Original Article

Design and construction of Beclin1-expressing plasmid as an autophagy inducing system: a novel strategy for enhancing the potency of DNA vaccines

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ABSTRACT

Introduction: Autophagy is a complicated process which is involved in many biological events such as antigen presentation by immune cells. Beclin1, as a key component of autophagic machinery, plays the main role in the induction and initiation of this process. In the present study, we hypothesized that overexpression of Beclin1 could be useful in autophagy induction as an immunostimulatory strategy for improving the efficiency of DNA vaccines. **Methods:** Beclin1 gene was cloned into pVITRO2 eukaryotic expression vector and was confirmed by agarose gel electrophoresis. Beclin1 expression was evaluated by Real-Time RT-PCR and Western blotting. The autophagy induction by pVITRO2 encoding Beclin1 in HEK293 cells was evaluated through detection of the expression levels of LC3II as a marker for autophagy induction by Western blotting. **Results:** Beclin1 expression by the constructed plasmid was detected in HEK293 cells. The pVITRO2 encoding Beclin1 showed autophagy enhancement as compared to the control condition. Our data showed significant increase in the expression of LC3II protein following Beclin1 transfection (P <0.05 or 0.01). **Conclusion:** Our results support a novel strategy for autophagy induction via up-regulation of Beclin1 expression. This could be useful for improvement of DNA vaccines through enhancing their antigen presentation.

KEYWORDS: Autophagy, Beclin1, DNA vaccine, Antigen presentation.

INTRODUCTION

The protein degradation process in eukaryotic cells mainly happen in two hydrolytic pathways, namely, proteasomal and lysosomal processes. Although, short-lived proteins degrade by proteasome machinery, the lysosomal machinery is associated with autophagy degradation of damaged cell organelles and long-lived proteins [1]. Initially, autophagy was introduced as an ancient response in unicellular eukaryotes and later its important role was discovered in the adjustment of cells to internal and external stresses. There are at least three types of autophagy: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. During CMA process, proteins are targeted via signal peptide and HSC70 for their translocation into the lysosome. Microautophagy involves in direct invagination of the cytoplasmic proteins by the lysosomal membrane. However, macroautophagy is frequently referred as autophagy and is considered the main pathway [2].

Autophagy is a continuous biological process that can enfold cytoplasmic material in a cup-shaped double membrane or multi-membrane-bound structure, known as autophagosome which will be subsequently recycled. Autophagosomes fuse then with lysosomes and late endosomes to form autolysosomes to degrade their contents [3]. The most common inducer of autophagy is nutrient starvation and activation of autophagy is a defense strategy by the host to maintain cell survival during the nutrient stress by recycling of amino acids and other intracellular nutrients [4]. Importantly, autophagy plays an essential role in the immunological control of bacterial, parasitic and viral infections. This process can deliver intracellular antigens for major histocompatibility complex class I and II (MHC-I and-II) presentations [5]. Furthermore, the autophagy process contributes to innate immune responses as the first line of defense by regulating pathogen detection and modifying the production of innate immune cytokines during the infections [6].

Beclin1, the mammalian orthologue of yeast autophagy-related gene 6 (Atg6), is a key factor for autophagy induction due to its essential role for autophagosome formation or autophagosome/endosome maturation [7]. Previous studies have indicated that over-expression of Beclin1 induces autophagy via marking membranes for phagophore formation [8]. Autophagy facilitates antigen presentation to CD4+ during

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immune responses in many viral infections, therefore, viruses have exploited multiple strategies to inhibit autophagy pathway. Interestingly, most of these viruses restrain autophagosome generation or maturation via binding to Beclin1 [9]. Limited immunogenicity of DNA vaccines is the main obstacle for DNA vaccines; however, several novel and promising approaches have been suggested to enhance the potency of such vaccines [10-13]. Based on the ability of autophagy in the augmentation of the immune responses, here we evaluated the induction of autophagy through constructing a plasmid expressing Beclin1 in an *in vitro* model. We envisage using this formula as an adjuvant in DNA vaccine strategy in our future studies. Our results provide evidence that the constructed Beclin1-expressing plasmid induces autophagy and may function as an immunostimulatory agent.

