Toll-like receptor 7 promotes the apoptosis of THP-1-derived macrophages through the CHOP-dependent pathway

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Abstract. Macrophage apoptosis is a prominent characteristic of advanced atherosclerotic plaques and leads to plaque destabilization. Certain studies have confirmed that influenza virus A (IVA) infection is related to acute myocardial infarction (AMI). However, it remains unknown as to whether this phenomenon is associated with Toll-like receptor (TLR)7, since single-stranded RNA (ssRNA) of IVA is a natural ligand of TLR7. Thus, in the present study, THP-1-derived macrophages were infected with IVA or treated with imiquimod (IMQ) in the presence or absence of pre-treatment with oxidized low-density lipoprotein (oxLDL). The macrophages were pre-treated with oxLDL (5 μg/ml) for 24 h to mimic high lipid conditions. Cell viability and apoptosis were detected by 3-(4,5-dimethylthiazol-2-y-1)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and flow cytometry, respectively. Our results revealed that TLR7 played an important role in macrophage apoptosis and cytokine secretion. Both IVA infection and IMQ treatment increased TLR7 expression, as well as the secretion of pro-inflammatory cytokines [interleukin (IL)-6, monocyte chemotactic protein (MCP)-1] and apoptosis. However, this increase in cytokine secretion occurred independently of cell apoptosis. oxLDL had potential synergistic pro-apoptotic effects combined with TLR7 activation. To determine whether endoplasmic reticulum (ER) stress plays a role in cell apoptosis, the mRNA and protein expression of known markers of ER stress [glucose-regulated protein (GRP)78 and C/EBP homologous protein (CHOP)] was detected by reverse transcription PCR (RT-PCR), quantitative reverse transcription PCR (qRT-PCR) and western blot analysis. Our results revealed that apoptosis aggravated ER stress, as shown by the overexpression of the pro-apoptotic sensor, CHOP. In conclusion, our study demonstrates the converging role of oxLDL pre-treatment, IVA infection and IMQ in ER stress-induced cell apoptosis.

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Introduction

Atherosclerosis (AS) is a chronic inflammatory disease and has been identified as the leading cause of mortality in the industrialized world. Cell apoptosis is a key event in the progression of advanced AS (1). Most importantly, the apoptosis of macrophage-derived foam cells, particularly in advanced lesions where the phagocytic clearance of apoptotic macrophages is defective, leads to the development of a necrotic core of atherosclerotic plaque, which may contribute to plaque instability and the majority of cardiovascular complications (2). A number of sero-epidemiological studies have demonstrated that human atherosclerotic diseases are related to previous exposure to pathogens, such as those of the human immunodeficiency virus (HIV) (3) and *Helicobacter pylori* (4). Influenza virus RNA (5) and antigen (6) have been found in human atherosclerotic plaques. A meta-analysis revealed that the influenza virus triggered acute myocardial infarction (AMI) and cardiovascular death (7), which was consistent with the results of our previous study (8); however, the mechanisms behind this remain elusive.

Influenza virus is a negative-stranded RNA virus, which is recognized by Toll-like receptor (TLR)7 and retinoic acid-inducible gene-I (RIG-I) (9,10). TLRs comprise an evolutionarily conserved receptor family that is capable of detecting and responding to microbial challenge (11). Single-stranded RNA (ssRNA) has been identified as a ligand for TLR7 (9). In fact, TLR7 has been verified to be expressed in macrophages (12). Imiquimod (IMQ) is a synthetic small nucleotide-like compound of imidazoquinoline, a well known TLR7 agonist. It was first identified as a compound that has antiviral activity and has been successfully used for the treatment of genital warts caused by human papilloma virus in clinical practice (13). In recent years, IMQ has been reported to exert direct pro-apoptotic effects against various tumor cell populations, such as oral squamous carcinoma cells (14) and basal cell carcinoma cells (15) by activating the caspase-dependent mitochondrial pathway.

The endoplasmic reticulum (ER) is a membranous organelle that plays a crucial role in cell homeostasis. Under certain conditions, such as those caused by chemical agents, adverse metabolic conditions induce protein misfolding and protein

assembly in the ER, leading to ER stress. Ample evidence implicates prolonged ER stress in the progression of a number of diseases, including AS (16). It has been considered that ER stress is linked to cellular processes with multiple risk factors in all stages of AS (17), and it is believed to play a critical role in cellular apoptosis (18). The majority of research on ER stress has focused on the unfolding protein response (UPR), a collection of intracellular signal transduction reactions designed to restore protein homeostasis. In UPR, ER-resident chaperones prevent ER stress and promote cell survival (19). Among these proteins, glucose-regulated protein (GRP)78, a member of the ER heat-shock protein (HSP)70 family that is the best characterized ER-resident chaperone, is indicative of UPR activation (20) and is used to determine whether the ER stress is activated (21). On the other hand, some mediators in ER stress have pro-apoptotic effects; the first of these is the transcriptional activation of the gene for C/EBP homologous protein (CHOP), which is ubiquitously expressed at very low levels, but robustly expressed by intense stimulation, ultimately leading to apoptosis.

