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KLF6 and iNOS regulates apoptosis during respiratory syncytial virus infection

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Abstract

Human respiratory syncytial virus (RSV) is a highly pathogenic lung-tropic virus that causes severe respiratory diseases. Enzymatic activity of inducible nitric oxide (iNOS) is required for NO generation. Although NO contributes to exaggerated lung disease during RSV infection, the role of NO in apoptosis during infection is not known. In addition, host trans-activator(s) required for iNOS gene expression during RSV infection is unknown. In the current study we have uncovered the mechanism of iNOS gene induction by identifying kruppel-like factor 6 (KLF6) as a critical transcription factor required for iNOS gene expression during RSV infection. Furthermore, we have also uncovered the role of iNOS as a critical host factor regulating apoptosis during RSV infection.

Keywords

Respiratory syncytial virus; nitric oxide; apoptosis; transcription factor; kruppel-like factor 6; inducible nitric oxide synthase

1. Introduction

Respiratory syncytial virus (RSV) is a lung tropic negative-sense, single-stranded RNA virus that causes severe respiratory diseases like pneumonia and bronchiolitis among infants, children and elderly (1, 2, 3, 4). RSV productively infects both lung epithelial cells and immune cells (e.g. macrophages) present in the respiratory tract. Innate immune response constitutes the first line of defense against pathogens, including respiratory viruses. Among the various innate immune mediators, nitric oxide (NO) plays a pivotal role in combating pathogen invasion and spread (5–10). Although NO is an important component of host defense, it can also exaggerate the disease pathology associated with infection. The ability of NO to launch a hyper-inflammatory response contributes to tissue damage (11). Action of NO induced inflammatory mediators and/or NO mediated apoptosis of target cells is responsible for exacerbating disease pathology. During virus infections, NO could play a dual role by acting as an antiviral molecule and at the same time contributing to disease progression by inducing tissue damage (12). Several studies have shown that RSV infection

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results in NO production due to induction of inducible nitric oxide synthase (iNOS) gene (13, 14, 15). However, the mechanism responsible for iNOS gene induction during RSV infection is not known. In that regard, cellular transcription factor(s) required for iNOS gene expression during RSV infection has not been identified. Interestingly, NO produced in the airway during RSV infection contributes to enhanced lung disease pathology associated with infection (14). Inhibition of iNOS activity in RSV infected mice significantly reduced clinical disease associated with RSV infection since diminished inflammation in the lung and reduced hyper-responsiveness was observed in iNOS inhibited animals (14). During non-infectious respiratory diseases [e.g. asthma and chronic obstructive pulmonary disease (COPD)] NO mediated apoptosis in the respiratory tract significantly contributes to enhanced inflammation and tissue damage (11, 16). Although RSV induce NO production (13, 14, 15), the role of NO in apoptosis during infection is not known. Moreover, the transcription factor(s) responsible for iNOS gene induction during RSV infection has not been identified yet.

Recently, we identified triple zinc finger containing DNA binding transcription factor Kruppel-like factor 6 (KLF6) (17) as a transcription factor required for iNOS gene induction during influenza A virus (flu) infection (18). Moreover, we demonstrated an important role of iNOS/NO in regulating apoptosis following flu infection (18). Since RSV is also a negative-sense, single-stranded RNA respiratory virus similar to flu, we studied the role of KLF6 in iNOS gene expression in RSV infected cells and investigated the contribution of iNOS/NO in apoptotic induction during RSV infection. Our studies have demonstrated that similar to flu, iNOS induction and subsequent production of NO contributes to apoptosis during RSV infection and KLF6 is a key transactivator of iNOS gene following RSV infection.

2. Material and methods

2.1. Virus and Cell culture

RSV (A2 strain) was propagated in CV-1 cells (19, 20, 21, 22). RSV was purified by centrifugation (two times) on discontinuous sucrose gradients (23). A549 cells were maintained as described previously (19, 20, 21, 22). Bone marrow-derived macrophages (BMDMs) were obtained from femurs and tibias of mice and were cultured for 6–8 days as described earlier (18, 19).

2.2. Generation of stable A549 cells expressing shRNAs

Stable KLF6 shRNA expressing cells were generated as described previously (18). A549 cells transduced with either scrambled shRNA (control) or KLF6-specific shRNA expressing lentivirus (Santa Cruz Biotechnology) were selected in the presence of puromycin dihydrochloride. The antibiotic resistant cells were expanded to generate a population of stable cells expressing either scrambled shRNA or KLF6-specific shRNA.

2.3. Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from A549 cells using Tri Reagent (Invitrogen). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed as described earlier (18, 19, 20). The primers used to detect the indicated genes by RT-PCR are shown below.

