Research Article

Potential Role of NLRP3 Inflammasome to Combat Pandemic 2009 (PH1N1) Influenza Virus Infection in Human Lung a549 Epithelial Cells

Nivedita Tiwari, Drirh Khare, Sheetal Verma, Tapan N. Dhole.
Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.
***Corresponding author's E-mail:** tndhole@gmail.com

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ABSTRACT

The role of NOD-like receptors (NLR) involvement in viral sensing has not been widely investigated and is not well understood. There is paucity of information about the innate immunity and role of inflammasomes in the airway epithelium against influenza virus infection. This study demonstrates the physiological role of NLRP3 during influenza virus infection *in-vitro*. Three different influenza virus strains (H1N1, Influenza A and pandemic H1N1 virus) were used as infectious agent in A549 human lung epithelial cells and expression of NLRP3 at mRNA level and protein level as well as IL-1β and IL-18 levels in the cell culture supernatant were evaluated. The recognition of influenza virus RNA triggers activation of the NLRP3 inflammasomes, which in turn modulates the severity of influenza pneumonia and elevation of IL-1β and IL-18. Therefore it is suggested that NLRP3 inflammasomes appears to be an essential component responsible for elevation of pro-inflammatory cytokines in host defence against infection through the sensing of viral RNA and would aid as a clue to make more effective vaccines and therapeutics against pH1N1.

Keywords: IL-1β, 1L-18, Influenza virus, NLRP3.

INTRODUCTION

Influenza virus infection is a highly contagious respiratory illness which is associated with significant morbidity and mortality worldwide. The annual epidemics typically affects 5–15% of the population and there were approximately 28,000–111,500 deaths attributable to influenza in children <5 years of age worldwide, and the burden was highest in the developing countries.[1] Influenza A virus is a negative single-strand RNA virus emerges sporadically as pandemic viruses and is responsible for annual seasonal epidemics worldwide. The primary target of influenza virus infection is the airway epithelial cells lining the respiratory mucosa but also infect alveolar macrophages and dendritic cells (DCs) that reside in the airways.

Influenza infection can be recognized by the innate immune system in multiple ways. These include endosomal recognition through the Toll-like receptor (TLR)-3 [2,3], TLR-7 [4, 5] and cytosolic recognition through the retinoic acid inducible gene I (RIG-I).[6-11] Recent studies also identified the role of NOD-like receptors (NLR) in innate recognition of influenza virus infection.[12,13] The NLR mediated signalling has emerged as a major pathway in which the innate immune system responds to microbial pathogens. Substantial evidence suggests that the NLR proteins serves as intracellular mediators of PAMP initiated host-cell signalling, although the exact mechanisms underlying NLR responses to pathogens are not completely understood. It has been demonstrated that the NLR protein 3 (NLRP3), together with its adaptor protein, PYD- and CARD-domain-containing protein (PYCARD) (which is also known as Apoptotic Speck protein containing a CARD [ASC]), regulates IL-1β and IL-18 maturation through the formation of a biochemical complex called the inflammasome.[14-16] This inflammasomes regulates the activation of caspase-1 and subsequent cleavage of the IL-1β and IL-18 precursor into their functional form, which is then released from the cell.[17]

One study has shown a role for NLRP3 in responding to dsRNA, poly (I: C), activates IL-1β through the NLRP3 pathway[18] but other failed to replicate this finding. [19] The role of NLRP3 and the inflammasomes during viral infection is also not understood. In the present study we assessed the role of NLR inflammasomes in viral pathogenesis and host response to the H1N1, influenza A virus and pandemic H1N1 (pH1N1) virus. In this study, the evidence of NLRP3 inflammasomes and induction of IL-1β and IL-18 production, as a model of human A549 lung epithelial cell cultures has been studied with different mutant strain of Influenza A virus to show the expression pattern of NLRP3 relation to disease severity. This report demonstrates that the NLRP3 inflammasomes is an essential component of the host immune response to viral infection in an *in vitro* model system that is physiologically relevant to human disease.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the Institutional Ethics Committee (IEC) of Sanjay Gandhi Postgraduate Institute of Medical Sciences Lucknow. All participants gave written informed consent before their samples were collected and processed in the laboratory.

