

# Mechanisms and consequences of phagocytosis on influenza virus-infected cells

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**Mechanisms and Consequences of Phagocytosis of Influenza Virus-Infected Cells**

Yoshinobu Nakanishi<sup>1,\*</sup>, Yumi Hashimoto<sup>1</sup>, Takenori Takizawa<sup>2</sup> and Akiko Shiratsuchi<sup>1</sup>

<sup>1</sup>*Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan,*  
and <sup>2</sup>*Department of Virology, Toyama Institute of Health, Imizu, Toyama 939-0362, Japan.*

\*Address correspondence to this author at Graduate School of Medical Science, Kanazawa University, Shizenken, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan; Tel: +81 76 234 4481; Fax: +81 76 234 4480; E-mail: nakanaka@kenroku.kanazawa-u.ac.jp

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**Abstract:** Influenza virus-infected cells are induced to undergo apoptosis and become susceptible to phagocytosis. Data from our *in vitro* and *in vivo* experiments have suggested that 1) alveolar macrophages and neutrophils phagocytose influenza virus-infected cells in an apoptosis-dependent manner; 2) the membrane phospholipid phosphatidylserine and viral neuraminidase-processed carbohydrates at the surface of target cells and phagocytes, respectively, are involved in the association of the two types of cells; and 3) phagocytic elimination of virus-infected cells leads to a reduction in the pathogenesis of influenza. These findings could lead to the development of a novel antiviral agent against influenza.

**Key Words:** apoptosis, influenza virus, innate immunity, macrophages, neutrophils, phagocytosis, phosphatidylserine.

## **INTRODUCTION**

The death of cells infected with viruses has been documented for many years and, until recently, was considered to be caused by viruses when their progeny burst out from host cells. However, the current understanding is that virus-infected cells undergo apoptosis [1], a physiological mode of cell death [2]. Viruses manipulate the host cell's machinery for protein synthesis to propagate and produce progeny. The death of host cells upon infection should hamper the replication of virus and thus serves as a defense mechanism. On the other hand, viruses seem to resist this action using anti-apoptotic proteins encoded by viral genes [3, 4]. However, accomplishment of the apoptotic process takes time, and some types of viruses may complete replication before the machinery for protein synthesis breaks down. To overcome this problem, apoptosis in host cells serves another purpose in acting against viral invasion. In general, apoptotic cells become susceptible to phagocytosis [5], a biological event where a type of cell known as phagocyte captures, engulfs, and in most cases digests other cells [6]. Cells undergoing apoptosis are recognized and engulfed by phagocytes at an early stage of the apoptotic pathway [5]. If infected cells are phagocytosed and digested before the completion of viral replication, the dissemination of viruses and the development of viral diseases may be prevented. In this article, we describe our previous and current studies on the mechanism and consequences of the phagocytosis of influenza virus-infected cells and propose that the phagocytosis plays an important role in the prevention of influenza.

## **INDUCTION OF APOPTOSIS IN HOST CELLS UPON INFECTION WITH INFLUENZA VIRUS**

In 1993, we reported for the first time that chromosomal DNA of HeLa cells or Madin-Darby canine kidney cells was cleaved into oligonucleosomal units following infection with influenza A/Udorn/72 (H3N2) virus [7]. Given that this cell death is associated with the condensation of chromatin [7] and the externalization of phosphatidylserine [8], a membrane

phospholipid normally confined to the cytoplasmic side, and requires the actions of caspases [9], we have concluded that the mode of death of influenza virus-infected cells is apoptosis. These apoptotic events were observed much earlier than the occurrence of cell lysis. It is most likely that cells infected with influenza virus are induced to undergo apoptosis and eventually lyse. A couple of papers reporting a similar observation followed [10, 11], and the occurrence of apoptosis in lung tissues upon infection with influenza virus was shown with an animal model of influenza virus infection [12]. Later on, many types of viruses were found to induce apoptosis in host cells, and the current understanding is that host cells die by apoptosis when infected with virus [1].

