Book Chapter

Overexpression of Bcl2 and Bcl2L1 Can Suppress Betanodavirus-Induced Type III Cell Death and Autophagy Induction in GF-1 Cells

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Abstract

Betanodavirus infection induces viral nervous necrosis (VNN) in fish. However, the role of cell death and autophagy in the pathogenesis of VNN remains unknown. This study aimed to investigate the effect of red-spotted grouper nervous necrosis virus (RGNNV) infection on Bcl2 downregulation and overexpression on asymmetric interaction between cell death and autophagy. The mRFP-LC3 reporter system was used to identify autophagosome formation in GF-1 (Grouper fin-1) fish cells. We found that the RGNNV can strongly induce autophagosome formation 36 hours post-infection (hpi) after autophagy inhibitor 3-MA had downregulated anti-apoptotic genes such as Bcl2 and Bcl2L1 (Bcl-xL). We proposed that overexpression of Bcl2 and Bcl2L1 can modulate both cell death and autophagy. Then, we found that it can also reduce either

type III cell death or autophagy, which mildly correlated to reduced viral replication. Our data suggest that RGNNV-induced Bcl2 downregulation correlates with the asymmetrical interaction between cell death induction and the autophagy process, which resembles viral replication.

Keywords

Betanodavirus; Bcl2; Autophagy; Type III Cell Death; Viral Gene

1. Introduction

Betanodaviruses are the causative agents of viral nervous necrosis (VNN), an infectious neuropathological condition characterized by necrosis of the brain and retina in fish [1]. RGNNV infection causes massive mortality in larval and juvenile populations of several marine teleost species [2,3]. The infection correlates with the modulation of innate or acquired immunity [4,5], but very little is known about the molecular mechanisms underlying the pathogenesis of VNN.

The betanodavirus genome also includes two single-strand RNA molecules with positive polarity: RNA1 (3.1 kb) and RNA2 (1.4 kb), which lacks a 3' poly (A) extension [4]. RNA1 encodes a 110 kDa protein acting on an RNA-dependent RNA polymerase (protein A) for replication of the viral genome. RNA2 encodes a 42 kDa capsid protein [4,6] that can trigger post-apoptotic necrotic cell death through a cytochrome c release-mediated pathway in GF-1 cells [7]. In the middle–late replication stage, betanodaviruses were also shown to synthesize a sub-genomic RNA3 from the 3’ terminus of RNA1 during genome replication, which encodes two non-structural proteins, B1 (absent in some strains) [8] and B2 (as a death gene) [1,9].

Recently, cell death has acquired multiple meanings. Apoptosis, type I cell death, has taken center stage as the principal mechanism of programmed cell death in mammalian tissues. Autophagy is belonging to type II cell death, which based on existing the massive accumulation of autophagic vacuoles in the

cytoplasm of effective cells. The autophagic process is regulated by a variety of extracellular and intracellular stresses, including pathogenic infection, nutrient deprivation, therapeutic treatment, and damaged organelles, hormonal, aggregated and misfolded proteins. Necroptosis is so-called as a programmed type III cell death, manifests with a distinctive morphology different from type I and type II [10-11].

A number of studies have investigated the molecular mechanisms by which the red-spotted grouper nervous necrosis virus (RGNNV) induces apoptosis or necrosis in host cells [12-13]. RGNNV infection induces cell death that correlates with the loss of mitochondrial membrane potential (MMP) and the release of cytochrome *c* at the mid-replication stage. This is regulated by the zebrafish anti-apoptotic protein Bcl-2 [13], a member of the BCL-2 family [14], and blocked by bongkrekic acid, an inhibitor of the mitochondrial permeability transition pore [12,14]. Furthermore, betanodavirus-induced mitochondria-mediated cell death can be blocked by the protein synthesis inhibitor cycloheximide [14], indicating that newly synthesized proteins from the viral genome are necessary for cell necrosis. Recent studies of the viral genome revealed the role of two viral death factors: protein α, which can induce the caspase-3- and caspase-9-dependent signaling pathway [15]; and protein B2, a non-structural protein that can induce BAX (BCL-2 associated X, apoptosis regulator)-mediated cell death [9] and cause ATP-depletion-mediated necrosis [16], which is dependent on the expression of the zebrafish anti-apoptotic gene *Bcl2L1* (BCL-2 like 1) [9,14]. A recent study showed that B2 can induce DNM1L/Drp1 (dynamin 1 like)-mediated mitochondrial fragmentation [17]. However, whether betanodaviruses induce necrosis or necroptosis is little known.