Reagents

Viral Transport Media (VTM), Minimum Essential Media (MEM), L-Glutamine, N-2hydroxyethyl piperazine N-2

Ethane sulphonic acid (HEPES), Sodium bicarbonate, antibiotics (Penicillin/Streptomycin), Trypsin, TPCK-Trypsin were procured from Sigma-Aldrich, fetal calf serum (FCS) (Gibco), QIAaMP viral RNA mini kit (QIAGEN, GmbH, Germany), Rabbit polyclonal anti-NLRP3 antibody (Sigma Aldrich) and a β-actin antibody (Cell Signalling Technology), ELISA kit for IL-1β was procured from BD Bioscience Pharmigen, San Diego, CA and for IL-18 from Medical & Biological Laboratories Co.

Virus Strains

In the study three different strains of Influenza A virus were used to assess the expression of NLRP3 inflammasomes. The H1N1 strain (laboratory adapted) which is similar to A/Brisbane/59/2007 was procured from NCDC Delhi. The Influenza A (wild strain) isolated from patient who had common cold and the pandemic H1N1 virus (mutant strain) isolated from patient who had pandemic 2009 influenza virus infection.This new strain appears to be a result of reassortment of human influenza and swine influenza viruses. The differentiation of Influenza A and pH1N1 virus were done by according to CDC kit given in Table 1. The confirmation of the Influenza A and pH1N1 virus was done by Real Time-PCR.

Real Time-PCR

The throat swab of patients suspected with influenza virus was collected in VTM (Viral Transport Media). Viral RNA was extracted from all samples by using the QI Aamp Viral RNA mini kit (Qiagen) according to the manufacturer's guidelines. Real Time-PCR of the extracts was performed by using Agpath-ID™ One-Step Real Time-PCR kit (Ambion U.S.A) according to the manufacturer's instructions, with the influenza gene primers given as depicted in Table-1 and thus classified accordingly.

Briefly, the 25µl reaction volume contained 5µl of 5X PCR buffer, 13µl of RNAse-free water, 1µl of 10mmol/L dNTPs, 1.5µl of 10 nmol/L reverse primer, 1.5µl of 10nmol/L forward primer, 1 µl of enzyme mix (Taq DNA polymerase and reverse transcriptase), and 2µl of viral RNA extract. Amplification was carried out in an Applied Biosystems Step One Real Time PCR with a single reverse transcription step of 50°C for 30 min, activation of hot start Taq at 95°C for 15 Sec followed by cycling step (95°C for 15 Sec. and 55°C for 30 sec).

Cell Culture

The Madin–Darby canine kidney (MDCK) cell line and human alveolar epithelial cell line A549 cells were procured from National Centre for Disease Control (NCDC) Delhi (India). The cells were cultured in Minimum Essential Media (MEM) with non-essential amino acids, containing glutamine (4mM), and penicillin–streptomycin (50IU/ml) with fetal bovine serum (10%) at 37°C in a humidified atmosphere of 5% CO_2.

Virus Propagation

The Influenza virus H1N1 lab adapted strain which is similar to A/Brisbane/59/2007 Influenza A and p-flu influenza viruses were propagated using MDCK cells. In brief, 200µl of throat swab samples which were positive for Influenza A virus and p H1N1 influenza viruses by reverse-transcription PCR assay RT-PCR were used for virus isolation in MDCK cells. After adsorption for 1 hour at 37°C under 5 % CO_2, cells were washed with phosphate buffer saline (PBS) and maintained in DMEM containing 2µg/ml TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)–Trypsin. Infected cells were observed daily for cytopathic effect (CPE) and harvested when ~75 % of the total cells in the monolayer showed CPE. After 3-4 passaging with tissue culture supernatants (TCF) were tested by hem agglutination (HA) assay using 0.5 % fowl red blood cells (RBCs). The TCF was aliquoted and stored at -70°C and was used as virus stock for further experiments.

