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Pro-inflammatory Effects of Influenza Type A Virus PB1-F2 Protein-derived Peptide in Lipopolysaccharide-treated Macrophages

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ABSTRACT

Influenza A virus (IAV) has the potential to cause pandemics with considerable health and socio-economic burdens. A viral protein, polymerase basic 1- frame2 (PB1-F2), as a virulence factor, has pro-apoptotic activity and contributes to viral pathogenesis by delaying viral clearance and inducing inflammation. Macrophages are susceptible to IAV infection and produce high levels of inflammatory cytokines and chemokines. In the present study, the pro-inflammatory effects of PB1-F2 derived peptide was evaluated by measuring the expression of key inflammatory mediators in murine macrophage cell line J774.1.

PB1-F2 treated macrophages were examined for nitric oxide (NO) production, inflammatory cytokines, and enzymes expression and pro-inflammatory cytokines secretion using Griess reagent, real-time polymerase chain reaction (PCR) and ELISA, respectively. Our results have shown that PB1-F2 peptide at non-cytotoxic concentrations (0.1–0.8 μmol/mL) had no effect on NO production.

When applied to Lipopolysaccharide (LPS)-treated macrophages, PB1-F2 peptide at 0.8 μmol/mLincreasedinducible NO synthase (iNOS), cyclooxygenase (COX)-2, and interleukin (IL)-6 genes expression to 2.02, 3.81, and 3.65 folds, respectively. PB1-F2 at concentrations of 0.4 and 0.8 μm/mL increased tumor necrosis factor (TNF)-α transcription by 4.15 and 5.55 fold. At posttranslational level, TNF-α increased from 166.5±13.88 in LPS-treated cells to 773.6±95.27 and 1485±76.31 at concentrations of 0.4 and 0.8 μmol/mL in PB1-F2 peptide, respectively. However, PB1-F2 Peptide did not have any significant effect on IL-6 production.

These findings suggest that PB1-F2 peptide may partly exert its enhancing role in viral pathogenicity through the induction of inflammatory mediators in macrophages. Hence, targeting PB1-F2 peptide would be helpful in the reduction of viral infection complications.

Keywords: Inflammation; Influenza A virus; Macrophage; Polymerase basic–frame-2

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INTRODUCTION

Highly pathogenic H1N1 and H5N1 influenza A virus (IAV) strains have been responsible for a number of pandemics during the last century and are still potential threats for global health. Infiltration of macrophages and neutrophils in the lung and overproduction of pro-inflammatory cytokines upon Fluviruses infection can disturb the lung function and play a crucial role in the respiratory and systemic pathology in the course of infection.[1,2] Production of pro-inflammatory cytokines such as interleukin (IL)-1β, interferon (IFN)-γ and tumor necrosis factor (TNF)-α is increased in the lung tissues of IAV-infected mice. Moreover, H5N1 viruses cause the production of high levels of chemokines and cytokines in the human macrophage and mouse lung.[3-5] The mentioned strains have been shown to express a virulence factor called PB1-F2 (Polymerase Basic 1- Frame 2).[6] It is a non-structural protein, which is translated by the +1 frameshifting of PB1 transcript of the majority of influenza viruses.[7] Among the amino acid sequence of PB1-F2 protein, location 61 to 87 known as an inflammatory motif is associated with the extent of inflammatory responses to the infection.[8,9] This protein is expressed maximally five hours after infection.[10] In spite of the dispensable role of virus replication, the PB1-F2 protein has several pathogenic functions including induction of apoptosis in the immune cells (macrophages), delaying viral clearance by modulation of the innate immune response, enhancing viral polymerase activity, and more importantly, escalating the inflammatory responses.[11,12] The presence of a mitochondrial targeting sequence (MTS) within PB1-F2 protein induces its translocation into the mitochondria membrane and release of cytochrome C, which leads to apoptosis via the endogenous pathway.[13] Some studies have shown that PB1-F2 induces infiltration of the immune cells and increases the cytokines level in the lung tissue of infected mice.[14,15] However, the exact mechanisms and pathways by which the inflammation is initiated and ensues in macrophages are not clear yet, and only a few studies attributed anti-inflammatory properties to PB1-F2.[16-18] Therefore, our study aimed at a better understanding of the role of the PB1-F2 in virus-mediated inflammation. Therefore, we specifically treated macrophage (as the main cell involved in the inflammation process) by the PB1-F2-derived peptide to investigate its impacts on the expression and release of various inflammatory mediators.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM) and non-essential amino acids were prepared from Gibco (Ashland, KY, U.S.A). Cell culture grade dimethyl sulfoxide (DMSO, MERK, Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, MERK, Germany), trypan blue, Griess reagent and lipopolysaccharide (LPS; type 0111:B4 from Escherichia coli, MERK, Germany) were purchased from Sigma (St. Louis, MO, U.S.A). Mouse IL-6 and TNF-α ELISA kits were purchased from eBioscience (San Diego, CA). Total RNA extraction kit (Parstous, Mashhad, Iran), High-capacity cDNA reverse transcription kit (ABI, U.S.A) and Prime Q-Mastermix (2X, Real-time PCR with SYBR Green I) (Genetbio Inc., Korea) were also used in this study.

