



OPEN Analysis of DNA methyltransferase 3 alpha expression during respiratory syncytial virus strain A infection

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Respiratory syncytial virus (RSV) is a seasonal pathogen known to cause lower respiratory tract infections, including bronchiolitis and pneumonia, and it is associated with an increased risk of developing asthma and recurrent wheezing in pediatric populations. Epigenetic mechanisms, such as DNA methylation and histone modifications, have been implicated in regulating host immune responses during viral infections. In this study, we investigated DNMT3A expression, a de novo DNA methyltransferase, during RSV infection. We queried the Gene Expression Omnibus (GEO) database for RNA-sequencing datasets related to RSV infection. Among the five relevant datasets identified, one demonstrated significantly elevated *DNMT3A* expression in RSV-infected samples compared to controls. To validate and further examine this finding, we infected MRC-5 cells, a human lung fibroblast cell line, with RSV-A strain (RSV-A2) and accessed *DNMT3A* expression at different time points using real-time RT-qPCR. We observed an upregulation of *DNMT3A* expression at 8 h post-infection; this induction was decreased in the presence of rapamycin, an inhibitor of the mTOR pathway. Immunofluorescence analysis confirms DNMT3A presence in RSV-infected cells. In vivo analysis revealed a significant increase in *Dnmt3a* expression in murine lung tissue five days following RSV infection. In ex-vivo RSV-infected pediatric lung tissue sample the *DNMT3A* gene expression was not increase compared to uninfected tissue. Our findings suggest that RSV modulates *DNMT3A* expression through mTOR pathway, but this effect is transient and dependent on the sample and the kinetics of infection. Our data highlights a potential epigenetic mechanism involved in the gene expression regulation of the host during infection

Keywords DNMT3A, Epigenetics, Respiratory syncytial virus, mTOR, Lung

Respiratory syncytial virus (RSV) is an infectious agent responsible for a considerable disease burden, especially among infants and elderly people^{1–3}. It is estimated that about 45% of hospital admissions and deaths of children under six months of age are due to RSV infection affecting the lower airways. In the elderly, RSV is estimated to cause around 200,000 hospitalizations, with the highest burden observed in adults aged 75 years and older⁴. This outcome leads to significant mortality and morbidity, generating suffering and a high impact in terms of costs to health systems. The disease burden is greater in low- and middle-income countries^{5,6}. RSV infection also increases the long-term risk of wheezing in infected children. This virus causes pneumonitis and acute

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bronchiolitis with generalized lung and bronchial inflammation, hypoxia, and respiratory distress. RSV triggers Th2 responses leading to bronchial hyperreactivity^{7,8}.

After decades of research, vaccines to prevent RSV infection are now available. Three vaccines have been approved for the protection of older adults, and a bivalent prefusion F vaccine is indicated for maternal immunization during pregnancy to confer passive protection to newborns through transplacental antibody transfer⁹. Approved prophylaxis interventions for RSV prevention are available, such as palivizumab, a humanized monoclonal antibody targeting the RSV F protein and nirsevimab, a more potent and long-acting humanized monoclonal, however, they are expensive and remain inaccessible to most of the at-risk population⁹. Consequently, the development of new interventions remains necessary, particularly for infants under 12 months of age, who are especially vulnerable to RSV-induced respiratory tract infections, making effective protection essential¹⁰. A deeper understanding of the virus and its interactions with the host is crucial for advancing novel strategies to prevent or treat RSV infection.

Epigenetic regulation refers to the heritable changes in the activation or suppression of gene transcription in a cell without changing the DNA sequence. The three main types of epigenetic regulation are histone modifications, microRNA expression and DNA methylation¹¹. Histone modifications, such as acetylation, methylation, or phosphorylation, alter the structure of chromatin and influence gene accessibility, thereby regulating transcription. MicroRNAs are small non-coding RNAs that post-transcriptionally regulate gene expression by binding to complementary mRNA sequences, leading to mRNA degradation or translational inhibition. DNA methylation is a common epigenetic modification that involves a methyl group addition at position 5 of the cytosine ring, in regions rich in cytosine and guanidine, known as CpG islands.

Histone modifications can contribute to ameliorate RSV infection. Methylation of histone H3K4 in regulatory T cells (Treg) is important for controlling lung inflammation during RSV infection. TGFβ active SMYD3, a histone methyltransferase, which perform H3K4 methylation in the *foxp3* loci, and maintenance of Foxp3 expression levels in Treg cells. SMYD3 knockout mice present an exacerbation of RSV induced disease¹². Also, interferon gamma pre-infection treatment in epithelial cells can result in changes to histone H3K9 methylation at the retinoid acid-inducible gene (RIG-I) promoter, leading to reduced RSV viral load¹³. RSV infection, in vitro, can modulate the expression of Histone Deacetylase in airway epithelial cells (BEAS-2B). Treatment with Histone deacetylase inhibitors, trichostatin and suberoylanilide hydroxamic acid, restricted RSV replication and modulated cytokine expression in vitro and in vivo¹⁴. Suggesting that Histone deacetylases have a role in the RSV replication and pathogenesis.

Emerging evidence suggests that microRNA is involved in the pathobiology of RSV infection. For example, miR-221, controls RSV replication in human bronchial epithelial cells¹⁵. Also, miR-146a mitigates RSV-induced lung injury in vivo¹⁶. Airway microRNA signatures are associated with severe RSV infection in Infants¹⁷.

Children who have been infected with RSV presented changes in DNA methylation. Children with a history of hospital admission for severe RSV bronchiolitis in the early two years of life, exhibit lower levels of DNA methylation compared to matched healthy controls. These differences in the DNA methylation were at two CpG loci within the perforin gene proximal enhancer, corresponding to a STAT5 element. The percentage of methylation was determined in the DNA of peripheral blood leucocytes collected from children by pyrosequencing of bisulfite-converted DNA¹⁸.

