodies overnight at 4°C, followed by washing with PBST and incubation with anti-rabbit IgG (H+L), an HRP-conjugated antibody (Promega), for 1 h. Finally, the blots were developed using the Western Lightning ECL Plus substrate (Thermo Fisher Scientific) using the CL-XPosure Films (Thermo Fisher Scientific).

Immunocytochemistry

HD11 (4.0×10^5 cells/well) and CU91 (4.0×10^4 cells/well) cells were plated in Nunc Lab-Tek Chamber Slides (Thermo Fisher Scientific) and incubated with PBS, exosomes from unstimulated HD11 cells (**CTRL-EXOs**, 50 μg/mL), or POLY-EXOs (50 μg/mL) for 6 h in a humidified incubator with 5% CO_2 at 41°C. The cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min and then incubated with ice-cold methanol for 10 min at 4°C. Following overnight incubation with primary antibodies (antibodies against p-TAK1 and p-NF-κB1) at 4°C, they were treated with Alexa Fluor 488-conjugated secondary antibodies for 2 h, and then stained with DAPI for 5 min. Finally, images were captured using the EVOS FLoid Cell Imaging Station (Life Technologies, Carlsbad, CA).

Table 1. Sequences of primers used for quantitative real-time PCR analysis of gene expression.

Gene	F/R	Primer sequences (5′-3′)	Accession No.
GAPDH	F	TGCTGCCCAGAACATCATCC	NM_204305
	R	ACGGCAGGTCAGGTCAACAA	
IFN-α	F	GAGCAATGCTTGGACAGCAG	GU119896.1
	R	GAGGTTGTGGATGTGCAGGA	
IFN-β	F	CTTGCCCACAACAAGACGTG	NM_001024836.1
	R	TGTTTTGGAGTGTGTGGGCT	
IFN-γ	F	AACAACCTTCCTGATGGCGT	NM_205149.1
	R	TGAAGAGTTCATTCGCGGCT	
IL-1β	F	TGCCTGCAGAAGAAGCCTCG	NM_204524.1
	R	CTCCGCAGCAGTTTGGTCAT	
IL-4	F	ATTGTTTGGGAGAGCCAGCA	NM_001007079.1
	R	GACATGGTGCCTTGAGGGAG	
IL-6	F	GCAGGACGAGATGTGCAAGA	NM_204628.1
	R	ATTTCTCCTCGTCGAAGCCG	
IL-17A	F	TGTCTCCGATCCCTTGTTCT	AM773756
	R	GTCCTGGCCGTATCACCTT	
CCL2	F	CATTGCCTCCGCCTACAT	XM_415780.5
	R	ACTCCTCGGGGGTTTACACATA	
CCL4	F	CTTCACCTACATCTCCCGGC	NM_001030360.2
	R	CTGTACCCAGTCGTTCTCGG	
CCL17	F	TCTCGAAGCGCTGAAGAGTT	NM_204596.1
	R	TTTCACCCAAGGTGCGTTTG	
TRAF3	F	CGTCTCGGCGCCACTTAGGA	XM_421378
	R	GGGCAGCCAGACGCAATGTTCA	
TRAF6	F	TGGAGGCCGAGACTTGGATA	XM_004941548.3
	R	CATCGTATCCCTGCGTCTCC	

Statistical Analysis

Data are presented as the mean ± standard error of the mean of 3 independent experiments. Statistical analyses were performed using the IBM SPSS software (SPSS 26.0 for Windows; IBM, Chicago, IL). Results with a P-value < 0.05 were considered statistically significant. Differences between groups were evaluated using Tukey's multiple comparison tests.

