



## **IFN- $\gamma$ Induction of Apolipoprotein A-I Expression is Mediated by NF- $\kappa$ B Signalling in HepG2 Cells**

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### **Highlights**

- IFN- $\gamma$  increases apolipoprotein A-I (APOA-I) expression.
- Inactivating NF- $\kappa$ B signalling pathway decreases the IFN- $\gamma$  induction of APOA-I expression.
- IFN- $\gamma$  regulates APOA-I through NF- $\kappa$ B p65 phosphorylation at Ser468 and Ser536.

## **IFN- $\gamma$ Induction of Apolipoprotein A-I Expression is Mediated by NF- $\kappa$ B Signalling in HepG2 Cells**

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**Abstrak:** Keradangan hati berkait rapat dengan perubahan dalam ekspresi lipoprotein dan apolipoprotein. Interferon- $\gamma$  (IFN- $\gamma$ ), wakil tunggal jenis kedua IFN, memainkan peranan yang penting dalam memodulasi dan mempergiatkan tindak balas keradangan. Justeru itu, kajian ini direka untuk mengenal pasti kesan IFN- $\gamma$  terhadap apolipoprotein A-I (APOA-I) dan penglibatan nuclear factor- $\kappa$ B (NF- $\kappa$ B) dalam laluan isyarat tersebut. Tindak balas rantai polimerase transkripsi berbalik kuantitatif (qRT-PCR) dan analisis blot western telah dilaksanakan untuk menguantifikasi ekspresi APOA-I dalam sel-sel HepG2 selepas dirawat dengan IFN- $\gamma$ . Kajian ini menunjukkan bahawa 50 ng/mL IFN- $\gamma$  merangsangkan ekspresi mRNA dan protein APOA-I. Walau bagaimanapun, pra-rawatan sel dengan inhibitor laluan isyarat NF- $\kappa$ B mengurangkan tahap ekspresi APOA-I. Kajian ini juga mendemonstrasikan penglibatan langsung isyarat NF- $\kappa$ B dalam ekspresi APOA-I akibat rangsangan IFN- $\gamma$ , di mana IFN- $\gamma$  meningkatkan tahap fosforilasi NF- $\kappa$ B p65 Ser468 dan Ser536 kepada 2.59-ganda and 1.63-ganda. Namun demikian, pra-rawatan sel dengan perencat laluan isyarat NF- $\kappa$ B melumpuhkan peningkatan tersebut dan kemudian mengurangkan ekspresi APOA-I dalam sel HepG2. Sebagai rumusan, kajian ini berjaya mengenalpasti peranan isyarat NF- $\kappa$ B dan pengaktifan p65 Ser468 dan Ser536 sebagai pengantara IFN- $\gamma$  ke atas induksi APOA-I di dalam sel-sel HepG2.

**Kata kunci:** IFN- $\gamma$ , Penglibatan Langsung NF- $\kappa$ B, Apolipoprotein A-I, Keradangan Hati

**Abstract:** Liver inflammation is associated with changes in lipoprotein and apolipoprotein expression. Interferon- $\gamma$  (IFN- $\gamma$ ), the sole representative of type II IFN, plays a pivotal role in modulating and intensifying inflammatory responses. This study was designed to identify the effect of IFN- $\gamma$  on apolipoprotein A-I (APOA-I) and to identify the involvement of nuclear factor- $\kappa$ B (NF- $\kappa$ B) in its regulation. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot analysis were performed to quantify the APOA-I expression in treated HepG2 cells. Here, we show that 50 ng/mL of IFN- $\gamma$  induced APOA-I mRNA and protein expression. Pretreatment of cells with NF- $\kappa$ B signalling pathway inhibitors, however, decreased the APOA-I expression levels. This study also demonstrated the direct involvement of NF- $\kappa$ B signalling in IFN- $\gamma$ -induced APOA-I expression, whereby

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IFN- $\gamma$  induced the levels of phosphorylated NF- $\kappa$ B p65 Ser468 and Ser536 expression to 2.59-fold and 1.63-fold, respectively. However, pretreatment of cells with NF- $\kappa$ B signalling pathway inhibitors attenuated their increment and subsequently reduced APOA-I expression in HepG2 cells. In summary, the present study successfully confirmed the role of NF- $\kappa$ B signalling and activation of p65 Ser468 and Ser536 in mediating IFN- $\gamma$  induction of APOA-I expression in HepG2 cells.