Autophagy flow is a common universal cell defense process for against intracellular pathogens that those are fused to lysosomes for degradation [18]. Many cases, autophagy play a role on the activation of antiviral innate immunity [19-20], which process can connect to the adaptive immune response by passing virus-derived peptides to T lymphocytes via major histocompatibility complex molecules [21-23]. Recently, special issues such as

autophagy process are correlated to play anti-viral and proviral roles in the virus life cycle, which caused the pathogenesis of many different virus families are well known [20]. Interestingly, a process termed xenophagy, play an anti-viral roles, by using autophagy proteins to targeting viral components or virions for lysosomal degradation. On the other hand, some DNA or RNA viruses appear to use components of the autophagic signals to foster their own intracellular growth or by non-lytic cellular egress to outside for second rounding infection. We based on the details of the role(s) of autophagy in viral pathogenesis become clearer, which new anti-viral therapies could be developed to inhibit or suppress the destructive aspects of autophagy during at different viral replication cycle [20].

The mechanistic target of rapamycin (MTOR) is a protein kinase that regulates autophagy by stimulating protein synthesis and inhibiting induction of autophagy [24].

The class III phosphatidylinositol 3-kinase (PtdIns3K) plays an important role in many biological processes, including the activation of MTOR. The chemical 3-methyladenine (3-MA) has been reported to inhibit autophagy by blocking autophagosome formation via the inhibition of class III Ptdlns3K [25]. However, 3-MA plays a dual role in autophagy. Prolonged treatment with 3-MA promotes autophagy under nutrient-rich conditions, whereas 3-MA inhibits starvation-induced autophagy [25]. Recently, regarding the molecular pathogenesis of RGNNV infection, the symmetrical or asymmetrical interaction between cell death induction and autophagy during viral replication has become a topic of interest.

Numerous viruses have evolved molecular mechanisms to allow them to escape or inhibit autophagy, thereby increasing their infectivity [20], but little has been discovered about how an RNNV virus induces autophagy. In the present study, we investigated the role of Bcl2 family crosstalk between cell death and autophagy. Our findings in the molecular pathogenesis of RGNNV infection may help to identify potential targets that could be used for the prevention or treatment of RNA viruses.

2. Materials and Methods

2.1 Cell and Virus Lines

Grouper fin-1 (GF-1), cells derived from the fin tissue of a grouper (*Epinephelus coioides*), were grown at 28 °C in Leibovitz's L-15 medium (ThermoFisher Scientic Inc., 11415-114) that included with 25 μg/mL gentamycin and 5% fetal bovine serum. **The** RGNNV TN1 strain was collected from the naturally infected red grouper larvae, in 2002 from the Tainan Prefecture in Taiwan that the strain was used to infect GF-1 cells. The virus was prepared and purified based on described by Mori et al. [26] and continued to store at –80 °C until use. The viral titer was counted by using the $TCID_{50}$ (50% Tissue culture Infective Dose) assay, according to reported by Dobos et al. [27].

2.2 Selection of mRFP-LC3-Producing GF-1 Cells

Vector-producing (pmRFP-C1 as a negative control) (Addgene, 21075; deposited by Tamotsu Yoshimori Lab) and pmRFP-LC3-producing cells were obtained by transfection and selection with G418 (800 μg/mL; Invivogen, ant-gn-1). Transcription of the inserted sequences in these vectors was driven by the immediate-early promoter of human cytomegalovirus. The selection time varied from 2 to 2.5 months according to cell properties [14].

2.3 Autophagosome Formation Assay

The mRFP and mRFP-LC3-producing cells (10^5 cells/mL) were cultured to monolayer confluence in 60 mm diameter Petri dishes for 20 h, rinsed twice with PBS (pH 7.4; Gibco, 10010023), and serum-starved for 48 h as a positive control. Cells were then pre-treated with autophagy inhibitor (2 mM 3-MA; Acros, 379791000) for 2 h, and infected with RGNNV (multiplicity of infection (MOI) = 1) for 0, 24, 36 and 48 h at 28 °C. At the end of each incubation time, the slides were observed under fluorescence microscopy (Olympus IX70). The red fluorescent images were captured at 300× to document formation of RFP-LC3 puncta or dots indicative of

autophagosomes or autolysosomes. The formation of RFP-LC3 puncta or dots was counted up to 10 as a positive cell. The number of mRFP dots was determined as previously described [28]. Fluorescent puncta were counted manually in at least 3 independent experiments, and at least 200 cells were examined in each experiment.