RESULTS

Characterization of Chicken Macrophage-Derived Exosomes

HD11 cells (chicken macrophage cell line) were incubated with or without 50 μg/mL poly (I:C) for 12 h and exosomes were purified from cell culture supernatants. Purified exosomes were characterized based on their size and the presence of exosomal biomarkers (CD9) (Figure 1). The size of purified CTRL-EXOs and POLY-EXOs ranged between 50.53 and 72.81 nm (Figure 1A). In addition, the exosomal biomarker CD9 was detected in CTRL-EXOs and POLY-EXOs using western blotting (Figure 1B). DiI-labeled POLY-EXOs were incubated with HD11 and CU91 cells to visualize the internalization of exosomes (Figure 2). DiI-labeled POLY-EXOs were successfully internalized in the cytoplasm of HD11 and CU91 cells.
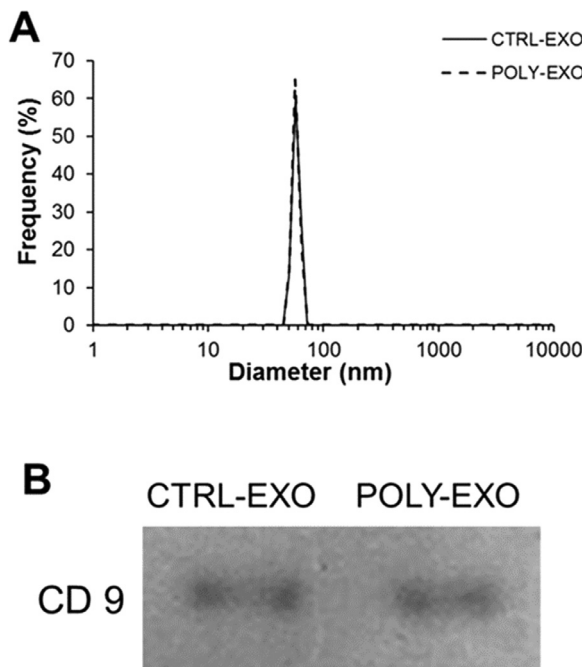

Figure 1. Characterization of purified exosomes. (A) Particle size distribution of exosomes from unstimulated HD11 (CTRL-EXO) and poly(I:C)-stimulated exosomes (POLY-EXO) as measured using a nanoparticle analyzer. (B) Western blotting for detecting the expression of the exosomal marker CD9.
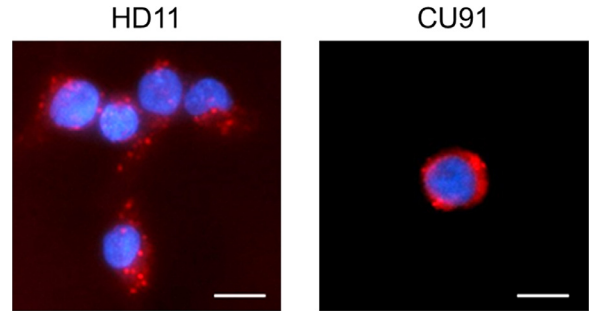

Figure 2. Cellular uptake of DiI-labeled poly(I:C)-stimulated exosomes following incubation with HD11 and CU91 cells. Scale bar = 10 μm.

POLY-EXOs Regulate the Expression of Cytokines and Chemokines

HD11 and CU91 cells were incubated with 50 μg/mL POLY-EXOs for 0, 6, 12, 24, 36, and 48 h. Then, the expression levels of cytokines and chemokines were evaluated using qRT-PCR (Figures 3 and 4). In the HD11 cell line, the expression of IFN-α and IFN-β, type I IFNs, was markedly upregulated upon treatment with POLY-EXOs and peaked at 36 h (3.91-fold) and 24 h (1.90-fold), respectively (Figure 3). The expression of the proinflammatory cytokines IFN-γ and IL-1β was also significantly upregulated upon treatment with POLY-EXOs and peaked at 36 h (6.39-fold) and 24 h (1.86-fold), respectively. The expression of IL-4 and IL-6 was not notably regulated upon treatment with POLY-EXOs. The expression of IL-17A was significantly upregulated upon treatment with POLY-EXOs and peaked at 24 h (2.18-fold). The expression of the chemokines CCL2, CCL4, and CCL17 was significantly upregulated upon treatment with POLY-EXOs and peaked at 24 h (1.23-fold), 48 h (2.19-fold), and 6 h (2.29-fold), respectively. The expression of TNF receptor-associated factor (**TRAF**) 3 and TRAF6, which are adaptor proteins of the TLR signaling pathways, was also significantly upregulated upon treatment with POLY-EXOs and peaked at 24 h (1.58-fold and 2.42-fold, respectively).