GAPDH forward, 5'-GTCAGTGGTGGACCTGACCT, *GAPDH reverse*, 5'-AGGGGTCTACATGGCAACTG,

Human iNOS forward, 5'- TCCGAGGCAAACAGCACATTCA, *iNOS reverse*, 5'-GGGTTGGGGTGTGGTGATGT,

Human KLF6 forward, 5'- CTCTCAGCCTGGAAGCTTTTAGCCTAC, KLF6 reverse, 5'-ACAGCTCCGAGGAAGTTTCTCCCA,

2.4. Chromatin immuno-precipitation (ChIP) assay

Standard ChIP assay protocol was followed (18, 24). Following isolation of the chromatin from infected human A549 cells and mouse BMDMs (after cross-linking with formaldehyde), it was immuno-precipitated with either human or mouse specific anti-KLF6 antibody or isotype matched control IgG (Santa Cruz Biotechnology). Human and mouse iNOS promoter-specific PCR primer pairs were used to analyze the antibody precipitated DNA. As a negative control, PCR primer pairs corresponding to iNOS promoter region that does not possess consensus KLF6 binding site was used. Primers used for the ChIP assay are shown in Table-1.

2.5. Viral infection of cells

A549 cells and BMDMs were infected with purified RSV. Following adsorption for 1.5h at 37°C, cells were washed and the infection was continued in the presence of serum containing medium. For some experiments, A549 cells were pre-treated with either water (vehicle) or L-NIL (Cayman Chemical) (200 µM) for 2h before infection. Infection was continued in the presence of L-NIL.

2.6. Nitric oxide quantification

NO production from infected cells was determined by measuring nitrite levels in the medium supernatant as described previously (18, 25). Medium supernatant collected from cells were mixed with Griess reagent for 10 min at room temperature. The absorbance (at 543 nm) was measured using a micro-plate reader.

2.7. Luciferase assay

Luciferase assay was performed as described previously (18, 19, 21). A549 cells were transfected (using Lipofectamine 2000 from Invitrogen) with pRL-null-renilla luciferase and luciferase (firefly) reporter gene fused to human iNOS promoter (iNOS-luc). At 24h post-transfection, cells were infected with RSV. At 24h post-infection, cells lysate was used to measure luciferase activity by using Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was normalized by measuring expression of renilla luciferase. Luciferase units were measured by standard methodology and presented as fold activation of luciferase activity.

2.8. Apoptosis assay

Cells infected with RSV were examined for apoptosis by annexin V labeling, using annexin V/propidium iodide apoptosis detection kit (BioVision) (18, 22). Apoptosis of RSV infected cells were also examined by TUNEL assay. TUNEL assay was performed as described previous by us (18). Formalin fixed BMDMs were utilized for TUNEL assay using DeadEnd Colorimetric TUNEL system (Promega, WI, USA). Digital images of the TUNEL stained cells were captured by light microscopy. The images were utilized to count the number of TUNEL positive cells by using Image J software from NIH (<http://rsbweb.nih.gov/ij/>) as described previously (18). Gross apoptotic area was expressed as pixels/micron (normalized to number of cells in a field) and this value was used to calculate percentage apoptotic area.

3. Results

3.1. KLF6 expression is required for nitric oxide (NO) production during RSV infection

The role of KLF6 during RSV infection was investigated by silencing KLF6 expression via shRNA in human lung epithelial A549 cells. A549 cells are routinely used as model type-II human alveolar epithelial cells and the alveolar cells are infected by RSV during productive infection of human airway. KLF6 silencing was achieved by using lentivirus mediated shRNA delivery system to generate stable A549 cells lacking KLF6. Stable A549 cells derived after transduction with scrambled shRNA expressing lentivirus was used as a control. RT-PCR analysis revealed efficient silencing of KLF6 since drastic loss of KLF6 expression was observed in KLF6 shRNA transduced stable A549 cells (Fig. 1A).

We next utilized control and KLF6 silenced cells to investigate whether KLF6 play any role in NO production following RSV infection. Control and KLF6 silenced cells were infected with RSV. At 12h post-infection, medium supernatant was collected to measure nitrite (the end product of nitric oxide) levels by Griess reagent. Drastic reduction in NO levels was noted in RSV infected KLF6 silenced cells, compared to infected control cells (Fig. 1B). This result suggested that KLF6 is an essential host gene involved in NO production during RSV infection.

3.2. iNOS gene expression is regulated by KLF6 during RSV infection

To further explore the mechanism regulating KLF6 dependent NO production, we next examined the role of KLF6 in iNOS gene expression. Control and KLF6 silenced cells were infected with RSV for 24h. RT-PCR analysis was performed to deduce iNOS mRNA levels in these cells. RT-PCR analysis demonstrated diminished levels of iNOS transcripts in KLF6 silenced cells compared to control cells (Fig. 2A). This result indicated that KLF6 regulates iNOS gene expression at the transcriptional level.

To further test the possible role of KLF6 in positively transactivating iNOS promoter . during RSV infection, we utilized luciferase reporter gene fused to human iNOS promoter (iNOS-luc). Control and KLF6 silenced cells were transfected with luciferase expressing plasmids. At 24h post-transfection, cells were infected with RSV and at 12h post-infection, luciferase expression was monitored. Luciferase assay revealed reduction in luciferase activity in RSV infected cells that lack KLF6 expression (Fig. 2B). These results showed that KLF6 directly regulates iNOS promoter during RSV infection. Thus, KLF6 may function as an important transcription factor regulating iNOS gene expression during RSV infection.

