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Oseltamivir inhibits both viral entry and release but enhances apoptosis of cells infected with influenza A H1N1

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ABSTRACT

Oseltamivir (Tamiflu) has been shown to be effective as a treatment and chemoprophylaxis for influenza virus infections. However, the safety consideration of oseltamivir should not be overlooked because adverse events associated with the drug have frequently been reported after the use of Tamiflu for the treatment of influenza patients. Here, we investigated oseltamivir's influence on viral load, apoptosis, and cytokine response of influenza A H1N1-infected A549 or THP-1 cells. In addition, we analyzed the effect of the drug on the function of influenza-infected NK cells after oseltamivir treatment. The results of our study suggest that oseltamivir may inhibit both viral entry and release but may also enhance apoptosis of infected cells and interfere with NK cell function during influenza infection.

1. Introduction

Prompt antiviral drug treatment and chemoprophylaxis are recommended for pandemic or likely pandemic influenza virus infection when no specific vaccine is available. Oseltamivir (Tamiflu) has been shown to be effective in the treatment and chemoprophylaxis of seasonal as well as 2009 pdmH1N1 influenza infections [1–3]. Clinical investigations have concluded that early therapy with oseltamivir may reduce the severity and duration of symptoms and the risk of complications associated with influenza [1,4–6]. However, the safety considerations of oseltamivir should not be overlooked because serious adverse events arising after the treatment of influenza patients with Tamiflu have been reported, including fatal neuropsychiatric adverse reactions [7] and sudden death resulting from cardiorespiratory arrest [8].

For acute influenza infection, the induction of apoptosis and cytokines is a crucial factor in pathogenesis. For many years, apoptosis was mainly regarded as a host cell defense mechanism because many viruses express antiapoptotic proteins to prevent this cellular response. However, more recent data have revealed that viruses can also take advantage of proapoptotic signaling events to increase the efficiency of their replication machinery within the apoptotic cell. Apoptosis, a crucial cellular response frequently observed in influenza virus-infected tissue, has been verified to play a key role in influenza-induced pneumonia or lung injury [9]. Several authors suggest alveolar epithelial cell apoptosis to be an underlying mechanism of alveolar damage in murine models or human of respiratory distress syndrome [10–12]. Cytokine response is another crucial host response that may play a protective role or induce immune injury in influenza infection. Influenza virus infection of blood monocyte-derived murine and human macrophages [13,14] and porcine alveolar macrophages [15] has been shown to result in the induction of pro-inflammatory cytokines. In recent years, it has become increasingly apparent that, following influenza A virus infection, innate immune cells, including natural killer cells, alveolar macrophages, and dendritic cells, are essential in the direct control of viral replication and in the induction and regulation of virus-specific adaptive immune responses. In addition, cytokine responses and apoptosis are involved in a complex host immune response web. However, to our knowledge, no study of oseltamivir's effects on influenza-induced apoptosis has been conducted.

To study the effect of oseltamivir on influenza H1N1 virus and infected cells, we investigated oseltamivir's effect on apoptosis, viral load, and cytokine response in H1N1-infected monocytes (THP-1) and lung epithelial (A549) cells. In addition, we analyzed the impact of oseltamivir on H1N1-infected primary NK cells.

2. Materials and methods

2.1. Cells and virus

Primary NK cells, A549 cells and THP-1 cells were used in this study. The A549 and THP-1 cells were donated by the US Center for Disease Control and Prevention. The influenza viruses used

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included 2009 pdmH1N1 A/Sichuan/1/2009 (H1/09) and A/Hunan/3/2009 (HN3) strains and the seasonal influenza H1N1 A/Brisbane/59/2007 (H1/07) and A/Hunan/1374/2009 (HN1374) strains. H1/09 and H1/07 are oseltamivir-sensitive viruses, and HN3 and HN1374 are oseltamivir-resistant viruses.

2.2. Isolation of primary human NK cells

Blood samples were obtained from 9 healthy adult volunteers (25–35 years old) after written informed consent was provided. Ethical approval was given by the Ethics Committee of the Chinese CDC. Peripheral blood mononuclear cells (PBMCs) were isolated by separation with a Ficoll–Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient. $CD56^{+}CD3^{-}$ NK cells were enriched from PBMCs via magnetic bead separation using an NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity was confirmed using a FACS Aria I flow cytometer (BD Biosciences, San Diego, CA). The resulting preparations routinely contained >95% $CD56^{+}CD3^{-}$ NK cells.