Cell Culture and Peptide Preparation

A mice macrophage line, J774.1 cell (ATCC TIB-67 TM), was cultured in Dulbecco's Modified Eagle Medium(DMEM) (Sigma, Germany) in a humidified atmosphere with 5% CO2 at 37ºC containing 10% FBS (Gibco (Ashland, KY,U.S.A)), 2mM L-glutamine and 1% streptomycin/penicillin antibiotics. A peptide derived from the C-terminus of the PB1-F2 protein (strain A/Puerto Rico/8/1934 H1N1, NCBI: txid211044) with sequence WLSLRNPILVFLKTRVLKRWRLFSKHE (amino acid 61-87) was purchased from Takapouzist Inc., Iran and suspended in Phosphate buffer saline (PBS) Takapouzist Inc., Iran at a concentration of 1mM/mL. This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1393.S7426).

Toxicity Assay

To find the non-toxic concentrations of the peptide, the J774.1 cells were cultured in 96-well plates (Nunclone, Thermo Scientific, CA) to a density of 7.5×10^3 cells/well in DMEM medium supplemented with 10% FBS. After overnight incubation, the cells were treated with different concentrations of the peptide (0.1-100 µmol/mL) for 12 hours in a final volume of 100 µL. A negative control containing cells

only were included. Then, 10 µL4,5-Dimethylthiazol-2-yl (MTT) solution (5 mg/mL) was added to each well and after 4 hours of incubation at 37°C, to dissolve the formazan crystals, the medium was replaced with 150 µLDMSO(dimethyl sulfoxide). The quantity of formazan was measured by recording the absorbance at 570 nm with background subtraction at 630 nm using a microplate reader (BioTek, Winooski, USA). The optical density (OD) of solubilized formazan in the negative control cells was considered as 100% viability and the percentage of viability of treated cells was calculated.

Measurement of NO Production

To study the effect of non-cytotoxic concentrations of the peptide on NO production, the cells were cultured in 24-well plates and incubated at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 and allowed to become confluent. The medium was replaced with a fresh one and the cells were treated with PB1-F2 peptide in the presence of LPS (1µg/mL) for 12 h. Positive control was LPS-only treated cells and negative control was the cells containing medium only. Then, Griess assay reagent (containing sulfanilamide, N-1-naphthylethylene diamine (NED) and H3PO4) was added according to the manufacturer's instruction, (PromegaCorporation, U.S.A) to culture the supernatants (ratio 1:1) and incubated 10 min at room temperature to form a purple azo dye. NO level was determined by reading the absorbance at a wavelength of 540 nm in a microplate reader (BioTek). A reference curve that was obtained with serial dilutions of sodium nitrite was prepared and NO concentration determined by comparison to the Nitrite Standard reference curve.