In the present study, we investigated the association between influenza virus A (IVA) infection and macrophage viability, as well as the underlying mechanisms of cell apoptosis. Furthermore, we investigated the synergistic role of TLR7 activation with lipid loading in cell apoptosis. Our results revealed that TLR7 activation either by IVA infection or IMQ promoted apoptosis and increased cytokine secretion. In addition, it was found that ER stress plays an important role in IMQ-induced cell apoptosis. Pre-treatment oxidized low-density lipoprotein (oxLDL) had a synergistic pro-apoptotic effect. These data suggest that a high lipid not only plays a crucial role in foam cell formation, but also acts as a 'second hit' to promote cell apoptosis with other detrimental stimulations.

Materials and methods

Reagents. RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Gibco-BRL (Gaithersburg, MD, USA). Tunicamycin (TM), dexamethasone (DEX), 3-(4,5-dimethylthiazol-2-y-1)-2,5-diphenyl-2H-tetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA) and monoclonal antibody to β-actin were from Sigma-Aldrich (St. Louis, MO, USA). IMQ was obtained from InvivoGen (San Diego, CA, USA). Monoclonal antibodies to GRP78 and CHOP were from Cell Signaling Technology (Beverly, MA, USA). The horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse secondary antibodies were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). TRIzol reagent was from Invitrogen (Carlsbad, CA, USA). Interleukin (IL)-6 and monocyte chemotactic protein (MCP)-1 enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Minneapolis, MN, USA). Enhanced chemiluminescence (ECL) kits were from Thermo Scientific Pierce (Rockford, IL, USA). The reagent kits for cDNA synthesis and quantitative reverse transcription PCR (qRT-PCR) were from Bioneer Corp. (Daejeon, Korea); the reagents for reverse trancription PCR (RT-PCR) reagents were from Tiangen Biotech (Beijing, China). The Annexin V/PI apoptosis kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). oxLDL was obtained from Union Biological Chemistry (Beijing, China). The reagents for western blot analysis were from Beyotime Institute of Biotechnology (Nantong, China). The other reagents were of analytical grade and were obtained from commercial sources.

Cell culture and differentiation. The THP-1 monocyte cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, and were maintained at (3-5)x10^5 cells/ml at 37˚C in a humidified incubator containing 5% CO_2. The THP-1 cells were seeded at 5x10^5 cells/ml in tissue culture dishes, and differentiation was induced by 5 ng/ml PMA for 48 h with maintenance medium (2% FBS). Our observation that 5 ng/ml of PMA stimulated the THP-1 cells to differentiate into macrophages and did not induce undesirable gene upregulation was in accordance with the results presented in the sudy by Park *et al* (22). Subsequently, the THP-1-derived macrophages were pre-incubated with or without oxLDL (5 μg/ml) for 24 h prior to the addition of the TLR7 agonist, IMQ, for 24 h at 1 μg/ml or infected with IVA at the indicated concentrations in the presence or absence of 1 μg/ml dexamethasone (DEX) for 24 h. TM was used as a positive control (10 μg/ml) for 24 h. The IVA infection model was as follows: cells were prepared in dishes and inoculated with the indicated viral titers (1x10^3 TCID50/ml - 1x10^4 TCID50/ml) for 1 h. The inoculums were removed after 1 h of viral adsorption, followed by washing the monolayer 3 times with PBS. The monolayer cells were maintained in a virus isolation medium for the indicated periods of time and used for the subsequent experiments.

Virus preparation. The influenza A strain PR/8/34 (H1N1) was provided by the Department of Epidemiology of Harbin Medical University, Harbin, China. The virus was propagated in MDCK cells and stored in a -80˚C freezer, following the standard procedure. To evaluate the presence of the virus in the cell culture, we used RT-PCR and a hemagglutination assay as previously described by Mehrbod *et al* (23) and tested the median (50%) tissue culture infective dose (TCID50).

ELISA. The cells were plated in 48-well plates at 2x10^4 cells per well overnight and treated with IVA or IMQ as described above. The supernatant was harvested to detect the levels of IL-6 and MCP-1 by ELISA. All assays were performed according to the manufacturer's instructions. The absorbance of the reaction solution was measured at 450 nm using a microplate autoreader (LUmo microplate luminometer; PHOmo microplate reader; Autobio Diagnostics, Henan, China).

Isolation of cell proteins and western blot analysis. The cells were seeded in 6-well plates, and after being subjected to the different treatments, whole cell protein extracts were prepared using RIPA lysis buffer following the manufacturer's instructions. The lysates were sonicated for 20 sec and kept at 4˚C for 15 min. Following 10 min of centrifugation (12,000 x g, 4˚C), the supernatant was saved as a whole-cell lysate and measured using the Micro BCA™ protein assay reagent kit (Thermo Scientific Pierce). Loading buffer was added to adjust the lysate followed by boiling for 10 min. The samples were resolved on SDS-polyacrylamide gels and transferred onto PVDF

Table I. Primers used for RT-PCR and qRT-PCR.