**Keywords:** IFN- $\gamma$ , NF- $\kappa$ B Signalling Pathway, Apolipoprotein A-I, Liver Inflammation

## INTRODUCTION

Apolipoproteins are amphipathic polypeptide that can be found in different types of lipoproteins and serve important roles in mediating lipoproteins assembly, maintaining their structure and directing their metabolism (Irshad & Dubey 2005; Dominiczak & Caslake 2011). Apolipoprotein A-I (APOA-I), the main protein found on the high-density lipoprotein (HDL), is synthesised in intestine and liver. Alteration in APOA-I level is often associated with inflammatory conditions such as hepatitis, atherosclerosis and arthritis (Yang *et al.* 2010; Terkeltaub 2014).

Interferons (IFNs) are a group of soluble proteins that induce antiviral state in cells upon virus infections. In addition, they also possess immunomodulatory and antiproliferative properties (Platanias 2005; Sen & Sarkar 2007; El Jamal *et al.* 2016). Interferon gamma (IFN- $\gamma$ ), the only type II interferon member, is produced by natural killer (NK) cells, antigen-presenting cells (APCs), CD8+ cells and T helper 1 (TH1) cells (Kindt *et al.* 2004). It is a well-known pleiotropic cytokine which plays essential roles in regulating inflammatory and immune responses against pathogens (Zhang 2007). In addition, past studies had shown the significance of this pro-inflammatory cytokine in triggering cell-mediated immunity and intensifying liver inflammation which exacerbates liver injury and ultimately, lead to hepatic cell death and fibrosis (Mihm *et al.* 1996; Mizuhara *et al.* 1996; Knight *et al.* 2007; Thomsen *et al.* 2013; Deng *et al.* 2015; Ramalingam *et al.* 2016).

Chronic inflammation causes significant lipid disturbances, such as the increase in hepatic triglyceride level, impairment of reverse cholesterol transport and changes in lipoproteins and apolipoproteins levels (Esteve *et al.* 2005; Masoodi *et al.* 2015; Ertunc & Hotamisligil 2016). Many studies have shown the significance of IFN- $\gamma$  in regulating and intensifying inflammatory process. IFN- $\gamma$  regulates the expression of several key players in cholesterol metabolism and it has been shown to decrease cholesterol efflux to HDL or APOE, which is a protein component of the lipoprotein that is responsible for the transport of cholesterol and other lipids between liver and other peripheral tissues (Panousis & Zuckerman 2000; Reiss *et al.* 2004; Yu *et al.* 2015; Pérez-Baos *et al.* 2017). However, the exact influence of IFN- $\gamma$  on APOA-I expression during liver inflammation is yet to be elucidated. It is hypothesised that IFN- $\gamma$  could regulate APOA-I gene expression through the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway. Understanding the underlying molecular mechanisms of IFN- $\gamma$  signalling pathway on the alteration of APOA-I profile would certainly provide a worthwhile platform for future therapeutic

development in treating inflammatory disorders of lipid metabolism. Here, we show that IFN- $\gamma$  increases APOA-I expression by acting through NF- $\kappa$ B signalling pathway.

## **MATERIALS AND METHODS**

### **Maintenance of Cells in Culture**

HepG2 cells (ATCC, USA) were grown in Minimum essential medium (MEM) (Gibco, USA) in 75 cm<sup>2</sup> tissue culture flasks (SPL Life Science, Korea). This medium was supplemented with 2 mM of L-glutamine, 10,000 Units/mL of penicillin, 10,000 mg/mL of streptomycin, 1 mM of sodium pyruvate, 0.1 mM of non-essential amino acid, and 2.2 g/L of sodium bicarbonate, with 10% (v/v) heat-inactivated (30 min, 56°C), filter-sterilised foetal bovine serum (FBS). The cells were maintained in a humid incubator of 5% (v/v) CO<sub>2</sub> at 37°C. The cell culture medium was replaced every three days to maintain a healthy culture. The medium in the tissue culture flasks was discarded, followed by the washing of the cells twice with 5 mL of phosphate buffered saline (PBS) each time. Sub-culturing of cells was performed after the cells achieved 80% confluence.