2.4 Western Blot Analysis

GF-1 cells were cultured in 60 mm Petri dishes (10^5/mL) for 20 h to monolayer confluence, rinsed twice with PBS, treated with 3-methyladenine (3-MA, 2 mM) for 2 h, and then infected with RGNNV (MOI = 1) for 0, 24, 36 or 48 h at 28 °C. In other experiments, Flag, Flag-Bcl2, and Flag-Bcl2L1-producing GF-1 cells were need to culture in 60 mm Petri dishes (10^5/mL) and continuing to culture for 20 h. At the end of time cells was rinsed twice with PBS, and infected with RGNNV (MOI = 1), then at different time points cultured such as 0, 24, 36 or 48 h under at 28 °C. At the end of each incubation period, the medium was aspirated, and washed the cells with PBS. Then, cells were lysed in 0.3 mL of lysis buffer, which included 10 mM Tris, pH 7.3, 20% glycerol, 10 mM SDS, 2% ß-mercaptoethanol, in pH 6.8. Cells lysates were separated by SDS-PAGE and furthermore analysis [29] and the proteins were transferred to nitrocellulose. Blots were incubated with polyclonal antibodies to protein α, protein B2, mouse BCL2 (Cell Signaling, 15071), BCL2L1 (Cell Signaling, 2764), LC3 (GeneTex, GTX127375), and ACTB/ß-actin, followed by peroxidase-labeled goat anti-rabbit conjugate (1:7500) (Calbiochem, MAB1501). Binding was detected by chemiluminescence, and the signals were recorded on Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) [30]. Protein expression was quantified using the Personal Densitometer (Molecular Dynamics).

2.5 ANXA5/Annexin-A5–FLUOS-Propidium Iodide (PI) Double Staining

To identify apoptotic cells, phosphatidylserine externalization on the outer layer of the apoptotic cell membranes was analyzed

using ANXA5-fluorescein (Invitrogen, V13242), and necrotic cells were identified by PI staining [9]. GF-1 cells (10^5/mL) were cultured to monolayer confluence in 60-mm diameter Petri dishes for 20 h, rinsed twice with PBS, and treated with the autophagy inhibitor 3-MA (2 mM) for 2 h. Cells were then infected with RGNNV (MOI = 1) for 0, 24, 36, and 48 h. In other experiments, Flag-, Flag-Bcl2, and Flag-Bcl2L1-producing GF-1 cells (10^5/mL) were cultured to monolayer confluence in 60 mm diameter Petri dishes for 20 h, rinsed twice with PBS, and then infected with RGNNV (MOI = 1) for 48 h. At each time point, the cells were harvested, washed with PBS, incubated 10–15 min with 100 µL of a HEPES-based ANAX5–fluorescein solution (Roche, 11858777001) and 100 µL of a staining solution (PI in HEPES buffer; Merck, H3375), and then evaluated by fluorescence microscopy (excitation: 488 nm; emission: 515 nm long-pass filter) [17].

2.6 Regulation of Autophagy by Overexpression of Bcl2 and Bcl2L1 in GF-1 Cells

Flag-Bcl2L1 and Flag-Bcl2-producing cells were obtained by transfection of GF-1 cells were transfected with pFlag (p3XFLAG-Myc-CMV), pFlag-Bcl2, and pFlag-Bcl2L1, respectively as previously described [31] by using Lipofectamine-Plus (Invitrogen, 11668500), according to the manufacturer's instructions. Positive clones were selected with G418 (800 mg/mL). Transcription of the *Bcl2 and Bcl2L1* genes in these vectors is driven by the immediate-early promoter of human cytomegalovirus. The selection time was about 2.5–3 months from a single colony, depending on cell properties [32].