2.3. Virus preparation, titration, and infection

The viruses were first proliferated and titrated in Madin–Darby canine kidney (MDCK) cells using a 72 h culture to determine TCID50. This titration was followed by a hemagglutination assay of the supernatant with Turkey red blood cells under 5-fold dilutions. THP-1 or A549 cells (seeded at 2×10^5 cells per well in 12-well tissue culture plates) were infected at 1 or 0.1 TCID50/Cells by H1/07, H1/09, HN3, or HN1374, respectively. The viruses were diluted with DMEM medium (containing 0.5% BSA and 1 μg/ml TPCK-treated trypsin) with or without 50 μM oseltamivir (Roche, Germany). After 1 h of virus adsorption, the infected cells were washed with PBS and incubated in DMEM medium with or without oseltamivir. Four duplicates were made for each infection or MOCK group. Cell supernatants were collected at 6 h, 24 h, 48 h, or 72 h POI. The supernatants were maintained at −80 °C for subsequent virus titration or cytokine analysis. The cells were lysed using RLT buffer (Qiagen) and maintained at −80 °C for subsequent RNA extraction. Additionally, primary NK cells were infected by H1/09 or H1/07 at 0.2 MOI. After 1 h of adsorption, the cells were washed with PBS to remove the residual non-adsorbed virus and incubated in RPMI1640 (Invitrogen, Camarillo, CA, USA) overnight at 37 °C.

2.4. Apoptosis detection

Two different techniques were used to evaluate apoptosis. (1) Annexin-V-FITC-PI staining was used. This stain detects the exposure of phosphatidylserine (PS) to the external leaflet of the plasma membrane in early apoptosis and allows differentiation between live, early apoptotic, and dead cells. The infected THP-1 cells at 24 h POI were washed with PBS, centrifuged and resuspended in 400 μL of a binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Annexin-V-FITC (5 μL, 10 μg/mL) was added to a sample of 195 μL of cell suspension, mixed, incubated for 10 min at room temperature in the dark, washed with PBS, and resuspended in 190 μL of binding buffer containing 10 μL of PI (1 μg/mL). The double-stained cells were analyzed using a FACS Aria I flow cytometer within 10 min (10^4 cells/sample). (2) Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was applied for the in situ detection of apoptosis of the infected A549 cells. The detection was performed with polyformalin-fixed cells using the Cell Death TUNEL POD Detection System (Roche, Germany) according to the manufacturer's instructions.

2.5. Cytometric bead array assay

Cytokines were assayed in cell-free supernatants using an IL-1beta, IL-4, IL-6, IL-8, IL-10, IFN-gamma, IP-10, MCP-1, MIP-1alpha, MIP-1beta, RANTES, TNF, and MIG cytometric bead array (CBA) assay (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. The data were collected with a FACS Aria I flow cytometer and analyzed with BD Cytometric Bead Array 1.4 software.

2.6. Standard RNA synthesis

In vitro transcribed RNA of the Matrix (M) gene RNA of pdmH1N1 and beta-2-microglobulin (B2-MG) was used to determine the detection limit of the assay and as an internal positive control. One micrograms of linearized plasmid DNA with the M and B2-MG gene segment was transcribed using a Riboprobe In Vitro Transcription System kit (Promega, USA) and the T7/SP6 promoter according to the manufacturer's instructions. The transcribed RNA was purified and quantified by copy number.

2.7. RNA extraction

RNA was extracted from cell lysates using a Qiagen RNA Easy Plus Mini Kit according to the kit's protocol. RNA was obtained in a 50 μL volume after genomic DNA was removed by use of a gDNA spin column and stored at −80 °C before use.

2.8. Quantitative real-time RT-PCR (qRT-PCR)

To quantify the influenza viral load in infected cells, quantitative real-time RT-PCR was performed using an Agilent Mx3005P detection system. The primer and probe for influenza A (Matrix gene) were WHO-released CDC primer sets [16]. The assays were performed in a total volume of 25 μL using the QuantiTect Probe PCR Kit (Qiagen, Germany), 5 μL total RNA, 20 μM of each primer and 10 μM of each probe. The thermal cycling conditions used for the assays were as follows: reverse transcription at 50 °C for 30 min, 1 cycle at 94 °C for 15 min, then 45 cycles at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s each. A standard curve was generated using serial dilution of in vitro-transcribed standard RNA (from approximately 10–10^7 copies).