Methylation profile of RSV-infected children who develop recurrent wheezing, subsequent asthma, and those experiencing complete recovery after infection are different. Analysis of the DNA methylome of RSV infected children showed around 5000 differentially methylated positions mainly located in inflammatory genes when comparing those children with sequelae (asthma or recurrent wheezing) or without sequelae¹⁹. These data suggested that DNA methylation might have a role in the respiratory morbidity caused after RSV infection.

DNA methylation is regulated by enzymes of the DNA methyltransferase (DNMT) family, including DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. DNMT1 is responsible for the maintenance and repositioning of methyl groups during DNA duplication. DNMT3A and DNMT3B perform the so-called 'de novo' methylation that acts as a transcriptional regulatory mechanism^{20–23}. DNMT3A has been described to play a role during virus infection. This enzyme can act as a host factor required for effective herpes simplex virus type 1 infection²⁴. Also, DNMT3A is involved in controlling immune responses during virus infection. DNMT3A maintains high expression of the histone deacetylase HDAC9 in a DNA-methylation-dependent manner in peritoneal macrophages, preparing the antiviral response of these cells, by the activation of TBK1-IRF3 signaling and the production of type 1 interferon²³. Additionally, DNMT3A is a crucial factor for CD8 memory T cell differentiation in experimental models of influenza virus and lymphocytic viral choriomeningitis²⁰. However, the role of DNMT3A during RSV infection has not yet been described. This study aimed to analyze DNMT3a expression during RSV infection using different approaches, analyzing RNAseq database at GEO, and performing experiments in vitro, in vivo, and ex vivo with RSV infection. By integrating these strategies, the work provides a comprehensive view of how RSV may influence DNA methylation pathways. A better understanding of DNMT3a regulation during infection could reveal novel mechanisms by which RSV modulates host responses, contributing to immune dysregulation and disease severity, and long-term consequences of the infection. These insights may ultimately open new avenues for identifying epigenetic biomarkers and therapeutic targets to improve the prevention and treatment of RSV-related diseases.

Materials and methods

DNA methyltransferase expression analysis collected from RNAseq dataset

We performed a systematic search for RNA-seq datasets related to Respiratory Syncytial Virus (RSV) infection using the Gene Expression Omnibus (GEO) DataSets repository²⁵. The search query was formulated as follows: "(Respiratory Syncytial Virus) OR RSV". This initial query yielded a total of 6662 datasets. We restricted the search results by filtering the data collection technique to "Expression profile by high-throughput sequencing"

and the studied species to “homo sapiens”, to find studies that performed RNA sequencing analysis and obtained a total of 43 datasets.

In a rigorous selection process, we excluded 38 datasets based on the following criteria: 1) infection with viruses other than RSV, 2) absence of uninfected control samples, 3) treatment studies without healthy control samples and untreated RSV, 4) data not available for reading via GEO2R, and 5) duplicate studies. The remaining five datasets, accession numbers: GSE146795 (normal human bronchial epithelial cells)²⁶, GSE179353 (small human airway epithelial cells)²⁷, GSE99298 (A549 cells)²⁸, GSE166161 (human nasal curettage samples), and GSE155237 (human nasal curettage samples)²⁹, were considered relevant and had their data analyzed.

The five datasets in question were analyzed using GEO2R, an extension of GEO designed to perform DEG (Differential Expression Gene) analysis with R code. Fold change (log2FC), adjusted p-value (padj), and standard error (lfcSE) were analyzed for all genes in each study. Volcano plots were generated for each study, estimating the quality of the analyses performed. The gene of interest, DNMT3A, was selected based on its known relevance to epigenetic regulation and placed in Table 1 with the values obtained in the analysis. Additionally, the time elapsed after infection at the time of analysis was duly recorded in the same Table. The statistical test employed was the Benjamini & Hochberg method (False discovery rate). Data with a padj < 0.05 were considered significant.

Cell culture

MRC-5 cells (Medical Research Council cell strain 5) (Source: ATCC CCL-171), a fibroblast cell line originally from the lung, and Vero cells (African green monkey kidney epithelial cells) (Source: ATCC CCL-81), were cultured in DMEM high glucose medium supplemented with 10% Fetal Bovine Serum (FBS) at 37 °C under an atmosphere of 5% CO₂.

Virus

The RSV A2 strain (generously provided by Dr. Fernando Polack, Fundación Infant, Argentina) was propagated in Vero cells maintained in Opti-MEM medium supplemented with 2% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO₂. Viral titers were determined by infecting Vero cells in serum-free medium, followed by plaque assay using carboxymethylcellulose overlay. Plaque titration was conducted using an anti-RSV antibody (Millipore, Cat# AB1128, RRID: AB_90477), and viral titers were expressed as plaque-forming units per milliliter (PFU/mL). Virus aliquots were stored at – 80 °C until use.

In vitro experiment

For in vitro analysis, 10⁵ MRC-5 cells were seeded separately in 24-well plates and after 24 h infected with RSV at 10² PFU/mL (MOI 0.001) or 10³ PFU/mL (MOI 0.01). After 0, 2, 8, and 24 h of virus infection, the cells were collected for RNA extraction and cDNA synthesis. UV-inactivated RSV was used as a control; the virus was exposed to UV light for 30 min at a distance of 20 cm. For more mechanistic insight, MRC-5 cells were inhibited with 10 ng/mL 3-methyladenine (3-MA) and 12 ng/mL rapamycin for 2 h, and then infected with RSV (10³ PFU) for 8 h. We also submitted cells to starvation to increase DNMT3A expression, using cell culture medium without fetal bovine serum supplementation, as previously described, to use as a positive control³⁰. The experiment was performed using 4 to 7 wells per condition, and it was repeated at least three times.