2.7 Statistical Analysis

The fraction of cells positive for ANXA5 and PI in each sample was determined from counts of 200 cells. Results are expressed as means ± SEMs. Data were analyzed using the paired or unpaired Student's *t*-test or ANOVA with multiple comparisons, as appropriate. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Betanodavirus Induces Autophagy Process In GF-1 Cells

The mRFP-LC3 reporter system was used to identify autophagosome formation in GF-1 fish cells and cells with serum starvation were used as a positive control. Autophagosome formation were found at 36 h in serum starving cells (Figure 1A) and RGNNV-infected cells (Figure 1A). In RGNNV-infected cells, autophagosome formation was significantly increased in a time-dependent manner, with a ~20% increase at 24 h, ~40% increase at 36 h, and ~60% increase at 48 h (Figure. 1B) compared to control cells.

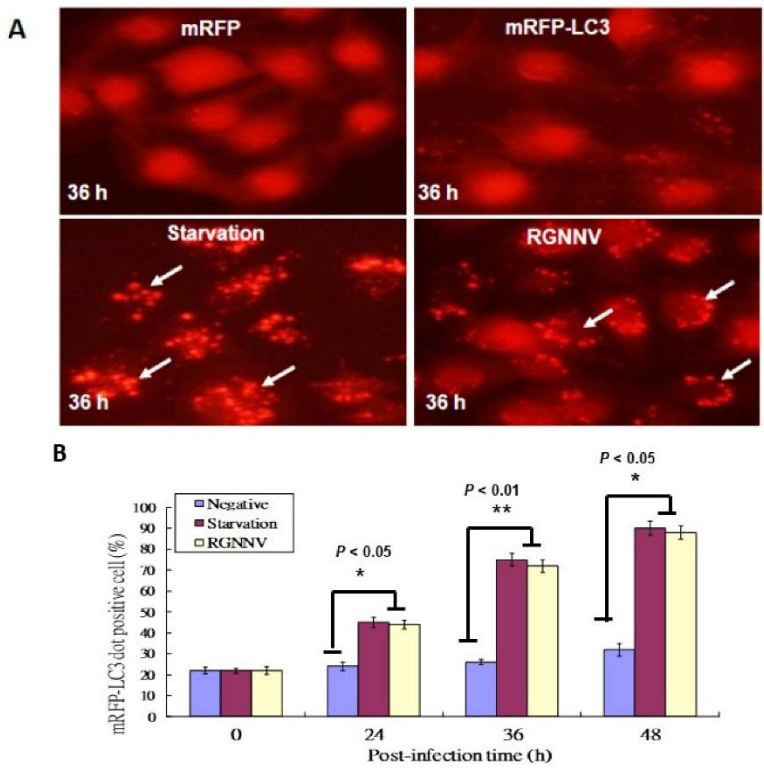

Figure 1: RGNNV-induced autophagosome formation in GF-1cells. (**A**) The formation of autophagosomes in GF-1 cells at 36 h detected by the mRFP-LC3 reporter system, which the formation of RFP-LC3 puncta or dots was counted up to 10 as a positive cell.; (a) mRFP (negative control), (b) mRFP-

LC3, (c) serum-starvation treatment, and (d) RGNNV infection. Arrows indicated autosomes. Scale: 10 μM. (**B**) Quantification of the results in A at 0 h, 24 h, 36 h and 48 h in three separate experiments, based on 200 cells per treatment. Results are shown as means ± SEMs *: $p < 0.01$.

3.2 3-Methyladenine (3-MA) Treatment Reduced RGNNV-Induced Autophagy and Blocked Bcl2 Family Downregulation in GF-1 Cells

There was a reduction of autophagosome formation in RGNNV-infected cells treated with 3-MA compared to untreated control by using the mRFP-LC3 reporter system (Figure 2A). The autophagy inhibitor 3-MA has titration and received 2 mM is an optimal condition, which did not induce cell damage, then for further testing. The 3-MA treatment significantly eliminated the autophagosome formation by ~20% at 24 h, ~40% at 36 h, and ~55% at 48 h (p <0.05) (Figure 2B).

Furthermore, treatment with 3-MA recovered RGNNV-induced BCL2 and BCL2L1 downregulation compared to untreated RGNNV-infected cells (Figure 2C). The 3-MA treatment significantly inhibited BCL2 downregulation ~0.6-fold and BCL2L 0.45-fold at 36 h ($p < 0.05$) (Figure 2D).