Target mRNA	Forward primer (5'→3')	Reverse primer (5'→3')
M1	TTCTAACCGAGGTCGAAAC	AAGCGTCTACGCTGCAGTCC
GRP78 (RT-PCR)	GAACGTCTGATTGGCGATGC	TCTTTGGTTGCTTGGCGTTG
GRP78 (qRT-PCR)	CCCGAGAACACGGTCTTTGA	TTCAACCACCTTGAACGGCA
CHOP (RT-PCR)	CACCACTCTTGACCCTGCTT	CTTTCTCCTTCATGCGCTGC
CHOP (qRT-PCR)	GCTCAGGAGGAAGAGGAGGA	CTCCTTCATGCGCTGCTTTC
TLR7	ATTGCCCTCGTTGTTATA	TTCCTGGAGTTTGTTGAT
GAPDH	GACCTGACCTGCCGTCTA	AGGAGTGGGTGTCGCTGT
β-actin	CCCAGCACAATGAAGATCAAGATCAT	ATCTGCTGGAAGGTGGACAGCGA

M1, influenza matrix protein; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; TLR7, Toll-like receptor 7.

membranes. Following blocking with 5% non-fat dry milk for 1 h at room temperature and washing with Tris-buffered saline with 0.05% Tween-20 (TBST), the PVDF membranes were incubated overnight at 4˚C with primary antibody in TBST containing 5% non-fat dry milk. The HRP-conjugated secondary antibody (1:5,000 dilution) was subsequently incubated with the membranes for 1 h at room temperature and washed extensively 3 times, 8-10 min each time with TBST at room temperature. The blots were probed using the ECL western blot detection system and visualized using X-ray film.

RNA extraction and RT-PCR, and qRT-PCR. Total RNA was extracted using TRIzol reagent. Equal amounts of total RNA were reverse transcribed using a cDNA kit following the manufacturer's instructions. The primers used for PCR are listed in Table I. For RT-PCR, initial denaturation was performed at 95˚C for 3 min, followed by 40 cycles of denaturation at 94˚C for 20 sec, then annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec, and a final extension at 72˚C for 10 min. The PCR products were visualized after electrophoresis on a 2% agarose gel containing ethidium bromide. qPCR was performed using Bioneer Corporation SYBR-Green qRT-PCR Master Mix kits. The incubation conditions were as follows: 95˚C for 20 sec, followed by 40 cycles of 15 sec at 95˚C, annealing/extension for 45 sec, at 60˚C. The data were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized against GAPDH. For each sample, qRT-PCR was performed in triplicate for both the target genes and GAPDH control.

MTT assay. The THP-1 cells were seeded at $5x10^3$ cells per well in 96-well flat-bottomed plates and incubated in 2% FBS-supplemented 1640 medium and treated with 5 ng/ml PMA for 48 h. Following treatment, MTT solution was added to a final concentration of 0.5 mg/ml, and the cells were incubated in a CO_2 incubator at 37˚C for 4 h. After discarding the supernatant, the reduced MTT was solubilized in 150 μl per well of dimethylsulfoxide (DMSO), and the absorbance was measured at 490 nm using a PHOmo microplate reader. Cell viability was expressed as the percentage of the untreated controls.

Detection of apoptosis by flow cytometry. Annexin V-FITC/PI double-staining was used to quantify the number of apoptotic cells by flow cytometry. Following treatment as described above, the cells harvested and washed 3 times with PBS. The concentration was adjusted to $1x10^6$ cells/ml, and 100 μl of cell suspension were stained with 5 μl Annexin V and 5 μl propidium iodide (PI) for 15 min in the dark at room temperature; the cells were then re-suspended in 400 μl calcium-binding solution and $1x10^4$ cells per sample were analyzed immediately using a BD FACSCantoII flow cytometer (BD Biosciences, Oxford, UK) and relevant software (BD FACSDiva). The identification of the different cell population patterns was as follows: live cells ($FITC^-$ PI^-), early apoptotic cells ($FITC^+$ PI^-), late apoptotic cells ($FITC^+$ PI^+) and necrotic cells ($FITC^-$ PI^+).

Statistical analysis. All data are presented as the means ± SD. One-way ANOVA was applied in order to detect statistically significant differences. Statistically significant differences between the treatment groups were calculated using SPSS 17.0 software for Windows. Values of $P<0.05$ were considered to indicate statistically significant differences.

Results

IVA or IMQ increases TLR7 mRNA expression and induces IL-6 and MCP-1 secretion. The mRNA expression of the influenza matrix protein (M1) was detected after the cells were infected with growing concentrations of IVA for 24 h to prove that IVA can infect THP-1-derived macrophages in our cell model of IVA infection. M1 is a conservative protein among the influenza viruses. The results of RT-PCR revealed that IVA infected the THP-1-derived macrophages as M1 mRNA expression was observed in the IVA-infected groups and not in the untreated controls (Fig. 1A).