3.3. KLF6 associates with iNOS promoter during RSV infection of epithelial cells

The role of KLF6 in iNOS gene transactivation during RSV infection was further validated by performing chromatin immuno-precipitation (ChIP) assay, an assay that can detect direct association of trans-acting factors with gene promoters. Human lung epithelial A549 cells were either mock infected or infected with RSV. At 6h, 12h and 24h post-infection time-periods, chromatin from these cells was immuno-precipitated by using either isotype matched control IgG (negative control) or anti-KLF6 antibody. PCR with primers corresponding to KLF6 consensus binding site on human iNOS promoter (25) was performed to analyze KLF6-DNA binding complex. As a negative control, a primer specific for non-KLF6 binding region on human iNOS promoter element (an upstream region in the human iNOS gene devoid of the KLF6 responsive region) was used. KLF6 binding to human iNOS gene was not detected in mock infected cells (Fig. 3). However, RSV infection led to binding of KLF6 to the human iNOS promoter (Fig. 3). It is interesting to note that optimal KLF6 association with iNOS promoter occurred at 12h postinfection, which

corresponds to the time-frame at which iNOS induction and NO production was observed during RSV infection of A549 cells (Fig. 1, 2). The binding of KLF6 to iNOS promoter was specific since immuno-precipitation with control IgG did not yield any amplified product. Similarly, amplification with primers that do not correspond to KLF6 binding site on human iNOS promoter did not generate any amplified product. These results demonstrated that KLF6 is a key transcription factor required for iNOS gene expression since it can directly associate with iNOS promoter during RSV infection.

3.4. Regulation of apoptosis by KLF6 during RSV infection

We have identified KLF6 as a critical host factor required for iNOS gene expression and subsequent NO production following RSV infection (Fig. 1, 2, 3). One of the mechanisms responsible for NO mediated tissue damage is induction of apoptosis. Since apoptosis in the respiratory tract play a crucial role in determining disease severity during virus infection (26, 27, 28, 29), we next examined the role of KLF6 in regulating apoptosis during RSV infection. For these studies, we infected control and KLF6 silenced cells with RSV. At 48h post-infection, the apoptotic status of the cells was determined by evaluating annexin V staining, which signifies early apoptosis. Annexin V staining assay showed significantly reduced apoptosis in RSV infected KLF6 silenced cells compared to infected control cells (Fig. 4). These results demonstrated that KLF6 regulates apoptosis during RSV infection.

3.5. iNOS expression and activity is required for apoptosis during RSV infection

We speculated that iNOS (by virtue of producing NO from infected cells) may contribute to apoptosis during RSV infection since KLF6, an iNOS gene trans-activation factor (Fig. 1, 2, 3) is required for apoptosis (Fig. 4). Moreover, the contribution of NO (and iNOS) in promoting apoptosis during RSV infection is unknown. In order to assess the role of iNOS, we inhibited the enzymatic activity of iNOS with specific iNOS inhibitor *N^G-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL)* (30). A549 cells were pre-treated with either vehicle (water) or L-NIL (200 μ M) for 2h, followed by RSV infection in the presence of water (control) or L-NIL. At 48h post-infection, apoptosis status was investigated by annexin V staining assay. iNOS is required for apoptosis, since blocking iNOS activity significantly diminished apoptosis of RSV infected cells (Fig. 5). Approximately 60% reduction in apoptosis was noted in L-NIL treated cells compared to control cells. These results uncovered the role of iNOS in inducing apoptosis during RSV infection.

RSV productively infects both human and mouse macrophages and they play an essential role in host defense against RSV infection. Therefore, the requirement of iNOS for optimal apoptosis during RSV infection was further confirmed by using primary bone marrow derived macrophages (BMDMs). BMDMs were isolated from wild type (WT) and iNOS knock-out (KO) mice. Apoptotic status of RSV infected (48h post-infection) WT and iNOS KO BMDMs was evaluated by annexin V staining. Similar to A549 cells, we observed that iNOS is a critical player regulating apoptosis following RSV infection of primary macrophages. Significant reduction (40% reduction in apoptosis) in apoptosis was noted in RSV infected iNOS KO BMDMs compared to WT BMDMs (Fig. 6A, B, C). The apoptosis status in WT and iNOS KO was further validated by TUNEL assay (18). Similar to annexin V staining, TUNEL assay revealed diminished apoptosis in RSV infected iNOS KO BMDMs compared to WT cells (Fig. 6D). Thus our studies have demonstrated that iNOS expression/activity is required for optimal apoptosis during RSV infection.