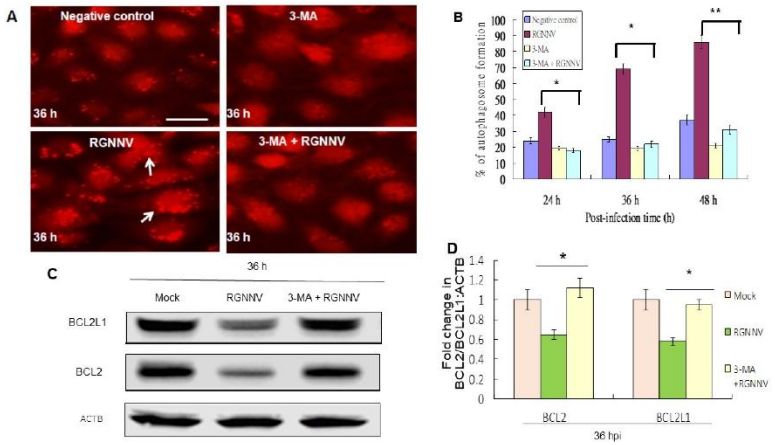

Figure 2: Elimination of RGNNV-induced autophagy by 3-MA treatment by reducing autophagosome formation and Bcl-2 family downregulation in GF-1 cells. (A) Autophagosome formation in GF-1 cells at 36 h after different treatments. a: mRFP-LC3 (negative control), b: 3-MA alone, c: RGNNV and d: 3-MA + RGNNV. Arrows indicated autophagosome-positive cells. (B) Percentage of autophagosome-positive cells at 36 h. *: $p < 0.05$; **: $p < 0.01$.

(C) Western blot analysis of BCL2 and BCL2L1 expression at 36 h after different treatments and (D) quantification of these results in three individual experiments. *: $p < 0.01$.

3.3 Overexpression of Zebrafish Bcl2 (Bcl2) and Zebrafish Bcl2 Like 1 (Bcl2L1) Reduced Autophagy and Protected Against Type III Cell Death

Whether the downregulation of proteins in the BCL2 family correlated with the RGNNV-induced autophagy and cell death was then examined. We designed and produced Bcl2 and Bcl2L1-producing GF-1 cells for these experiments and confirmed that the expression of Bcl2 (Figure 3A) and Bcl2L1 (Figure 3B) were stable in GF-1 cells. Then, the effect of Bcl2 and Bcl2L1 overexpression on the regulation of autophagy-mediated cell death was examined. The results from the LC3-II:LC3-1 ratio at 36 and 48 h showed that the upregulation of Bcl2 and Bcl2L1 had eliminated the RGNNV-induced increase in LC3-II:LC3-1 ratio, indicating that overexpression of Bcl2 and Bcl2L1 may inhibit RGNNV-induced autophagy (Figure 3C and D).

Upregulation of RIPK3 during necroptosis was suppressed more efficiently by Bcl2L1 overexpressed cells than Bcl2 at 36 h and 48 h, compared with untreated RGNNV-infected cells (Figure 3E).