To confirm the effects of IVA or IMQ on TLR7 expression, we examined the mRNA expression of TLR7 in macrophages co-incubated with IVA or IMQ by RT-PCR. As shown in Fig. 1B, the mRNA expression of TLR7 was detected in all groups. In addition, both IVA infection ($1x10^3$ TCID50/ml) and IMQ treatment (1 μg/ml) for 24 h increased TLR7 mRNA expression. At the same time, the production of IL-6 and MCP-1 was determined by ELISA following treatment with IVA or IMQ. The expression of IL-6 and MCP-1 was significantly increased following treatment with IVA or IMQ (Fig. 1C and D). These findings indicate that TLR7 is actually activated during IVA infection or IMQ treatment.

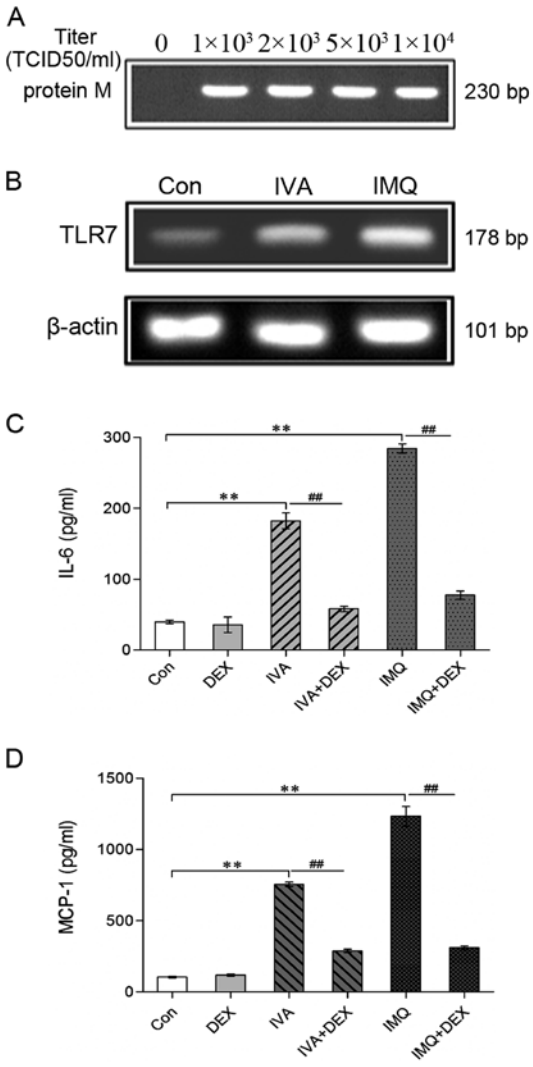

Figure 1. Influenza virus A (IVA) infection or imiquimod (IMQ) treatment activated Toll-like receptor (TLR)7 and induced pro-inflammatory cytokine secretion in THP-1-derived macrophages. (A) M1 mRNA was detected in the IVA-infected groups ($1x10^3$-$1x10^4$ TCID50/ml) and not observed in the uninfected group by RT-PCR. (B) TLR7 was increased by IVA infection ($1x10^3$ TCID50/ml for 24 h) or IMQ treatment (1 μg/ml for 24 h). (C and D) Interleukin (IL)-6 and monocyte chemotactic protein (MCP)-1 secretion was augmented by IVA infection ($1x10^3$ TCID50/ml for 24 h) or IMQ treatment (1 μg/ml for 24 h). Co-incubation of the cells with dexamethasone (DEX) (1 μg/ml for 24 h) markedly inhibited cytokine secretion. The results are the means ± SD of at least 3 independent experiments. **P<0.01, compared to control; $^{\#\#}$P<0.01, compared with DEX treatment.

Pro-apoptotic effect of IVA infection or IMQ treatment. The THP-1 macrophages were co-incubated with increasing IVA concentrations ($1x10^3$, $2x10^3$, $5x10^3$, $1x10^4$ TCID50/ml) as described above and collected at 8, 24 and 48 h post-inoculation (pi). The results revealed a pronounced decrease in cell viability in the infected groups, as determined by MTT assay (Fig. 2A). In early infection (8 h pi), cell viability fluctuated slightly, and the difference was not statistically significant (P>0.05). The decline in viability was more pronounced at 24 h pi. For further confirmation of this phenomenon, we detected the percentage of apoptotic cells ($FITC^+$ PI^- and $FITC^+$ PI^+) by flow cytometry at 24 h pi. Similar results were observed, except that the number of apoptotic cells following treatment with $1x10^4$ TCID50/ml IVA was significantly higher compared with the apoptosis observed at the other IVA concentrations (Fig. 2B), suggesting that flow cytometry was more sensitive in detecting apoptotic cells than MTT assay.

A diversity of concentrations of IMQ (0.1, 1, 10 μg/ml) incubated with the THP-1 macrophages for 24 h induced cell apoptosis in a dose-dependent manner (Fig. 2C and D). These results suggest that TLR7 activation is important in cell apoptosis, which represents a host defense mechanism to limit viral replication.