Immunofluorescence

MRC-5 human lung fibroblast cells (10⁵ cells) were seeded in 24-well plates containing medium with 5% FBS medium or without FBS to use as a positive control. After 24 h, the cells were inhibited with the inhibitors 3-methyladenine (3-MA) and rapamycin for 2 h at 37 °C and 5% CO₂, then the cells were infected with RSV at 10³ PFU/mL (MOI 0.01). After 8 h of infection, the wells were washed with PBS and then fixed with paraformaldehyde. Cells were stained with mouse anti-human monoclonal primary antibody DNMT3A (1:200; Invitrogen*), followed by R-phycoerythrin conjugated goat anti-mouse IgG1 (gamma 1) secondary antibody (1:1000 Invitrogen* by ThermoFisher Scientific*). Nuclear staining was performed with Hoechst 33,342 (ThermoFisher Scientific*) for analysis under a fluorescence microscope (Olympus). Images were acquired using QCapture Pro version 6,0 (QImage). Fluorescence intensity was measured using ImageJ version 1.5.4 (Java).

RSV infection in mice

To access DNMT3A gene expression during RSV infection in vivo, female BALB/c mice between 6 and 18 weeks of age were bred and acquired from the Center for Experimental Biological Models (CeMBE, PUCRS). The protocol was approved by the animal research ethical committee (protocol #10,180). Mice were anesthetized with 5% isoflurane and then intranasally infected with an inoculum (10⁷ PFU RSV A2) or given a saline solution

Sample type	Accession number	n	Time of analysis after infection	log2FC	padj	lfcSE	RSV strain
Normal human bronchial epithelial cells	GSE146795 -	6 (2 RSV, 4 Controls)	144 h	0.1072891	0.648	0.145	RSV A2
Small human airway epithelial cells	GSE179353	8 (4 RSV, 4 Controls)	24 h	0.9617056	<0.001	0.0896	RSV A long
A549 cells	GSE99298	5 (3 RSV, 2 Controls)	96 h	0.095382	0.816	0.287	RSV A line19
Human nasal curettage samples	GSE166161	13 (9 RSV, 4 Controls)	72 h	0.1236458	0.999	0.1999	RSV A M37
	GSE155237	39 (23 RSV, 16 Controls)	72 h	0.1177311	0.565	0.1012	RSV A M37

Table 1. Differential gene expression analysis for the DNMT3A genes in RNAseq data base. Fold change (log2FC), adjusted p-value (padj), and standard error (lfcSE).

and left for 3 or 5 days until harvesting. The experiments were performed twice, using 5 mice per group. At the end of the experiment the animals were euthanized with a lethal dose of anesthetic through intraperitoneal route.

RSV infection in pediatric human lung tissue

To access *DNMT3A* gene expression in pediatric samples, macroscopically normal lung tissue fragments were collected from three neonates (both genders) who underwent pulmonary resection for pulmonary malformation at the Santo Antônio Children's Hospital. There were no respiratory infection symptoms at the moment of the samples collection. Written informed consent was obtained from all relatives or authorized legal guardians. The samples were sectioned, and frozen in 1 mL of CytoSofree™ DMSO-free medium (Sigma-Aldrich). The samples were thawed, the weight was average 60 mg, and infected or not with 10^4 RSV A2/mL for 24 h in a 24-well plate in DMEM-F12 with 2% of FBS and 1% of MEM vitamin solution (Gibco), under 5% of CO₂. Lung tissues were collected from culture and RNA extraction was performed for gene expression analysis using Real-time PCR.

Real-time PCR analysis

Mouse and human lung samples and MRC-5 cells (4–7 wells of 10^5 cells) were then accessed for *DNMT3A* gene expression. RNA was extracted using Brazol® reagent (LGC biotecnologia) following the manufacturer's instructions. Complementary DNA (cDNA) was generated from the RNA using the GoScript® Reverse Transcription Kit (Promega®, Madison, WI, USA). The synthesized cDNA was quantified in Qubit® 2.0 Fluorometer (Invitrogen™, by life technologies™) using the Qubit™ dsDNA HS Assay Kit and subsequently stored at -20 °C until PCR analysis. The relative mRNA expression levels were determined by real time RT-qPCR using 12 ng of cDNA to amplify the normalised genes human GAPDH using the follow primers (forward (5'-C TCTCTGCTCCTCCTGTTCGAC -3') and reverse (5'- TGAGCGATGTGGCTCGGCT -3')) and mouse B2M (forward (5'-CCCCAGTGAGACTGATACATACG-3') and reverse (5'-CGATCCCAGTAGACGGTCTTG-3')). *DNMT3A* gene amplification was performed using forward (5'-GCACATGCCTCCAATGAAGA-3) and reverse (5'-GCCAAGAAACCCAGAAAGAGC-3') primers for mouse; and forward (5'-TTCTACCGCCTCCT GCATGAT-3') and reverse (5'-GCGAGATGTCCCTCTTGTCATA-3') primers for human. PCR conditions followed PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™) protocol. PCR analysis was conducted using StepOne™ (Applied Biosystems) platform. The analysis was performed using StepOne Software version 2.3 using $2^{-\Delta\Delta C_t}$ or $2^{-\Delta C_t}$ to calculate gene expression.

The RSV viral load in the human lung samples was accessed by RSV F protein gene expression using the indicated specific primers and probes: forward primer 5'-AACAGATGTAAGCAGCTCCGTTATC-3', reverse primer 5'-GATTTTATTGGATGCTGTACATTT-3', and probe 5'-FAM/TGCCATAGCATGACACAATGGCT CCT-TAMRA/-3'. Primer sequences were synthesized and cloned into pUC57 plasmids (GenScript, Piscataway, NJ, USA) to perform a tenfold dilution to generate a standard curve for viral load calculation.