2.9. The analysis of NK cell-surface receptor expression and NK cell function by flow cytometry

To monitor changes in NK cell-receptor expression, the influenza-infected NK cells were stained. The staining was followed by flow cytometric analysis using anti-NKp46 PE, anti-NKp44 PE, anti-NKp30 APC, and anti-CD56 PE-cy5 (BD Biosciences San Diego, CA) after stimulation of influenza viruses with or without oseltamivir treatment. NK cell function was accessed by intracellular CD107a staining. NK cells were stimulated with influenza virus in the presence of CD107a Abs (BD Biosciences, San Diego, CA), monensin, and brefeldin A (Sigma–Aldrich, Saint Louis, MO). After a 6-h incubation, the cells were treated with 20 mM EDTA, pH 7.2–7.4 for 15 min at RT. Surface marker staining was performed as described above. The cells were then permeabilized with Cytofix/Cytoperm buffer (BD Biosciences, San Diego, CA) for 20 min and washed in washing solution. An isotype control Ab was used for each staining combination.

2.10. Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by paired or nonparametric tests using Graph Prism 5

software (GraphPad Software), and $p < 0.05$ was considered significant.

3. Results

3.1. The enhanced induction of apoptosis in oseltamivir-treated cells

The annexin V/PI assay indicated that the number of cells exhibiting both early apoptosis (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+) in the oseltamivir-treatment group was higher than that of the non-treatment H1/09- or H1/07-infected THP-1 cells at 24 h POI (Fig. 1). There was no difference in the MOCK group cells (data not shown) or in the HN3- or HN1374-infected THP-1 cells for either the oseltamivir treatment group or the non-treatment group ($p > 0.05$). The TUNEL assay indicated that apoptotic cells were more frequently observed in H1/09- or H1/07-infected A549 cells treated with oseltamivir (Fig. 1E) than in untreated cells (Fig. 2D). The number of apoptotic cells significantly increased with infection progression ($p < 0.05$, Fig. 2F).

3.2. Decreased cytokines response in infected THP-1 cells with oseltamivir-treatment

To determine the relationship between oseltamivir-induced apoptosis and cytokine response, the expression of 13 cytokines (including IL-1β, IL-4, IL-6, IL-8, IL-10, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF, and MIG) was detected using CBA in infected THP-1 cells with or without oseltamivir-treatment. Compared with the non-treatment group, H1/09-infected THP-1 cells presented significantly decreased levels of MIP-1β, MCP-1,

