Role for autophagy in cellular response to influenza virus infection

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KEY MESSAGES

1. A differential induction of autophagy was noted between influenza virus A/Hong Kong/54/98 (H1N1) and A/Quail/Hong Kong/G1/97 (H9N2/G1) infections.
2. The H9N2/G1 virus, which shows a delay in apoptosis activation, induces autophagy to a greater extent than the H1N1 virus.
3. Autophagy is not involved in H9N2/G1 virus replication in primary human blood macrophages.
4. Using 3-methyladenine to inhibit autophagy and small interfering RNA to silence the autophagy gene (Atg5), autophagic responses play a role in influenza virus-induced CXCL10 and interferon-α expression in human macrophages.

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Introduction

The outbreak of avian influenza A (H5N1/97) virus infection in Hong Kong is the first documented case of direct transmission of the H5N1 virus from birds to humans. The high mortality rate of >60% caused great concern to the public and government. In addition, avian influenza H9N2 viruses have become highly prevalent in poultry in many countries and have caused human infections. The high prevalence, wide geographic range, rapid evolution, and frequent reassortment of H9N2 viruses in poultry,[1] combined with the increasing number of avian influenza infections in humans, highlights their pandemic and zoonotic potential.

Apoptosis and autophagy constitute the two processes through which superfluous, damaged, or aged cells or organelles are eliminated.[2] Apoptosis has been shown to be one of the most effective host defence mechanisms against microbial pathogens. The cell death process results in inhibition of virus replication, limitation of virus dissemination, and minimisation of uncontrolled inflammatory responses. Avian influenza (H5N1/97) and its precursors trigger a caspase-dependent but delayed apoptotic response in human macrophages, compared with human influenza viruses (H1N1 and H3N2).[3]

Autophagy is a tightly regulated cellular homeostatic process involving the sequestration of parts of the cytosol and intracellular organelles within double-membraned autophagic vacuoles that are delivered to lysosomes for degradation. Autophagy plays essential roles in physiological processes such as cellular responses to starvation, cell survival, and death. Additionally, autophagy has been implicated in cancer, neurodegeneration, and myopathies, and it is involved in both innate and acquired immunity.[4] Autophagy is involved in the host defence mechanism against pathogens, including bacteria and viruses.[5] The antiviral role of autophagy is strengthened by the involvement of the autophagic process in the induction of interferon (IFN) in virus infection.[5]

To investigate the mechanisms underlying the diversion of cell death processes, we hypothesised that avian influenza viruses divert the cell death pathways from apoptosis, which ultimately involves whole cell death and degradation of cellular organelles, to that of autophagy, which is a self-sustained process resulting in removal of unwanted subcellular structures leading to prolonged survival of the avian virus-infected cells. With longer survival of virus-infected cells resulting from autophagy activation, there are opportunities for completion of virus replication and consequent immune dysregulation.

Methods

This study was conducted from October 2009 to September 2011. Human blood macrophages from healthy donors (Hong Kong Red Cross Blood Transfusion Service) were prepared as described previously.[3] Differentiated macrophages were obtained after culturing for 14 days. Influenza viruses A/Quail/Hong Kong/G1/97 (H9N2/G1) and A/Hong Kong/54/98 (H1N1) were grown in Madin-Darby canine kidney (MDCK) cells and were purified by pre-adsorption to and elution from turkey red

blood cells. Virus infectivity was determined by titration on MDCK cells.

Cells were infected with the viruses at a multiplicity of infection (MOI) of two or at the indicated dose for 30 minutes at 37°C. The supernatant containing the virus inoculum was then removed, and the cells were incubated in serum-free medium supplemented with 0.6 μg/mL penicillin and 60 μg/mL streptomycin. The mock-treated control was incubated with the buffer under parallel conditions.

To measure intracellular protein expression, whole-cell lysates were prepared by using total lysis buffer (50 mM Tris-hydrochloride, pH 7.4, 150 mM sodium chloride, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 0.1 mM ethylene diamine tetraacetic acid, 10% glycerol, 1% Triton X-100) containing a protease inhibitor cocktail. For western blot analysis, 30 μg of protein was heat-denatured and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane for assaying protein levels by using enhanced chemiluminescence solution.

