TFF-3 Modulates Responsiveness to Bronchodilators in Airways

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Abstract

Rhinovirus (RV) is the major cause of exacerbations, or worsening of symptoms, in asthmatic children and adults. This often reduces the efficacy of therapeutic interventions such as bronchodilators – a type of medication used to promote airflow and alleviate asthma symptoms. The exact mechanisms through which RV exposure decreases responsiveness to bronchodilators remain unclear. Previous data demonstrates that airway cells release a specific signature of inflammatory mediators following RV exposure. Other research has shown that Trefoil Factor 3 (TFF-3), one of the mediators identified by our screen, regulates cell motility in other cell types. We show that RV exposure attenuates relaxation in both the airway and human airway smooth muscle (HASM). Given our data, we aim to examine whether or not TFF-3 attenuates the relaxation of HASM and airways.

Primary non-diseased human airway smooth muscle (HASM) was used to examine the consequences of TFF-3 in modulating HASM and airway relaxation. Following RV-C15 exposure, it was found that the airway and HASM relaxation was attenuated. TFF-3 exposure also attenuated both airway and HASM relaxation. Additionally, TFF-3 exposure partially weakened iso-induced reversal of carbachol-induced phosphorylation of the myosin light chain. Within the cADDis Live Cell Assays, which provide real-time kinetic measurements of cyclic Adenosine Monophosphate (cAMP) production, TFF-3 attenuated formoterol-induced cAMP production. Researching how bronchodilation pathways change following RV infection can lead to the development of effective treatments and pharmaceutical solutions to alleviate worsening asthma symptoms during a viral exacerbation of the disease.

1 Introduction

Rhinovirus, or RV, is the major cause of exacerbations in asthmatic children and adults and can lead to reduced efficacy of therapies for symptomatic relief. Previous research has found that RV produces unique effects on asthmatics in comparison to other viral infections, causing increased symptom severity and stronger effects on treatment response (Reddel et al. 2011). Another study revealed that bronchodilator treatments have reduced efficacy in both adult and pediatric patients once exposed to RV (James & Collins 2012). Although the mechanisms of RV induced asthmatic exacerbations are well-studied, research has yet to uncover exactly how RV may affect treatment efficacy.

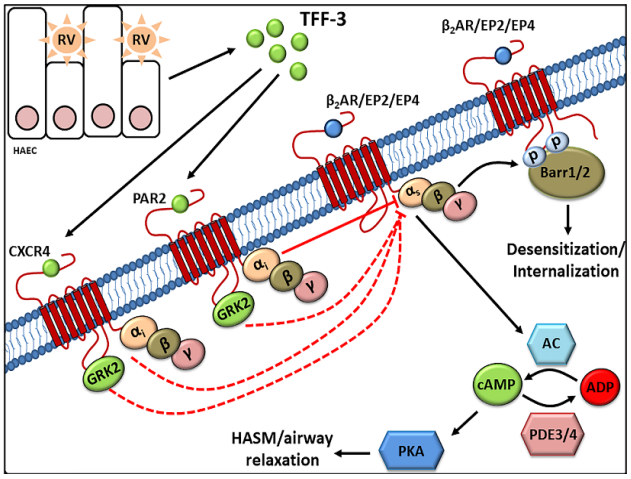

Figure 1: Proposed Trefoil Factor-3 Cell Signaling Pathway

The proposed pathway for when TFF-3 is released into the body post-infection with rhinovirus, activating cell receptors as a response.

We identified one of the mediators released following RV infection of airway epithelial cells as Trefoil Factor 3 (TFF-3). TFF-3 exhibits various properties, including forming dimers which interact with other proteins and aid in signaling within the body (Weste et al. 2022). Research has demonstrated that TFF-3 plays a role in the wound healing response and has established functions in mucosal cells in the gastrointestinal

tract, specifically aiding in their repair and regeneration post-damage (Braga et al. 2020) through enhanced cell migration and anti-apoptotic effects (Fan et al. 2023). Given that TFF-3 engages in reparative responses, it likely plays a similar role within the airways following RV exposure. Our findings suggest that TFF-3 is present in a post-RV stimulation of primary human airway epithelial cells and intact in human lung slices. Figure 1 depicts a proposed signaling pathway by which TFF-3 can modulate airway function. As shown, the Human Airway Epithelial Cell (HAEC) releases ligand TFF-3 post-exposure to RV, which activates Protease-Activated Receptor-2 (PAR2) and/or C-X-C Motif Chemokine Receptor 4 (CXCR4) cell receptors on the human airway smooth muscle (HASM). Previous studies have established a connection between PAR2 and both inflammation and inflammatory diseases (Kennedy et al. 2020), while CXCR4 is related to tissue regeneration and cell proliferation. This in turn leads to the inhibition of alpha s, which is linked to the G protein and adenylyl cyclase (AC), decreasing cAMP production and airway relaxation. (Bianchi & Mezzapelle 2020).