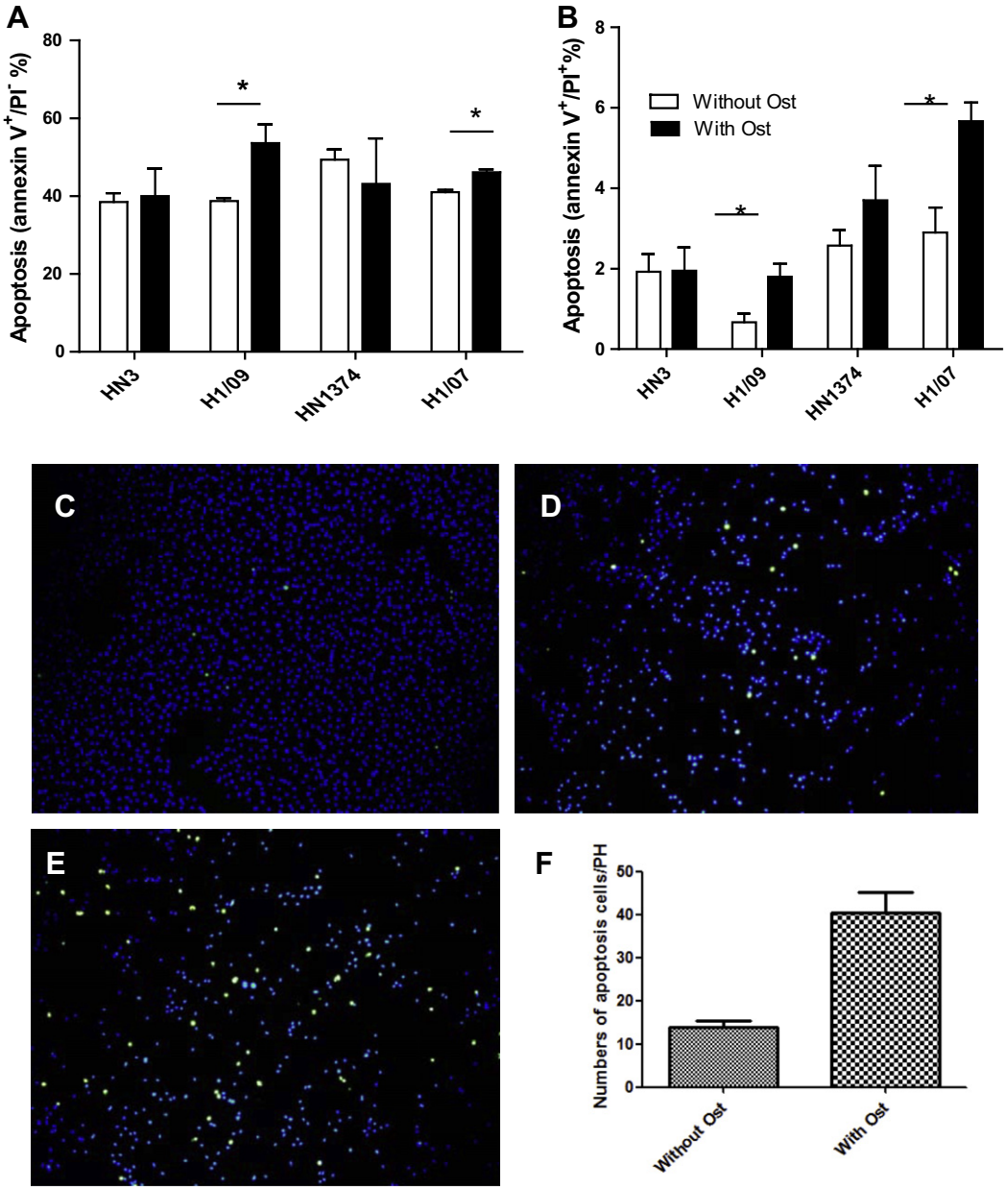

Fig. 1. Apoptosis of infected THP-1 or A549 cells, with or without oseltamivir treatment, as evaluated by FCM or TUNEL assay. The representative columns display the ratio of early apoptotic cells with annexin V^+/PI^- stains (A) and the ratio of late apoptotic cells with annexin V^+/PI^+ stains (B). The images present representative H1/09-infected cells from three experiments. The variable numbers of apoptotic cells presented in the MOCK (C), pH1N1-infected A549 cells without (D) or with oseltamivir-treatment (E) groups, respectively. Data in the bar graph are from three experiments (F). Statistical analyses with *t*-tests were performed; $^*p < 0.05$ and $^{**}p < 0.01$.

and RANTES in the oseltamivir-treatment group (Fig. 2). Similar to H1/09, oseltamivir decreased cytokine response, including IL-8, IP-10, MCP-1, TNF, and RANTES, in H1/07-infected THP-1 cells. No significant differences in cytokine expression were found with oseltamivir-resistant viruses HN3 and HN1374 between the two groups.

3.3. Oseltamivir inhibits both viral entry and release

Quantitative RT-PCR assay indicated that the viral copy number in the H1/07- or H/09-infected THP-1 or A549 cells was significantly lower in the oseltamivir-treatment group than in the non-treatment group at 0 h POI (Fig. 3A–F). The two cells lines also presented differences in viral load. The viral copies decreased more slowly in infected THP-1 cells with oseltamivir treatment but increased more quickly in infected A549 cells with oseltamivir treatment. Viral loads in the two cells were much higher in the oseltamivir treatment group than in the non-treatment group at 6 h and 24 h POI (Fig. 3A–F). No difference was found with oseltamivir-resistant viruses HN3 or HN1374 between the two groups ($p > 0.05$). To ensure consistency of the kinetics of viral load in the two cells, a validation of viral kinetics was performed. The validation demonstrated that THP-1 cells are not as susceptible to H1N1 viruses compared with the good replication of the viruses in A549 cells (Supplementary Fig. S1). In addition, the viral titration of the supernatant of infected cells indicated that oseltamivir may inhibit H1/09 and H1/07 replication (Fig. 3G–J). Compared with the non-treatment group, H1/09 titers in A549 cells in the oseltamivir-treatment group presented similar levels at 6 h or 24 h POI then presented lower levels at 48 h POI, but the levels increased to a significantly higher level at 72 h POI (Fig. 3G). H1/07 titers in oseltamivir-treated A549 cells were markedly lower than the titers observed in the non-oseltamivir treatment group at 24 h POI. The titers were similar at 48 h and 72 h POI between the two groups (Fig. 3H). In contrast to the A549 cell results, H1/09 and H1/07 titers in THP-1 cells decreased with the progression of infection, and H1/09 titers decreased more slowly in the oseltamivir-treatment group than in the non-treatment group (Fig. 3I). H1/07 titers in THP-1 cells did not present a significant difference (Fig. 3J).