Cell apoptosis is independent of pro-inflammatory cytokine secretion. It has been proven that IMQ has no direct antiviral activity *in vitro* and is not virucidal; rather, the antiviral activity is indirect through cytokine induction and immune activation (24). To investigate whether pro-inflammatory cytokines play a role in cell apoptosis, we used DEX to decrease inflammation as described above. As shown in Fig. 1C and D, DEX inhibited pro-inflammatory cytokine secretion to a large extent, although apoptosis did not decline (Fig. 3). These data indicate that cell apoptosis is independent of pro-inflammatory cytokine secretion *in vitro*.

oxLDL plays a synergistic role in cell apoptosis with TLR7 activation. oxLDL is a potent atherogenic factor in the progression of AS. It has been proven that a high concentration of oxLDL (200 μg ApoB/ml) triggers a prolonged ER stress activation that switched towards apoptosis, as supported by the increased expression of the pro-apoptotic mediator, CHOP, and JNK (25). Our results, which suggest that oxLDL had contradictory pro-apoptotic effects that depended on the oxLDL concentrations, are consistent with those of Hundal *et al* (26). In our study, oxLDL at 5 μg/ml did not influence cell viability and foam cells formed from macrophages (data not shown). To mimic IVA infection in obese individuals who are in a sensitive pro-atherogenic situation, we pre-treated the THP-1 macrophages with oxLDL (5 μg/ml) prior to treatment with IVA or IMQ. As shown in Fig. 4, oxLDL pre-treatment increased cell apoptosis as compared with IVA or IMQ treatment alone. This phenomenon demonstrates that oxLDL has a potential pro-apoptotic effect and acts as a 'second hit' to promote cell apoptosis with other detrimental stimulations.

Upregulation of GRP78 and CHOP in IVA infection. Previous studies have produced controversial results on ER stress occurring in influenza infection (27-29). To investigate this, in this study, GRP78 and CHOP mRNA were detected by RT-PCR. As shown in Fig. 5A, the mRNA level of GRP78 and CHOP markedly increased. In agreement with this finding, the results produced by qRT-PCR (Fig. 5B) showed that the GRP78 levels markedly increased at first. It is interesting to note however that the GRP78 levels declined following treatment with $1x10^4$ TCID50/ml IVA. In contrast to GRP78, CHOP mRNA expression was induced in a dose-dependent manner and paralleled with the number of apoptotic cells (Fig. 2B). Collectively, these data verified that IVA infection induced ER stress and that CHOP played a role in IVA-induced apoptosis. Additionally, it is generally accepted that GRP78 expression suppresses cell apoptosis. In the present study, we found that GRP78 was inhibited when the cells were exposed to strong