Hem agglutination (Ha) Assay

A549 cells were grown in 6-well flat-bottom plates to obtain monolayer of 80 % confluency. Cells were then inoculated with 0.1MOI different virus stock (H1N1, Influenza A and p H1N1 virus) maintained at 4 X 10^7 pfu/ml. Unabsorbed virus was removed, and cells were washed with serum-free medium. After 48 h, 100µl media was removed from each well, and HA assay was performed.

Briefly, HA assays were performed on 'U' bottom plates and 50µl of PBS was added in the wells of microtiter plates. In the first row 50µl of the test sample was taken, and serially diluted by transferring 50µl from the first well to the successive well and so on 50µl of the 0.5% fowl red blood cell (RBCs) suspension was added to each well. Cell control and virus control were kept on the same plate. The plate was incubated at room temperature and the titres were read after 60 min. Hemagglutination units (HAU) were expressed as the reciprocal of the maximum dilution of virus that resulted in complete agglutination.[20]

RNA Extraction and Real-Time PCR

Total RNA was extracted from the infected as well as control A549 cells after washing twice with PBS and harvested with TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the supplier's protocol. RNA quality and quantity were determined by gel electrophoresis and spectrophotometry. Total RNA (2µg) was reverse-transcribed to cDNA using Thermo Script RT-PCR kit as per the manufacturer's instructions. Briefly, RNA was reverse-transcribed in cDNA with oligo (dT) primers and 200 U of Superscript II (Invitrogen) following manufacturer's instructions. Real-time analysis for NLRP3 and normalizing gene HPRT was performed using SYBR Green Master Mix as per the manufacturer's instruction (Applied Biosystems). This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labelled PCR product. Briefly, cDNA corresponding to 100 ng of RNA

served as a Δtemplate in a 20-µl reaction mixture containing 4 mM MgCl2, 0.2 nM (each) primers, and 10 µl Fast Start DNA Master SYBR Green mix (ABI). The samples were loaded into 96-well plate format and incubated in the fluorescence thermo cycler 7500 (ABI System). Initial denaturation at 95˚C for 10 min was followed by 45 cycles, each cycle consisting of 95˚C for 15s, touchdown of 1˚C/cycle from the primer-specific starting to end annealing temperatures for 5s, and 60˚C for 10s. The primer sequences used for specific genes were NLRP3 F-5′GAAGAAAGATTACCGTAAGAAGTACAGAAA3′ and NLRP3 R-5′CGTTTGTTGAGGCTCACACTCT3′. All quantifications were normalized to the housekeeping HPRT gene, which showed a very stable expression in A549 cells. The number of fold changes for specific genes expression in virus treated group against control was calculated using 2-ΔΔCT method.

Cellular Protein

Whole-cell protein extracts were prepared by lysis of cell monolayer's harvested in T25 flasks. Lysis buffer, contained protein and phosphatase inhibitors (50 mM Tris [pH 7.4], 50 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 0.1% SDS, and 1% Triton-x100, 0.2% protease inhibitor cocktail, 1 mM PMSF, 2 mM NaF, and 2.5 mM sodium pyrophosphate). The harvested cells were collected and treated with 20 µl of lysis [21] buffer and vortexed. The lysates were sonicated for 20s and kept at 4˚C for 30 min. After 5 min of centrifugation (3,000 rpm at 4 ˚C), the supernatant was saved as a whole-cell lysate.

Western Blot Analysis

Fifty microgram of whole-cell lysate proteins were resolved on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane in electro-transblot apparatus (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated with primary antibody-rabbit polyclonal anti-NLRP3 antibody (Sigma Aldrich) β-actin antibody (Cell Signaling Technology) was used as internal control. The bands were detected by using horseradish peroxidise conjugated secondary antibodies to primary immunoglobulin using enhanced chemiluminescence system (Amersham Biosciences). Densitometric analysis was performed using Image J software.