### **Cell Treatment with IFN- $\gamma$ and NF- $\kappa$ B Signalling Pathway Inhibitors**

HepG2 cells were seeded into 6-well tissue culture plates (TPP, Switzerland) and allowed to grow to 70% confluence. The cells were treated with 50 ng/mL IFN- $\gamma$  (Millipore, USA) for another 24 h for the investigation of the impact of IFN- $\gamma$  on *APOA-I* expression. Prior to the treatment, the growth medium was discarded and all the cells were starved with 0.5% (v/v) FBS at 37°C, in a 5% (v/v) CO<sub>2</sub> incubator for 2 h. To inhibit the NF- $\kappa$ B signalling pathway, the cells were pretreated with 200 nM of NF- $\kappa$ B Activation Inhibitor IV, 50  $\mu$ M of SC-514 or 10  $\mu$ M of Wedelolactone respectively, for 2 h prior treatment with 50 ng/mL IFN- $\gamma$  for another 24 h. For vehicles control, DMSO (diluent of inhibitors) was used to stimulate the cells, whereas no mediator was added for the negative control.

### **Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

Total cellular RNA was extracted from cells cultured in 6-well tissue culture plate using Tri-Reagent<sup>®</sup> LS (Sigma Aldrich, USA) according to the manufacturer's instruction. To determine the integrity and purity of the extracted RNA, 1% (w/v) denaturing agarose gel electrophoresis and the optical density at 260 nm and 280 nm were performed. DNase treatment of RNA was carried out using RQ1 RNase-free DNase (Promega, USA). qRT-PCR was then carried out using One-step Quantifast SYBR Green RT-PCR Kit (Qiagen, Germany) according to the manufacturer's instruction using iCycler MyIQ Real-Time PCR Detection System

(Bio-Rad, USA). All reactions were assembled on ice and performed in a final volume of 20  $\mu$ L. Parameters used were reverse transcription step at 50°C for 600 s, followed by an initial denaturation step at 95°C for 300 and a 35-cycle consisting of 95°C for 10 s, 65°C for 30 s and 72°C for 30 s, before a melt-curve analysis. The nucleotide sequences of APOA-I primers were obtained from Matsuura *et al.* (2007), whereas  $\beta$ -actin primers were adopted from Chew *et al.* (2007).

## Western Blot Analysis

Total cellular protein was extracted using Tri-Reagent<sup>®</sup> LS (Sigma-Aldrich, USA) and the concentration of protein extracted was determined using the Bio-Rad Dc Protein Assay reagent kit (Bio-Rad, USA) according to the manufacturers' instruction. Eighty (80)  $\mu$ g of protein sample were loaded into the SDS-PAGE and electrophoresed. Electrophoretic transfer of proteins to polyvinylidene fluoride (PVDF) transfer membrane was done using Bio-Rad Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, USA). The primary antibodies used were APOA-I antibody,  $\beta$ -actin antibody, phospho-NF- $\kappa$ B p65 Ser468 antibody, and phospho-NF- $\kappa$ B p65 Ser536 antibody (Cell Signaling, USA), followed by a secondary antibody, anti-rabbit IgG HRP-linked antibody (Cell Signaling, USA) according to protocols (Lim *et al.* 2013). Chemiluminescence detection of the antibodies was performed using the ChemiDoc<sup>™</sup> MP Imaging System Cabinet (Bio-Rad, USA) and Immobilon<sup>™</sup> Western Chemiluminescence HRP substrate (Milipore, USA). Immunodetected protein bands were quantified using the Image Lab TM version 4.1 software (Bio-Rad, USA). The protein expression of APOA-I and phosphorylated NF- $\kappa$ B p65 Ser468 and Ser536 were quantified and normalised against the  $\beta$ -actin protein expression.