For the detection of autophagosome formation, cells were fixed with 4% paraformaldehyde and permeabilised with 0.25% Triton X-100 for 5 minutes. The cells were stained with antibodies specific to LC3B and rhodamine-conjugated anti-rabbit secondary antibodies. Nuclei were counterstained with 4,6-diamidine-2-phenylindole dihydrochloride, and the immunocytochemical staining was examined under fluorescence microscopes.

The production of chemokines and interferons upon influenza infection were assayed by TaqMan Gene Expression Assays (Applied Biosystems; Life Technologies, Carlsbad, CA, USA) and enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, USA).

In studying the involvement of autophagy in virus replication and cytokine production, expression of Atg5 in human blood macrophages was knocked down by short interfering RNA (siRNA) oligos specific to Atg5 (siAtg5). Non-targeting siRNA oligos (siCtrl) were used as controls. Macrophages were transfected with either the siAtg5 or siCtrl at indicated concentrations using jetPEI transfection reagent (Polyplus-transfection, Illkirch, France). At 48 hours after transfection, cells were infected with the viruses, and then cells were harvested at indicated time points for further analyses.

Results

H9N2/G1 is a stronger autophagy inducer than H1N1

To investigate whether influenza virus can induce autophagy, differentiated primary human blood

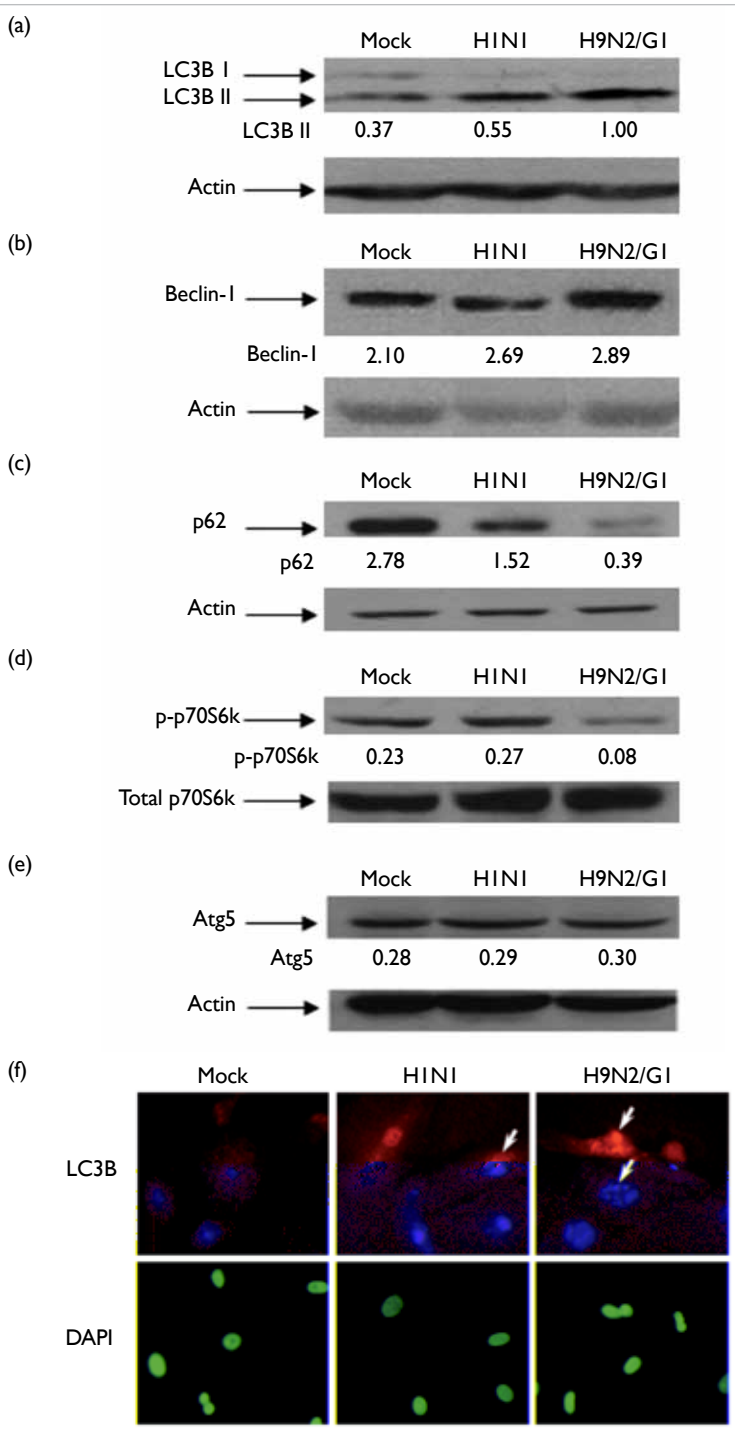

FIG 1. Autophagy is induced by H1N1 and H9N2/G1 influenza viruses. Human blood macrophages are mock-infected or infected with H1N1 or H9N2/G1. Whole-cell lysates are harvested and expressions are examined by western blot analysis. Actin is used as a loading control. (a) LC3B, (b) Beclin-1, (c) p62, (d) phosphorylation levels of p70S6K (Thr389), and (e) Atg5 are examined. Total p70S6K is used as a loading control. The band intensities of the indicated proteins are measured and then normalised to those of the corresponding actin. The normalised values are presented. Representative figures of experiments from three independent blood donors are shown. (f) Autophagosomes are examined by staining the mock-, H1N1-, or H9N2/G1-infected cells with antibodies specific to LC3B. DAPI is used to stain the nuclei. Arrows indicate LC3B-positive vesicles (x3400). (Figures were adapted with permission from: Law AH, Lee DC, Yuen KY, Peiris M, Lau AS. Cellular response to influenza virus infection: a potential role for autophagy in CXCL10 and interferon-alpha induction. Cell Mol Immunol 2010;7:263-70.)