BD Opteia Elisa

Levels of IL-18 and IL-1β were estimated in the supernatants of the infected cells using commercially available ELISA kits (BD Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. In brief, wells were coated with 100 µl of capture antibody and incubated overnight at 4˚C. Next day, wells were washed three times with 300 µl /well wash buffer. Wells were blocked with 200 µl of blocking solution at RT for 1h followed by washing (3X). Hundred microliters each of pre-diluted standards, controls, and cell culture supernatants were added in duplicates and incubated at RT for 2 h. After repeating washing step, 100 µl of working detector (specific enzyme labelled antibody conjugate) was added to each well and plates were incubated at RT for 1 h. After repeated washing, 100 µl of substrate solution was added to each well, and incubated for 30 min at RT in the dark followed by addition of 50 µl of stop solution. After 30 min, absorbance was measured first at 570 nm and then at 450 nm wavelength. 570 nm readings were subtracted from 450 nm reading, and a standard curve was plotted. The amount of cytokine in the culture medium sample was calculated by extra poling the values on from the standard curve. The sensitivities and linear ranges of the cytokine OptEIA human ELISA were as follows: IL-18 sensitivity 1-15.0 pg/ml and IL-1β sensitivity 3.9–250 pg/ml.

Statistical Analysis

When two groups were compared, we used a two-tailed Student's t-test. To compare three groups, we used repeated measures ANOVA followed by Bonferroni's method to control for multiple comparisons. Data are presented as mean ± SEM. p values of, 0.05 were considered statistically significant.

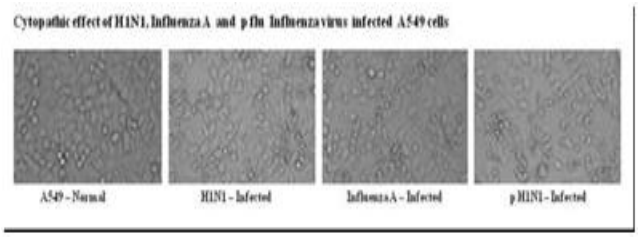

Figure 1: Cytopathic effect of HIN1, Influenza A and pH1N1 Influenza virus infected A549 cell. Phase contrast picture of A549 cells (10X) after 48 h of infection with H1N1, Influenza A and p-flu Influenza virus (1/64 HA unit) demonstrates cytopathic effect in A549 cell.

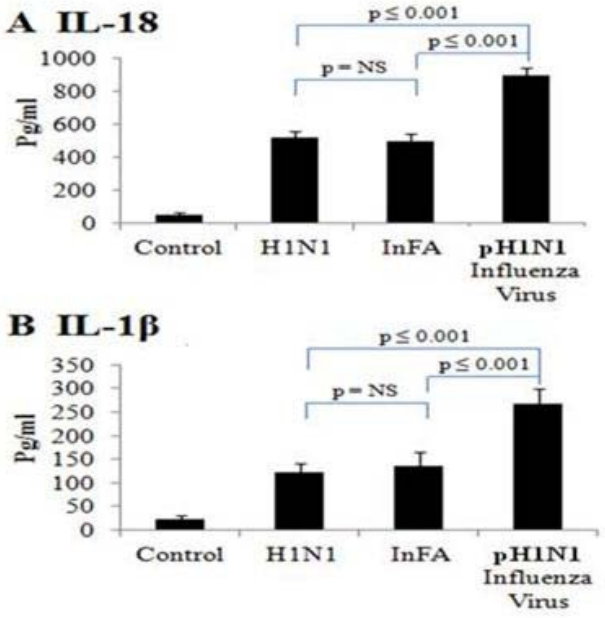

Figure 2: Anti-inflammatory properties of HIN1, Influenza A and pH1N1 Influenza virus. Expression levels of (A) IL-1β and (B) IL-18 were measured by ELISA in A549 cellular

protein after HIN1, Influenza A and pH1N1 Influenza virus infection in A549 cell. Data are expressed as mean of triplicate samples ± S.E. Significant up-regulation in expression of IL-1β and IL-18 in pH1N1 Influenza virus infection was observed in H1N1-infected cells