Statistical analysis of in vitro and in vivo experiments

The in vitro experiments were repeated at least three times. The in vivo experiments were performed twice, using 5 mice per group. The Kruskal–Wallis test was applied to compare statistical significance among multiple experimental groups, and to compare two groups, the Mann–Whitney was used. Spearman's rank correlation (ρ) was used to find an association between viral load and DNMT3a expression. The results were analyzed using the GraphPad Prism 8 statistical software package. All data are expressed as mean $\pm$ standard error of mean (SEM), and the significance level was set at $p < 0.05$.

Ethical procedures

The study followed the ARRIVE guidelines' (Animal Research: Reporting of In Vivo Experiments) during the planning, execution, and writing of the study. Animals procedures were performed in accordance with protocols approved by Animal Research Ethical Committee of PUCRS (protocol #10,180). For neonates sample collection, the parents or legal guardians of the participants signed an informed consent form before sample collection. The study was reviewed and approved by the Research Ethics Committee of Santa Casa de Misericórdia (Porto Alegre) under protocol number 59156122.5.0000.5335 and followed the standards established by the Declaration of Helsinki.

Results

RNAseq analyses of DNMT3A gene expression in human samples infected with RSV

Methylome analysis of blood samples from children who have been infected with RSV showed differential methylation positions compared to uninfected ones¹⁹. However, the role of DNMT3A in this context has not yet been described. To investigate the *DNMT3A* gene expression during RSV infection, we performed a systematic search for RNA-seq datasets in the Gene Expression Omnibus (GEO) datasets repository, and a total of 6662 datasets were found in the initial query. After filtering the data collection five datasets remained and the summarized results are showed at Table 1. The analysis of one RNAseq dataset (GSE179353), from RSV-infected human airway epithelial cells, demonstrated a significant increase in the expression of *DNMT3A* compared to uninfected cells (Table 1 and supplementary Fig. 1). The study included eight samples (4 RSV-infected and 4 uninfected controls), in which infection was performed using the RSV-A long, and samples were collected 24 h post-infection. The analysis revealed a log₂ fold change (log₂FC) of 0.96, indicating that DNMT3A expression in infected cells was approximately 94% higher compared to uninfected controls. The associated adjusted p -value (p_{adj}) was < 0.001 , confirming that this upregulation remained statistically significant after correction for multiple testing using the Benjamini–Hochberg method. Additionally, the standard error of the log₂FC (lfcSE) was 0.0896, indicating low variability in the estimated fold change. The remaining four datasets, which evaluated

different cell types and later time points postinfection (ranging from 72 to 168 h), showed smaller effect sizes (log2FC values between 0.09 and 0.12) and were not statistically significant (padj values ranging from 0.565 to 0.999). It is also important to note that the strain of RSV used for infection is not the same in all studies which might interfere in the variability of the results (Table 1).

The data suggests that the upregulation of DNMT3A expression during RSV infection might be dependent on the kinetics of the infection when the sample was collected, indicating a temporary expression of this enzyme.

DNMT3A gene expression is increased in MRC-5 cell line infected with RSV

To confirm these RNAseq results and further explore DNMT3A expression during RSV infection lung fibroblast cell line (MRC-5) were infected with RSV (10^2 PFU) and analyzed by real-time PCR at different time points. RSV significantly changed *DNMT3A* gene expression 8 h after infection comparing to UV-inactivated virus, suggesting that a productive infection is essential for this outcome (Fig. 1A). However, the upregulation of DNMT3A gene in RSV-infected cells is transient, since 24 h after infection the levels of DNMT3A gene expression return to similar to those found in cells incubated with UV-inactivated virus (Fig. 1A).

We also measured DNMT3A at the protein level in RSV-infected cells using immunofluorescence after 8 h of infection with 10^2 PFU of virus (Fig. 1B). RSV significantly enhanced DNMT3A expression compared to uninfected cells corroborating with the mRNA data found using real-time PCR (Fig. 1A and B). This effect again was absent when the virus was UV-inactivated (Fig. 1A, B and C). A more pronounced response was found with a higher virus concentration (10^3 PFU), indicating an effect dependent on viral load (Fig. 1B and C). Epigenetic mechanism controls cellular starvation in human fibroblasts, increasing the levels of DNMT3A³⁰. As a positive control for DNMT3A expression, MRC-5 cells were cultured in medium without fetal bovine serum (FBS) supplementation to induce growth arrest and nutrient starvation. RSV-infected cells presented a similar expression of DNMT3A to the levels observed in starved cells (Fig. 1B and C).

mTOR is associated with DNMT3A expression mediated by RSV

Further we want to better understand the mechanism associated with the increase in expression of DNMT3A by RSV. We and other research groups demonstrated that RSV can modulate the mechanistic target of rapamycin (mTOR) pathway^{31–33}. mTOR is a fundamental cellular signaling pathway that integrates diverse environmental signals to regulate cellular growth, metabolism, and proliferation. mTOR interacts with epigenetic regulators to control gene expression³⁴, including DNA methylation³⁵. Different cellular receptors can activate phosphoinositide 3-kinase (PI3K), triggering the protein kinase (Akt), which in turn phosphorylates and activates mTOR, configuring PI3K/Akt/mTOR activation. This pathway has been previously described to be associated with DNMT3A expression³⁶. To investigate whether PI3K/Akt/mTOR was involved in DNMT3A expression mediated by RSV, we pre-treated MRC-5 cells with the PI3K inhibitor 3-methyladenine (3-MA) or the mTOR inhibitor rapamycin and infected the cells with RSV. Our results show that the expression of DNMT3A induced by RSV infection was partially inhibited by rapamycin, but not by the PI3K inhibitor (Fig. 2A and B). These data indicate that RSV increases the expression of DNMT3A, and this effect is partially mediated by mTOR signaling pathway.