4. Discussion

Expression of iNOS gene is tightly regulated because its product, NO can exaggerate disease phenotype by promoting tissue damage by inducing apoptosis and/or enhancing local

inflammation (5–12). Due to this reason, NO produced during virus infection can also significantly contribute to development of disease state. Infection with lung-tropic viruses like RSV and influenza A virus results in NO production due to induction of iNOS gene expression (13, 14, 15, 30, 31, 32, 33). The resulting NO produced in the respiratory tract of influenza A virus and RSV infected host contributes to exacerbated lung disease due to enhanced damage of the lung tissue (14, 31, 32, 33). NO produced in the airway during RSV infection contributes to RSV-associated clinical disease since inhibition of iNOS activity in mice lungs during RSV infection resulted in diminished lung inflammation and reduced hyper-responsiveness (14). Although blocking NO production in RSV infected mice drastically improved lung disease state, it is not clear what role NO play during human RSV infection. In that regard, decreased NO levels was noted in infants and children suffering from RSV-mediated bronchiolitis and exhaled NO has been used as an indicator of the severity associated with RSV-induced lung disease (34, 35). Further systematical studies with mice models and human clinical samples are required to investigate the relationship of KLF6/NO in RSV-induced disease in mice and humans.

Although iNOS is a key host factor determining disease severity, it is not known whether iNOS is required for apoptosis during RSV infection. Moreover, the mechanism by which RSV induces iNOS gene expression during infection is not known. Among the respiratory viruses, iNOS gene expression mechanism has been investigated only for flu. Previous studies have shown that “inflammatory” (e.g. $\text{NF-}\kappa\text{B}$) and “non-inflammatory” (e.g. KLF6) transcription factors are required for iNOS expression during flu infection (18, 30). In that context, we have uncovered two mechanisms associated with iNOS gene expression (and NO production) during RSV infection. Our studies demonstrated a role of iNOS in regulating apoptosis during RSV infection. Furthermore, we have identified a “non-inflammatory” transcription factor KLF6 as a trans-activator of iNOS gene during RSV infection. We propose that RSV infection promotes binding of KLF6 to iNOS promoter leading to expression of iNOS gene/protein and NO generated by iNOS is a key contributing factor for induction of apoptosis during infection.

Apoptosis is one of the essential contributing factors responsible for enhanced pathogenesis and exaggerated lung-injury/disease associated with RSV and flu infection (26, 27, 28, 29, 36). Apoptosis of lung epithelial cells during RSV infection results in their sloughing and these cells aggregate along with fibrin, mucin to form plugs associated with airway obstruction, a condition prevalent among RSV infected infants and children (37, 38). Apart from airway obstruction, a high degree of apoptosis of both lung epithelial cells and immune cells (e.g. macrophages) during respiratory virus infection results in delayed clearance of dead cells from the airway lumen, which culminates in induction of necrosis and subsequent inflammation of the airway (27).

Various mechanisms are involved in the induction of apoptosis following RSV infection. p53 (39), TRAIL (36, 40) and Fas (41) mediates and regulates apoptosis of RSV infected cells. Since early apoptosis is detrimental for viral growth, RSV utilizes various molecules/ pathways including activation of $\text{NF-}\kappa\text{B}$ (42, 43) and reduction in p53 protein levels (44) to suppress apoptosis during infection. Endoplasmic reticulum resident caspase-12 has also been implicated during apoptosis of RSV infected cells (45). NO mediated apoptosis triggers enhanced inflammation and tissue damage (11, 16). Although RSV infection results in iNOS gene expression and NO production (13, 14, 15), the role of NO in apoptosis during RSV infection has not been examined. Our studies have illustrated an essential role of iNOS/NO in inducing apoptosis during RSV infection of epithelial cells and macrophages. Furthermore, we have also identified KLF6 as a critical transcription factor required for iNOS gene expression during RSV infection.

It is important to mention that a study have shown that TGF- β (1) can negatively regulate NO production from lung epithelial A549 cells (46). Negative regulation by TGF- β (2) was also noted in macrophages (47, 48). We have previously shown that KLF6 is required for TGF- β production from RSV infected cells (49). Therefore, one may envision that KLF6 controls two mechanisms that can auto-regulate itself via paracrine/autocrine mechanism. However, it is possible that such regulation is temporal in nature. It was shown that pre-treatment of cells with TGF- β results in down-regulation of NO production following treatment with cytokines (IL-1 β , TNF- α and IFN- γ) (46). It is not know whether TGF- β will act in a similar fashion if it is added after iNOS gene expression. In that regard, we have demonstrated that while iNOS is induced by KLF6 in RSV infected cells early (within 6h–12h post-infection) (Figs. 1B, 3), TGF- β gene expression (KLF6 mediated) does not occur before 16h (49). Thus in RSV infected cells, iNOS is induced prior to TGF- β expression, and it would be interesting to examine whether in that scenario TGF- β could negatively regulate NO production from RSV infected cells. In the future we will study in depth the relationship between KLF6 mediated TGF- β production vs. KLF6-dependend NO production from RSV infected cells.