macrophages (PBMacs) were infected with avian influenza virus H9N2/G1 or seasonal H1N1 virus at MOI of two. Expressions of key autophagy genes were investigated. At 19 hours post-infection, whole-cell lysates were prepared and conversion of LC3B I to II, a hallmark of autophagy, was analysed by western blot using antibodies specific to LC3B. Actin was used as a loading control. LC3BI was converted to LC3B II at a higher efficiency in cells infected with H9N2/G1 virus than with H1N1 virus (Fig 1a). Consistent with the conversion of LC3B, the expression level of Beclin-1, another autophagic marker, was also increased in cells infected with H9N2/G1 compared with H1N1 (Fig 1b). In addition, the level of p62 protein, a polyubiquitin-binding protein was also examined. The p62 protein can bind directly to LC3 proteins that enhance the degradation of ubiquitinated proteins in the lysosome through the autophagic processes. The p62 protein can also be degraded by autophagy. Its expression level, therefore, indicates the autophagic status of the cell. The p62 expression decreased by approximately 45% in macrophages with H1N1 infection when compared with mock-treated cells, whereas the level was decreased by about 84% in H9N2/G1-infected cells (Fig 1c). Furthermore, autophagy was negatively regulated by mTOR. Inhibition of mTOR led to induction of autophagy. As p70S6K is a direct target of mTOR, the phosphorylation of p70S6K at threonine 389 was examined to determine the activity of the rapamycin-sensitive mTOR complex. Phosphorylation of p70S6K at threonine 389 in H9N2/G1-infected macrophages decreased at 2 hours post-infection when compared with mock-treated cells (Fig 1d), whereas there was no significant change in H1N1-infected cells. In contrast, the expression level of Atg5, another autophagy-related protein involving in autophagosome formation, was not affected by the influenza virus infections (Fig 1e).