Real-time PCR

Changes in the levels of IL-6, TNF-α, iNOS and COX-2 genes transcription in peptide-treated cells RT-PCR were investigated using qPCR. Briefly, the cells were cultured overnight in DMEM as described above. After overnight incubation, different concentrations (0.1, 0.2, 0.4, 0.8 µmol/mL) of peptide were added to the cells in the presence of LPS (1 µg/mL) and further incubated for 12 h. The culture supernatants were collected for cytokine measurement and stored at -80°C until analysis. Cells were collected and total RNA extracted using a total RNA extraction kit according to the manufacturer's protocols. The concentration of RNA samples was determined by the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized by a high-capacity cDNA reverse transcription kit according to the manufacturer's instructions and quantified by RT-PCR analysis. For each gene, 4 µL cDNA was amplified in the presence of 10 µl SYBR Premix Ex Taq II, 0.5 µL of each primer (10 pM), 0.3 µL ROX reference dye-2, 4.7 µL doubly-distilled water. The sequences of primers are listed in Table 1. Amplification was carried out for 40 cycles using an Applied Biosystems 7500 Real-Time PCR system. An initial denaturation step at 95˚C for 30 s, was followed by 40 cycles at 95°C for 5 s and 56.3ºC (GAPDH, iNOS),53˚C (TNF-α, COX-2), 56.2°C (IL-6) for 18 s and 72˚C for 30 s. The target mRNA expression relative levels were normalized against GAPDH (Glyceraldehyde 3-phosphate

Table1. Primer sequences for Real-time PCR

Genes	Primer Sequences	Size (bp)
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	F: 5′-CGGTGTGAACGGATTTGGC-3′ R: 5′-GTGAGTGGAGTCATACTGGAAC-3′	142
iNOS	F: 5′- CTGGAGGTTCTGGATGAG-3′ R: 5′- CTGAGGGCTGACACAAGG-3′	193
COX-2	F: 5′-CAGCACTTCACCCATCAG-3′ R: 5′-GATACACCTCTCCACCAATG-3′	181
TNF-α	F: 5′-GTC-TCAGCC-TCTTCTCATTC-3′ R: 5′-GGAACTTCTCATCCCTTTGG-3′	99
IL-6	F: 5′-ACCTGTCTATACCACTTCAC-3′ R: 5′-GCATCATCGTTGTTCATAC-3′	117

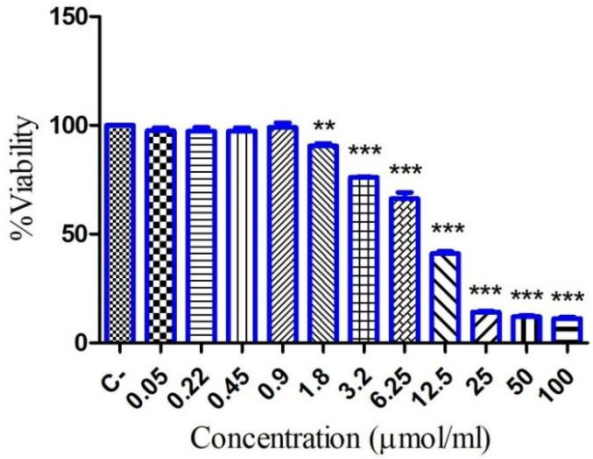

Figure 1. Effect of PB1-F2 peptide on J774.1 cell viability. The cells were treated with different concentrations of peptide for 12 h. Viability of cells was determined using MTT colorimetric assay. Negative control (C-) was cells containing medium only. Values are reported as mean±SE of three independent experiments in triplicate. *****p*<0.01, ******p*<0.001 versus negative control.

dehydrogenase) using the $2^{-\Delta\Delta CT}$ method.[19] Results were expressed as relative fold change (RFC) to a positive control (cells treated with only LPS).

Cytokine Analysis

The concentration of interleukin (IL)-6 and TNF-α cytokines in the culture of supernatants was determined using ELISA assay kits according to the manufacturer's instructions. The sensitivity of these kits was determined to be 4 and 8 pg/mL for IL-6 and TNF-α, respectively.

Statistical Analysis

All experiments were performed in triplicates and repeated three times. All data are presented as mean±standard error of the mean (SEM). Significant differences between the groups were evaluated using Graphpad software (San Diego, CA), and analysis of variance (ANOVA; Tukey's post hoc test) was used to compare various concentrations of the peptide. *p* values<0.05 were considered as statistically significant.

RESULTS

Effect of PB1-F2 Peptide on J774.1 Cell Viability

Based on MTT assay, the peptide at concentrations of 1.56-100 µmol/mL significantly decreased the cell viability in a dose-dependent manner, while at lower concentrations (0.1-0.8 µmol/mL) it had no effect on cell viability (Figure 1). Therefore, the 0.1-0.8 µmol/mL range was selected for further experiments.