In the initial study [7], we found that the fragmentation of host cell DNA required newly synthesized proteins, and that the amount of Fas, the receptor for the apoptosis-inducing Fas ligand [13], increased at the surface of virus-infected cells. An increase in Fas expression was caused at the level of transcription [14] involving the transcription factor C/EBP $\beta$  [15]. The activity of C/EBP $\beta$  was post-translationally augmented upon infection with influenza virus, possibly through the action of double-stranded RNA-activated protein kinase [16]. Virus-infected cells with an increased level of Fas became susceptible to treatment with an apoptosis-inducing anti-Fas monoclonal antibody [14]. Furthermore, the production of Fas ligand was also stimulated with a time course similar to that of Fas, and both proteins were expressed at the surface of influenza virus-infected HeLa cells [8]. Finally, we found that the presence of an antagonizing anti-Fas monoclonal antibody inhibited apoptosis in virus-infected cells [8, 14]. These results have allowed us to propose a mechanism for the induction of apoptosis in influenza virus-infected cells [8] (Fig. (1)); the level of surface expression of both Fas and Fas ligand increases upon infection with the virus, and the cells induce apoptosis each other when they come into contact. Another mechanism has recently been proposed for the induction of apoptosis in influenza virus-infected cells [17], and there seems to be multiple pathways for this apoptosis.

## PHAGOCYTOSIS OF INFLUENZA VIRUS-INFECTED CELLS

Replication of influenza virus, assessed by the level of the viral component hemagglutinin, appeared to continue irrespective of the occurrence of apoptosis in host cells [7], and we reasoned that viral propagation was completed before the onset of host cell apoptosis, at least prior to the breakdown of the protein synthesis machinery. Apoptotic cells are in general rapidly and selectively removed from the body by phagocytosis, and this is important for the morphological and functional development of organisms, renewal of tissues and organs, and prevention of diseases [18–20]. We thus examined if cells infected with influenza virus were subject to phagocytosis *in vitro* and found that infected HeLa cells were efficiently phagocytosed by peritoneal [21] or alveolar [22] macrophages of mice. The recognition of virus-infected cells by macrophages involved phosphatidylserine [21], a phospholipid that is normally confined to the cytoplasmic side and externalized during apoptosis [23], on the surface of target cells, and carbohydrates on the surface of macrophages, which are presumably desialylated by viral neuraminidase [24, 25] (Fig. (2)). This mode of recognition is unique in that both a general “eat-me” signal, i.e. phosphatidylserine, and a signal specific for influenza virus infection are involved. To further investigate this event *in vivo*, mice that had been intranasally challenged with influenza virus, A/WSN (H1N1), were analyzed [22]. We found that influenza virus-infected, apoptotic cells present in the bronchoalveolar lavage and lung tissues were phagocytosed by macrophages and neutrophils (Fig. (3)). Influenza virus-infected cells seemingly produced not only surface molecules to be recognized by phagocytes but also a secreted factor(s) that stimulates phagocytosis by macrophages [22]. In addition, the involvement of Toll-like receptor 4, a pattern recognition receptor regulating innate immune reactions including phagocytosis of apoptotic cells [26], in the regulation of phagocytic elimination of influenza virus-infected cells in mice was suggested [22].

## CONSEQUENCES OF PHAGOCYTTIC ELIMINATION OF INFLUENZA VIRUS-INFECTED CELLS

Influenza virus-infected HeLa cells, though undergoing apoptosis, allowed the invading virus to propagate before they lysed [7]. We examined if these events proceed in the presence of macrophages in cultures that are capable of phagocytosing apoptotic cells. Influenza virus-infected HeLa cells were maintained in cultures in the presence and absence of mouse peritoneal macrophages, and the titer of virus released into culture media was determined. We found that the presence of macrophages completely inhibited virus growth, and this inhibition required direct contact of macrophages with infected cells, suggesting that influenza virus is phagocytosed together with host cells and digested by macrophages [27]. We then assessed if the same is true *in vivo* using a mouse model of influenza virus infection. Histochemical analyses of lung tissues of virus-inoculated mice showed that the levels of phagocytosis and virus titer were roughly inversely related [22]. Furthermore, when mice infected with influenza virus were administered inhibitors of phagocytosis such as the phosphatidylserine-binding protein annexin V, both the level of inflammation in the lung and the lethality were enhanced [28]. These results support the conclusion drawn from *in vitro* experiments and suggest that apoptosis-dependent phagocytosis of influenza virus-infected cells by macrophages and neutrophils in the lung acts to reduce the pathogenesis of influenza.