Autophagosomes were also examined by immunocytochemistry at 19 hours post-infection (Fig 1f). Consistent with the western blots, LC3B-positive vesicles with a punctate-staining pattern were found in cells infected with H9N2/G1 or H1N1. Moreover, more prominent staining was found in cells infected with H9N2/G1 when compared with those infected with H1N1. The H9N2/G1 virus induced more autophagic responses than H1N1.

Autophagy is not involved in influenza virus replication in macrophages

Association between concomitant activation of autophagy and significant enhancement of influenza virus replication was investigated. We treated the PBMacs with rapamycin, which is a well-known autophagy inducer through inhibition of mTOR, and then infected the cells with H1N1 virus. Cell culture supernatant was harvested at 48 hours post-infection for viral titre determination. Treatment with rapamycin did not significantly change the virus titres (Fig 2a).

The effect of inhibiting the virus-induced autophagy on virus replication was also investigated. We pre-treated the PBMacs with a chemical inhibitor, 3-methyladenine (3-MA), which inhibits autophagy by suppressing class III phosphatidylinositol-3-

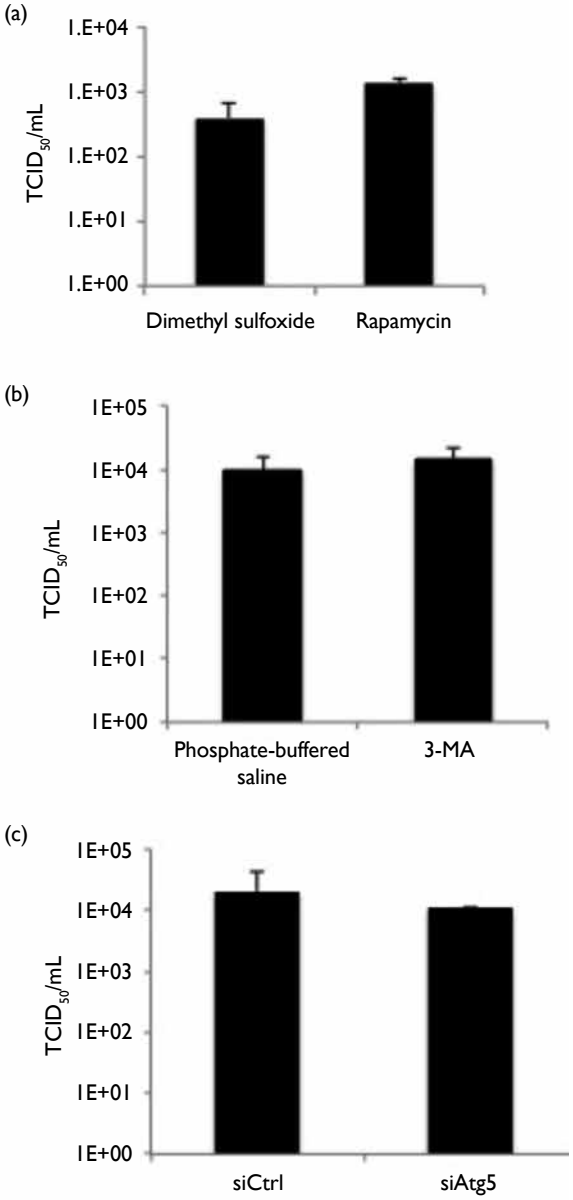

FIG 2. Autophagy is not involved in influenza virus replication. (a) Macrophages are pre-treated with 10 nM of rapamycin for 1 hour and then infected with H1N1 at multiplicity of infection of two. (b) Macrophages are pre-treated with 10 mM of 3-methyladenine (3-MA) for 1 hour or (c) transfected with the Atg5-specific siRNA oligos (siAtg5) or the control oligos (siCtrl) at 48 hours before infection. Cells are then infected with H9N2/G1 virus at MOI of two. At 48 hours post-infection, culture supernatants are collected. The virus titres are calculated using $TCID_{50}$ assay.