2 Materials and Methods

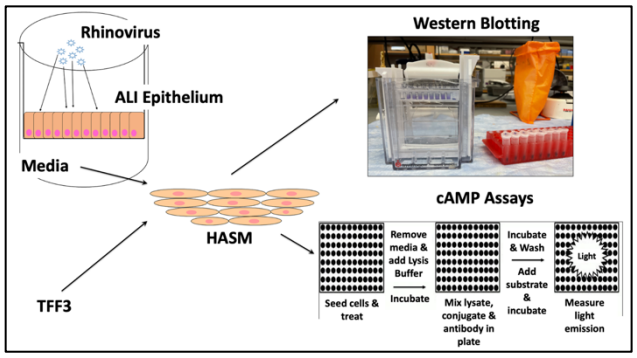

Figure 2: Human Airway Smooth Muscle Analysis Methods (Left) Cells cultured on both sides of the transwell insert until confluency reached (Top Right) Western Blotting (Bottom Right) cAMP assay workflow.

This study utilized primary HASM cells to conduct the various procedures seen in Figure 2. We obtained human lung tissue samples from the National Disease Research Interchange (NDRI) and from the International Institute for the Advancement of Medicine (IIAM). Primary HASM cell lines originated from the trachea of both healthy, non-asthmatic donors with no history of chronic diseases or smoking, and from diseased, aborted-transplant human lung donors. Cells were cultured in F-12 medium supplemented with 10% Fetal Bovine Serum (FBS) to encourage growth, along with 100 U/mL penicillin, 0.1 mg/ml streptomycin, and 0.1% Primocin from Invitrogen to prevent contamination. To split the cell lines, we washed the cells with phosphate buffered saline (PBS) and aspirated the solution. Then, we added the enzyme 0.05% 1X Trypsin-EDTA (Gibco) to detach and release the cells from the bottom of the plates or flasks. Later in the procedure, we added in feeding media to prevent the trypsin from lysing the cells and causing complete damage.

The experiment utilized the HASM cells in subculture during passages 1–5, as these cells retain the expression of native contractile protein. HAEC was derived from the same tracheas as the HASM cells. Cells were on transwell inserts from Corning and were fed on both the upper and lower side of the insert with Pneumacult Ex Basal Media from StemCell Technologies until they reached the appropriate level of confluency. The cells then underwent air-liquid interface (ALI) differentiation for 21 days prior to stimulation and were fed with Pneumacult ALI Media. We fed and maintained cell lines by routinely replacing media in order to prevent the cells from drying out.

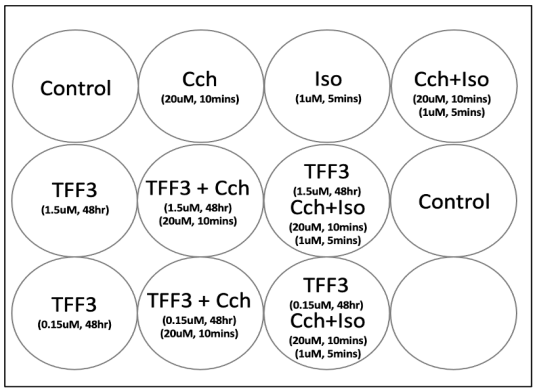

Figure 3: Western Blotting Media Treatments

HASM underwent treatment to test the effects of TFF-3 on protein modifications that are surrogates for bronchodilation/relaxation. The cells grew to confluence until the desired percentage of cells covered the surface of the plate, then were serum starved with stock media 24-74 hours before treatment. This Western blot

utilized four different cell donor lines, with cells receiving a treatment of 0.15μM and 1.5μM of TFF-3 48 hours before short-time point treatments. After the 48-hour period, specific wells then received a treatment of either carbachol (carbamoyl choline - Cch) solution at 20μM for 10 minutes, a bronchodilator known as isoprenaline hydrochloride/isoproterenol (Iso) at 1μM for 5 minutes, or both (Figure 3). Sigma Aldrich (St. Louis, MO) supplied Carbachol (Cch), Iso, and perchloric acid. RNA transfection produced Rhinovirus C15, followed by a RNase A treatment of clarified lysates and ultracentrifugation through a 30% sucrose cushion to purify the virus. Cell Signaling Technologies supplied Radio-Immunoprecipitation Assay (RIPA) buffer, utilized to lyse the cells. A quick addition of stop buffer (perchloric acid) then halted intracellular signaling, followed by lysate collection for Western blotting.