## CONCLUSION AND PERSPECTIVE

Based on the data from our *in vitro* and *in vivo* experiments, we propose that influenza virus-infected cells in the lung are induced to undergo apoptosis and become susceptible to phagocytosis by macrophages and neutrophils, and that phagocytic elimination of the infected cells contributes to the inhibition of viral dissemination (Fig. (4)). The recognition of target apoptotic cells by phagocytes does not seem to involve antigen receptors or antibodies [5, 29]. Therefore, phagocytosis of apoptotic cells, when accomplished as a self-defense mechanism, is considered to be an innate immune response [19, 30]. Phagocytes that have phagocytosed influenza virus-infected cells contribute to the prevention of influenza in other ways as well [31] (Fig. (4)); they may process viral components and present antigens to T cells [32, 33] or secrete cytokines that

regulate inflammation [18–20]. These findings mean that the phagocytosis of influenza virus-infected cells acts in a variety of ways to protect organisms from the development of influenza. Considering the mechanistic part of this phenomenon, a similar mode of self-defense could be accomplished by phagocytes upon infection with other viruses, but so far we have not come across studies from such a point of view. It would be our pleasure if this article evokes attention from researchers investigating viral diseases.

A large number of deaths due to influenza have been reported for many years worldwide [34], and new pandemics of influenza virus are predicted [35]. Due to high genetic variability, the development of vaccines and antiviral agents against influenza virus faces difficulties [34]. New strategies are thus needed for developing novel antiviral agents, and our findings suggest that one aiming at a raise in the level of phagocytosis of influenza virus-infected cells is a good candidate. Substances could be developed that augment either the susceptibility of virus-infected cells to phagocytosis or the phagocytic activity of macrophages and neutrophils. It is apparent that the mechanism underlying the phagocytosis of influenza virus-infected cells is not unique, but at least a part of it is common to the phagocytosis of all apoptotic cells. Therefore, the development of such antiviral agents depends on how soon we gain a deeper understanding of the mechanism of the phagocytic clearance of apoptotic cells.

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**Figure Legends****Fig. (1). Proposed model for mechanism of apoptosis in influenza virus-infected cells.**

The surface expression of both Fas and Fas ligand increases in host cells upon infection with influenza virus, and those cells induce apoptosis each other when they come into contact.

**Fig. (2). Proposed model for recognition of influenza virus-infected, apoptotic cells by phagocytes.**

Phosphatidylserine (PS) externalized in influenza virus-infected cells during apoptosis and carbohydrates at the surface of macrophages after being desialylated by viral neuraminidase (NA) are involved in the recognition of target cells by phagocytes. This cell-cell association induces the phagocytosis of virus-infected cells. Molecules responsible for the recognition of PS and desialylated carbohydrates remain to be identified.

**Fig. (3). Evidence for phagocytosis of influenza virus-infected cells by macrophages and neutrophils in mouse lung.**

Micrographs show a macrophage and a neutrophil present in the bronchoalveolar lavage of mice infected with influenza virus. Phase contrast and fluorescence views of the same microscopic fields are presented. The arrowheads denote engulfed influenza virus-infected cells that are identified by an immunocytochemical analysis with anti-influenza virus antibody. Scale bar = 10  $\mu\text{m}$ .