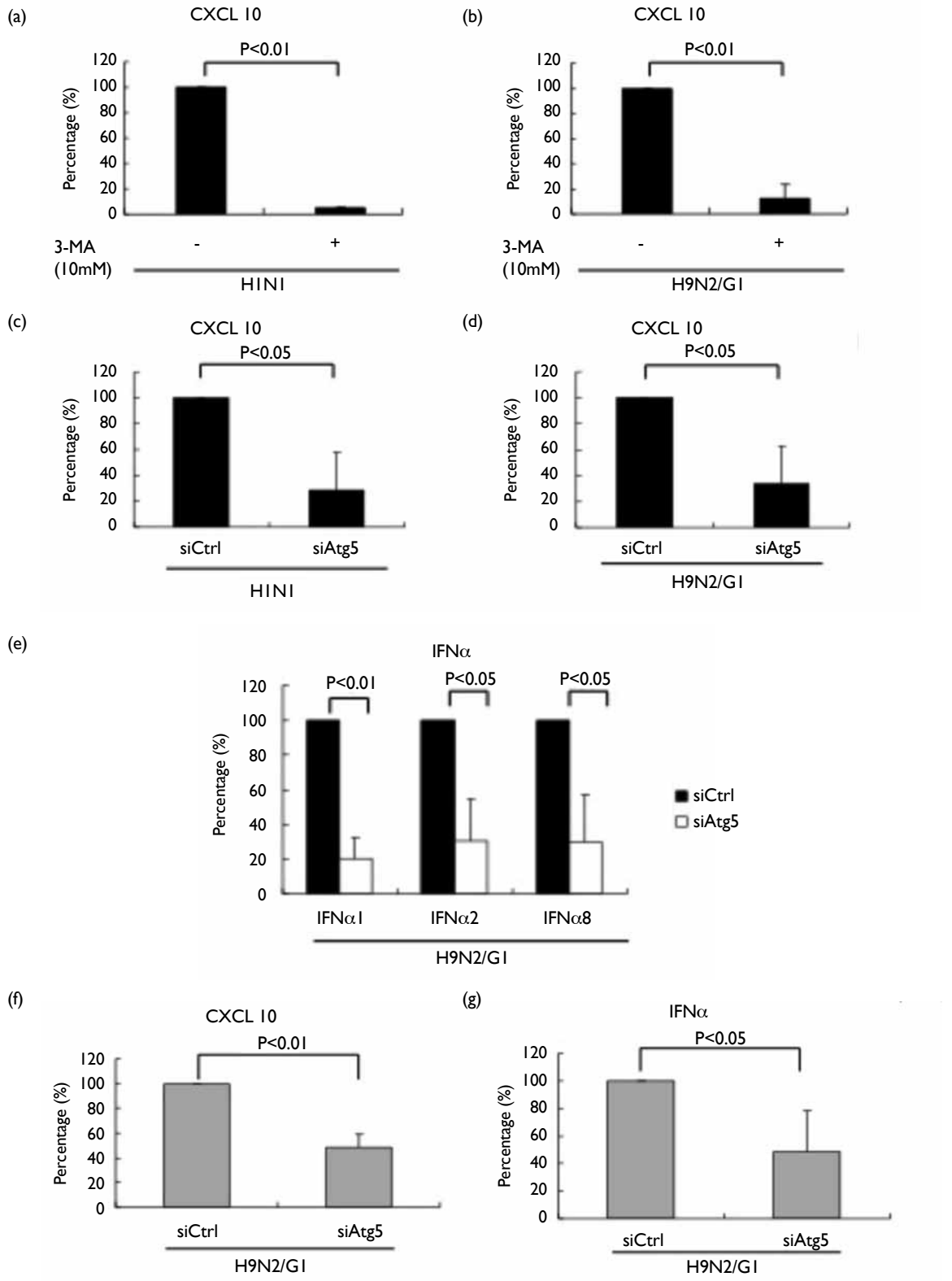

FIG 3. Inhibition of autophagy suppresses influenza-induced CXCL10 and IFN-α expression. Macrophages are pre-treated with 10 mM of 3-methyladenine (3-MA) before infection or transfected with siAtg5 or siCtrl at 200 nM. Cells are then infected with (a, c) H1N1 or (b, d) H9N2/G1. The relative CXCL10 mRNA levels are examined at 3 hours post-infection by TaqMan Gene Expression Assay, as are (e) relative mRNA levels of IFN-α1, -α2, and -α8 in siRNA-transfected cells with H9N2/G1 infections. Macrophages are transfected with siAtg5 or siCtrl at 200 nM. At 48 hour post-transfection, cells are infected with H9N2/G1. Quantities of (f) CXCL10 and (g) IFN-α in the culture supernatants at 8 hours post-infection are measured by enzyme-linked immunosorbent assay. (Figures were adapted with permission from: Law AH, Lee DC, Yuen KY, Peiris M, Lau AS. Cellular response to influenza virus infection: a potential role for autophagy in CXCL10 and interferon-alpha induction. Cell Mol Immunol 2010;7:263-70.)

kinase. Cells were then infected with H9N2/G1 virus and the virus titres were measured at 48 hours post-infection. No significant difference was found in the viral titre after the 3-MA treatment (Fig 2b). In addition to the chemical inhibitor, the effects of enhanced activation of autophagy in H9N2/G1 virus replication were further investigated by inhibiting the autophagic pathway using the siRNA silencing method. At 48 hours after transfecting the PBMacs with Atg5-specific siRNA oligos or control oligos, the cells were infected with H9N2/G1 virus at MOI of two and viral loads in the cell culture supernatant were measured by $TCID_{50}$ assay at 48 hours post-infection. No significant difference was found in the cells transfected with the Atg5-specific oligos and the control oligos (Fig 2c). Therefore, with the inhibition of the autophagic pathway by 3-MA or Atg5-specific siRNA oligos, autophagy did not involve H9N2/G1 replication in PBMacs.