PB1-F2 Peptide had no Effect on NO Production

The level of NO production in the peptide-treated cells was determined by Griess reagent. As shown in Figure 2, no significant differences were observed in the production of NO as a result of exposure to the PB1-F2- derived peptide.

Effect of PB1-F2 Peptide on iNOS, COX-2, TNF-α and IL-6 Gene Expression

As shown in Figure 3 (A) and (B), the transcription of iNOS and COX-2 followed similar patterns, where PB1-F2 at the concentration of 0.8 µmol/mL significantly increased the transcription of both inducible enzymes, iNOS to 2.02(±025)-folds (p<0.05); and COX-2 to 3.81(±0.74)-folds (p<0.001) compared to the positive control (LPS-only treated cells).Interestingly, lower concentrations of peptide somehow exhibit an inhibitory/modulatory role on macrophages which were activated by LPS (Figure 3). These differences were significant for iNOS (p=0.0037), COX2 (p=0.0069), and TNF-α (p=0.0127), but not for IL-6 (p=0.001). Treatment of cells with PB1-F2 at concentrations of 0.4 and 0.8 µmol/mL increased TNF-α transcription by4.15 and 5.55 fold, respectively (p<0.001; Figure 3C). Only at the concentration of 0.8 µmol/mL of PB1-F2 peptide, IL-6 gene expression increased to 3.65 fold compared to the positive control (p<0.001; Figure 3D).

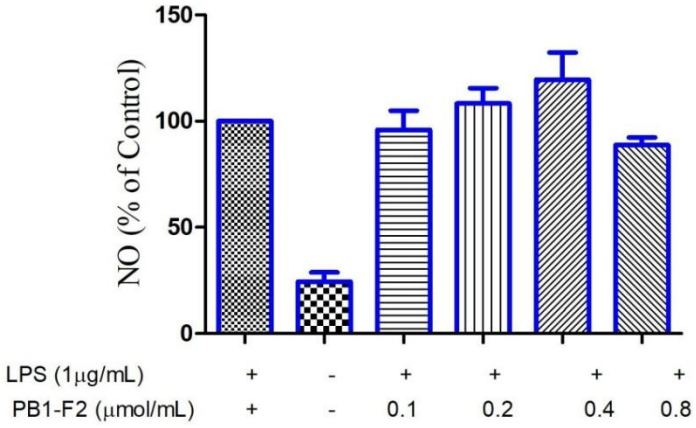

Figure 2. Effect of PB1-F2 peptide on nitric oxide production. The J774.1 cells were cultured in DMEM medium and exposed with non-cytotoxic concentrations of peptide in the presence of LPS (1µg/mL) for 12 h. Level of NO production was measured by Griess reagent. The positive control was LPS-only treated cells and cells containing medium only were used as the negative control. Values represent as mean ± SE of three independent experiments.