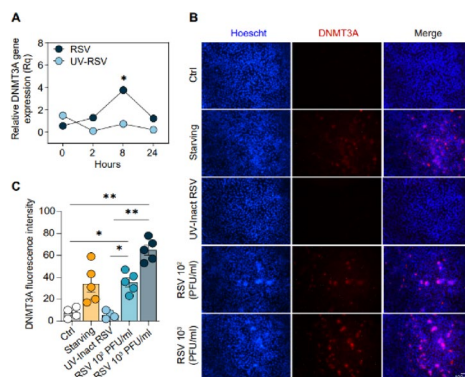


Fig. 1. RSV modulates DNMT3A expression in lung fibroblast cells infected with RSV. (A) 10^5 MRC-5 cells were seeding in 24-well plate and after 24 h incubated with RSV 10^2 PFU/mL (0.001 MOI) or UV-inactivated RSV and collected in different times and *DNMT3A* was accessed by Real-time PCR-. Gene expression analysis were performed by calculating $2^{-\Delta\Delta C_t}$ (Rq), using *GAPDH* expression to normalize. (B) MRC-5 cells were infected with RSV (10^2 PFU/mL (0.001 MOI) and 10^3 PFU/mL (0.01 MOI) or left in the starving state (cell culture medium without fetal bovine serum supplementation) for 8 h. DNMT3A was stained with anti-human DNMT3A antibodies, shown in red. Cells were fixed to perform the immunofluorescence assay. Representative panels show nuclei stained in blue (Hoescht), DNMT3A methylation in red and the merged channels. (C) Quantification of immunofluorescence detected by the number of DNMT3A (red) positive cells (second column staining for DNMT3A, third column merged). All data are expressed as mean $\pm$ SEM. The experiments were repeated at least three times. All data are expressed as mean $\pm$ SEM. In (A) data were compared using the Mann–Whitney test. In (B) and (C), multiple groups were compared using the Kruskal–Wallis test. * $p < 0.05$, ** $p < 0.01$.

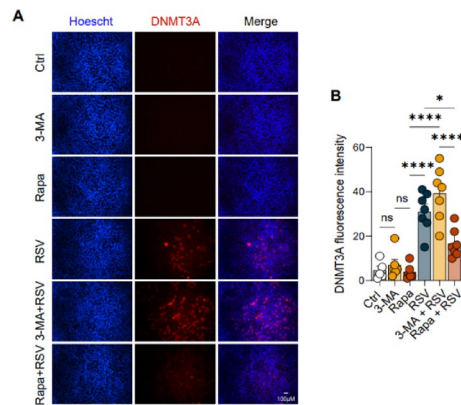


Fig. 2. MRC-5 cells were inhibited with 10 ng/mL of 3-MA (apoptosis PI3K-related inhibitor) or with 12 ng/mL of rapamycin (mTOR inhibitor) for 2 h prior to infection. Afterwards, cells were infected with RSV at 10^3 PFU/mL (MOI 0.001) and left for 8 h. Cells were fixed to perform the immunofluorescence assay. **A**, Representative panels show nuclei stained in blue (Hoescht), DNMT3A methylation in red and the merged channels. **B**, Quantification of immunofluorescence detected by the number of DNMT3A (red) positive cells. All data are expressed as mean $\pm$ SEM. Statistical significance between groups was determined by the Kruskal–Wallis test. A p value < 0.05 was considered significant.

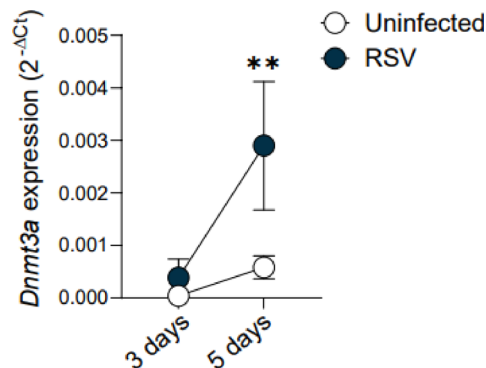


Fig. 3. *Dnmt3a* gene expression in the lungs of RSV-infected mice. Analysis of murine lungs 3 and 5 days after RSV infection (10^7 PFU) by real-time PCR. Data are from two independent experiments ($n = 5$ per group). All data are expressed as mean $\pm$ SEM. Statistical significance between groups was determined by the Mann–Whitney test. ** $p < 0.01$.

RSV-infected mice present increased *Dnmt3a* expression in the lung

The majority of RNAseq datasets that we found were from human cell samples infected with RSV in vitro. Two of the datasets were from samples from the upper respiratory tract (nasal curettage) of RSV-infected adults. We next sought to investigate whether RSV can induce *Dnmt3a* gene expression in lower respiratory tract tissue in vivo. To test that, Balb/c mice were infected with 10^7 PFU of RSV intranasally as we previously described³⁷, and the lung tissue was collected for analysis of *Dnmt3a* gene expression by real-time PCR. At the beginning of the infection (day 3), enzyme gene expression in the lung was not different between uninfected and RSV-infected mice (Fig. 3). However, five days after infection, expression of the *Dnmt3a* gene was significant increase in the lungs of infected mice (Fig. 3). These data suggested the *Dnmt3a* expression in lung of RSV-infected mice is different at different times after infection.