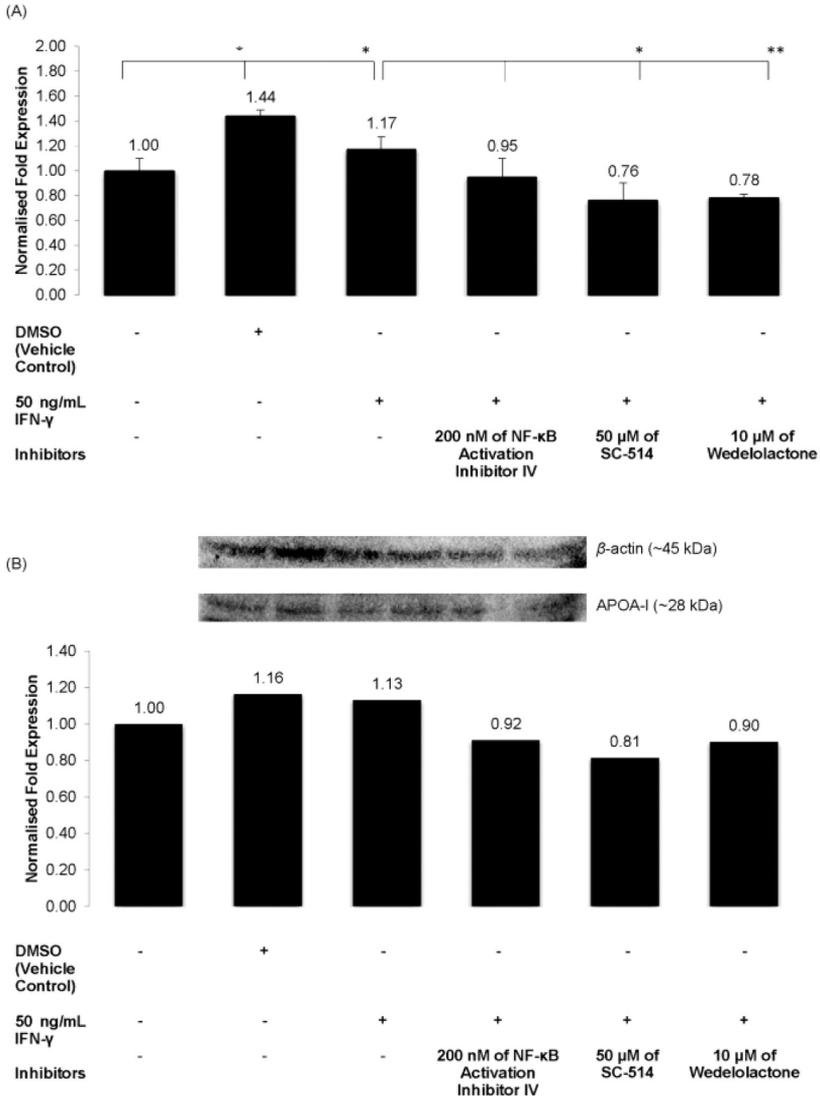
## RESULTS

### APOA-I Expression was Increased by IFN- $\gamma$ but Reduced by the Presence of NF- $\kappa$ B Signaling Pathway Inhibitors

According to Fig. 1, treatment of cells with 50 ng/mL of IFN- $\gamma$  significantly increased the APOA-I expression, when compared with untreated. On the other hand, APOA-I expression decreased significantly to 0.76-fold ( $p < 0.05$ ) and 0.78-fold ( $p < 0.01$ ) in the cells pretreated with 50  $\mu$ M SC-514 and 10  $\mu$ M Wedelolactone prior to 50 ng/mL of IFN- $\gamma$  treatment. Cells inhibited with 200 nM of NF- $\kappa$ B Activation Inhibitor IV showed only slight decrement in APOA-I mRNA expression, which was 0.95-fold related to untreated.