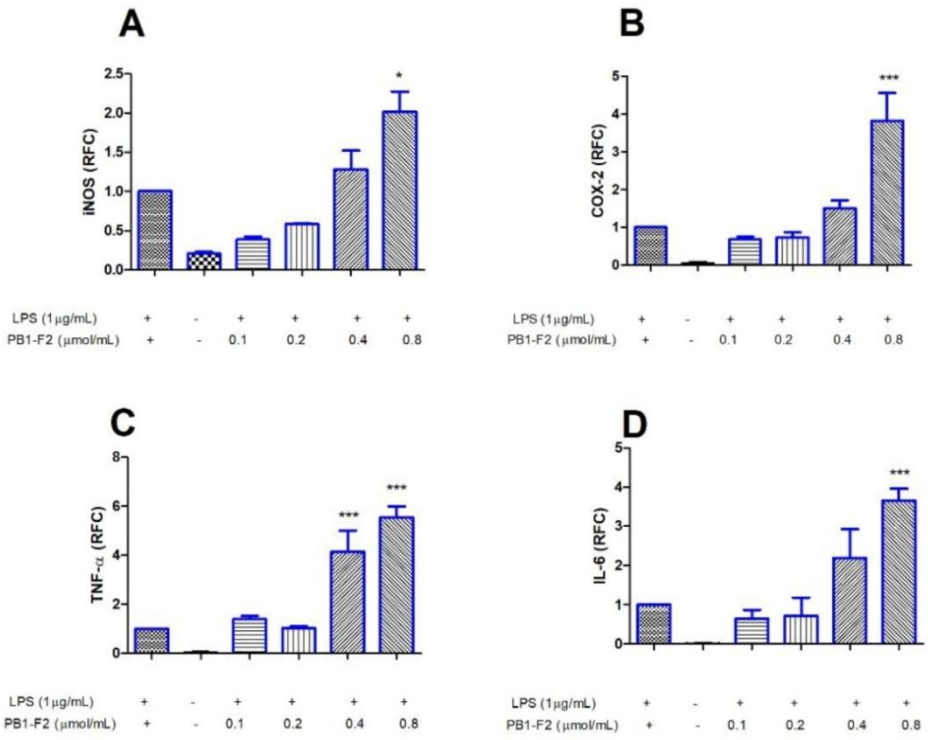

Figure 3. Effect of PB1-F2 peptide on inflammatory genes expression. The J774.1 cells were treated with different concentrations of peptide in the presence of LPS (1µg/ml). After 12 h incubation at 37 °C and 5% CO_2, gene expression levels of iNOS (A), COX-2 (B), TNF-α (C) and IL-6 (D) was determined using RT-PCR. Negative control was cells in the absence of peptide and LPS; Positive control was LPS-only treated cells. Values shown are mean ± SE of three independent experiments.*p<0.05, **p<0.01, ***p<0.001 versus positive control. RFC, relative fold-change.

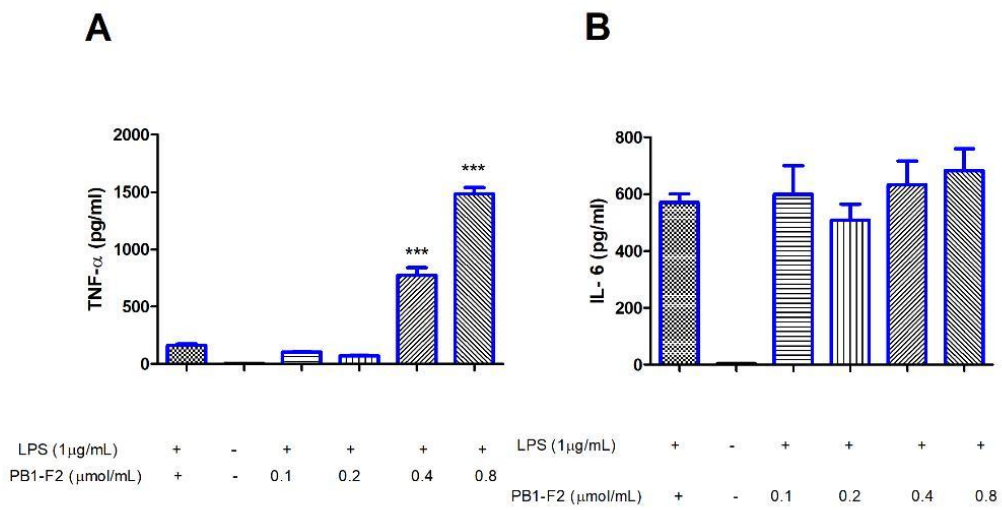

Figure 4. Effect of PB1-F2 peptide on the secretion of inflammatory cytokines. TNF-α (A) and IL-6 (B) protein levels were determined in the culture supernatant of peptide treated cells using ELISA. Negative control was cells containing medium only; LPS-only treated cells were used as the positive control. Values represent as mean ± SE of three independent experiments. ***p<0.001 versus positive control.

PB1-F2 Peptide Induced Inflammatory Cytokine Production in Macrophage

The concentration of TNF-α in the presence of 0.4 and 0.8 μmol/ml of peptide increased from 166.5±9.81 pg/mL in LPS-only treated cells to 73.5±6.4 and 1484.7±54 pg/mL, respectively (p<0.001) (Figure 4A). PB1-F2 Peptide did not have any significant effect on IL-6 production (Figure 4B).

DISCUSSION

In the current study, we investigated the effect of PB1-F2 derived peptide of IAV, which is known as an inflammatory motif, on key inflammatory mediators produced by the macrophage. Briefly, in LPS-treated macrophages PB1-F2 peptide at 0.8 μmol/mL increased iNOS, COX-2, TNF-α, and IL-6 gene expression by two to six times. The protein level of TNF-α also increased dose-dependently by five to nine times, but IL-6 did not show a significant increase. The protein level should usually be interpreted in terms of gene expression, but this correlation is not very strong so that some studies have shown a 40% correlation (depending on the system).[20] Higher concentrations of the peptide displayed immunostimulatory effects.