Kruppel-like factor (KLF) family of transcription factors regulates various biological processes like cellular growth, proliferation and differentiation (17). KLF6 has been identified and characterized as a critical trans-activator of genes in multiple tissues. Expression of genes like E-cadherin, TGF- β receptors, collagen, urokinase, plasminogen activator requires transactivating function of KLF6 (50, 51, 52, 53). Previously it was reported that KLF6 regulates human iNOS gene expression following stress-related pathophysiological stimuli like heat shock, serum starvation, and hypoxia (54). We have shown that KLF6 also play a critical role in regulating host gene expression during virus infection. KLF6 is required for iNOS and TGF- β gene expression following flu and RSV infection, respectively (18, 49).

In summary, we have identified KLF6 as a critical transcription factor required for iNOS gene expression during RSV infection. Moreover, our studies have revealed an essential role of iNOS (via NO production) in contributing to induction of apoptosis during RSV infection

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Abbreviations

RSV	respiratory syncytial virus
iNOS	inducible nitric oxide synthase
NO	nitric oxide
KLF6	Kruppel-like factor 6

BMDMs	bone marrow-derived macrophages
WT	wild type
KO	knock-out
ChIP	Chromatin immuno-precipitation
L-NIL	N ^G -(1-iminoethyl)-L-lysine dihydrochloride

References

- Hall CB. Respiratory syncytial virus and parainfluenza virus. *N. Engl. J. Med.* 2001; 344:1917–1928. [PubMed: 11419430]
- Collins, PL.; McIntosh, K.; Chanock, RM. Respiratory syncytial virus. Knipe, DM.; Howley, PM., editors. Philadelphia: Fields Virology, Lippincott Williams & Wilkins; 2007. p. 1601-1640.
- Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med.* 2005; 352:1749–1759. [PubMed: 15858184]
- Ruuskanen O, Lahti E, Jennings LC, Murdoch DR. Viral pneumonia. *Lancet.* 2011; 377:1264–75. [PubMed: 21435708]
- Pautz A, Art J, Hahn S, Nowag S, Voss C, Kleinert H. Regulation of the expression of inducible nitric oxide synthase. *Nitric Oxide.* 2010; 23:75–93. [PubMed: 20438856]
- Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses. *Clin Microbiol Rev.* 2011; 24:210–29. [PubMed: 21233513]
- O'Reilly P, Hickman-Davis JM, McArdle P, Young KR, Matalon S. The role of nitric oxide in lung innate immunity: modulation by surfactant protein-A. *Mol Cell Biochem.* 2002; 234–235:39–48.
- Richardson AR, Libby SJ, Fang FC. A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity. *Science.* 2008; 319:1672–1676. [PubMed: 18356528]
- Zheng S, De BP, Choudhary S, Comhair SA, Goggans T, Slee R, Williams BR, Pilewski J, Haque SJ, Erzurum SC. Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis. *Immunity.* 2003; 18:619–630. [PubMed: 12753739]
- Zheng S, Xu W, Bose S, Banerjee AK, Haque SJ, Erzurum SC. Impaired nitric oxide synthase-2 signaling pathway in cystic fibrosis airway epithelium. *Am J Physiol Lung Cell Mol Physiol.* 2004; 287:L374–L381. [PubMed: 15107292]
- Bove PF, van der Vliet A. Nitric oxide and reactive nitrogen species in airway epithelial signaling and inflammation. *Free Radic Biol Med.* 2006; 41:515–527. [PubMed: 16863984]
- Akaike T, Maeda H. Nitric oxide and virus infection. *Immunology.* 2000; 101:300–308. [PubMed: 11106932]
- Song W, Liu G, Bosworth CA, Walker JR, Megaw GA, Lazrak A, Abraham E, Sullender WM, Matalon S. Respiratory syncytial virus inhibits lung epithelial Na⁺ channels by up-regulating inducible nitric-oxide synthase. *J Biol Chem.* 2009; 11:7294–7306. [PubMed: 19131335]
- Stark JM, Khan AM, Chiappetta CL, Xue H, Alcorn JL, Colasurdo GN. Immune and functional role of nitric oxide in a mouse model of respiratory syncytial virus infection. *J Infect Dis.* 2005; 191:387–395. [PubMed: 15633098]
- Kao YJ, Piedra PA, Larsen GL, Colasurdo GN. Induction and regulation of nitric oxide synthase in airway epithelial cells by respiratory syncytial virus. *Am J Respir Crit Care Med.* 2001; 163:532–539. [PubMed: 11179135]
- Maa SH, Wang CH, Liu CY, Lin HC, Huang KH, Kuo HP. Endogenous nitric oxide downregulates the Bcl-2 expression of eosinophils through mitogen-activated protein kinase in bronchial asthma. *J Allergy Clin Immunol.* 2003; 112:761–767. [PubMed: 14564359]
- McConnell BB, Yang VW. Mammalian Krüppel-like factors in health and diseases. *Physiol Rev.* 2010; 90:1337–1381. [PubMed: 20959618]