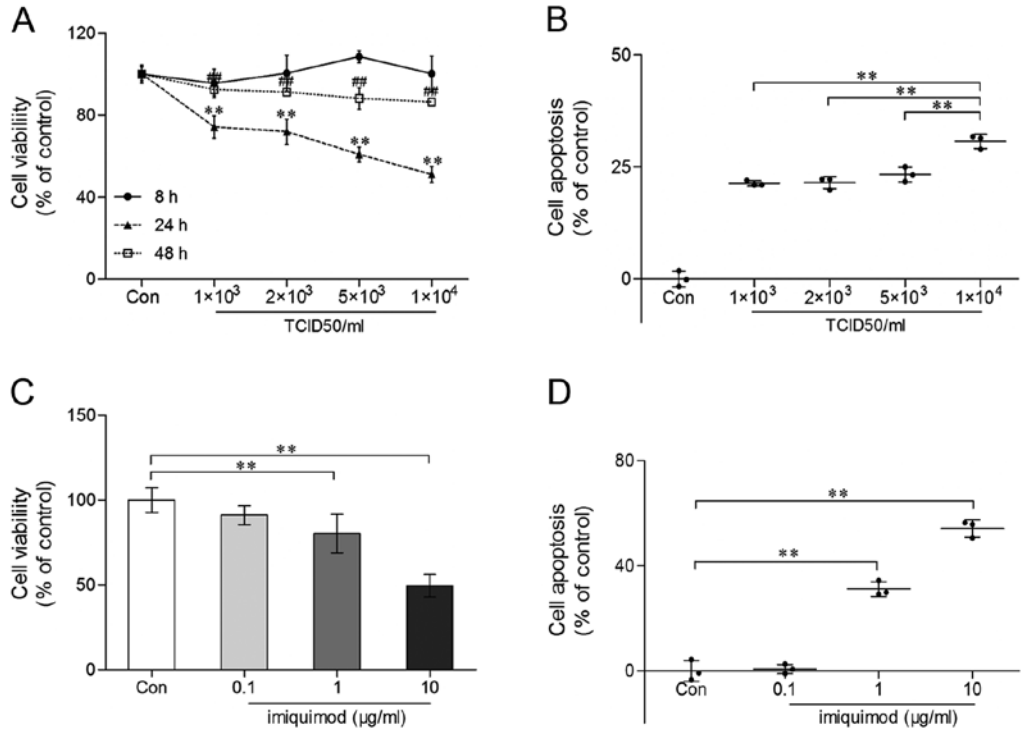

Figure 2. Effect of influenza virus A (IVA) infection or imiquimod (IMQ) treatment on cell apoptosis. Following absorption in the indicated IVA concentrations ($1x10^3$, $2x10^3$, $5x10^3$, and $1x10^4$ TCID50/ml) for 1 h, the cells were cultured in maintenance medium for an additional 24 h. (A) Cell viability was detected by MTT assay after the cells were infected with increasing IVA concentrations for 8, 24 and 48 h. (B) Flow cytometry confirmed the results of apoptosis when the macrophages were infected with the indicated concentrations of IVA for 24 h. (C and D) Cell viability or apoptosis were detected when the THP-1-derived macrophages were co-incubated with IMQ (0.1-10 μg/ml) for 24 h. Results were normalized to the control and expressed as the means ± SD; **P<0.01, $^{\#\#}$P<0.01. The representative results from 3 independent experiments are shown. **P<0.01, values are the means ± SD of 3 independent experiments.

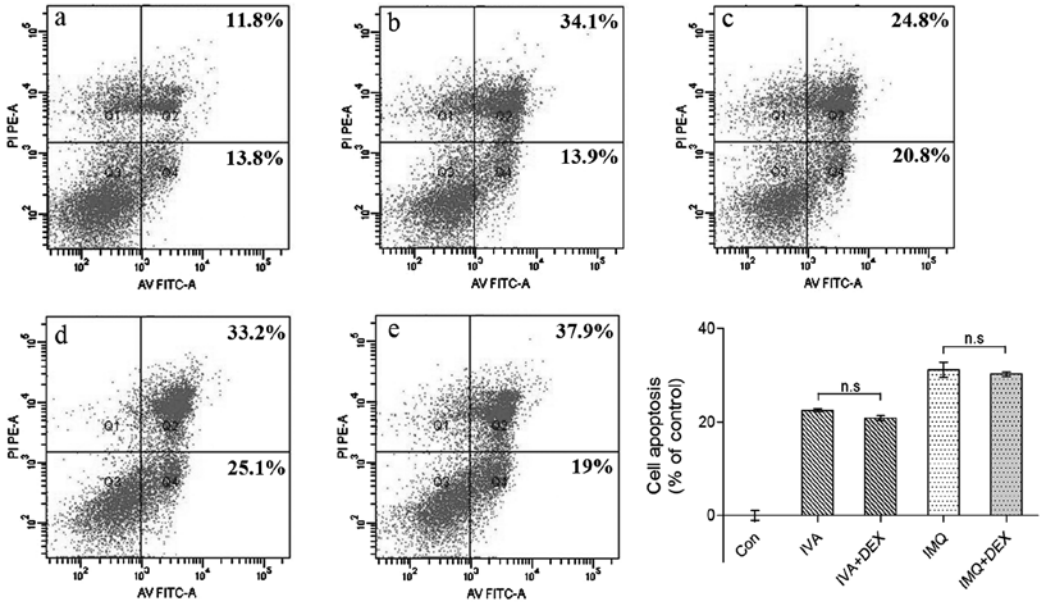

Figure 3. Dexamethasone (DEX) did not inhibit cell apoptosis. (A) Control, (B) influenza virus A (IVA) infection, (C) IVA combined with DEX, (D) imiquimod (IMQ), (E) IMQ combined with DEX. The IVA infection was at $1x10^3$ TCID50/ml for 24 h and IMQ treatment was at 1 μg/ml for 24 h. DEX was co-incubated with IVA or IMQ at 1 μg/ml for 24 h. n.s., no statistical difference. Values are the means ± SD of 3 independent experiments.

stimuli ($1x10^4$ TCID50/ml); this resulted in the ratio of CHOP/GRP78 becoming much higher. The same phenomenon was observed in the cells treated with TM, a strong inducer of ER stress. It faintly induced GRP78 (10-fold) expression, but significantly increased CHOP (>2,000-fold) mRNA expression (data not shown). This phenomenon indicated that CHOP/GRP78 was a more sensitive index in predicting cell apoptosis. We also observed an induction in GRP78 and CHOP protein

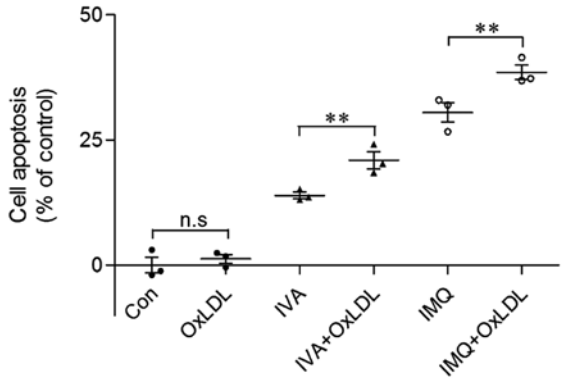

Figure 4. Effect of oxLDL pre-treatment on cell apoptosis. oxLDL (5 μg/ml) pre-treatment did not increase cell apoptosis under normal conditions, but increased cell apoptosis along with influenza virus A (IVA) infection or imiquimod (IMQ) treatment. The IVA infection was at $1x10^3$ TCID50/ml for 24 h and IMQ treatment was at 1 μg/ml for 24 h. **P<0.01, values are the means ± SD of 3 independent experiments. n.s., not significant.

