Upregulated A20 (TNFAIP3) expression during respiratory syncytial virus infection in mice

Running title: A20 gene expression in RSV mouse model

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Abstract

Background: Severe lower respiratory tract infections in infants and young children are frequently caused by respiratory syncytial virus (RSV), with the degree of illness strongly associated with disproportionate inflammatory activity. The signaling protein A20 (TNFAIP3) functions to inhibit NF-κB pathway activation, suggesting a possible role in tempering RSV-triggered lung inflammation. In this research, we assessed how infection with RSV alters A20 gene expression in the lungs using mice model system.

Methods: Of the twelve female BALB/c mice allocated for the study, half were administered RSV intranasally at a concentration of 3×10^6 plaque-forming units (PFU), while the remaining six served as uninfected controls. All animals were humanely euthanized five days post-infection. Upon collection, lung tissue samples were immediately processed. The relative expression levels of messenger RNA (mRNA) for the gene TNFAIP3, which encodes the A20 protein, were subsequently quantified using real-time reverse transcription polymerase chain reaction (RT-PCR).

Results: Analysis via quantitative PCR revealed that A20 expression was significantly higher in the lungs of RSV-infected mice relative to uninfected controls at the 5-day post-infection mark ($p=0.0048$).

Conclusions: The upregulation of A20 in RSV-infected mice points to its potential function in modulating post-viral pulmonary inflammation.

Keywords: Respiratory Syncytial Virus, Human, Tumor Necrosis Factor Alpha-Induced Protein 3 (A20), NF-kappa B, Gene Expression Regulation, Mice

Introduction

RSV represents a leading etiology of acute lower respiratory tract illness in infants and young children, frequently resulting in clinical diagnoses of bronchiolitis and pneumonia (1). Infection initiates within the respiratory epithelium, provoking a robust inflammatory cascade. This response is characterized by the secretion of numerous pro-inflammatory signaling molecules, which in turn mediate the influx of innate immune cells, including macrophages and monocytes, into pulmonary tissue (2). Although this immunological activity is crucial for controlling viral replication, an overly aggressive or dysregulated response can contribute to significant tissue pathology and exacerbate clinical outcomes. Consequently, the central challenge in RSV immunopathology lies in achieving effective viral clearance while minimizing collateral inflammatory damage (3).

The protein A20, produced by the TNFAIP3 gene, acts as a vital governor of inflammatory and immune signaling pathways. Its primary function is to serve as a negative feedback regulator, dampening activity in the NF-κB pathway-a key circuit that switches on genes responsible for producing inflammatory molecules. Through this modulation, A20 plays an essential role in restraining overactive immune reactions and preserving a balanced state within tissues (4). Impairments in A20 function are associated with numerous inflammatory and autoimmune disorders, underscoring its significance in limiting immune-related tissue injury during infections (5). Considering its fundamental position as a molecular checkpoint against inflammation, we proposed that RSV infection would modify A20 expression levels. Accordingly, this research was conducted to examine how RSV infection influences the expression of the A20 gene in the lungs of mice.

Methods

This laboratory experiment investigated differences between mice infected with respiratory syncytial virus (RSV) and a non-infected control group. Female BALB/c mice, aged five to seven weeks and weighing 15–18 grams, were used in the study. A total of twelve mice were obtained from the Pasteur Institute in Karaj, Iran, and were equally divided into two groups of six: one for RSV infection and one for the control. The animals were maintained in a controlled environment with unrestricted access to standard food and water. Prior to the study, all procedures received approval from the Animal Ethics Committee at Golestan University of Medical Sciences, under the ethical code IR.GOUMS.REC.1397.341. The work was subsequently carried out in the university's animal housing facility, adhering strictly to established guidelines for research involving animals.