3.4. Oseltamivir induces polarization of NK cells towards cytotoxicity after influenza infection

Primary $CD56^{+}/CD3^{-}$ NK cells at 90% purity were obtained from PBMCs where the CD3+ and CD14+ lymphocytes were removed. As shown in our previous published study [17], FACS analysis indicated that the NK cells compromised an obvious $CD56^{dim}$ and $CD56^{bright}$ subgroup (Fig. 4A). NKp44 and NKp30 expression presented no surface change on influenza-infected NK cells overnight. However, NKp46 presented decreased expression on the surface of $CD56^{dim}$ NK subgroup cells following infection and lower levels in the oseltamivir-treatment group than in the non-treatment group (Fig. 4B). Additionally, the decreased NKp46 expression level was more significant with H1/09-infected $CD56^{dim}$ NK cells than with H1/07-infected $CD56^{dim}$ NK cells. For intracellular CD107a staining, CD107a stains were observed with both $CD56^{dim}$ and $CD56^{bright}$ subgroup-NK cells after the primary NK cells were infected by influenza viruses. As Fig. 4C depicts, an increased ratio of NK cells with CD107a was noted in both the $CD56^{dim}$ and $CD56^{bright}$ subgroups after infection with H1/07 or H1/09 overnight. The effect of oseltamivir on CD107a expression was mainly observed in the $CD56^{dim}$ subgroup, although a mildly increased ratio of NK cells with CD107a expression was noted with the $CD56^{bright}$ subgroup ($p > 0.05$). A decrease in the expression of CD107a was observed with the H1/07-infected $CD56^{dim}$ subgroup of NK cells with

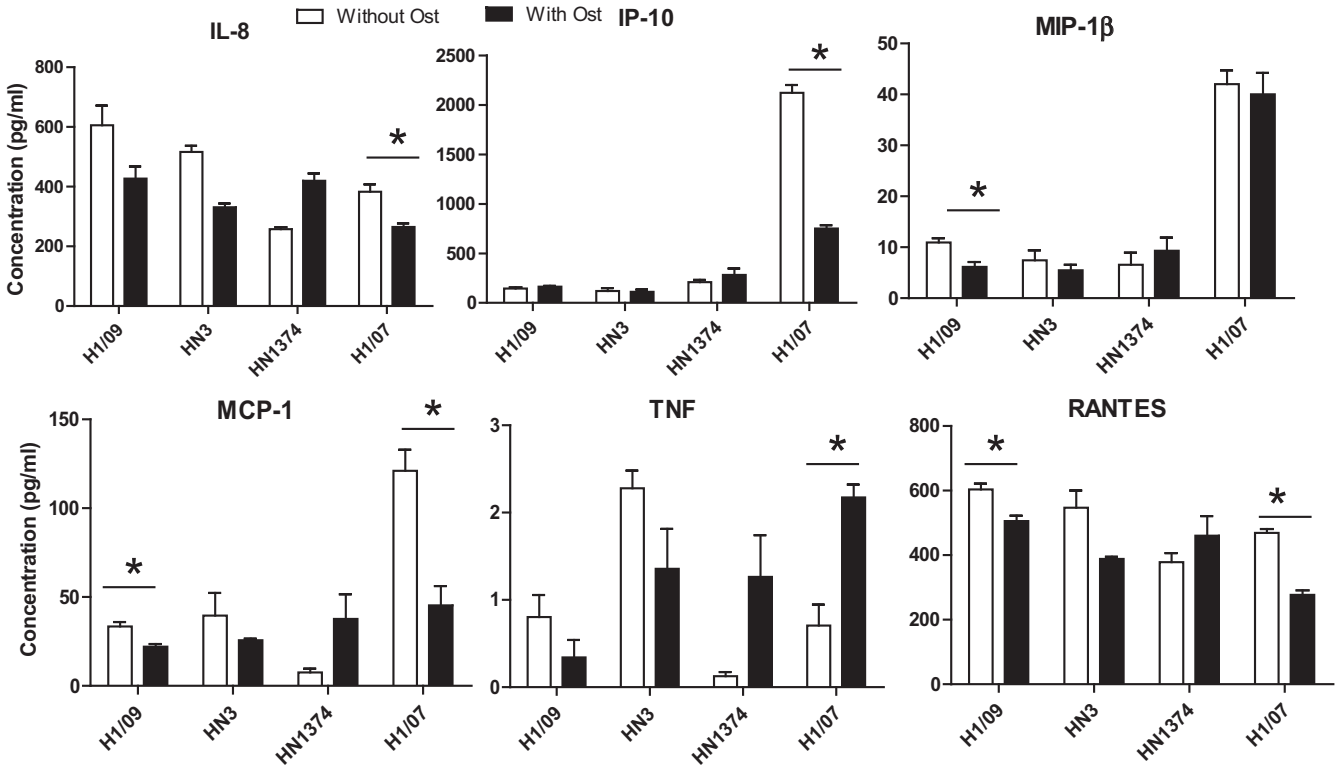

Fig. 2. Cytokine levels in the supernatant of infected THP-1 cells with or without oseltamivir treatment. The representative column depicts the levels of IL-8, IP-10, MCP-1, MIP-1β, TNF or RANTES in THP-1 infected by pH1/09, pH1/09 (r), H1/07 or sH1/09 (r) with or without oseltamivir treatment, respectively. Statistical analyses with *t*-tests were performed; $^{*}p < 0.05$ and $^{**}p < 0.01$.