Similarly, HepG2 cells treated with 50% (v/v) of DMSO (vehicle control for the cell signaling inhibitors) had 1.16-fold increment of APOA-I protein expression. The APOA-I was increased to 1.13-fold by 50 ng/mL of IFN- $\gamma$  treatment as compared to untreated. Pretreatment with NF- $\kappa$ B signalling pathway inhibitors, 200 nM of NF- $\kappa$ B Activation Inhibitor IV, 50  $\mu$ M SC-514 and 10  $\mu$ M Wedelolactone

with IFN- $\gamma$  decreased the APOA-I expression to 0.92-fold, 0.81-fold and 0.90-fold, respectively (Fig. 1). These results proved the direct link between IFN- $\gamma$  and NF- $\kappa$ B signalling pathway in mediating the APOA-I expression.



**Figure 1:** Effects of IFN- $\gamma$  and NF- $\kappa$ B signalling pathway inhibitors on APOA-I (A) mRNA expression and (B) protein expression in HepG2 cells.

Y-axis represents the normalised fold expression of APOA-I/APOA-I, whereas X-axis represents the different treatment conditions. Value above each bar signifies the fold value of APOA-I/APOA-I expression which was normalised to  $\beta$ -actin and then relative to untreated HepG2 cells (assigned as 1.00-fold). Error bars are expressed as standard deviation. Data represent the mean SD; n=3 (triplicate data) for all experiments. \* $p$  < 0.05 and \*\* $p$  < 0.01 represent the statistically significant alteration in expression.

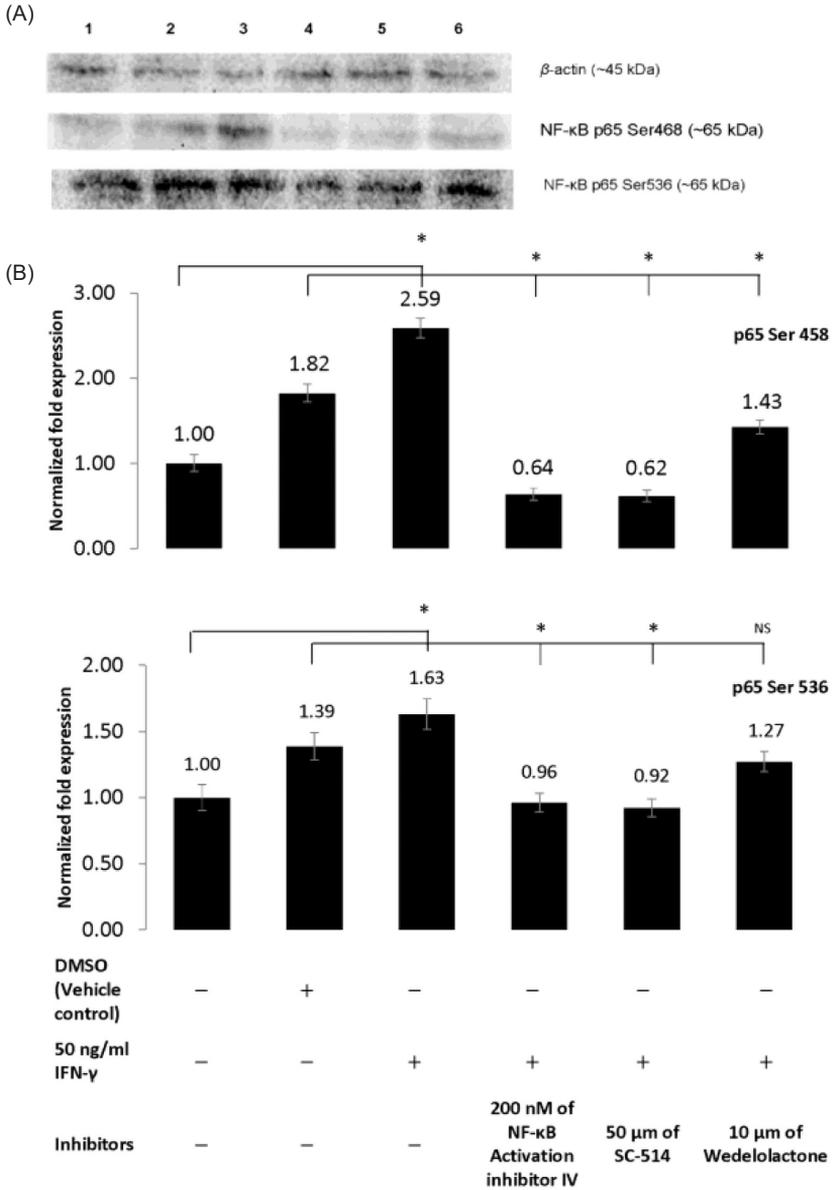
## **IFN- $\gamma$ Increased Phosphorylation of NF- $\kappa$ B p65 Protein Expression at Ser468 and Ser536 in HepG2 Cells**