After collection, lysate either underwent sonication or freezing to break the cell membranes and release the contents, followed by a heating process at 70°C for 10 minutes to denature the samples. The samples then underwent centrifugation at 14000 rpm for 5 minutes. Using a micropipette, we added 3uL of the latter, 15uL of the samples, and 5uL of the sample buffer into 15 4-12% Bis-Tris polyacrylamide gel wells. After running the gels, iBlot facilitated the transfer of samples onto the membrane for staining with antibodies against phosphorylated myosin light chain (pMLC) and total myosin light chain (MLC) to compare the fold change of expression between the two. Antibodies for detection of pMLC came from Cell Signaling Technologies. EMD Millipore supplied the MLC antibodies. We then used ImageStudio to visualize membranes and analyze band intensities.

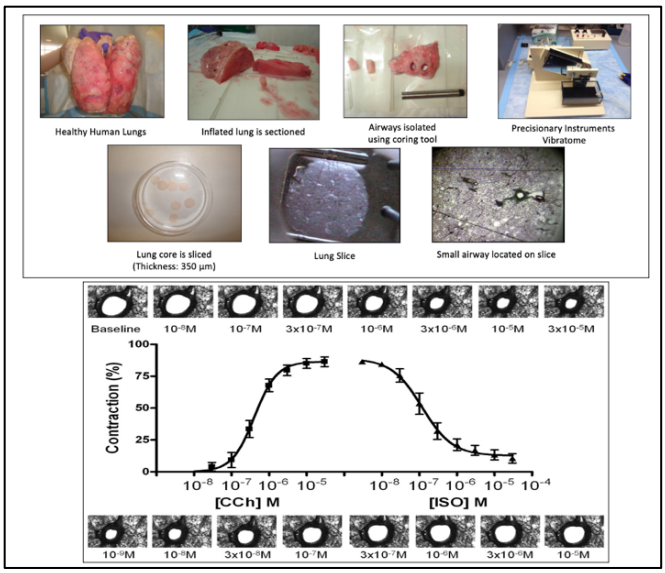

Figure 4: Generation and Measurement of Human Precision Cut Lung Slices

(Top) Method shown in images depicting how hPCLS are generated from human lung samples. (Bottom) hPCLS are then measured to measure contraction (when Cch stimulated) and relaxation (when Iso stimulated).

Human precision cut lung slices (hPCLS) shown in the top section of Figure 4 originated from the lungs of diseased or non-diseased patients and received an injection of 2% low melting agarose into the bronchi and branched airways to inflate them. After the agarose set in ice, we sectioned the lung lobes and isolated the airways into 8mm diameter cores using the coring tool and sliced at a thickness of 350μm using the Precisionary Instruments VF300 Vibratome. The best intact airways provided samples for PCLS assays and separate measurements for contraction and relaxation as shown in the bottom of Figure 4. Any other airway slices contributed to supernatant experiments.

Muscle relaxation was tracked by measuring cAMP production. To measure the total cellular cAMP production in cell lysates, we plated sub-confluent HASM cells and allowed them to grow to ~80% confluence using cAMP assays purchased from the Applied Biosystems cAMP-Screen Immunoassay System, adhering to the manufacturer provided instructions. After aspirating the media, we replaced it with conditioned media from a control buffer or with RV-C15 stimulated

epithelial cells for 48 hours prior to stimulation with isoproterenol. The cells underwent lysis and were incubated for 30 minutes at 37°C. We then added Conjugate Dilution Buffer, cAMP-AP Conjugate, anti-cAMP antibody, and the samples to a pre-coated assay plate to incubate for 1 hour on a plate shaker. After a 30 minute incubation period with CSPD®/Sapphire-II RTU Substrate, a luminometer measured light emission from the plates. We derived data from standard curves and reported cAMP levels following standard dilutions.

When performed with the cell lines, the cADDis Live Cell Assays detected Gs protein activation, which when stimulated produces cAMP. This allowed for the determination of TFF-3's effect on this signal. This assay was able to measure cAMP production in real time, showcasing the response kinetics with greater dimming and indicating a higher cAMP concentration in the cells.