In CU91 cells, the expression of IFN-α and IFN-β was upregulated upon treatment with POLY-EXOs, compared to the case in the HD11 cells, peaking at 48 h (2.89-fold and 2.83-fold, respectively) (Figure 4). In contrast to that seen in HD11 cells, the expression of IFN-γ was downregulated in CU91 cells upon treatment with POLY-EXOs. The expression of IL-1β was upregulated in CU91 cells upon treatment with POLY-EXOs and peaked at 48 h (3.17-fold). The expression of IL-4, IL-6, and IL-17A in CU91 cells was also upregulated upon treatment with POLY-EXOs and peaked at 48 h (1.47-fold) and 6 h (4.06-fold and 3.37-fold), respectively. The chemokines CCL2 and CCL4 showed high expression in CU91 cells upon treatment with POLY-EXOs and their expression levels peaked at 6 h (1.93-fold) and 48 h (1.90-fold), respectively. However, the expression of CCL17 in CU91 cells was downregulated upon treatment with POLY-EXOs. The gene expression of TRAF3 and TRAF6 in CU91 cells was upregulated
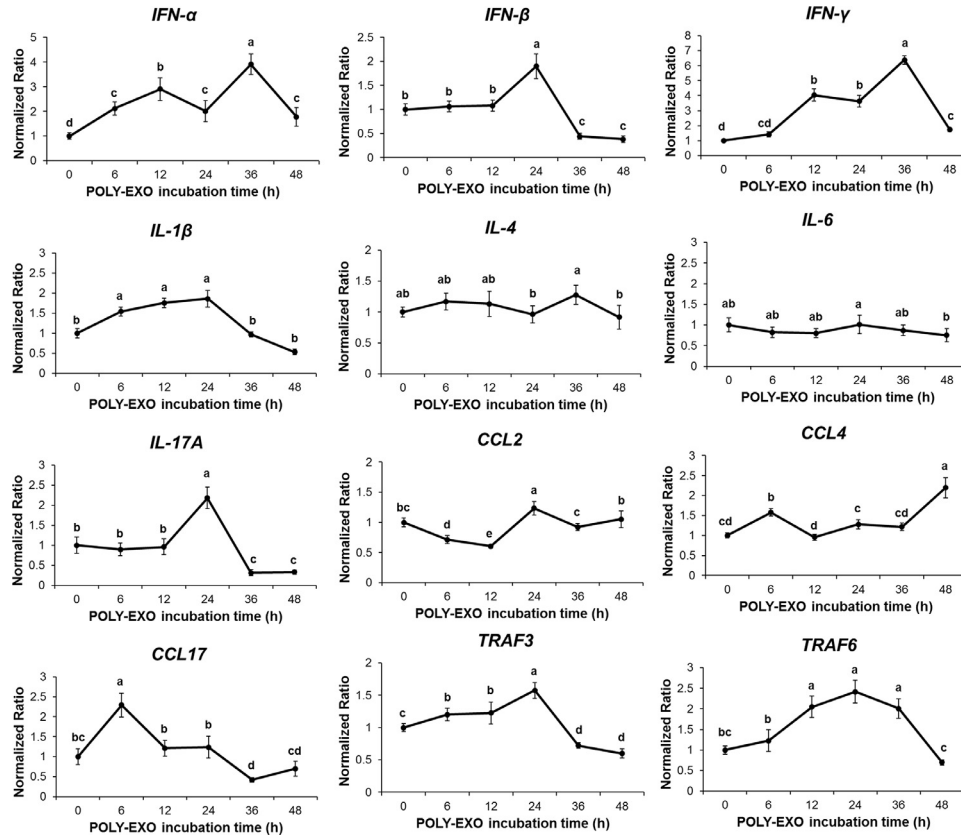
Figure 3. Effects of poly(I:C)-stimulated exosomes (POLY-EXOs) on cytokine and chemokine production in HD11 cells. HD11 cells were treated with POLY-EXOs (50 μg/mL) for 0, 6, 12, 24, 36, or 48 h and the expression level of cytokines and chemokines was determined using quantitative real-time PCR. Expression levels of the target genes were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All culture conditions were tested in triplicate. The data are expressed as the mean ± standard error of the mean and are representative of three independent experiments. Different lowercase letters above the dots indicate significant differences (*P*< 0.05) as determined by analysis of variance with Tukey's multiple comparison test.