18. Mgbemena V, Segovia J, Chang T, Tsai S-Y, Cole GT, Hung C-Y, Bose S. S, Transactivation of inducible nitric oxide synthase gene by Krüppel-like factor 6 regulates apoptosis during influenza A virus infections. *J Immunol.* 2012; 189:606–615. [PubMed: 22711891]
19. Sabbah A, Chang T, Harnack R, Frohlich V, Dube PH, Tominaga K, Xiang Y, Bose S. Activation of innate immune antiviral response by Nod2. *Nature. Immunol.* 2009; 10:1073–1080. [PubMed: 19701189]
20. Kota S, Sabbah A, Chang TH, Harnack R, Xiang Y, Meng Y, Bose S. Role of human beta-defensin-2 during tumor necrosis factor-alpha/NF-kB mediated innate anti-viral response against human respiratory syncytial virus. *J. Biol. Chem.* 2008; 283:22417–22429. [PubMed: 18567888]
21. Bose S, Kar N, Maitra R, Didonato J, Banerjee AK. Temporal activation of NF- κ B regulates an interferon independent innate anti-viral response against cytoplasmic RNA viruses. *Proc. Natl. Acad. Sci. USA.* 2003; 100:10890–10895. [PubMed: 12960395]
22. Echchgadda I, Kota S, DeLa Cruz I, Sabbah A, Chatterjee B, Bose S. Anticancer oncolytic activity of respiratory syncytial virus. *Cancer. Gene. Ther.* 2009; 16:923–935. [PubMed: 19444304]
23. Ueba O. Respiratory syncytial virus - concentration and purification of the infectious virus. *Acta. Med. Okayama.* 1978; 32:265–272. [PubMed: 153087]
24. Yao H, Hwang J, Moscat J, Diaz-Meco MT, Leitges M, Kishore N, Li X, Rahman I. Protein Kinase C₃ Mediates Cigarette Smoke/Aldehyde- and Lipopolysaccharide-induced Lung Inflammation and Histone Modifications. *J. Biol. Chem.* 2010; 285:5405–5416. [PubMed: 20007975]
25. Kolodziejeki PJ, Koo JS, Eissa NT. Regulation of inducible nitric oxide synthase by rapid cellular turnover and cotranslational down-regulation by dimerization inhibitors. *Proc Natl Acad Sci U S A.* 2004; 101:18141–18146. [PubMed: 15601772]
26. Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K, Velozo L, Jafri H, Chavez-Bueno S, Ogra PL, McKinney L, Reed JL, Welliver Sr RC. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. *J Infect Dis.* 2007; 195:1126–1136. [PubMed: 17357048]
27. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest.* 2006; 129:1673–1682. [PubMed: 16778289]
28. Zhang C, Yang Y, Zhou X, Liu X, Song H, He Y, Huang P. Highly pathogenic avian influenza A virus H5N1 NS1 protein induces caspase-dependent apoptosis in human alveolar basal epithelial cells. 2010; 7 *Viro J.* 51.
29. Herold S, Steinmueller M, von Wulffen W, Cakarova L, Pinto R, Pleschka S, Mack M, Kuziel WA, Corazza N, Brunner T, Seeger W, Lohmeyer J. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J Exp Med.* 2008; 205:3065–3077. [PubMed: 19064696]
30. Uetani K, Der SD, Zamanian-Daryoush M, de La Motte C, Lieberman BY, Williams BR, Erzurum SC. Central role of double-stranded RNA-activated protein kinase in microbial induction of nitric oxide synthase. *J Immunol.* 2000; 165:988–996. [PubMed: 10878375]
31. Karupiah G, Chen JH, Mahalingam S, Nathan CF, MacMicking JD. Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonitis in nitric oxide synthase 2-deficient mice. *J Exp Med.* 1998; 188:1541–1546. [PubMed: 9782132]
32. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci U S A.* 1996; 93:2448–2453. [PubMed: 8637894]
33. Burggraaf S, Bingham J, Payne J, Kimpton WG, Lowenthal JW, Bean AG. Increased inducible nitric oxide synthase expression in organs is associated with a higher severity of H5N1 influenza virus infection. *PLoS One.* 2011; 6:e14561. [PubMed: 21283521]
34. Gadish T, Soferman R, Merimovitch T, Fireman E, Sivan Y. Exhaled nitric oxide in acute respiratory syncytial virus bronchiolitis. *Arch Pediatr Adolesc Med.* 2010; 164:727–731. [PubMed: 20679163]