Mice were allocated into two experimental groups. Animals within the RSV-infected cohort received an intranasal inoculation, under anesthesia, with the RSV-A2 strain. This viral strain was provided as a gift by Dr. Salimi, and the challenge dose was standardized to 3×10^6 plaque-forming units (PFU). All infected subjects were humanely euthanized on the fifth day following infection, which corresponds to the documented peak of viral replication. Euthanasia was performed via ketamine injection (Ketamine 10%, Alfasan, Woerden, Netherlands), after which lung tissues were harvested for subsequent RNA isolation. Before inoculation, the RSV virus was cultured and amplified in HEp-2 cells (ATCC® CCL-23™). The titer of the infectious viral stock was quantified using a standard plaque assay methodology, performed on Vero cell monolayers (ATCC® CCL-81™), as has been reported in earlier work (6).

To evaluate gene expression, we first isolated total RNA from lung tissue samples. This extraction was conducted using RNX-Plus solution, a product of CinnaGen in Iran. The purified RNA was then converted into complementary DNA (cDNA). For this reverse transcription step, we

employed the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems in the USA. Subsequent quantitative polymerase chain reaction (qPCR) analysis was carried out on an ABI PRISM 7900 sequence detection system, also from Applied Biosystems. The reactions utilized SYBR Premix Ex Taq II, manufactured by Takara Bio in Japan. The qPCR protocol involved an initial denaturation phase at 95°C for 10 seconds. This was followed by 40 amplification cycles, each consisting of a 5-second denaturation at 95°C and a 40-second annealing/extension step at 60°C. We designed and used specific primer sets for both the target gene, A20, and the reference gene, β-actin. To determine relative expression levels, we applied the 2-ΔΔCt calculation method. All target gene expression data were normalized to the endogenous β-actin control before final analysis (7).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism software, version 6.0, developed by GraphPad Software in San Diego, California. All outcome data are summarized as the mean plus or minus the standard error of the mean (SEM). To determine differences between experimental cohorts, we employed unpaired, two-tailed Student's t-tests. A probability (P) value of less than 0.05 was considered indicative of statistical significance.

Results and Discussion

The core aim of this research was to determine whether RSV infection alters A20 gene expression in mouse lungs. Using quantitative PCR, we observed a clear and statistically significant increase in A20 expression in RSV-infected mice compared to healthy controls ($p=0.0048$; see Fig. 1). This upregulation implies that RSV infection directly modifies transcription of the A20 gene, a pivotal player in immune regulation.

The pathogenesis of RSV is largely driven by the host's immune response, highlighting the importance of endogenous regulatory mechanisms (1). Our investigation reveals that infection with RSV induces a substantial increase in the expression of A20 (TNFAIP3) messenger RNA within the pulmonary tissue of murine models. This marked upregulation is specifically observed during the acute infectious stage, which corresponds to day five post-infection, the documented peak of viral load. This *in vivo* finding confirms and extends previous *in vitro* work, such as that by Martín-Vicente et al., which showed that RSV induces a strong, delayed increase in A20 expression in human lung epithelial cells (4). This collective evidence firmly establishes A20 as a pivotal negative regulator of inflammatory signaling during RSV infection.

A20 functions as a molecular brake on the NF-κB and IRF3 signaling pathways. Mechanistically, it acts in complexes with partners like TAX1BP1 and ABIN1 to deubiquitinate key kinases such as TBK1 and IKKε, thereby attenuating the RIG-I-initiated signaling cascade that drives the production of interferons, cytokines, and chemokines (8, 9).

The functional consequences of A20 induction, however, present a dualistic nature. Studies show that genetic ablation of A20 in epithelial cells leads to a heightened early innate immune response to RSV, characterized by elevated cytokines, interferons, and ISGs, which correlates with a marked reduction in viral titers (10). This indicates that A20's anti-inflammatory role may inadvertently create a cellular environment more permissive for viral replication. Furthermore, A20 depletion enhances apoptosis in infected cells, a process that can serve as an antiviral defense (11). Thus, the upregulation we report may also function to inhibit this cell death pathway, potentially prolonging the survival of infected cells.