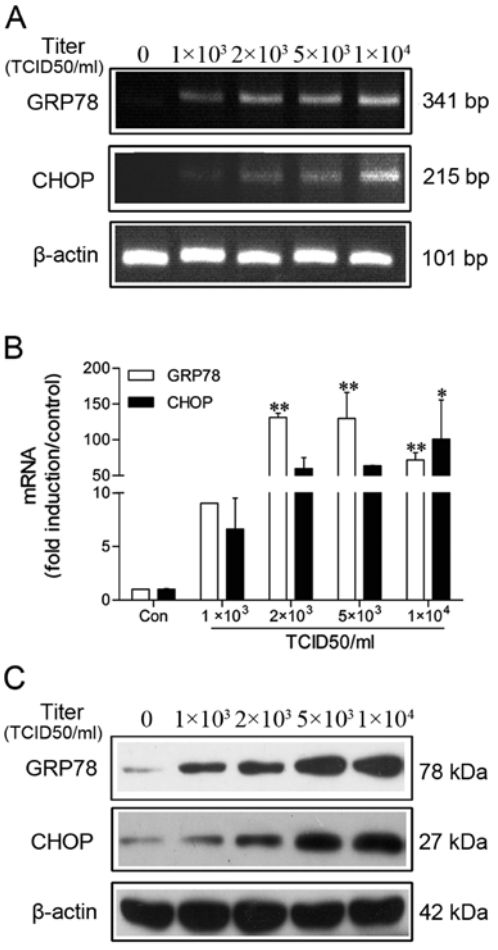

Figure 5. Expression levels of glucose-regulated protein (GRP)78 and C/EBP homologous protein (CHOP) on influenza virus A (IVA)-infected THP-1 macrophages. After bing absorbed in the indicated IVA concentrations ($1x10^3$, $2x10^3$, $5x10^3$, $1x10^4$ TCID50/ml) for 1 h, the cells were cultured in maintenance medium for an additional 24 h. The levels of GRP78 and CHOP mRNA were detected by (A) RT-PCR and quantified by (B) qRT-PCR. For RT-PCR, the housekeeping gene, β-actin, was used as a control. In qRT-PCR, mRNA expression was measured relative to the controls and calculated after adjusting to GAPDH using the $2^{-\Delta\Delta ct}$ method. (C) The protein expression of GRP78 and CHOP was analyzed by western blot analysis and normalized to β-actin. The reported values represent the means ± SD of triplicate experiments. The asterisks (*P<0.05; **P<0.01) indicate the values that are significantly different from the control.

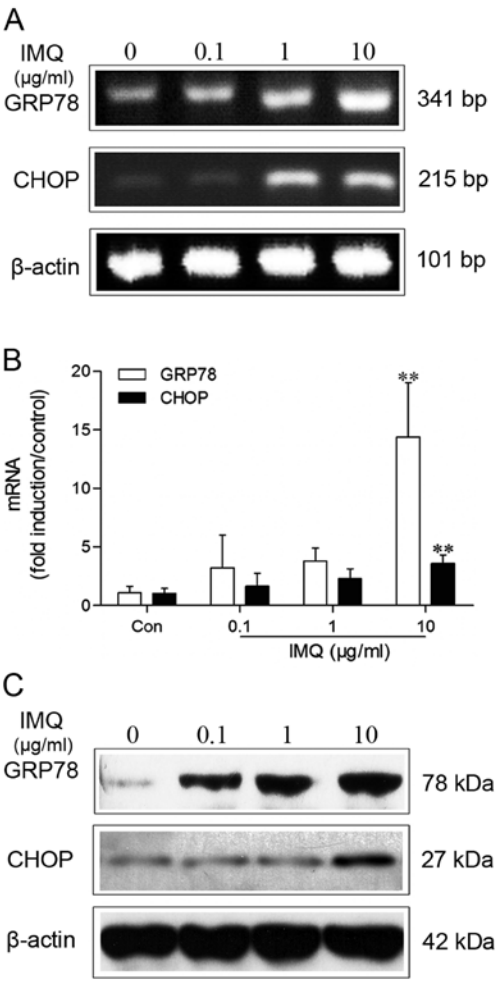

Figure 6. Endoplasmic reticulum (ER) stress played a pro-apoptotic role in imiquimod (IMQ)-induced THP-1 macrophage apoptosis. Growing concentrations of IMQ (0.1-10 μg/ml) were co-incubated with THP-1 macrophages. The levels of glucose-regulated protein (GRP)78 and C/EBP homologous protein (CHOP) mRNA were detected by (A) RT-PCR and quantified by (B) qRT-PCR. For RT-PCR, the housekeeping gene, β-actin, was used as a control. In qRT-PCR analysis, mRNA expression was measured relative to the controls and calculated after adjusting to GAPDH using the $2^{-\Delta\Delta ct}$ method. (C) The protein expression of GRP78 and CHOP was analyzed by western blot analysis and normalized to β-actin. The reported values represent the means ± SD of triplicate experiments. The asterisks (**P<0.01) indicate the values that are significantly different from the control.

levels in the IVA-infected cell lysates, thus confirming the qRT-PCR results (Fig. 5C).