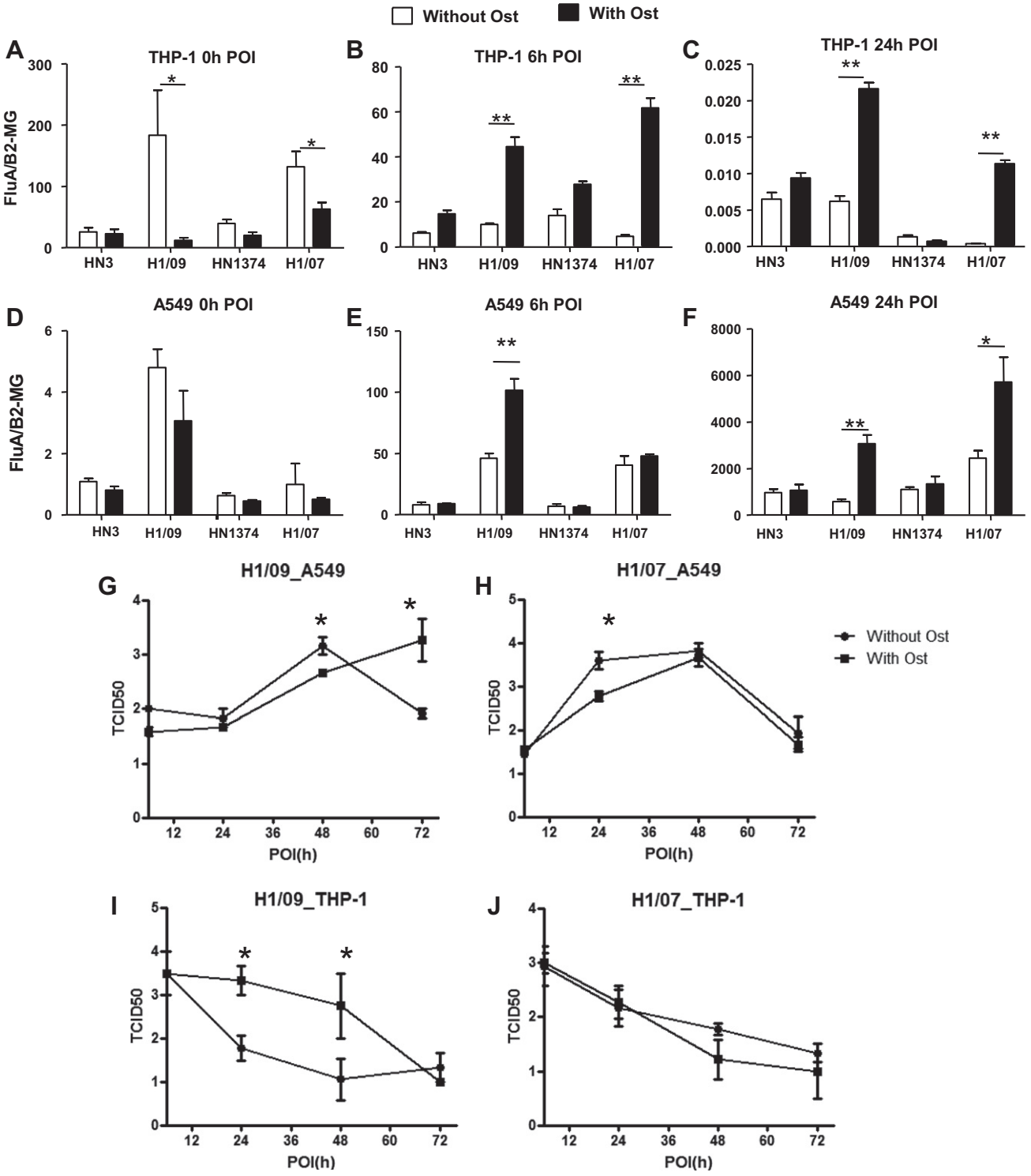

Fig. 3. Viral kinetics in infected THP-1 or A549 cells with or without oseltamivir treatment. The relative viral quantification is presented by the ratios between the influenza Matrix gene and B2-MG gene copies of infected A-549 (A–C) or THP-1 cells (D–F) at 6 h, 12 h or 24 h POI, respectively. Viral titration of H1/07 or H1/09 in supernatant is presented as TCID50 at 6 h, 24 h, 48 h or 72 h POI, respectively (G–J). The viral load in infected cells or viral titers in supernatant were compared between in the groups with and without oseltamivir treatment. Statistical analyses with *t*-tests were performed; $^{*}p < 0.05$ and $^{**}p < 0.01$.

oseltamivir treatment ($p < 0.05$), but an increase was noted with the H1/09-infected NK cells ($p < 0.01$).