On Day 0, cells underwent preparation for transduction and seeding. We decanted T75 containing confluent HASMCs by removing the media within the flask, and then we washed the cells with 1X PBS (Gibco). We then trypsinized the cells to release them from the bottom of the flask and isolated them for plating in F12 feeding medium supplemented with 10% FBS and Primocin (Gibco). We counted the cells in suspension using a hemocytometer and 0.4% Trypan Blue (Gibco). Cell density varied per experimental target. Cells were plated in a 96 well black-walled tissue culture treated assay plate (Corning COSTAR). The cells grew and proliferated for two days until transduction.

To perform the transduction, we supplemented the media with a combination of the cADDis Green Down cAMP Sensor Assay (Montana Molecular) and Trichostatin A (Sigma). The cells incubated for another two days until imaging. We took images once per second using an inverted fluorescent microscope to compile a cohesive TIFF file that can later undergo analysis using Python for image analysis and Prism for generating time course curves. One minute into the sequence, we used formoterol fumarate dihydrate (Sigma) to invoke the cAMP response, leading to a downward shift in green fluorescence.

3 Results

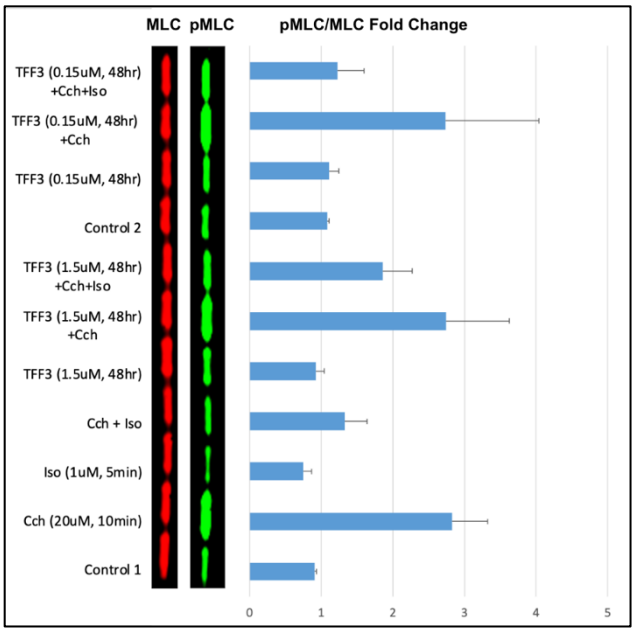

Figure 5: TFF-3 Stimulation of HASM Partially Attenuates Isoproterenol-Induced Reversal of Carbachol-Induced Phosphorylation of Myosin Light Chain (pMLC)

HASM were stimulated with TFF-3, and then with Cch ± Iso, lysates collected for Western blot analysis. (Left) Immunoblot of pMLC and MLC from n=1 donor cell line (Right) Band density measurements of pMLC normalized to MLC, n=4 distinct donors

Figure 5 depicts a representative immunoblot from one of the four cell lines utilized within the experiments. The red shows the total MLC and the green shows the phosphorylated MLC. The group mean data from Figure 5 shows the average fold change between the four cell lines used in the experiments, indicating minimal change in the pMLC signal in the Cch + TFF-3 treatment. Compared to the Cch + Iso treatment, addition of the highest concentration of TFF-3 (1.5μM) appeared to slightly attenuate the ability of Iso to reduce Cch-induced pMLC expression.

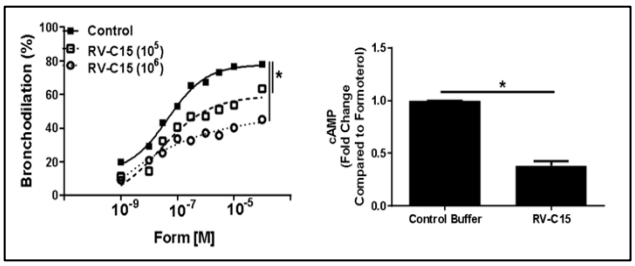

Figure 6: RV Stimulation Attenuates Formoterol (Form)-Induced Airway/HASM Relaxation

*(Left) hPCLS stimulated with RV and airway relaxation measured to form dose responses. n=10-18 distinct donors, *p < 0.05 (Right) HASM stimulated with media from control or RV exposed epithelial cells, then stimulated with form – cAMP production assessed by ELISA. n=7 distinct donors, *p < 0.05*