MATERIALS and METHODS

Plasmid construction and propagation

The Beclin1-encoding plasmid was generated by retrieving Beclin1 sequence from Genbank database (accession number NM_003766.3). To ensure efficient digestion, *BamH*I and *Sal*I restriction sites were inserted for sub-cloning. For translation efficiency in a eukaryotic expression system, the Kozak sequence (CCACCAUGG) was inserted and for purification of the protein, a polyhistidine-tag was added at the 5' end of the gene sequence. The gene sequences was completely synthesized and cloned into Puc19 vector by GenScript, USA, Inc. The synthetized Beclin1 gene was digested from Puc19 vector using suitable restriction enzymes and was sub-cloned into multiple cloning site 1 of the eukaryotic expression vector pVITRO2-neo-mcs (InvivoGen, France). *Escherichia coli* DH5-α (Pasteur Institute, Iran) was used for propagation and preparation of the naked pVITRO2 plasmid and plasmid-encoding Beclin1 gene. The plasmids were extracted by a QIAprep Spin Miniprep Kit according to the manufacturer's instructions (Qiagen, Germany) and were confirmed by agarose gel electrophoresis.

Cell line and transfection

HEK293 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/ml streptomycin and incubated at 37°C in 5% CO2. The pVITRO2 and pVITRO2-encoding Beclin1 plasmids were transfected in duplicate into HEK293 cells by Lipofectamine 3000 Transfection Reagent according to the manufacturer's instructions (Invitrogen, Germany) in a 6 well cell culture plate. Cells treated with rapamycin were used as positive control. Rapamycin activates autophagy pathway by inhibition of the mTOR which is the mammalian target of this reagent. The cells with or without plasmids treatment were harvested at 48 h after the transfection for experimental analyses.

Real-Time RT-PCR

Beclin1 expression level was detected by Real-Time RT-PCR. Briefly, Total RNA was extracted from cell lysate 48 h post-transfection using RNeasy plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA was synthesized using a cDNA Reverse Transcription kit (Bio-Rad, Canada). The expression levels of Beclin1 were determined by Real-Time RT-PCR on the Rotor-Gene 6000 (Corbett Life Science, Australia) and EvaGreen (Bio-Rad, Canada) using qPCR master mix kit (YTA, Iran). The reaction conditions were as follow: 95°C for 15 s, followed by 40 cycles 95°C for 20 s and 60°C for 40 s. The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer. Levels of genes expression were normalized to those of the housekeeping gene (GAPDH) using the $2^{-\Delta\Delta Ct}$ method and expressed in the graphs as ''relative expression''. The following primer pairs were used: Beclin1: 5'-GGAGAGACCCAGGAGGAAGAG-3' (F),
5'-GCCTCCCCAATCAGAGTGAAG-3' (R) and
GAPDH: 5'-CTCTGCTCCTCCTGTTCGAC-3' (F),
5'- TTAAAAGCAGCCCTGGTGAC-3' (R).

Flow cytometric analysis

HEK293 cells with or without plasmids treatments were harvested and washed. The cells were then resuspended to approximately 2.5×10^6 cells/ml in ice-cold PBS, 10% FCS and 1% sodium azide. The cells were then permeabilized with 0.1% Triton X-100 (Sigma, USA) and incubated for 15 min at 4ºC. The primary antibody against LC3II was added to the cells and incubated for at least 30 min at 4ºC in the dark. Subsequently, cells were washed and labelled with FITC-conjugated secondary antibody (2µg/ml; dilution 1:1000; Abcam, USA) for 20 min at 4ºC in the dark. . Flow cytometric analysis was performed by a Partec PAII flow cytometer equipped with a mercury arc lamp as the excitation light source after final washing with PBS. FlowJo® software (Tree Star, Inc., Ashland, OR, USA) was used to analyze the data.