4. Discussion

The morbidity and mortality associated with the influenza virus are global health concerns, especially since the 2009 global pandemic H1N1 [18]. Antiviral drugs will continue to play a significant role in the management of influenza given the inherent limitations of vaccines. Frequent mutations of influenza viruses make the manufacture of influenza vaccines exceptionally challenging. The antiviral oseltamivir, a neuraminidase (NA) inhibitor, is the most widely used antiviral for the treatment and prophylaxis of influenza infection [19]. However, the clinical safety of oseltamivir

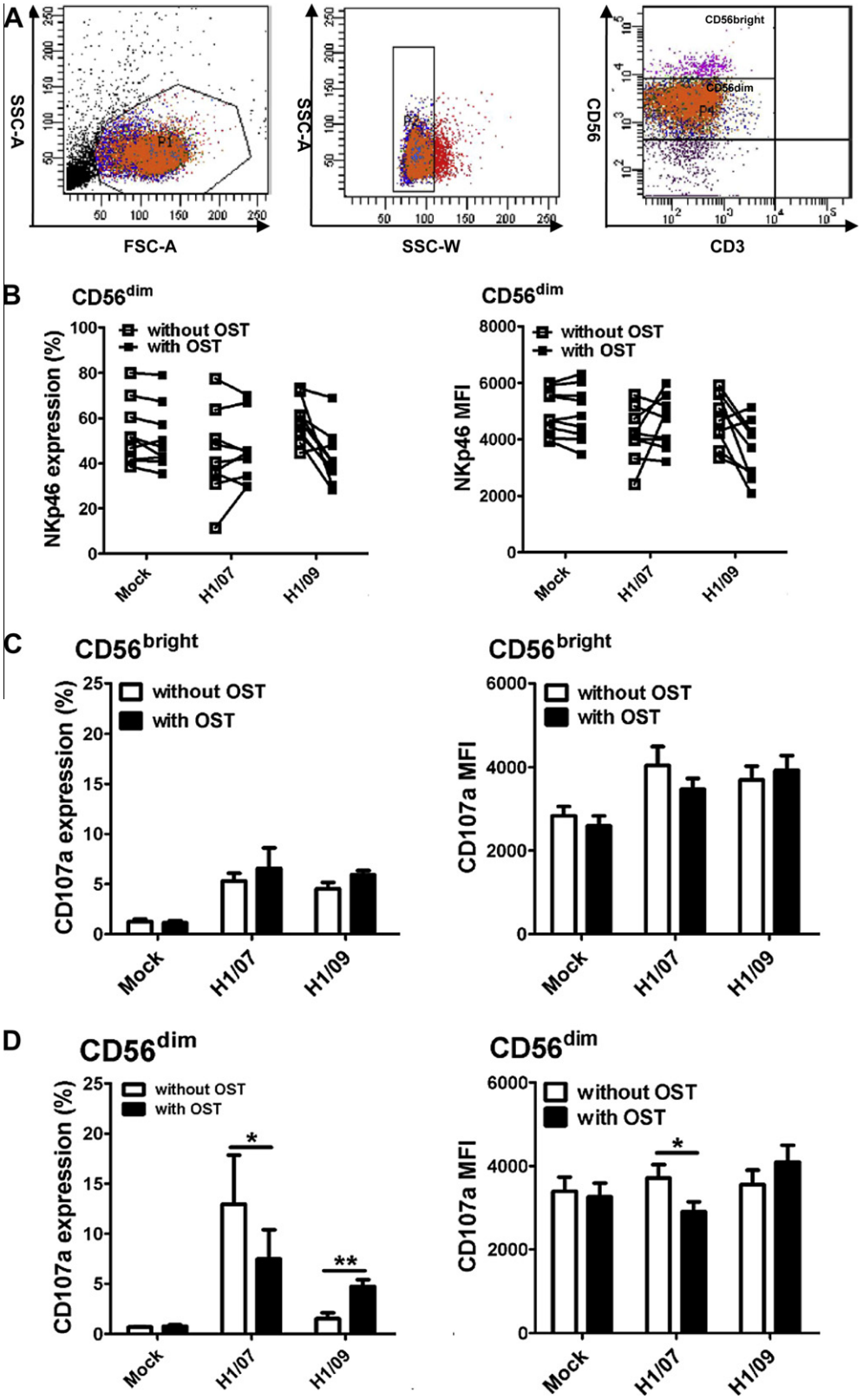

Fig. 4. Impact of oseltamivir on CD107a and NKp46 expression with influenza virus-infected primary NK cells. (A) Isolation and purification of human primary NK cells. Two groups of $CD56^{bright}$ and $CD56^{dim}$ were separated into CD56+/CD3− NK cells. (B) The expression of NKp46 in $CD56^{dim}$ NK cells. The percentage of $NKp46^{+}$ cells (left panel) and the NKp46 median fluorescence intensity (MFI) (right panel) are shown. (C) CD107a expression in $CD56^{bright}$ NK cells. The percentage of $CD107a^{+}$ cells (left panel) and the median fluorescence intensity (MFI) (right panel) are shown. (D) CD107a expression in $CD56^{dim}$ NK cells. The percentage of $CD107a^{+}$ cells (left panel) and the MFI (right panel) are shown. $^{*}p < 0.05$; $^{**}p < 0.01$.

for influenza patients should not be overlooked, given adverse event reports.

Influenza NA is a mushroom-shaped tetramer anchored in the viral envelope by a transmembrane domain [20,21]. As previously

published studies have shown, NA possess receptor-destroying activity, cleaving terminal sialic acid residues from cell-surface glycoproteins and gangliosides to release viral progeny from host cells [22]. In accordance with previous studies, our results suggest that oseltamivir may inhibit viral release and subsequently induce accumulation of viral copies in infected cells. Additionally, our results indicate that oseltamivir may decrease viral entry into cells as well. These results are consistent with the present knowledge in the field concerning the biological function of influenza NA, even if our particular results have not been previously reported. In viruses with inactive or absent NA, or in the presence of neuraminidase inhibitors, viral particles clump at the cell surface, and infectivity is consequently reduced. NA also removes sialic acid residues from the viral envelope itself, which prevents viral particle aggregation to enhance infectivity [23,24].