upon POLY-EXO treatment and peaked at 6 h (1.40-fold) and 48 h (2.27-fold), respectively.

Taken together, the expression of various cytokines such as type I IFNs, IL-1β, IL-4, IL-6, IL-17A, CCL2, CCL4, TRAF3, and TRAF6 were regulated by POLY-EXO treatment in HD11 and CU91 cells.

POLY-EXOs Induce the NF-κB Signaling Pathway

In order to examine induction of the NF-κB signaling pathway, HD11 and CU91 cells were incubated with 50 μg/mL POLY-EXOs for 0, 6, 12, 24, 36, and 48 h. The phosphorylation of TAK1 and NF-κB1 was investigated using western blotting and immunocytochemistry. We found that POLY-EXOs induced the phosphorylation of TAK1 (Ser^{192}) and NF-κB1(Ser^{933}) in HD11 and CU91 cells (Figure 5). In HD11 cells, the phosphorylation of TAK1 was induced by POLY-EXO treatment after 24 h and peaked at 36 h (Figure 5A). In addition, the phosphorylation of NF-κB1 was induced by POLY-EXO treatment and peaked at 48 h. In CU91 cells, the phosphorylation of TAK1 and NF-κB1 was induced upon treatment with POLY-EXOs and peaked at 24 h and 48 h, respectively (Figure 5B).

Furthermore, to verify the expression of the NF-κB signaling pathway-related molecules, HD11 and CU91 cells were incubated with PBS or 50 μg/mL of CTRL-EXOs or 50 μg/mL of POLY-EXOs for 6 h for immunocytochemistry analysis. The expression of p-TAK1 and p-NF-κB1 was upregulated by POLY-EXO treatment in the cytoplasm of HD11 and CU91 cells, and the signal was higher in HD11 cells than in CU91 cells (Figure 6).

DISCUSSION

The immune system is regulated through complex mechanisms and interactions of various cells. In particular, immune cell-derived exosomes regulate various immune responses by delivering molecules such as proteins and nucleic acids from donor cells. In this study, we examined the immunomodulatory functions of poly (I:C)-stimulated exosomes in chicken macrophages and T cell lines.