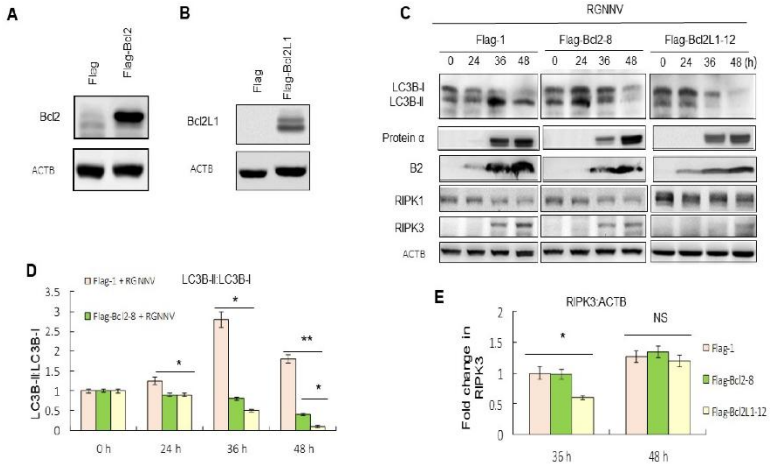

Figure 3: Overexpression of Bcl2 and Bcl2L1 suppressed autophagy and necroptosis. (A and B) Western blot analysis of Bcl2- and Bcl2L1-producing cells was identified by Bcl2 and Bcl2L1 PolyAbs. (A) Lanes: 1, Flag alone; 2, Bcl2-producing cells. (B) Lanes: 1, Flag alone; 2, Bcl2L1-producing cells. (C) Western blot analysis showed the effect of Bcl2 and Bcl2L1 overexpression on autophagy, necroptosis and viral replication, based on the LC3-II:LC3-I ratio, RIPK3 expression and viral protein expression. Lanes: 1–4, RGNNV-infected Flag-producing GF-1 cells at 0, 24, 36, and 48 h; 5–8, RGNNV-infected Bcl-2-producing GF-1 cells at 0, 24, 36, and 48 h; 9-12, RGNNV-infected Bcl2L1-producing GF-1 cells at 0, 24, 36, and 48 h. (D) Quantitation of the results in C for LC3-II:LC3-I ratio. (E) Quantitation of the results from C showed the effect of Bcl2 and Bcl2L1 overexpression on necroptosis, based on RIPK3 expression at 0, 24, 36, and 48 h in three separate experiments. Results are shown as means ± SEMs. *: $p < 0.05$; **: $p < 0.01$.

3.4 Blockage of Cell Death By Bcl2 and Bcl2L1 Overexpression and Reduced Viral Titers

Results from the ANXA5/annexin A5 and PI staining analysis indicated that Bcl2 and Bcl2L1 overexpression also significantly abolished RGNNV-induced cell death (Figure 4). Our data showed that RGNNV-induced autophagy regulated host cell death by increasing signals that promote cell death and decreasing signals that prevent cell death. In addition, Bcl2 and Bcl2L1 overexpression in cells had significantly inhibited RGNNV-induced the viral death factors (B2 and protein α) expression by about 25% (Figure 3C) at 48 h and decreased viral titers (Figure 5) at 24 h (~0.5-fold) and 48 hpi (~0.3-fold).

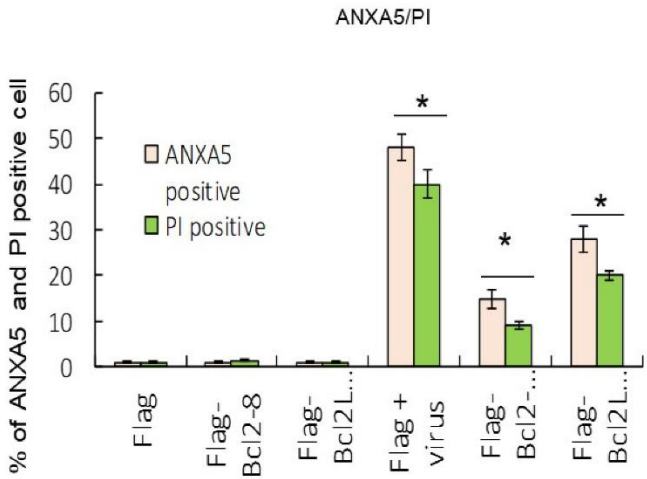

Figure 4: Overexpression of Bcl2 and Bcl2L1 suppressed cell death markers. Dual labeling with ANXA5 and PI stains indicated that Bcl2 and Bcl2L1 overexpression suppressed RGNNV-mediated autophagy at 48 h. The percentages of ANXA5- and PI-positive cells were determined by the number of green (ANXA5) and red (PI) fluorescent cells in three separate experiments. *: $p < 0.05$.

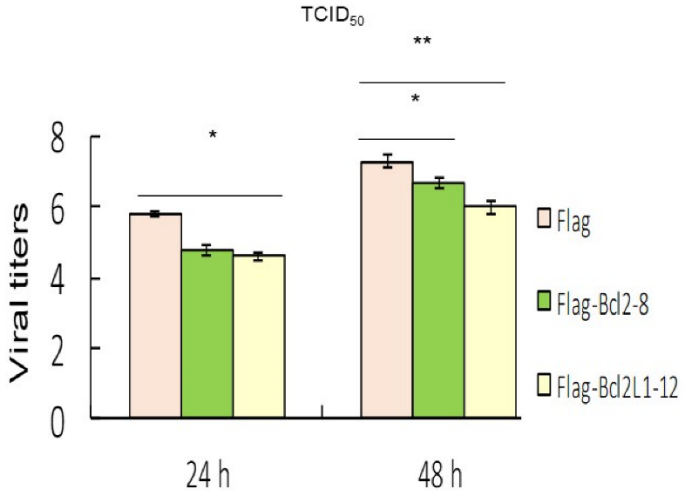

Figure 5: Overexpression of Bcl2 and Bcl2L1 suppressed viral replication. Viral titers (determined by the $TCID_{50}$ assay) in the supernatants of different groups at 24 and 48 h, in which received best effect on reducing titter between 50 to 100 times by Bcl2 or Bcl2L1 overexpression in three separate experiments. *: $p < 0.05$; **: $p < 0.01$.