35. Kato M, Hayashi Y, Kimura H. Oxygen radicals in inflammation and allergy related to viral infections. *Curr Drug Targets Inflamm Allergy*. 2005; 4:497–501. [PubMed: 16101528]
36. Bem RA, Bos AP, Wösten-van Asperen RM, Bruijn M, Lutter R, Sprick MR, van Woensel JB. Potential role of soluble TRAIL in epithelial injury in children with severe RSV infection. *Am J Respir Cell Mol Biol*. 2010; 42:697–705. [PubMed: 19635930]
37. Aherne W, Bird T, Court SD, Gardner PS, McQuillin J. Pathological changes in virus infections of the lower respiratory tract in children. *J. Clin. Pathol*. 1970; 23:7–18. [PubMed: 4909103]
38. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod. Pathol*. 2007; 20:108–119. [PubMed: 17143259]
39. Eckardt-Michel J, Lorek M, Baxmann D, Grunwald T, Keil GM, Zimmer G. The fusion protein of respiratory syncytial virus triggers p53-dependent apoptosis. *J Virol*. 2008; 82:3236–3249. [PubMed: 18216092]
40. Kotelkin A, Prikhod'ko EA, Cohen JI, Collins PL, Bukreyev A. Respiratory syncytial virus infection sensitizes cells to apoptosis mediated by tumor necrosis factor-related apoptosis-inducing ligand. *J Virol*. 2003; 77:9156–9172. [PubMed: 12915532]
41. O'donnell DR, Milligan L, Stark JM. Induction of CD95 (Fas) and apoptosis in respiratory epithelial cell cultures following respiratory syncytial virus infection. *Virology*. 1999; 257:198–207. [PubMed: 10208933]
42. Bitko V, Shulyayeva O, Mazumder B, Musiyenko A, Ramaswamy M, Look DC, Barik S. Nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by an NF-kappaB-dependent interferon-independent mechanism and facilitate virus growth. *J Virol*. 2007; 81:1786–1795. [PubMed: 17151097]
43. Thomas KW, Monick MM, Staber JM, Yarovsky T, Carter AB, Hunninghake GW. Respiratory syncytial virus inhibits apoptosis and induces NF-kappa B activity through a phosphatidylinositol 3-kinase-dependent pathway. *J Biol Chem*. 2002; 277:492–501. [PubMed: 11687577]
44. Groskreutz DJ, Monick MM, Yarovsky TO, Powers LS, Quelle DE, Varga SM, Look DC, Hunninghake GW. Respiratory syncytial virus decreases p53 protein to prolong survival of airway epithelial cells. *J Immunol*. 2007; 179:2741–2747. [PubMed: 17709487]
45. Bitko V, Barik S. An endoplasmic reticulum-specific stress-activated caspase (caspase-12) is implicated in the apoptosis of A549 epithelial cells by respiratory syncytial virus. *J Cell Biochem*. 2001; 80:441–454. [PubMed: 11135374]
46. Jiang J, George SC. TGF- β 2 reduces nitric oxide synthase mRNA through a ROCK-dependent pathway in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2011; 301:L361–L367. [PubMed: 21685242]
47. Takaki H, Minoda Y, Koga K, Takaesu G, Yoshimura A, Kobayashi T. TGF-beta1 suppresses IFN-gamma-induced NO production in macrophages by suppressing STAT1 activation and accelerating iNOS protein degradation. *Genes Cells*. 2006; 11:871–882. [PubMed: 16866871]
48. Mitani T, Terashima M, Yoshimura H, Nariai Y, Tanigawa Y. TGF-beta1 enhances degradation of IFN-gamma-induced iNOS protein via proteasomes in RAW 264.7 cells. *Nitric Oxide*. 2005; 13:78–87. [PubMed: 15964225]
49. Mgbemena V, Segovia J, Chang T, Bose S. Krüppel-like factor 6 regulates transforming growth factor-beta gene expression during human respiratory syncytial virus infection. *Virology J*. 2011; 8:409. [PubMed: 21849067]
50. DiFeo A, Narla G, Camacho-Vanegas O, Nishio H, Rose SL, Buller RE, Friedman SL, Walsh MJ, Martignetti JA. E-cadherin is a novel transcriptional target of the KLF6 tumor suppressor. *Oncogene*. 2006; 25:6026–6031. [PubMed: 16702959]
51. Kim Y, Ratzu V, Choi SG, Lalazar A, Theiss G, Dang Q, Kim SJ, Friedman SL. Transcriptional activation of transforming growth factor beta1 and its receptors by the Kruppel-like factor Zf9/core promoter binding protein and Sp1. Potential mechanisms for autocrine fibrogenesis in response to injury. *J Biol Chem*. 1998; 273:33750–33758. [PubMed: 9837963]
52. Ratzu V, Lalazar A, Wong L, Dang Q, Collins C, Shaulian E, Jensen S, Friedman SL. Zf9, a Kruppel-like transcription factor up-regulated in vivo during early hepatic fibrosis. *Proc Natl Acad Sci USA*. 1998; 95:9500–9505. [PubMed: 9689109]

53. Kojima S, Hayashi S, Shimokado K, Suzuki Y, Shimada J, Crippa MP, Friedman SL. Transcriptional activation of urokinase by the Kruppel-like factor Zf9/COPEB activates latent TGF-beta1 in vascular endothelial cells. *Blood*. 2000; 95:1309–1316. [PubMed: 10666204]
54. Warke VG, Nambiar MP, Krishnan S, Tenbrock K, Geller DA, Koritschoner NP, Atkins JL, Farber DL, Tsokos GC. Transcriptional activation of the human inducible nitric-oxide synthase promoter by Kruppel-like factor 6. *J Biol Chem*. 2003; 278:14812–14819. [PubMed: 12590140]

Highlights

- Transactivation of iNOS gene by KLF6 during respiratory syncytial virus infection
- KLF6 is required for optimal apoptosis during respiratory syncytial virus infection
- iNOS/nitric oxide promotes apoptosis during respiratory syncytial virus infection

Fig. 1. KLF6 is required for nitric oxide (NO) production during RSV infection. (A) KLF6 expression (RT-PCR) in stable A549 cells generated following transduction with scrambled shRNA (control) and KLF6 specific shRNA (KLF6 silenced cells) expressing lentivirus. (B) NO production from RSV infected (12h post-infection) control and KLF6 silenced cells. Mock; uninfected cells. NO was measured by monitoring nitrite production using Griess reagent. Each value represents the mean $\pm$ standard deviation from three independent experiments.