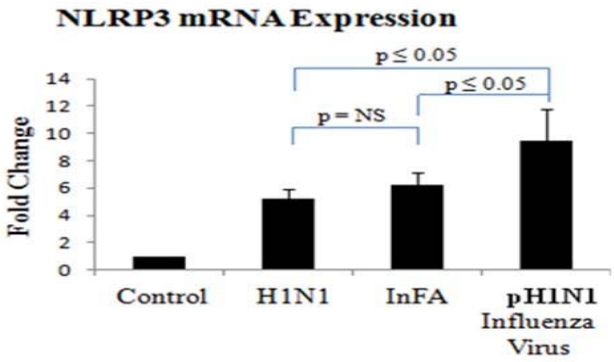

Figure 3: Regulation of NLRP3 mRNA level in HIN1, Influenza A and pH1N1 Influenza virus infection in A549 cell. Graphic presentation of NLRP3 mRNA levels by real-time PCR analysis showed that significant up regulation in p-flu Influenza virus infection. The relative mRNA levels were determined after normalization with HPRT. The data are expressed as mean of triplicate samples ± S.E with P≤ 0.01, P≤0.001 versus normal control**.**

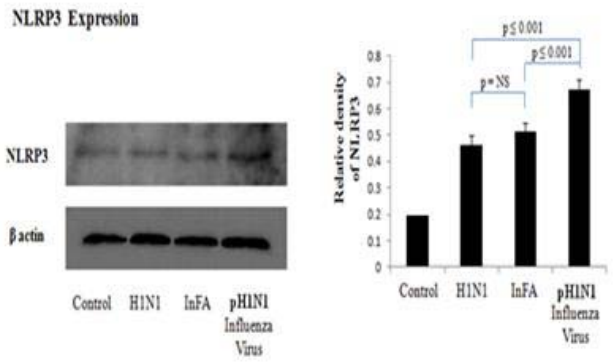

Figure 4: Regulation of NLRP3 protein HIN1, Influenza A and pH1N1 Influenza virus infection in A549 cell Representative Western blot results and graphic presentation showed the relative NLRP3 protein levels were normalized to β actin. The data are expressed as mean of triplicate samples ± S.E with P≤ 0.01, P≤0.001 versus normal control**.**

Table 1: Primer sequences for RT- PCR

Target gene	Direction	Sequence
InfA	Forward	GAC CRA TCC TGT TAC CTC TGA C
	Reverse	AGG GCA TTY TGG ACA AAK CGT C TA
	Probe	FAM-TGC AGT CCT CGC TCA CTG GGC ACG-MGB
pH1N1	Forward	GTG CTA TAA ACA CCA GCC TYC CA
	Reverse	CGG GAT ATT CCT TAA TCC TGT RGC
	Probe	FAM-CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A-MGB
RnaseP	Forward	AGA TTT GGA CCT GCG AGC G
	Reverse	GAG CGG CTG TCT CCA CAA GT
	Probe	FAM-TTC TGA CCT GAA GGC TCT GCG CG-MGB

RESULTS

The phase contrast picture of A549 cells (10X) demonstrated the cytopathic effect after 48 h of infection with H1N1, Influenza A and p H1N1 influenza virus (at constant viral titer that of 1/64 HA unit) (Fig. 1) and same HA titer was found in cell culture supernatant at this end point. This shows that there was no difference in the rate of replication of these viruses. The effect of various influenza virus strains on the mature (active) forms of IL-18 and IL-1β cytokines were estimated by OptEIA human ELISA assay [Figure 2(A) and 2(B)]. The result showed different patterns of IL-18 and IL-1β expression in the cell culture supernatant. The expression of IL-18 was observed to be average of 10 fold up regulation in H1N1 and Influenza A virus infected A549 cells but there was 17 fold up-regulation in pH1N1 Influenza virus. There was no significant difference between H1N1 and Influenza A virus infected A549 cells but statistically significant up-regulation was seen in pH1N1 Influenza virus (p ≤ 0.001) as compared to H1N1 and Influenza A virus infected A549 cells [Figure 2(A)]. The expression of IL-1β was observed to be average of 5 fold up regulation in H1N1 and Influenza A virus infected A549 cells but there was 11 fold up regulation in pH1N1 Influenza virus. Similar to IL-18 there was no significant difference between H1N1 and Influenza A virus infected A549 cells but significant up-regulation was seen in pH1N1 Influenza virus (p ≤ 0.001) compared to H1N1 and Influenza A virus infected A549 cells [Figure 2(B)].