**Fig. (4). Phagocytosis of virus-infected cells as self-defense mechanism against influenza.**

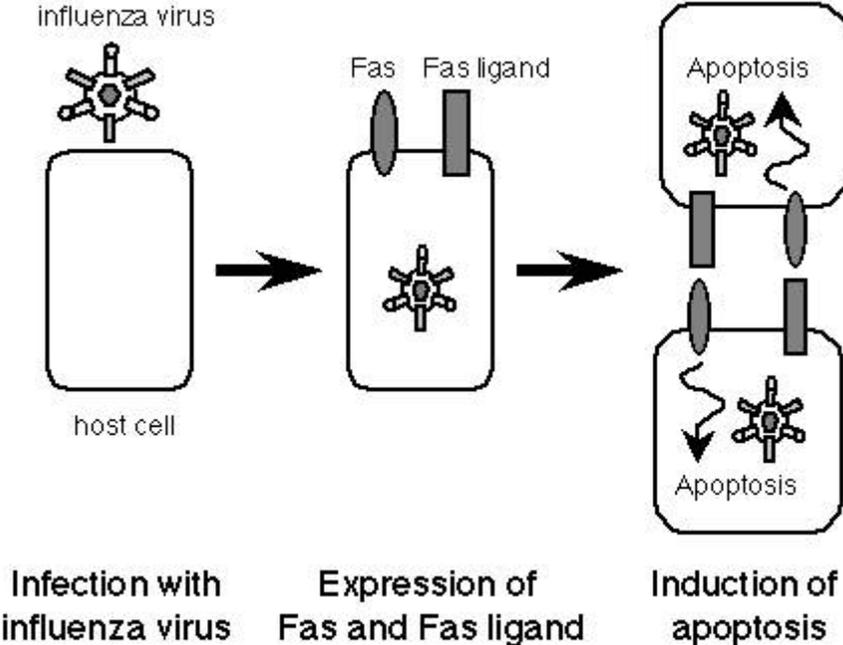
Phagocytosis of influenza virus-infected cells contributes in many ways to the prevention of influenza. Influenza virus is digested together with host cells in phagocytes (A); viral antigens are processed in phagocytes and presented to T cells, leading to the activation of adaptive immunity

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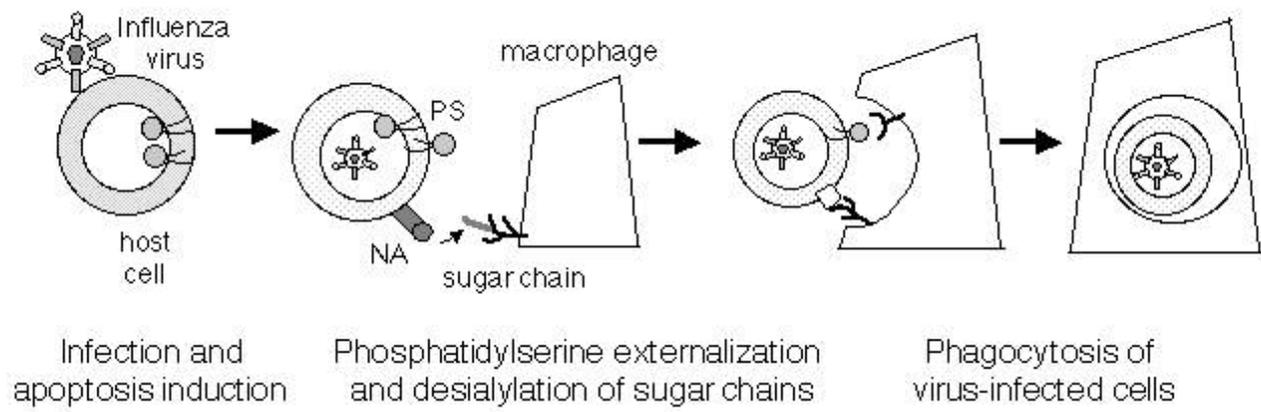
(B); phagocytes that have engulfed apoptotic cells secrete anti-inflammatory cytokines (C).

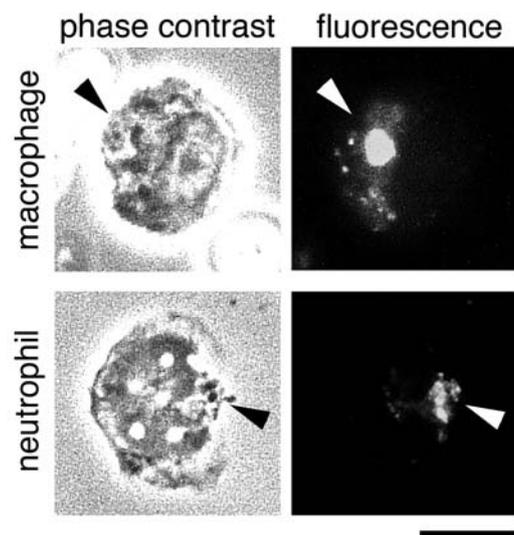
Consequences of the phagocytosis may vary depending on the type of phagocytes.

Nakanishi et al. Fig. (1)



Nakanishi et al. Fig. (2)





Nakanishi et al. Fig. (4)