TLRs expressed on immune cells, such as macrophages and dendritic cells, play important roles in the innate immune system by recognizing PAMPs from pathogens (Beutler, 2004). In particular, TLR3 recognizes the dsRNA of some viruses, such as retroviruses. Upon the recognition of the dsRNA, TLR3 induces
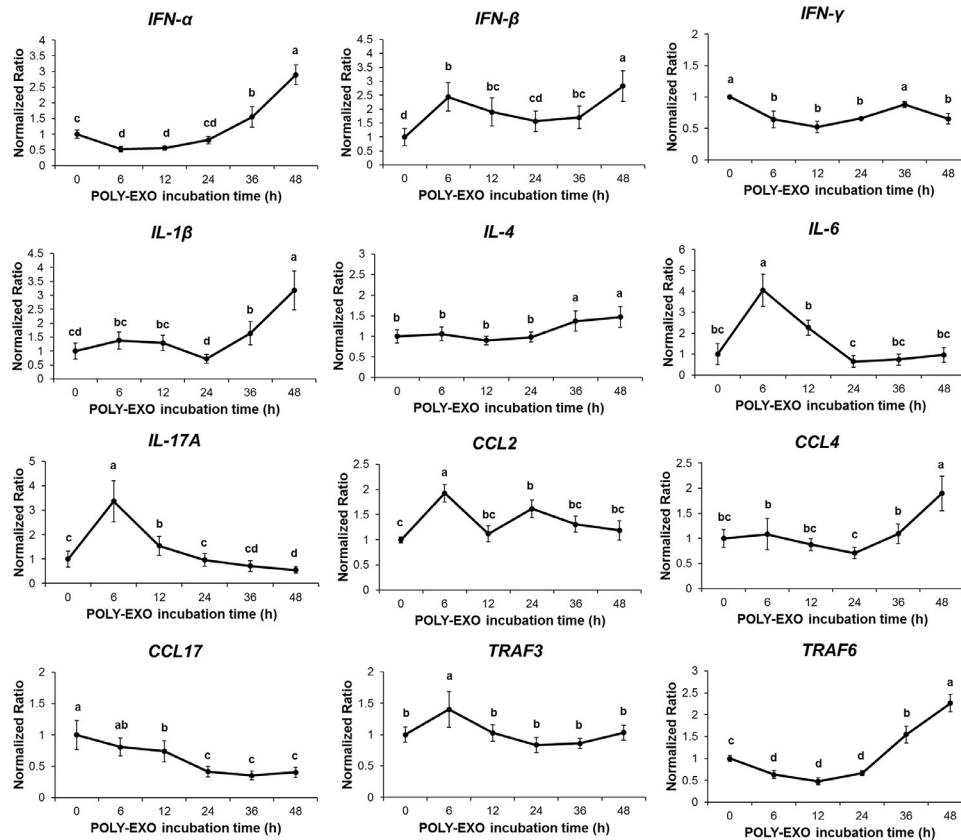
Figure 4. Effects of poly(I:C)-stimulated exosomes (POLY-EXOs) on cytokine and chemokine production in CU91 cells. CU91 cells were treated with POLY-EXOs (50 μg/mL) for 0, 6, 12, 24, 36, or 48 h and the expression level of cytokines and chemokines was determined using quantitative real-time PCR. Expression level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All culture conditions were tested in triplicate. The data are expressed as the mean ± standard error of the mean and are representative of three independent experiments. Different lowercase letters above the dots indicate significant differences ($P < 0.05$) as determined by analysis of variance with Tukey's multiple comparison test.

IRF3 and NF-κB activation via the TIR-domain-containing adapter-inducing interferon-β adaptor (Takeda et al., 2004). Activation of IRF3 induces the expression of type I IFNs, such as IFN-α and IFN-β, and antiviral cytokines; NF-κB induces the expression of proinflammatory cytokines (Liu et al., 2017). Therefore,

Figure 5. Western blotting for detecting the expression of p-TAK1, and p-NF-κB1. (A) HD11 and (B) CU91 cells were incubated with poly(I:C)-stimulated exosomes (POLY-EXOs) (50 μg/mL) for 0, 6, 12, 24, 36, or 48 h. Total cell lysates were analyzed by western blotting using antibodies against p-TAK1, p-NF-κB1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