The critical balance A20 maintains is underscored by a genetic association study linking specific *TNIP1* variants (associated with lower TNIP1 expression) to a reduced risk of severe RSV

bronchiolitis in infants (10). This suggests that a less efficient A20/TNIP1 "brake" might permit a more potent, contained innate immune response, highlighting that the activity level of this complex is a finely tuned determinant of disease outcome.

A notable constraint of this research is its focus on a solitary timepoint-specifically, day five following infection. Although this phase represents the infection's peak, the chosen design cannot illuminate the complete chronological expression pattern of the A20 protein. While the increased A20 levels detected at this juncture imply its involvement in the host's defensive regulation, its activity during the initial or concluding stages of infection is not defined by our data. Furthermore, the investigation lacked confirmatory protein-level analyses, such as Western blotting, and did not incorporate functional experiments to determine the consequences of altering A20 activity. Subsequent research employing longitudinal timelines and mechanistic evaluations will be essential to elucidate the precise function of A20 in RSV disease progression.

Conclusions

Our data validate A20 as a key endogenous modulator of inflammation in a physiologically relevant model of RSV. Its role is complex, limiting harmful inflammation at the potential cost of aiding viral survival, positioning it as a promising candidate for therapeutic intervention. Future investigations employing kinetic analyses and functional studies in conditional knockout models are essential to define the therapeutic window for modulating A20 to reduce RSV-related lung damage without compromising viral clearance.

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Ethical statement

The experimental protocol for this investigation received formal ethical clearance from the institutional review board of Golestan University of Medical Sciences (Ethics Approval Code: IR.GOUMS.REC.1397.341).

Conflict of interest

No potential conflicts of interest, including financial interests, are reported by the authors of this work.

Author contributions

Bahman Aghcheli was responsible for the study design and data collection, and prepared the initial manuscript draft. Romina Yavarinamini contributed to the study design and investigation, and also helped in drafting the first version of the paper. Alireza Tahamtan developed the core concept, oversaw the methodology, and performed validation, formal analysis, and investigation. He also

provided resources, supervised the project, secured funding, and handled the editing and review of the manuscript**.**

Data availability statement

The data underpinning the primary conclusions of this study can be provided by the corresponding author upon receipt of a justified request.

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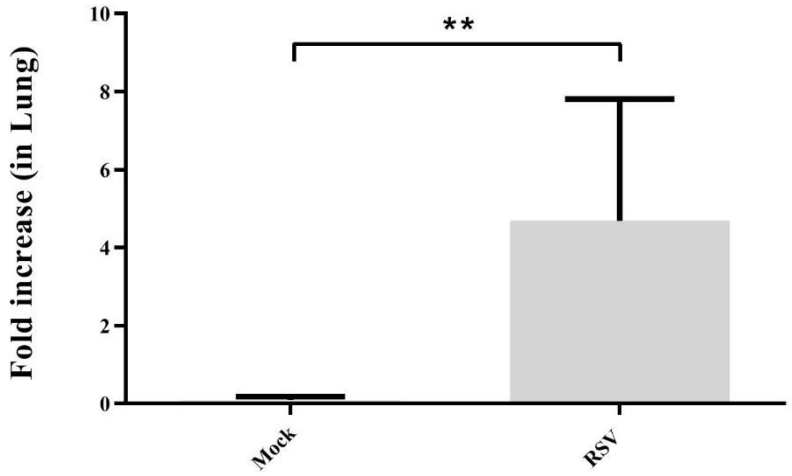

Figure 1. The effect of RSV infection on A20 expression in lung tissue of mice. Relative expression of A20 was evaluated in lung tissue of mice five days after infection, using specific primers targeting the A20 gene and normalised to those of the housekeeping gene (β-actin). Results represent the mean±SEM of six animals for each group (**p=0.0048).