SDS-PAGE and Western blotting

The expression of LC3II and Beclin1 proteins in HEK293 cells were evaluated by SDS-PAGE and Western blot analysis. The extracted total proteins were lysed in SDS-PAGE sample loading buffer, and the lysates were separated by SDS-PAGE and stained by Coomassie blue R250. The separated proteins were transferred onto nitrocellulose membrane (Amersham, UK) and hybridized with the monoclonal LC3II antibody and anti-6X His-tag antibody (Abcam, UK).

Detection was performed using an enhanced chemiluminescence (ECL), purchased from Amersham Company. The β-actin expression was used as an internal control.

Statistical analysis

Results are represented as mean ± SD. Comparisons between groups were conducted with Student's t test. Differences were considered significant when P value was less than 0.05.

RESULTS

The Beclin1 expression through constructed plasmid

Agarose gel electrophoresis analysis was used to confirm the constructed plasmid by double-digestion using *BamH*I and *Sal*I restriction enzymes (Fig.1).

Changes on the expression level of Beclin1 mRNA in transfected cells were confirmed by Real-Time RT-PCR as compared to non-transfected cells, used as a control. The GAPDH gene mRNA expression was not significantly different between the control and the transfected cells and served as optimal housekeeping gene. The results were evaluated by relative quantification and melting curve analyses. The transfection with 5 µg/µl of pVITRO2 encoding Beclin1 for 48 h resulted in more than threefold increase in the Beclin1 expression ($p < 0.05$; Fig. 2).

We also examined the expression of pVITRO2-Beclin1 plasmid in transfected HEK293 cells by Western blotting using anti-6X His-tag monoclonal Ab. We observed that Beclin1 protein was expressed effectively with clear bands as compared to the

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internal control (β-actin; $P < 0.05$). Lower expression of Beclin1 was detected in cells which were transfected with naked pVITRO2 (Fig. 3).

The induction of autophagy through Beclin1 expression

Autophagy induction in the transfected cells was determined through targeting the expression of LC3II which is situated in the autophagosome of cells experiencing autophagy, at protein level by Flow cytometry and Western blotting. The results indicated that transfection with pVITRO2 encoding Beclin1 for 48 h caused significant increase in LC3II protein level compared to the naked plasmid. Western blotting results also exhibited that LC3II protein could be clearly detected, compared to the naked plasmid ($P < 0.05$; Fig. 4).

Autophagy induction in transfected cells were also detected by immunofluorescent labeling of LC3II. The fluorescence signals of the treated cells were compared to un-transfected control cells as well as the rapamycin-treated cells. As shown in Fig. 5, LC3II levels in cell samples undergoing autophagy treatments are up-regulated.

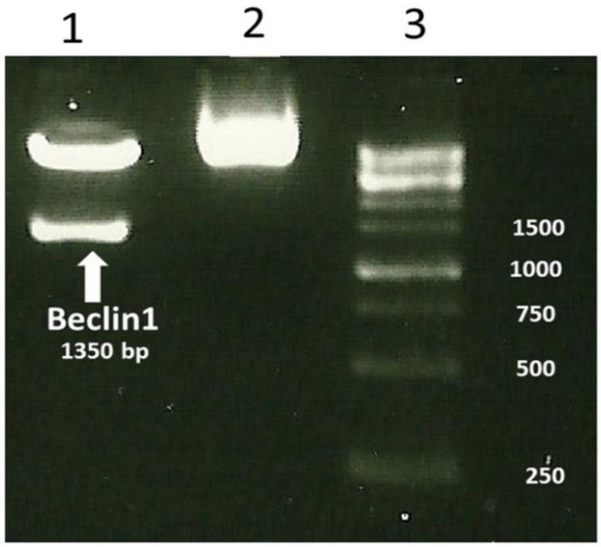

Fig 1. Agarose gel electrophoresis analysis of the constructed plasmid. The plasmid preparation encoding Beclin1 was digested by *BamH*I and *Sal*I restriction enzymes and was analyzed on 1.5% agarose gel by electrophoresis at 40 V for 60 min. (1: double-digested pVITRO-Beclin1, 2: undigested pVITRO-Beclin1, 3: Ladder)