The effects of NF- $\kappa$ B signalling pathway inhibitors and IFN- $\gamma$  on the expression of phosphorylated NF- $\kappa$ B p65 Ser468 and p65 Ser536 were examined. As shown in Fig. 2, treatment of HepG2 cells with IFN- $\gamma$  alone dramatically increased the expression of phosphorylated NF- $\kappa$ B p65 Ser468 subunit to 2.59-fold. On contrary, pretreatment of cells with NF- $\kappa$ B signalling pathway inhibitors significantly abrogated the effects of IFN- $\gamma$  on phosphorylated NF- $\kappa$ B p65 Ser468 subunit expression to 0.64-fold, 0.62-fold and 1.43-fold respectively, when compared to the vehicle control. Parallel to p65 Ser468 subunit, the expression of phosphorylated NF- $\kappa$ B p65 Ser536 subunit was also up-regulated following treatment 50 ng/mL of IFN- $\gamma$  to 1.63-fold. The expression of phosphorylated NF- $\kappa$ B p65 Ser536 subunit under treatment of signalling pathway inhibitors showed the similar expression pattern as p65 Ser468 subunit, in which pretreatment of cells with 200 nM of NF- $\kappa$ B Activation Inhibitor IV, 50  $\mu$ M of SC-514, and 10  $\mu$ M of Wedelolactone suppressed the p65 Ser536 expression to 0.96-fold, 0.92-fold and 1.27-fold respectively, as compared to IFN- $\gamma$  treated cells.

## **DISCUSSION**

The exact mechanism used by IFN- $\gamma$  to regulate the expression of APOA-I was unclear till now. Here, we show that IFN- $\gamma$  increased APOA-I expression and IFN- $\gamma$  was shown to activate NF- $\kappa$ B p65 subunit, while the inhibition of the NF- $\kappa$ B pathway abrogated the IFN- $\gamma$  upregulation of APOA-I. Collectively, the present results showed that IFN- $\gamma$  was capable of activating NF- $\kappa$ B signalling pathway by triggering the phosphorylation of p65 subunit. In general, IFN- $\gamma$  is known to be signalled through JAK-STAT pathway (Sizemore *et al.* 2004). In fact, IFN- $\gamma$  required IKK, a major activator NF- $\kappa$ B signaling pathway to induce expression of a major subset of IFN- $\gamma$ -stimulated chemokine gene. A study by Rani *et al.* (2009) showed that even though activation of certain subset of IFN- $\gamma$ -induced genes required components of normal NF- $\kappa$ B pathway, IFN- $\gamma$  did not significantly activate NF- $\kappa$ B in mouse embryo fibroblasts (MEFs).

Although JAK-STAT signalling pathway symbolises the most extensive-studied pathway for IFN- $\gamma$  signal transduction, there are evidences of alternative ancillary signalling pathways such that those involving MAPK or NF- $\kappa$ B which could be essential in mediating the response to IFN- $\gamma$  induction (Ramana *et al.* 2002; Jaramillo *et al.* 2003). According to a study conducted by Deb *et al.* (2001), IFN- $\gamma$  alone was able to induce the activation of NF- $\kappa$ B subunits, specifically the p50 and p65 subunits in a JAK-1-mediated, STAT-1-independent pathway. Besides, they also demonstrated that activation of this STAT1-independent pathway in HeLa S3 cell line required a dsRNA-activated protein kinase, known as protein kinase R (PKR). Importantly, PKR plays a crucial role in triggering the degradation



**Figure 2:** (A)  $\beta$ -actin and phosphorylated NF- $\kappa$ B p65 protein bands on blotted PVDF membrane under chemiluminescent detection and (B) graphical representation of phosphorylated NF- $\kappa$ B p65 Ser468 and Ser536 protein expression under different treatment conditions.