Autophagic responses play a role in influenza virus-induced CXCL10 and IFN-α expressions

Whether autophagy was involved in the hyperinduction of chemokine and IFN was examined. The autophagic pathway 3-MA was blocked, or the PBMacs were transfected with siRNA oligos specific to Atg5. With the 3-MA pre-treatment, mRNA levels of CXCL10 induced by H1N1 or H9N2/G1 were reduced significantly as determined by TaqMan Gene Expression Assays (Figs 3a-b). At 48 hours post-transfection of the siRNA oligos, the cells were infected with H1N1 and H9N2/G1. Total RNA was collected at 3 hours post-infection, and the mRNA level of CXCL10 was measured by TaqMan Gene Expression Assays. The mRNA levels of CXCL10 induced by the two viruses were significantly reduced upon knockdown of Atg5 (Figs 3c-d). In siAtg5-transfected cells at 3 hours post-infection, CXCL10 mRNA levels were decreased by 72% and 67% in H1N1- and H9N2/G1-infected cells, respectively, when compared with those transfected with siCtrl. The mRNA levels of IFN-α1, -α2, and -α8 were measured in H9N2/G1-infected cells by TaqMan Gene Expression Assays. Similarly, in cells transfected with siAtg5, the IFN-α1, -α2, and -α8 mRNA levels were significantly decreased by around 70% (Fig 3e). The CXCL10 and IFN-α protein levels in culture supernatants of Atg5 knockdown cells were measured. At 8 hours post-infection with H9N2/G1, the level of CXCL10 in cell culture supernatants was decreased by about 50% (Fig 3f), concomitant with reduced Atg5 expression. Similarly, IFN-α expression was decreased by approximately 50% in cells transfected with siAtg5 (Fig 3g).

Conclusion

In human macrophages, autophagy played a role in H1N1- and H9N2/G1-induced CXCL10 and IFN-α production but not in virus replication. Our results may provide new insights into the underlying mechanisms of the pathogenesis of avian influenza virus infections.

Acknowledgement

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