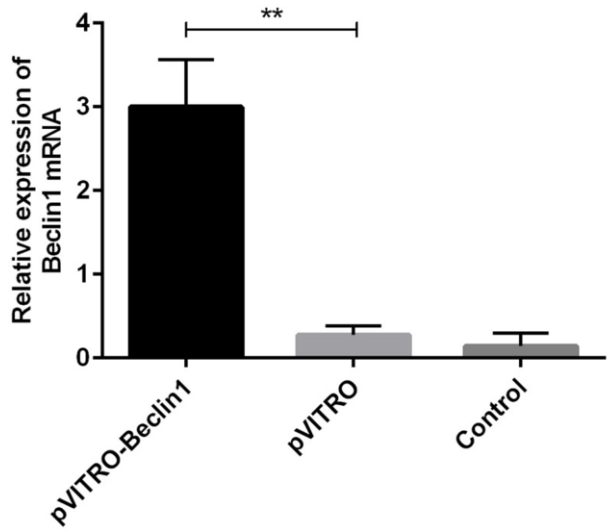

Fig 2. The expression level of Beclin1 mRNA in HEK293 through constructed plasmid. Relative expression of Beclin1 was evaluated at 48 h after the transfection, using specific primers targeting Beclin1 gene, and was normalized to the housekeeping gene (GAPDH). The results are averages of three independent experiments ($P < 0.05$).