TLR3-stimulated macrophages inhibit viral replication within infected cells and stimulate the antiviral activity of natural killer cells and CD8$^+$ lymphocytes via type I IFNs and various proinflammatory cytokines, resulting in an inflammatory response (Lester et al., 2014). In the present study, poly(I:C)-stimulated exosomes from chicken macrophages induced the expression of type I IFNs, proinflammatory cytokines, anti-inflammatory cytokines, and chemokines in macrophages and T cells (Figures 3 and 4). Especially, type I IFN and IFN-γ are known to induce the expression of interferon stimulated genes (ISGs) such as double-stranded RNA-activated protein kinase (PKR), the 2 isploligoadenylate synthetases (OAS), and the Mx protein (Fritsch et al., 2016). PKR phosphorylates the Eukaryotic Initiation Factor 2 (eIF2), which inhibits viral translation in the cells. OAS is an antiviral enzyme that degrades viral RNA. The Mx protein, which is part of the dynamin family of large GTPases, interferes with the replication of RNA viruses by inhibiting trafficking or the activity of viral polymerases. Besides IFN, various proinflammatory cytokines stimulate antiviral activity by boosting immune responses. Therefore, we suggest that exosomes released from chicken macrophages stimulated by viral dsRNA induce antiviral activity by increasing the expression of type I IFNs and proinflammatory cytokines.
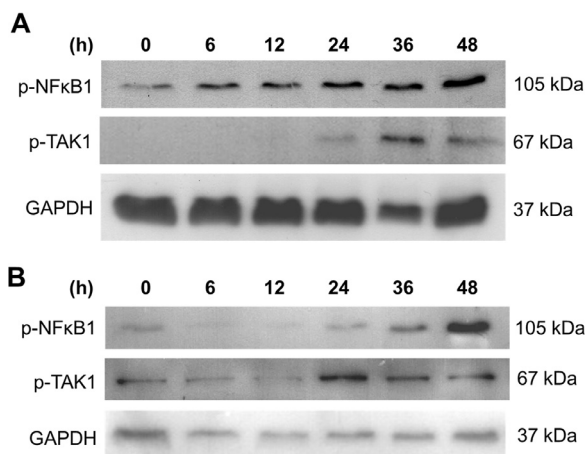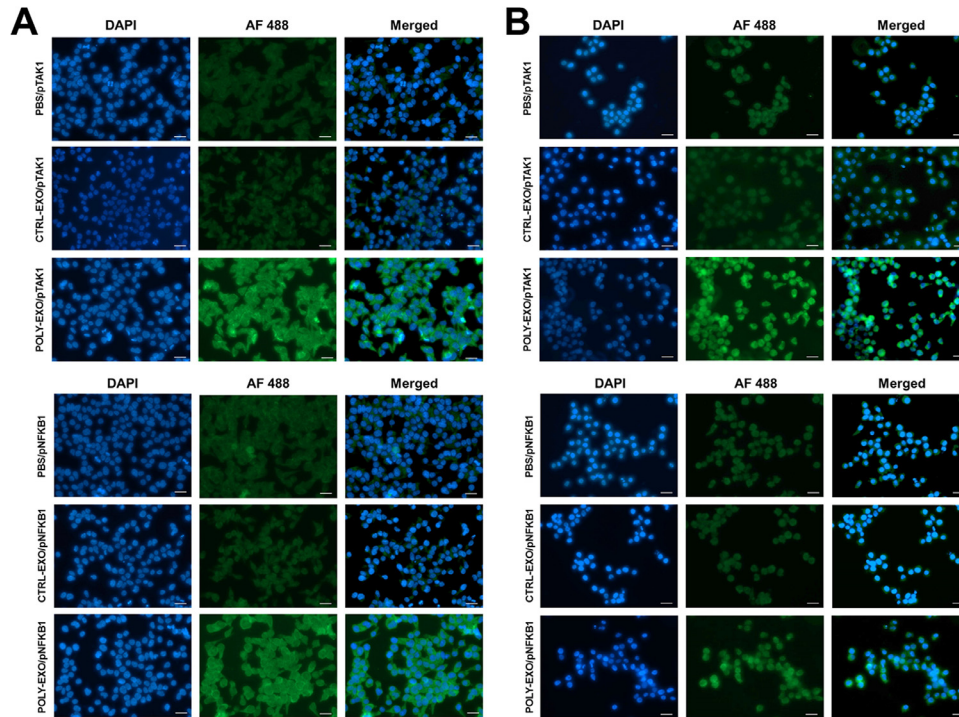
Figure 6. Immunocytochemistry analysis of p-TAK1, and p-NF-κB1. (A) HD11 and (B) CU91 cells were incubated with phosphate-buffered saline, CTRL-EXOs (50 μg/mL), or POLY-EXOs (50 μg/mL) for 6 h and analyzed via immunocytochemistry using antibodies against p-TAK1 and p-NF-κB1. Then, the cells were incubated with the primary antibodies, followed by incubation with Alexa Fluor 488 goat anti-rabbit IgG (H +L) secondary antibody and staining with 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar = 20 μm.