DNMT3A expression in pediatric lung tissue samples

A notable limitation of the RNA-seq dataset analysis is the absence of pediatric samples in the available repositories. To address this gap, lung tissue samples were collected from neonates who underwent lung recession. These samples were sectioned and infected with RSV for 24 h and the expression of DNMT3A were evaluated. The 24-h time point was selected because RNA-seq analysis indicated that RSV induces DNMT3A expression in primary human airway epithelial cells at this stage (Table 1). When comparing the uninfected and infected lung slices the mean expression of DNMT3A were slightly higher in the infected group, however, this difference did not reach statistical significance (Fig. 4 A). Virus replication was also accessed in these samples by quantifying the viral load using Real-Time PCR (Fig. 4 B). No correlation was observed between the viral load and the expression of DNMT3A (Fig. 4 C). Together, these findings suggest that RSV does not significantly increase

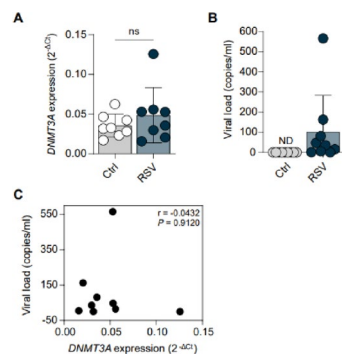


Fig. 4. DNMT3A expression in pediatric lung tissue infected *ex vivo* with RSV. Lung tissue sections from children were either left uninfected or infected with RSV (10^4 PFU) for 24 h. Samples were obtained from three different children, with three sections from each used for RSV infection. **(A)** DNMT3A expression. **(B)** Viral load quantification. **(C)** Correlation between viral load and DNMT3A expression. Statistical analysis was performed using an unpaired t-test (Mann–Whitney) and Spearman's rank correlation (ρ).

DNMT3A expression in neonatal lung tissue at the 24-h time point, and that the extent of viral replication is not directly associated with DNMT3A expression.

Discussion

In this study, we provide evidence that RSV infection is associated with changes in the expression of DNMT3A. Our data suggest that the expression of DNMT3A during RSV infection is transient and dependent on the kinetics of infection. DNMT3A expression during RSV infection may mediate epigenetic modifications that regulate host gene expression. Viruses are intracellular pathogens that have coevolved with their host to become masters of operating cellular systems so that they can replicate and transmit efficiently. Consequently, viruses can alter the epigenetic landscape to promote their regulation and interaction with host proteins. Influenza A virus, for example, can inhibit the methylation of genes associated with suppression of the JAK-STAT signaling pathway, an effect mediated by the NS1 protein³⁸. Also, the DNA methylation profile is unique in HIV-infected subjects. Patients infected with HIV present with increased expression of DNMT3A compared to uninfected individuals³⁹. During HSV-1 infection, the viral capsid protein interacts with DNMT3A; reduced levels of DNMT3A are associated with reduced virus propagation³⁴. In a murine model similar to human Epstein-Barr virus infection, DNMT3A was shown to play a critical role in transcription repression for gamma herpesvirus latency⁴⁰. Corroborating with our findings, the hepatitis B virus upregulates the expression of DNMT3A. HBx protein from hepatitis B virus mediates an increase in DNMT3A, which is related to increasing SOCS-1 gene promoter CpG island methylation⁴¹.

The immune response induced by viral infection can also modulate DNMT3A. Inflammation and inflammatory cytokines, such as IL6 and TNF α , induce DNMT3a expression via NF- κ B⁴². Also, the increase of specificity protein 3 (Sp3) transcriptional factor increases the expression of DNMT3a in human embryonic kidney 293 T cells⁴³. TGF- β 1 increases the expression of DNMT3A via mTOR/Akt signaling pathway³⁶. mTOR is a serine/threonine protein kinase that regulates cell growth, proliferation, motility, and survival, as well as gene transcription, and it is a member of the phosphoinositide 3-kinase (PI3K)-related kinase family. mTOR exerts its activities organized into two multiple-protein complexes, mTORC1 and mTORC2. We have previously demonstrated that RSV can activate mTORC1³³, and this pathway is associated with virus replication in immune cells³¹. The role of mTORC1 during RSV replication has been confirmed in other studies³². In the present study, we found that the induction of DNMT3A expression mediated by RSV was partially dependent on mTORC1 signaling pathway. PI3K is also an important pathway involved in RSV replication^{32,44,45}; however, in our model, inhibiting PI3K signaling was not associated with a reduction in DNMT3A, suggesting that DNMT3A expression induced by RSV may not be directly related to controlling viral replication. An alternative hypothesis is that RSV regulates DNMT3A expression to modulate the gene expression of the antiviral response, such as the one mediated by type 1 interferon. Further studies are needed to confirm the exact role of DNMT3A during RSV infection.

Our analysis of the RNA-seq data did not reveal a consistent increase in *DNMT3A* gene expression across all datasets included in the study. This variability may be attributed to differences in sample types and collection time points post-infection, suggesting that DNMT3A expression during RSV infection is likely transient. This observation aligns with our *in vitro*, *in vivo* and *ex-vivo* findings with lung pediatric samples infected with RSV strain A (Fig. 5). Subsequent investigations should aim to evaluate the expression of DNMT3 in airway samples from patients at several ages infected with different RSV strains.