These findings reconfirm the results of previous researchers who reported that infected macrophages respond to IAV-derived PB1-F2 through high expression of IL-6, TNF-α, IL-1, IFN-α, IFN-β and chemokines such as MIP-1α, MIP-1β, and RANTES.[21-23] A recent study has shown that PB1-F2 from H7N9 IAV induces lung inflammation through activation of NLRP3 inflammasome.[24] Others have reported elevated expression of IFN-β in human respiratory epithelial cells by full-length PB1-F2 through activation of NF-κB and it, later on, was confirmed by *in vivo* infection in a mouse model.[25,26] However, it has been indicated that PB1-F2 inhibits the activation of the canonical pathways of NF-κB in transfected cells. Also, the interaction of PB1-F2 with IKK (a key element of pro-inflammatory cytokines signaling) modulates the NF-κB signaling pathway.[27] PB1-F2 has an anti-IFN activity that is mediated by the C-terminus region which also has an apoptotic activity. IFN antagonist activity of PB1-F2 is due to its interference with the retinoic acid-inducible gene (RIG-I)/MAVS protein complex and inhibition of downstream transcription factor IFN regulatory factor 3 (IRF-3).[18,28] It has been shown that a single N66S mutation in the PB1-F2 protein of H5N1 and H1N1 influenza virus strains dramatically increased the pathogenicity through delaying the IFN response.[17,29]

Nitric oxide (NO) produced by the Inducible nitric oxide synthase (iNOS) enzyme exhibits a potent anti-viral property. However, elevated production of iNOS and NO during influenza infection results in the

induction of excessive inflammation that contributes to the viral pathogenesis in mice.[30,31] Activated macrophages produce high levels of NO during the inflammation.[19] Infection with influenza virus induces IL-1β, TNF-α and IFN-γ, which in turn have an effect on iNOS expression and subsequent NO production.[32] It has been reported that H5N1 influenza infection increased iNOS expression in various tissues and NO serum levels in infected animals. Increased iNOS production in H5N1 infection is associated with diseases severity.[33]

Our results have shown that the treatment of macrophages with different concentrations of PB1-F2 peptide increases the iNOS gene transcription in LPS-treated cells. Interestingly, this peptide did not show any significant additive effect on NO (nitrite) production. It was observed that PB1-F2 also significantly increased the COX-2 enzyme gene transcription. This inducible enzyme plays an important role in prostaglandin production that is a potent inflammatory mediator.[34]

Other studies have also reported that IAV strongly induces COX-2 expression in infected bronchial epithelial cells and macrophages.[35,36] It has been shown that COX-2 expression is induced at the early stages of IAV infection that are mediated by retinoic acid-inducible gene (RIG-I) expression and is down-regulated thereafter.[37] COX-2 can be induced rapidly by growth factors, LPS and cytokines such as IL-1β and TNF-α.[38] We reported a dose-dependent response about this protein both in prevention or induction of inflammation; indicating anti-inflammatory properties for PB1-F2.

In contrast to findings from previous studies, one study suggested that PB1-F2 reduced the pathogenicity and the expression of the tissue damage associated genes in chicken. However, we showed a decreased level of cytokines when a low concentration of PB1-F2 was used. In addition, this controversy could be attributed to differences in the immune response in mammalians versus avians.[16]

Our study had some limitations and strengths. We did not evaluate the changes in major transcription factors which are important in the process of inflammation during exposing the cells with this peptide. However, we used various concentrations of the peptide and showed that the dose of the peptide was a possible source of controversy in the literature regarding its inflammatory activity.

The PB1-F2 peptide exhibited inflammatory activity by increasing the expression of the main inflammatory mediators in macrophages in a dose-dependent manner. Nevertheless, we did not detect post-translational IL-6 and iNOS in our experiments. Our results suggest that this peptide contributes to viral pathogenesis.

Together, these findings suggest that the sequence type of PB1-F2 peptide is involved in the inflammogenesis of influenza and may predict disease severity/outcome. Strategies that target this molecule or block its paths of action would be rational for reducing viral pathogenesis and disease complications.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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