However, oseltamivir may enhance apoptosis of infected cells. Influenza-induced apoptosis has been presented in a variety of cell types, both in vitro and in vivo [25–28]. Several previous studies suggest that alveolar epithelial cell apoptosis is an underlying mechanism of alveolar damage in murine and human models of adult respiratory distress syndrome [29–31]. Recently, apoptosis dysregulation (characterized by too frequent or too infrequent apoptosis) has emerged as a new concept to explain important features in the development of several poorly understood diseases. Unregulated excessive apoptosis may be the cause of various degenerative and autoimmune diseases that are characterized by an excessive loss of normal or protective cells. Our results indicate that oseltamivir enhances the apoptosis of not only epithelia but also monocytes with influenza virus infection. Thus, oseltamivir may induce excessive apoptosis, especially in high viral load infections.

Oseltamivir may affect the activation or function of influenza-infected innate immune cells. Macrophages are the only innate immune system cell type susceptible to influenza virus infection [32,33]. NK cells may kill these infected cells in the early stage of influenza infection, though NK cells are not susceptible to the influenza virus [34]. Mouse-model studies have highlighted inflammatory monocytes as the major infiltrate and have identified a role for invariant NK T cells in control of influenza virus-infected cells and lung immune pathology [35]. In this study, our results indicated that oseltamivir may enhance the apoptosis of monocytes and induce the polarization of NK cells towards cytotoxicity after oseltamivir-sensitive influenza virus infection. Clinical studies indicate that patients with severe influenza infection present depletion of innate immune cells in peripheral blood or lung tissue [36,37]. Our results suggest that the dysfunction of infected NK cells due to oseltamivir treatment could be induced by inhibiting the release of virus or/and enhancing apoptosis of infected cells. Additionally, Influenza A virus is capable of infecting both epithelial cells and tissue macrophages and inducing cytokines/chemokines [38]. These cytokines may affect the interplay of innate and adaptive immunity [39]. However, our results indicate that the cytokine levels significantly decrease in H1/07 and H1-09 infected-THP cells treated with oseltamivir. These results might be explained by the high rate of apoptosis among infected cells, as excessive apoptosis may decrease the expression of cell proteins with DNA fragmentation of cells, and the high apoptosis rate is related to the viral load in infected cells. Taken together, oseltamivir could induce an adverse effect in severe influenza patients with high viral loads.

In conclusion, we investigated the effect of oseltamivir on epithelia and immune cells infected with H1N1 influenza viruses. Our results indicate that oseltamivir may inhibit both viral entry and release, and enhance the apoptosis of infected cells. Additionally, oseltamivir may induce polarization of NK cells towards cytotoxicity after influenza infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.019.

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