Fig. 2. KLF6 regulates iNOS gene expression following RSV infection. (A) iNOS expression (RT-PCR) in mock infected and RSV infected (24h post-infection) scrambled shRNA (control) and KLF6 shRNA expressing stable A549 cells. (B) iNOS-luciferase reporter gene activation in RSV infected (12h post-infection) control and KLF6 silenced cells. The luciferase assay results are presented as mean $\pm$ standard deviation from three independent experiments.

Fig. 3. KLF6 association with human iNOS promoter during RSV infection. Chroma immunoprecipitation (ChIP) assay on A549 cells infected with RSV for the indicated time-points. Mock; uninfected cells. Both anti-KLF6 antibody and isotype matched control antibody (control Ab) was used. An upstream region in the human iNOS gene devoid of the KLF6 responsive region was probed as a negative control.

Fig. 4.

KLF6 regulates apoptosis following RSV infection. (A) Percentage of annexin V positive cells in RSV infected (48h post-infection) control (scrambled shRNA expressing cells) and KLF6 silenced (KLF6 shRNA expressing cells) stable A549 cells. Annexin V staining quantified by FACS represents mean $\pm$ SEM, $n = 3$, * $p < 0.05$ by two-tailed t test. (B) The values presented in Fig. 4A were utilized to calculate % apoptosis in RSV infected control and KLF6 silenced cells. Apoptosis in RSV infected control cells are denoted as 100% apoptosis

Fig. 5.

Apoptosis of RSV infected epithelial cells is regulated by iNOS activity. (A) Percentage of annexin V positive cells were detected by FACS analysis of RSV infected (48h post-infection) A549 cells incubated with water (vehicle) or iNOS inhibitor L-NIL (200 μ M). Annexin V staining quantified by FACS represents mean $\pm$ SEM, $n = 3$, * $p < 0.05$ by two-tailed t test. (B) The values presented in Fig. 5A were utilized to calculate % apoptosis in RSV infected cells (+/- L-NIL). Apoptosis in RSV infected vehicle treated cells are denoted as 100% apoptosis. (C) A representative FACS showing percentage of apoptotic (lower right quadrant – annexin V positive and PI negative cells) and necrotic (upper right quadrant – annexin V and PI positive cells) cells following RSV infection of cells treated with vehicle or L-NIL.

Fig. 6.

iNOS expression is required for apoptosis during RSV infection of primary macrophages. (A) Percentage of annexin V positive cells were detected by FACS analysis of RSV infected (48h post-infection) wild-type (WT) and iNOS knock-out (KO) primary bone marrow derived macrophages (BMDMs). Annexin V staining quantified by FACS represents mean $\pm$ SEM, $n = 3$, * $p < 0.05$ by two-tailed t test. (B) The values presented in Fig. 6A were utilized to calculate % apoptosis in RSV infected WT and iNOS KO BMDMs. Apoptosis in RSV infected WT BMDMs are denoted as 100% apoptosis. (C) A representative FACS showing percentage of apoptotic (lower right quadrant – annexin V positive and PI negative cells) and necrotic (upper right quadrant – annexin V and PI positive cells) cells following RSV infection WT and iNOS KO BMDMs. (D) RSV infected (48h post-infection) WT and iNOS KO BMDMs were used for TUNEL staining. Image J software was used to calculate TUNEL positive areas (representing apoptosis) as detailed in the methods section. The data is presented as percent apoptotic area (which represents % of total area). The data represents mean $\pm$ SEM, $n = 3$, * $p < 0.01$ by two-tailed t test.

Table-1**ChIP primers**

Encompassing the KLF6 binding site at the human iNOS promoter	<u>Human iNOS forward</u> 5' CAGAGAGCTCCCTGCTGAGGAAA 3' <u>Human iNOS reverse</u> 5' GAGAGTTGTTTTGCATAAAGGTCTC 3'	Product: 321 bp (encompassing -45 to -366 region of human iNOS promoter)	Optimal annealing temp: 55.7°C
Encompassing the region in human iNOS promoter that does not bind to KLF6 (negative control)	<u>Human iNOS forward</u> 5' GGAGAACATTCAACCCAGGTAATC 3' <u>Human iNOS forward</u> 5' ACCAATAAGGAAACCGAGACACAG 3'	Product: 249 bp (encompassing -4417 to -4666 region of human iNOS promoter)	Optimal annealing temp: 56.0 °C.