Our study showed that RGNNV induced autophagy of GF-1 cells during the early stage of viral replication and led to a dramatic increase in viral protein expression. Viral infection was correlated with the increased necroptosis in host cells and the downregulation of the anti-apoptotic BCL2 and BCL2L1 proteins. Our finding suggested that overexpression of the Bcl2 family can regulate host cell death and the viral-controlling autophagosome, which correlated to reduced viral expression.

4. Discussion

DNA and RNA viruses have evolved molecular mechanisms to allow them to escape or inhibit autophagy, thereby increasing their infectivity [20], but RNNV virus-induced autophagy is still rare. As a result, how the autophagy process interacts with necroptosis is relatively unknown. In the present study, we investigated the role of Bcl2 family asymmetric crosstalk between cell death type III and autophagy. Our results in the molecular pathogenesis of RGNNV infection may help to identify potential targets that could be used for the prevention or treatment of RGNNV.

4.1 Betanodavirus-Induced Autophagy

Very recent, blockage of autophagy process and its singling pathways has emerged as an important mechanism for modulating intracellular bacteria or viruses. Some bacteria, such as *Listeria*, *Salmonella*, *Shigella*, and *Mycobacteria*, can induce autophagic flow, but the specific autophagy signals is mediated for triggering by intracellular pathogens is still need to defined [31]. On the other hand, in a viral pathogen infection, such as with *Herpes simplex type 1* (*HSV-1*), infection can induce autophagy, which suppress viral gene expression at late replication stage. Then, with *HSV-1* infection at early replication stage is almost did not required a newly synthesis pathway. [32].

Although certain RNA viruses have been previously shown to induce autophagy (e.g., the human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza) [35-37], there are few reports that examine the effect of betanodaviruses on autophagy. We showed that RGNNV infection induced the formation of autophagosomes (Figure 1) in GF-1 cells by

mRFP-LC3 at 36 hpf. In our analysis, 3-MA inhibited phosphatidylinositol 3-kinase (PI3K), which plays an important role in many biological processes, including controlling the activation of mTOR, a key regulator of autophagy. Apparently 3-MA blocked the formation of autophagosomes, which was consistent with previous studies where RGNNV-induced autophagy was inhibited by 3-MA [25]. On the other hand, several lysosomal inhibitors such as bafilomycin A_1 ($BafA_1$), protease inhibitors and chloroquine (CQ), was identified and used to blockage of autophagy flow during fused with lysosome at late stage for blocking lysosomal degradation. Up to now, CQ and its derivate hydroxychloroquine (HCQ) are approved as drugs by FDA that those used in clinical trials aimed to treat tumors on inhibition of autophagy process. Therefore, the precise mechanism of how CQ blocks autophagy process remains to be uncover. [38].

4.2 Crosstalk Between Autophagy and Necroptosis Cell Death Signals

The MTOR and BECN1 proteins play major roles in the regulation of autophagy [24,39-40]. BECN1 is part of a complex that regulates class III phosphatidylinositol 3-phosphate (Ptdlns3P) and hVps34 (human vacuolar protein sorting 34), whose activity is essential during autophagosome formation. Activators such as UVRAG (UV radiation resistance associated), Bif-1 (Bax-interacting factor) and Ambra-1 (the activating molecule in Beclin-1-regulated autophagy-1), bind with the BECN1 complex to increase phosphatidylinositol 3-monophosphate production. In contrast, proteins in the BCL2 family of anti-apoptotic proteins, such as BCL2 and BCL2L1, bind to BECN1 and act as inhibitors [24,40-41].

The antiapoptotic protein Bcl2 functions either as an antiapoptotic protein or as an anti-autophagy protein via its inhibitory interaction with Beclin 1 [42], which recently functioned in the lysosomal degradation pathway of autophagy and induced autophagic cell death in cancer cells. Furthermore, Bcl2/Bcl2L1 can bind Beclin 1 and inhibit Beclin 1-dependent autophagic cell death in cancer cells. Then, the BH3 domain on

Beclin binds to the BH3 binding groove of Bcl2L1 to block Bcl2L1 function.