In summary, in the present study, we showed that RSV infection can modulate the expression of DNMT3A, and this effect is associated with mTOR pathway activation (Fig. 5). RSV infection has been shown to alter host gene expression through various molecular mechanisms, including epigenetic regulation. One key factor potentially involved in this process is DNMT3A, an enzyme responsible for *de novo* DNA methylation. The observed upregulation of DNMT3A during RSV infection suggests a role in mediating epigenetic modifications, such as DNA methylation at promoter regions of immune-related genes. These changes may contribute to the

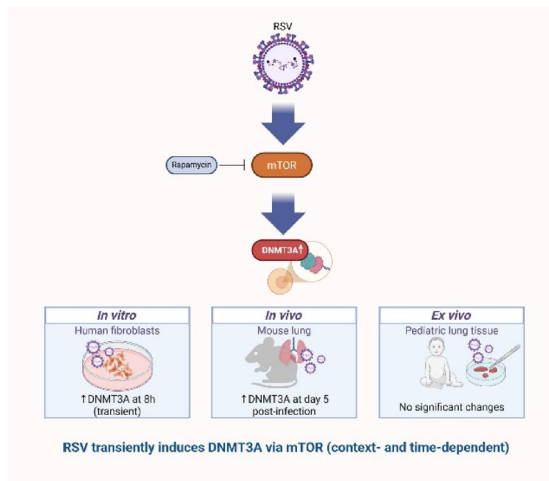


Fig. 5. RSV transiently induces DNMT3A. DNMT3A expression increased after 8 h of RSV infection in MRC-5 cells and this effect was partially inhibited by rapamycin. RSV-infected mice presented an increased gene expression of *Dnm3a* 5 days after infection. Ex-vivo RSV-infected pediatric lung tissue did not present an increase *DNMT3A* gene expression compared to uninfected tissue 24 h after infection. Image created with <https://biorender.com/>.

suppression or activation of specific transcriptional programs, thereby influencing the host's antiviral response and disease progression. Further investigation into DNMT3A's target sites and regulatory interactions during RSV infection could provide insights into viral strategies for immune evasion and the long-term impact of infection on the host epigenetic landscape.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

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References

- Shay, D. K. Bronchiolitis-associated hospitalizations among US children, 1980–1996. *JAMA* **282**, 1440–1446 (1999).
- Shay, D. K., Holman, R. C., Roosevelt, G. E., Clarke, M. J. & Anderson, L. J. Bronchiolitis-associated mortality and estimates of respiratory syncytial virus-associated deaths among US children, 1979–1997. *J Infect Dis.* **183**, 16–22 (2001).
- Rima, B. et al. ICTV virus taxonomy profile: Pneumoviridae. *J. Gen. Virol.* **98**, 2912–2913 (2017).
- Havers, F. P. et al. Burden of respiratory syncytial virus-associated hospitalizations in US adults, October 2016 to September 2023. *JAMA Netw Open.* **7**, e2444756 (2024).
- Rossey, I., Sedeyn, K., De Baets, S., Schepens, B. & Saelens, X. CD8+ T cell immunity against human respiratory syncytial virus. *Vaccine.* **32**, 6130–6137 (2014).
- Shi, T. et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: A systematic review and modelling study. *The Lancet.* **390**, 946–958 (2017).
- Stein, R. T. et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *The Lancet.* **354**, 541–545 (1999).
- Jares, B. S. & Polack, F. P. The long road to protect infants against severe RSV lower respiratory tract illness. *Fl1000Res* **8**, 610 (2019).
- da Silva, G. S., Borges, S. G., Pozzebon, B. B. & de Souza, A. P. D. Immune responses to respiratory syncytial virus vaccines: Advances and challenges. *Microorganisms.* **12**, 2305 (2024).
- Panjwani, A., Katz, J. S. & Katz, R. K. Nirsevimab and maternal respiratory syncytial virus vaccine recommendations for the pediatric population. *Pediatr Ann.* **54**, e160–e166 (2025).
- Allis, C. D. & Jenuwein, T. The molecular hallmarks of epigenetic control. *Nat Rev Genet.* **17**, 487–500 (2016).
- de Almeida Nagata, D. E. et al. Epigenetic control of Foxp3 by SMYD3 H3K4 histone methyltransferase controls iTreg development and regulates pathogenic T-cell responses during pulmonary viral infection. *Mucosal Immunol.* **8**, 1131–1143 (2015).
- Spalluto, C. M. et al. IFN- γ influences epithelial antiviral responses via histone methylation of the *RIG-I* promoter. *Am J Respir Cell Mol Biol.* **57**, 428–438 (2017).
- Feng, Q. et al. Histone deacetylase inhibitors suppress RSV infection and alleviate virus-induced airway inflammation. *Int J Mol Med.* **38**, 812–822 (2016).
- Othumpangat, S., Walton, C. & Piedimonte, G. MicroRNA-221 modulates RSV replication in human bronchial epithelium by targeting NGF expression. *PLoS ONE* **7**, e30030 (2012).
- Huang, Z., Liu, X., Wu, X., Chen, M. & Yu, W. MiR-146a alleviates lung injury caused by RSV infection in young rats by targeting TRAF-6 and regulating JNK/ERKMAPK signaling pathways. *Sci Rep.* **12**, 3481 (2022).
- Kyo, M. et al. Nasal microRNA signatures for disease severity in infants with respiratory syncytial virus bronchiolitis: A multicentre prospective study. *BMJ Open Respir Res.* **11**, e002288 (2024).
- Elgizouli, M. et al. Reduced PRF1 enhancer methylation in children with a history of severe RSV bronchiolitis in infancy: An association study. *BMC Pediatr.* **17**, 65 (2017).
- Pischedda, S. et al. Role and diagnostic performance of host epigenome in respiratory morbidity after RSV infection: The EPiRESVi study. *Front Immunol.* **13**, 875691 (2022).