ER stress plays a crucial role in the IMQ pro-apoptotic effects. Having established that the caspase-dependent mitochondrial pathway was activated in IMQ-induced cell apoptosis (30,31), we wished to determine whether ER stress also participates in IMQ-induced apoptosis. To examine this hypothesis, GRP78 and CHOP expression was detected by RT-PCR, qRT-PCR and western blot analysis. As shown in Fig. 6A and B, the levels of GRP78 and CHOP mRNA were increased following treatment with IMQ, particularly at a high concentration (10 μg/ml). The protein levels of GRP78 and CHOP were consistent with the mRNA levels (Fig. 6C). These results suggest that ER stress plays a pro-apoptotic role in IMQ treatment, particularly with a potent stimulation.

Discussion

AS and its complications are a major threat to human health, and there is a strong correlation between apoptotic macrophages and plaque rupture (32). In this study, we demonstrated that TLR7 activation aggravated ER stress and promoted apoptosis in THP-1-derived macrophages. Collectively, cell apoptosis was independent of pro-inflammatory cytokines. Pre-treatment with oxLDL at a dose of 5 μg/ml aggravated apoptosis along with TLR7 activation. The mechanism responsible for this phenomenon involved the increase in the epxression of the pro-apoptotic sensor, CHOP, in ER stress.

As already mentioned in the 'Introduction', certain pathogens, including IVA, correlate with the development of AS. In clinical practice, during influenza epidemics, there are many deaths and serious complications in vulnerable populations, particularly in cold wheather (33). The American Heart Association and American College of Cardiology recommend influenza immunization with inactivated vaccine as part of a comprehensive secondary prevention in individuals with coronary and other atherosclerotic vascular diseases (34). These findings led to the hypothesis that influenza infection may be a predisposing factor for cardiovascular events; however, the underlying mechanisms remain elusive. Van Lenten *et al* proved that the influenza infection contributed to high-density lipoprotein losing its anti-inflammatory properties and promoting macrophage traffic into the arteries of mice (35,36). On the other hand, our study demonstrated that IVA infection induced pro-inflammatory cytokine secretion and cell apoptosis. The greatest number of apoptotic cells was observed at 24 h pi; this result was in accordance with the results presented in the study by Mitchell *et al* (37). Therefore, the early treatment of IVA infection is an important measure to reduce serious cardiovascular incidents.

Several lines of evidence have suggested that IVA infection activates the distinct ER stress pathway in different cell types (27,28). Our data confirmed that IVA infection induced GRP78 and CHOP expression in a dose-dependent manner (Fig. 5). Furthermore, it accelerated macrophage apoptosis by increasing CHOP expression. However, the pro-inflammatory cytokines did not affect cell apoptosis *in vitro* (Fig. 3). IMQ had the same effect on macrophages (e.g., pro-apoptotic effects and pro-inflammatory cytokine secretion) as IVA. As expected, ER stress also played a role in IMQ-induced cell apoptosis in addition to the mitochondrial-dependent death pathway, particularly in potent stimulation (Fig. 6). It is vague whether IMQ induced cell apoptosis via the TLR7-dependent pathway (12,38). Our results showed that TLR7 activation promoted cell apoptosis; however, cannot reach a final conclusion regarding this matter as we did not inhibit TLR7 activation.

Generally speaking, oxLDL is a potent atherogenic factor in AS progression. Cells incubated with a lower cytotoxic concentration of oxLDL (5 μg/ml), followed by TLR7 activation, showed an increase in cell apoptosis which was more significant than that of the cells not treated with oxLDL (Fig. 4). Two reviews have suggested that obesity is a risk factor for influenza infection (39) and impaired immune function (40). Smith *et al* demonstrated that obesity increased the risk of death from influenza infection *in vivo* (41). As mentioned above, obesity is not only a cause of AS but also has pleiotropic effects on the progression of AS.

As mentioned above, TLR7 activation can promote cell apoptosis by CHOP expression, and oxLDL pre-treatment aggravates apoptosis. Therefore, the control of influenza infection and obesity is an important precaution for preventing AS. Our study is not without limitations. First, we did not demonstrate the concrete pathway of ER stress in IVA infection or IMQ treatment. Secondly, we did not knock down TLR7 to elucidate its effect. These, however, are our main aims for future studies.

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