Further RNA extracted from A549 cells to check the NLRP3 mRNA transcript level in A549 cells infected with H1N1, Influenza A and pH1N1 Influenza virus. The expression of NLRP3 mRNA was observed to be average of 5 and 6 fold up regulation in A549 cells infected with H1N1 and Influenza A virus but there was 9 fold up regulation in pH1N1 Influenza virus infection. There was no significant difference in NLRP3 mRNA transcript level between H1N1 and Influenza A virus infected A549 cells but significant up regulation was seen in p H1N1 Influenza virus (p ≤ 0.001) compared to H1N1 and Influenza A virus infected A549 cells (Figure 3). This was further confirmed by NLRP3 expression at protein level by western blot. The expression of NLRP3 was observed to be average of 2 and 2.5 folds higher in A549 cells infected with H1N1 and Influenza A virus but there was 9 fold up regulation in p H1N1 Influenza virus infection. Similarly it was found that there was no significant difference in NLRP3 mRNA transcript level between H1N1 and Influenza A virus infected A549 cells but significant up regulation in pH1N1 Influenza virus (p ≤ 0.001) compared to H1N1 and Influenza A virus infected A549 cells (Figure 4)

DISCUSSION

The present study showed that the replication rate of different influenza virus strains (H1N1, Influenza A and pH1N1 Influenza virus) in A549 cell culture was similar as depicted by HA titre and it appears that the various inflammatory responses of these viruses could be

predictors of severity. The viral replication and clearance can be arrested by adequate immune responses launched by enhanced circulating cytokine/chemokine by recognition through families of pathogen recognition receptors (PRRs).[22] The role of innate immunity against the pathogenic influenza viruses is complex and intricate. Our results indicate that NLRP3 which belongs to the family of PRRs, nucleotide-binding domain and leucine-rich-repeat containing (NLR) proteins was activated in response to these viral pathogens *in vitro* A549 cells. NLRP3 forms a multi-protein inflammasome complex, after its expressions leads to the maturation of several key pro-inflammatory cytokines, such as IL-1β and IL-18.[23-27] In this study, both IL-1β and IL-18 were expressed within the normal A549 human lung epithelium cell and then released into the medium. IL-1β and IL-18 also have very important roles for antimicrobial host defence. IL-1α and IL-1β, binds and activates the same receptor[28] and release of other proinflammatory cytokines such as TNF and IL-6, also induce a Th17 bias in the cellular adaptive responses[29], while IL-18 is essential for the induction of IFNγ and Th1 responses.[30] Through these mechanisms, cytokines of the IL-1 family are a crucial component of the host defence against infections. Viral mediated by the NLR containing inflammasomes (and independent of the TLRs) that induce cleavage of cytokine precursors into the active IL-1β and IL-18.[31,32]

The components necessary for mounting of a rapid protective inflammatory response via inflammasome activation were all expressed in the A549 human lung epithelium cells. Our finding of induced IL-1β and IL-18 is supporting the hypothesis that the airway epithelium is an important sentinel in the innate immune response and is primed for a rapid response when exposed to various Influenza virus strains but has yet to undergo inflammasome activation. The findings suggest that increased expression pattern of NLRP3 inflammasome; IL-1β and IL-18 activation could possibly be related to the severity of disease.[33-35]

Thus, our findings demonstrate that there are various licensing requirements for NLRP3 inflammasome-dependent processing and secretion of IL-1β and IL-18. Notably, these studies were performed using A549 cells, type II alveolar cells that represent the distal bronchiolar and alveolar epithelium of humans to evaluate the host cell response to H1N1 infection. Thus, these findings have important implications in understanding the mechanisms linked to different Influenza virus mediated disease pathogenesis and treatment. Thus addressing questions regarding Influenza virus recognition through NLRs would aid as a clue to make more effective vaccines and therapeutics against impending pH1N1 pandemics.

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