Viruses, such as γ-herpesvirus-68 (γHV68), modulate this effect [43-45]. In our system, RGNNV-induced autophagy correlated with the downregulation of BCL2 and BCL2L1 at the middle stage of viral replication (36-48 h) (Figure 2C), and a subsequent increase in RIPK3-mediated necroptosis of host cells. Interestingly, Bcl2/Bcl2L1 overexpression reduced autophagy and downstream events and the expression of viral death factors and host cell necrosis (Figure 3C), which suggests their potential as therapeutic agents.

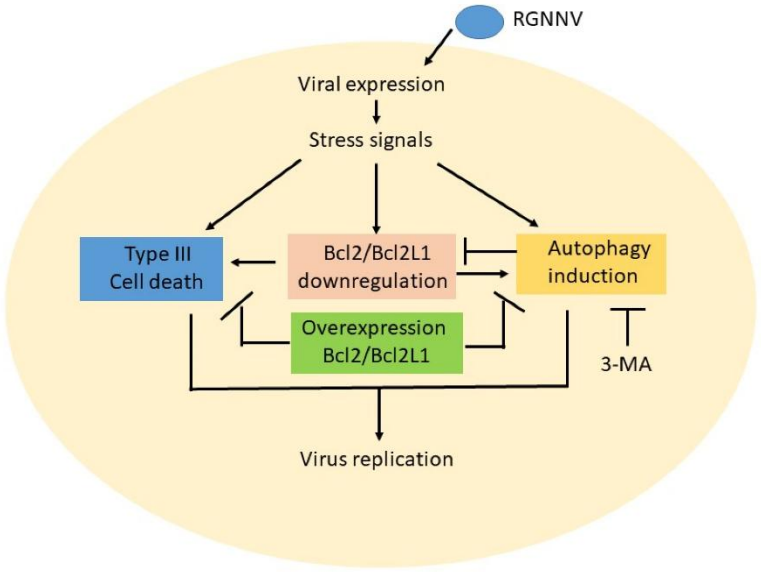

Figure 6: Hypothesized effect between triggering type III cell death and autophagy induction is through asymmetric crosstalk during RGNNV infection in GF-1 cells. RGNNV infection can also stress signals during viral expression and viral replication at the early replication stage (12–24 hpi). Then, those stress signals can regulate multiple signaling pathways either to induce cell death or trigger an autophagy flow in the middle replication stage that correlates with anti-apoptotic member Bcl2 and Bcl2L1 downregulation. In autophagy inhibitor treatment, 3-MA can suppress autophagy initiation. Furthermore, if overexpression of Bcl2 and Bcl2L1 genes can also either block cell death and reduce the viral titer or suppress the autophagy process. The correlations between cell death and autophagy, appear to be asymmetrical upstream or downstream correlation interactions.

In summary (Figure 6), we found that RGNNV infection can induce host cell death and autophagy, which also correlated with anti-Bcl2 member downregulation such as Bcl2 and Bcl2L1 at the early–middle viral replication stage (12–24 hpi). Then, blockage of autophagy initiation by 3-MA apparently reduced autophagy and Bcl2 downregulation at 36 hpi. On the other hand, overexpression of the Bcl2 family members Bcl2 and Bcl2L1 either dramatically reduced type III cell death or suppressed autophagy initiation. We hypothesized that RGNNV-induced cell death and autophagy by asymmetrical interaction, not by direct crosstalk. There seemed to be an upstream or downstream correlation in this system, but this requires further research.

5. Conclusions

RGNNV infection in GF-1 cells can induce autophagosome formation and Bcl-2 downregulation at different post-infection times; however, Bcl-2 downregulation is blocked by the autophagy inhibitor 3-MA. Then, extracellular Bcl-2 and Bcl2L1 overexpression can suppress host cell death type III and autophagy caused by with RGNNV infection. Our results suggest that RGNNV-induced cell death and autophagy by asymmetrical interaction was regulated by the anti-apoptotic members Bcl2 and Bcl2L1 and autophagy inhibitor 3-MA via the inhibition of class III Ptdlns3K.

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