IL-6 induces IL-4 production in naive CD4$^+$ T cells by polarizing T cells to Th2 cells (Rincón et al., 1997). Interestingly, in the present study, the expression of some genes in HD11 and CU91 cells showed varying patterns. The expression levels of type I IFNs (IFN-α and IFN-β), IL-1β, IL-17A, CCL2, and CCL4 increased in both macrophages and T cell lines; however, the expression patterns of IL-4, IL-6, IFN-γ, and CCL17 were different between macrophages and T cell lines. In particular, the expression levels of IL-6 were only increased in CU91 cells, and IFN-γ, one of the proinflammatory cytokine, was increased in HD11 cells. Therefore, we suggest that the initial increase in the expression of IL-6 upon treatment with POLY-EXOs at 6 h in CU91 cells stimulated the expression of IL-4 from 36 h, thereby inducing Th2 polarization.

Exosomes from TLR-stimulated immune cells can also regulate immune responses. For example, Srinivasan et al. (2017) demonstrated that exosomes derived from poly(I:C)-stimulated cells induce macrophage M1-like polarization in murine lymph nodes and activation of the NF-κB signaling pathway. Moreover, TLR3-stimulated exosomes from dendritic cells show more enhanced cancer immunotherapy than that shown by TLR4-and TLR9-stimulated exosomes (Damo et al., 2015). Tang et al. (2016) also reported that human monocyte-derived exosomes stimulated with LPS induce the expression of CCL2, ICAM-1, and IL-6 in endothelial cells through the NF-κB signaling pathway. In the present study, poly(I:C)-stimulated exosomes derived from chicken HD11 macrophages induced the NF-κB signaling pathway by increasing the expression of cytokines and chemokines in chicken macrophages and T cells. Therefore, we suggest that when chickens are infected with dsRNA viruses, the TLR3 ligand is activated, thereby inducing the TLR3 signaling pathway and increasing the expression of type I IFNs, proinflammatory cytokines, and chemokines.

Exosomes derived from APCs such as macrophages can communicate with not only neighboring cells but also distant cells through biological fluids by delivering information via molecules such as proteins and miRNAs. Because TLR3 exists in endosomes, which subsequently develop into exosomes, TLR3 can be internalized within exosomes and delivered to recipient cells. Therefore, we suggest that TLR3 delivered by exosomes can induce the TLR3 signaling pathway in recipient cells. Moreover, TLR signaling pathway-induced changes in miRNA composition in exosomes can regulate gene expression in recipient cells. For example, the miRNA composition of exosomes from LPS-stimulated mouse macrophages was different from that of unstimulated macrophages, and LPS-stimulated exosomes induced the NF-κB signaling pathway in naive cells (McDonald et al., 2014).

In summary, this is the first study to identify the immunomodulatory functions of poly(I:C)-stimulated exosomes in chicken macrophages and T cells. POLY-EXOs regulated the expression of type I IFNs, proinflammatory cytokines, anti-inflammatory cytokines, and chemokines through the NF-κB signaling pathway. Although HD11 and CU91 cell lines are not enough to represent like primary cells, this study will provide an understanding of the mechanisms underlying the action

of exosomes from poly(I:C)-stimulated macrophages in the chicken immune system. Moreover, POLY-EXOs have the potential to be used as immunostimulators.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2021.101247.

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