20. Ladle, B. H. et al. De novo DNA methylation by DNA methyltransferase 3a controls early effector CD8 + T-cell fate decisions following activation. *Proc. Natl. Acad. Sci.* **113**, 10631–10636 (2016).
21. Gu, T. et al. DNMT3A and TET1 cooperate to regulate promoter epigenetic landscapes in mouse embryonic stem cells. *Genome Biol.* **19**, 88 (2018).
22. Wu, H. & Zhang, Y. Reversing DNA methylation: Mechanisms, genomics, and biological functions. *Cell* **156**, 45–68 (2014).
23. Li, X. et al. Methyltransferase Dnmt3a upregulates HDAC9 to deacetylate the kinase TBK1 for activation of antiviral innate immunity. *Nat Immunol.* **17**, 806–815 (2016).
24. Rowles, D. L. et al. DNA methyltransferase DNMT3A associates with viral proteins and impacts HSV-1 infection. *Proteomics* **15**, 1968–1982 (2015).
25. Anon. Gene expression Omnibus.
26. Talukdar, S. N. et al. RSV-induced expanded ciliated cells contribute to bronchial wall thickening. *Virus Res.* **327**, 199060 (2023).
27. Xu, X. et al. Bromodomain Containing protein 4 (BRD4) regulates expression of its interacting coactivators in the innate response to respiratory syncytial virus. *Front Mol Biosci.* **8**, 728661 (2021).
28. Chatterjee, S. et al. Structural basis for human respiratory syncytial virus NS1-mediated modulation of host responses. *Nat Microbiol.* **2**, 17101 (2017).
29. Habibi, M. S. et al. Neutrophilic inflammation in the respiratory mucosa predisposes to RSV infection. *Science* **1979**, 370 (2020).
30. Galetzka, D., Tralau, T., Stein, R. & Haaf, T. Expression of DNMT3A transcripts and nucleolar localization of DNMT3A protein in human testicular and fibroblast cells suggest a role for de novo DNA methylation in nucleolar inactivation. *J Cell Biochem.* **98**, 885–894 (2006).
31. de Freitas, N. D., Gassen, R. B., Fazolo, T. & de Souza, A. P. D. Rapamycin increases RSV RNA levels and survival of RSV-infected dendritic cell depending on T cell contact. *Toxicol Vitro.* **36**, 114–119 (2016).
32. Huynh, H., Levitz, R., Huang, R. & Kahn, J. S. mTOR kinase is a therapeutic target for respiratory syncytial virus and coronaviruses. *Sci Rep.* **11**, 24442 (2021).
33. de Souza, A. P. D. et al. Respiratory syncytial virus induces phosphorylation of mTOR at ser2448 in CD8 T cells from nasal washes of infected infants. *Clin Exp Immunol.* **183**, 248–257 (2016).
34. Larabee, R. N. Transcriptional and epigenetic regulation by the mechanistic target of rapamycin complex 1 pathway. *J Mol Biol.* **430**, 4874–4890 (2018).
35. Chen, M. et al. The activation of mTOR signalling modulates DNA methylation by enhancing DNMT1 translation in hepatocellular carcinoma. *J Transl Med.* **21**, 276 (2023).
36. Koh, H. B., Scruggs, A. M. & Huang, S. K. Transforming growth factor- β 1 Increases DNA methyltransferase 1 and 3a expression through distinct post-transcriptional mechanisms in lung fibroblasts. *J. Biol. Chem.* **291**, 19287–19298 (2016).
37. Antunes, K. H. et al. Microbiota-derived acetate protects against respiratory syncytial virus infection through a GPR43-type 1 interferon response. *Nat Commun.* **10**, 3273 (2019).
38. Liu, S. et al. Epigenetic Modification is regulated by the interaction of influenza A virus nonstructural protein 1 with the De Novo DNA methyltransferase DNMT3B and subsequent transport to the cytoplasm for K48-linked polyubiquitination. *J Virol.* **93**, e01587–e1618 (2019).
39. Bogoi, R. N. et al. Expression profiling of chromatin-modifying enzymes and global DNA methylation in CD4+ T cells from patients with chronic HIV infection at different HIV control and progression states. *Clin Epigenetics.* **10**, 20 (2018).
40. Gray, K. S., Forrest, J. C. & Speck, S. H. The *de novo* Methyltransferases DNMT3a and DNMT3b target the murine gammaherpesvirus immediate-early gene 50 promoter during establishment of latency. *J Virol.* **84**, 4946–4959 (2010).
41. Fu, X., Song, X., Li, Y., Tan, D. & Liu, G. Hepatitis B virus X protein upregulates DNA methyltransferase 3A/3B and enhances SOCS-1CpG island methylation. *Mol Med Rep.* **13**, 301–308 (2016).
42. Wang, J. et al. Prenatal exposure to lipopolysaccharide alters renal DNA methyltransferase expression in rat offspring. *PLoS ONE* **12**, e0169206 (2017).
43. Jinawath, A., Miyake, S., Yanagisawa, Y., Akiyama, Y. & Yuasa, Y. Transcriptional regulation of the human DNA methyltransferase 3A and 3B genes by Sp3 and Sp1 zinc finger proteins. *Biochem J.* **385**, 557–564 (2005).
44. Chen, L.-F. et al. Inhibitory effect of PIK-24 on respiratory syncytial virus entry by blocking phosphatidylinositol-3 kinase signaling. *Antimicrob Agents Chemother.* **64**, e00608–e620 (2020).
45. Thomas, K. W. et al. Respiratory syncytial virus inhibits apoptosis and induces NF- κ B activity through a phosphatidylinositol 3-kinase-dependent pathway. *J. Biol. Chem.* **277**, 492–501 (2002).

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Author contributions

A.B., S.G.B., L.G.R.P., D.N.F., L.D., E.N., G.C., J.P., M.S.C., R.P.S., K.H.A.V.R.R., conducted the experiments and analyzed the data. A.B., S.G.B., L.G.R.P., R.S., J.O.E., C.F.A., wrote the manuscript. A.P.D.S. conceived the study, funding acquisition, project administration, design the experiments, wrote and revised the manuscript. All authors read and approved the final version of the manuscript

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Additional information

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