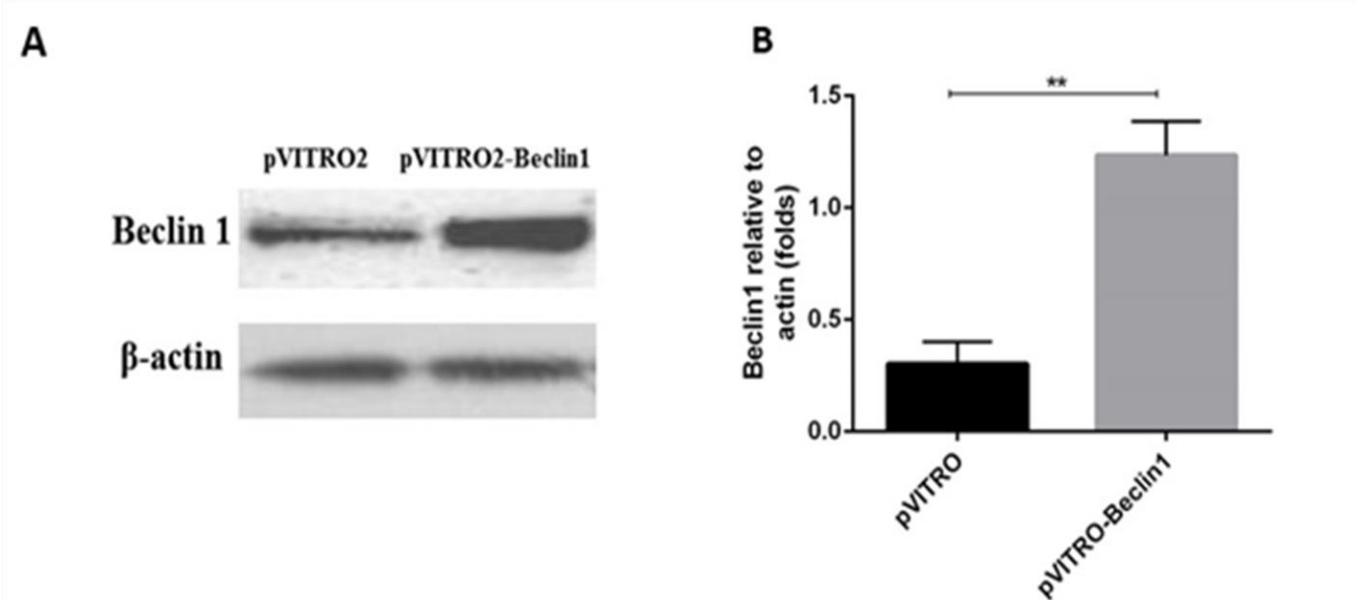

Fig 3. The expression level of Beclin1 protein in HEK293 cells through constructed plasmid. A) Western blot analysis of Beclin1 expression using anti-6X His tag antibody (Abcam, UK) in cells transfected with plasmid encoding Beclin1. B) Relative expression of Beclin1. The results are averages of three independent experiments ($P < 0.05$).

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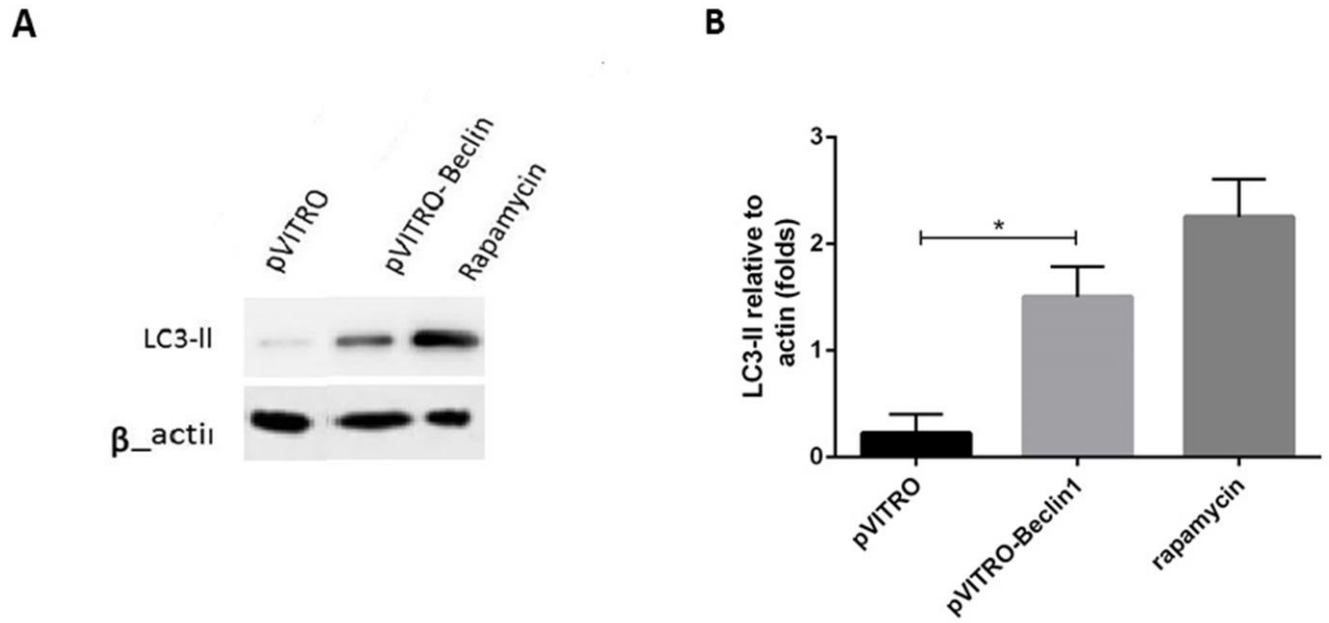

Fig 4. The expression levels of LC3II protein in HEK293 cells through up-regulation of Beclin1. A) Western blot analysis of LC3II expression using anti-LC3-II antibody (Abcam, UK) in cells transfected with plasmid encoding Beclin1. B) Relative expression of LC3II. The results are averages of three independent experiments ($P < 0.05$).