Y-axis represents the normalised fold phosphorylated protein expression of p65 Ser468 subunit while X-axis represents the different treatment conditions. Value above each bar indicates the fold value of phosphorylated subunit protein expression which was normalised to  $\beta$ -actin and relative to untreated HepG2 cells (assigned as 1.00-fold). \* $p < 0.05$  represents the statistically significant, while NS signifies non-significance alteration in expression.

of I $\kappa$ B protein specifically by activating IKK complex (Zamanian-Daryoush *et al.* 1999; Ishii *et al.* 2001; Yoshida *et al.* 2017). Thus, it is speculated that upon IFN- $\gamma$  stimulation, PKR activates IKK complex, which in turn phosphorylates I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$  and targets them for ubiquitin-proteasome-mediated degradation. This would free NF- $\kappa$ B subunits from sequestration which would then enter the nucleus, and trigger the activation of transcriptional activity of IFN-stimulated genes (ISG) with  $\kappa$ B response elements in their promoter sites (Stark *et al.* 1998; Shultz *et al.* 2007; Hayden & Ghosh 2014). All these studies support the present finding in this study. IFN- $\gamma$  by itself has been shown to activate IKK-dependent canonical NF- $\kappa$ B to regulate transcription process that is particularly involved in protecting the cells from cell death (Thapa *et al.* 2011). The activation of NF- $\kappa$ B by IFN- $\gamma$  was independent to the presence of STAT-1 and the main NF- $\kappa$ B heterodimer complex activated by IFN- $\gamma$  was comprised of both p65 and p50. In agreement with these observations, treatment of HepG2 cells with IFN- $\gamma$  in this study was shown to increase the phosphorylated NF- $\kappa$ B p65 Ser468 and Ser536 protein expression level significantly from the untreated sample and thus, confirming the roles of IFN- $\gamma$  in activating NF- $\kappa$ B signalling pathway.

In this present study, pretreatment with the NF- $\kappa$ B inhibitors not only decreased the phosphorylated NF- $\kappa$ B protein expression, but also suppressed the expression of APOA-I in HepG2 cells. This shows the link between NF- $\kappa$ B activation and APOA-I expression triggered by IFN- $\gamma$ . Since there is no direct NF- $\kappa$ B site present on the APOA-I promoter, it will be impossible for NF- $\kappa$ B to mediate the IFN- $\gamma$  responses on its own and intermediate signalling mediators are required. Past researches have elucidated that p65 was capable of inhibiting the expression and activation of Peroxisome Proliferator Activated Receptor Alpha (PPARA), (Delerive *et al.* 1999; Mogilenko *et al.* 2009; Lim *et al.* 2013, Zhang *et al.* 2014). Inhibition of PPARA in turn, could trigger a rise in APOA-I expression. In support of this hypothesis, Peters *et al.* (1997) demonstrated that liver APOA-I mRNA expression of *Ppara*-knockout mice was much higher than that in *Ppara*-wild type mice. Besides, L'opez-Vel'azquez *et al.* (2011) also reviewed that PPARA, could be a possible repressor of APOA-I, by acting through farnesoid X receptor (FXR). In other words, inhibition of *PPARA* would free APOA-I from suppression by IFN- $\gamma$  and thus, an increase in the APOA-I expression. This provides a possible explanation for the increment of APOA-I expression in IFN- $\gamma$  treated HepG2 cells. However, the role of PPARA in the regulation remains to be investigated.

## CONCLUSION

IFN- $\gamma$  increased APOA-I expression in HepG2 cells by activating NF- $\kappa$ B p65 phosphorylation at Ser468 and Ser536. Inhibition of these activation abrogated IFN- $\gamma$  induction of APOA-I expression.

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