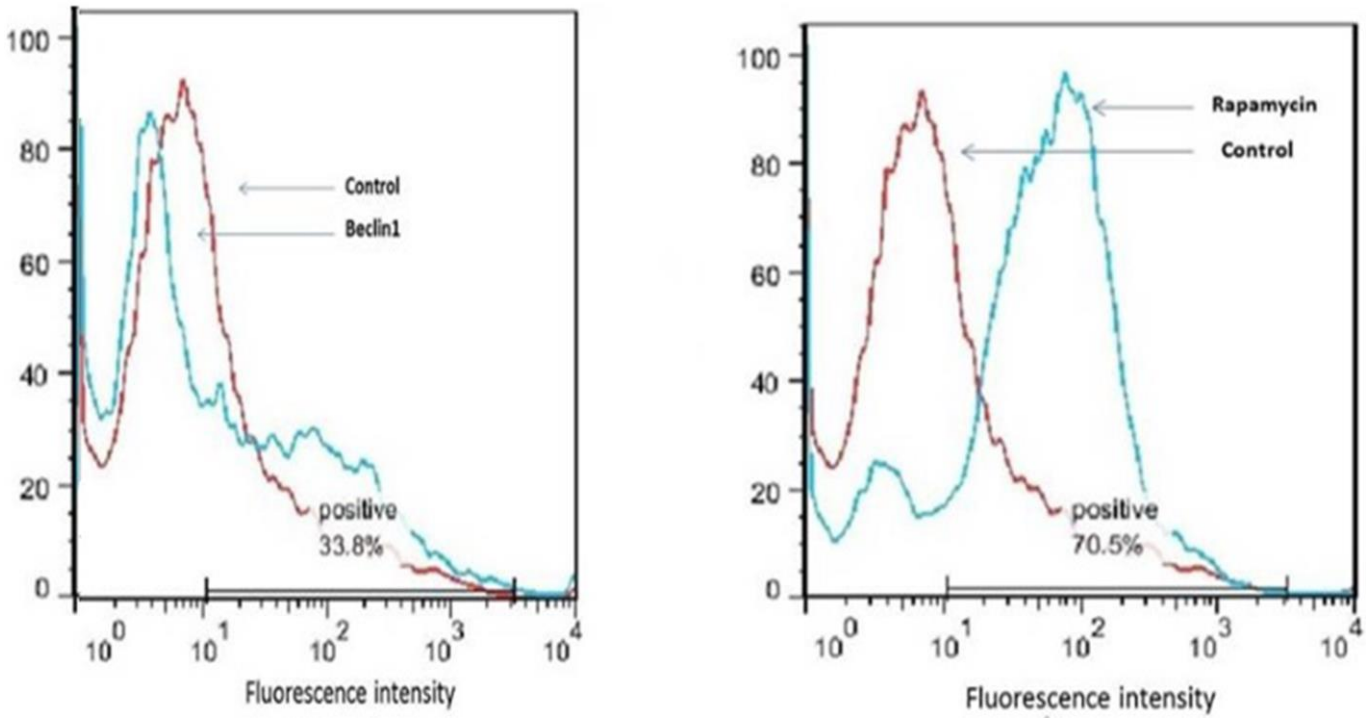

Fig 5. The expression levels of LC3II in HEK293 cells through up-regulation of Beclin1. Histogram plot presentation of FITC intensity vs. cell count. Flow cytometric analysis of HEK293 cells in MEM (control), rapamycin-treated and pVITRO2-Beclin1 transfected cells for 5 h. These data show that the LC3II signals from HEK293 cells at 48 h post-transfection compared to resting control levels. Transfected cells exhibited a detectable LC3II level compared to the controls.

DISCUSSION

Autophagy is a highly conserved catabolic pathway by which the cells deliver their intracellular constituents to lysosomes for degradation. The crucial role of Beclin1 in activation of autophagy has been determined and is deemed necessary for this biological process [14, 15]. Here, we further studied the overexpression of Beclin1, leading to induction of autophagy in HEK293 cells. Considering that Beclin1 can be an inducer of autophagy led us to hypothesize whether it may represent a novel strategy for vaccine improvement. Activation of autophagy by different methods is related to augmentation in

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antigen presentation *in vitro* and increased levels of humoral and cell mediated immune responses in mice models [16]. Previous works have determined that Beclin1 is important in autophagy induction following viral infections. Beclin1 is an essential autophagy protein in class III phosphatidylinositol 3-OH kinase (PI(3)K) due to its major role in autophagic vesicle formation and phagosome-lysosome fusion [17]. It is a vital component for the initiation of the autophagy pathway, and the cells lacking Beclin1 expression show reduction in autophagy and increased sensitivity to viral infections [9]. Intracellular pathogens such as viruses and bacteria can be removed by autophagy when they get trapped within an autophagosome, followed by lysosomal destruction [18].

In the present study, to understand the ability of Beclin1 to induce autophagy, we investigated whether Beclin1 transfection in HEK293, could increase LC3II levels (i.e. the marker for autophagy induction), in cultured cells. Our obtained results were in line with previous reports which had indicated the overexpression of Beclin1 gene enhances starvation-induced autophagy [19, 20]. However, further researches are required to determine whether pVITRO2 encoding Beclin1 is effective in animal models. The induction of autophagy by overexpression of Beclin1 in HEK293 cells was consistent with previous studies which had observed Hepatitis C Virus (HCV) up-regulated Beclin1 for stimulation of autophagy. Over-activation of autophagy in HCV infection has been determined by upregulating Beclin1 and mTOR signaling pathway activation [21]. Previous studies have also demonstrated that autophagy has a vital role in delivery of intracellular antigens for MHC-II presentation. Furthermore, several current studies have implicated the role of autophagic pathway in processing and presentation of extracellular antigens for both MHC-I and II presentations [22-25]. Based on the implication between autophagy and the immune response, we designed a construct which included Beclin1 expression to stimulate autophagy. In order to facilitate antigen-presentation through autophagy pathway, we hypothesize utilization of an autophagy-inducing plasmid into a DNA vaccine can probably increase the efficacy of this vaccine which may lead to better induction of antigen-specific CD8+ and CD4+ T lymphocytes.

Using a murine model, Xue and colleagues have demonstrated that an autophagosome-based hepatitis B virus (HBV) vaccine can be used as a therapeutic vaccine and they have found an enhancement of the antiviral functions of multi-specific anti-HBV T-cell responses, significant reduction in HBV replication and the expression of HBcAg in the liver [26]. In other studies, for evaluation of autophagy induction as an immunostimulatory model in the development of an efficient candidate DNA vaccine against *Mycobacterium tuberculosis* (MTB), co-administration of a plasmid containing MTB antigen 85B with a plasmid encoding the kinase defective mammalian target of rapamycin (mTOR-KD) have been performed in mice. They have found elevated antibody production, higher level of IFN-γ secretion, and augmented Ag85B-specific cytotoxic T-lymphocyte response in the murine splenocytes [27]. In agreement with the results of the present study, it has been previously shown that Beclin1 activates autophagy in human MCF7 breast carcinoma cells by gene-transfer techniques. Decreased endogenous Beclin1 protein expression has been detected in human breast epithelial carcinoma cell lines and increased induction of autophagy can lead to the treatment of breast cancer [28]. To further support our hypothesis, Hu and co-workers have shown using a murine model that incorporating LC3 gene in an autophagy inducing vaccine causes efficient and protective immunity against MTB where enhanced IgG2a in serum, IFN-γ and IL-2 produced by splenocyte have been detected in the immunized mice [29].

Taken together, although we are aware of the evidences, indicating a vital role for Beclin1 in autophagy induction, the value of the current study weighs on the construction of a plasmid encoding Beclin1 as a novel strategy for vaccine development. More importantly, our data indicated the overexpression of Beclin1 results in enhancement of LC3II (marker for induction of autophagy) rather than its control. There are still many questions remained to be answered regarding the consequences of autophagy induction in the body. More studies including animal model experiments are needed to further prove our hypothesis and to determine the interaction between autophagy and the immune responses.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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