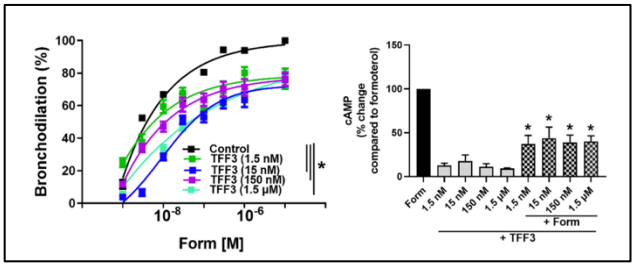

Figure 7: TFF-3 Stimulation Attenuates Formoterol-Induced Airway/HASM Relaxation

*(Left) hPCLS stimulated with TFF-3 and airway relaxation measured to formoterol dose responses. n=4 distinct donors, *p < 0.05 (Right) HASM stimulated TFF-3, then stimulated with formoterol – cAMP production assessed by ELISA. n=5 distinct donors, *p < 0.05*

Figures 6 and 7 depict results from the PCLS assays, graphing the percentage of bronchodilation while comparing the control to treatments with either RV or TFF-3 at varying concentrations. Both RV-C15 and TFF-3 treatments of PCLS attenuated formoterol-induced airway relaxation. These figures also depict cAMP assay results, showing that both RV and TFF-3 exposure attenuates cAMP production by formoterol.

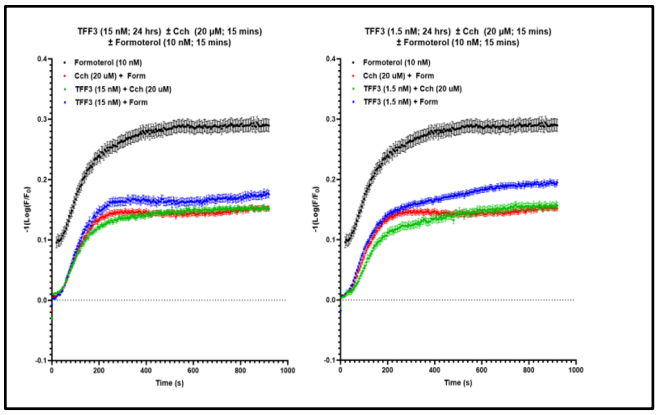

Figure 8: cADDis assay to measure cAMP in live cells

HASM cells received treatment with TFF-3 in varying concentrations, and cADDis assay measured the effects in expression when exposed to formoterol and/or carbochol.

In addition to the ELISA-based cAMP assays, we performed cADDis assays which measured the effect of TFF-3 treatment (24 hour pre-treatment) in the presence and absence of Cch (20 µM, 15 minutes pre-treatment). TFF-3 pre-treatment appears to attenuate formoterol-induced cAMP production, which further decreases with co-stimulation from Cch (Figure 8).

4 Discussion

TFF-3 is known to for its ability to form trimers, which can enhance its role in signaling. Generally, it is recognized for promoting epithelial cell health in the gastrointestinal tract and assisting with epithelial repair during wound healing by reducing cell death through the activation of multiple receptors. However, it remains unclear whether TFF-3 affects the biology of airway smooth muscle. Notably, TFF-3 release increased following stimulation of airway epithelial cells with RV-C15. Since TFF-3 appears to be associated with RV stimulation of the airways, its release may lead to activation of one or more receptors. This potentially causes changes in the smooth muscle biology, resulting in decreased cAMP production and reduced airway relaxation.

We used four different cell donor lines to analyze the Western blot results, providing a larger pool of data. The group mean fold change from the Western blot results reveals the overall effects of phosphorylation of MLC. This serves as a readout for muscle contraction

following exposure to Cch and Iso reversing the phosphorylation. The data shows that TFF-3 partially inhibits the Iso-dependent reversal of carbachol induced pMLC despite not augmenting carbachol-induced pMLC. It is possible that pMLC readout is not a sensitive enough to measure the effects of TFF-3 on airway smooth muscle relaxation. Hence, it is important to examine other targets involved in relaxation going forward.

We used precision cut lung slices (PCLS) to assess changes in formoterol-induced relaxation following RV or TFF-3 exposure since formoterol is a mainstay bronchodilator used in asthma therapy. RV and TFF-3 was able to significantly reduce formoterol's effectiveness in relaxing the airways. Treatment with RV or TFF-3 at varying concentrations resulted in significantly less bronchodilation in response to formoterol compared to the control buffer stimulation. Future experiments are needed to determine whether RV-dependent attenuation of relaxation is TFF-3-dependent.

Additionally, we used human airway smooth muscle to measure cAMP generation to indicate muscle relaxation. Results demonstrated that exposure of airway smooth muscle to either media from RV-stimulated epithelial cells or direct exposure to TFF-3 reduced cAMP production by formoterol show a decrease in the amount of cAMP generated by formoterol exposure. This indicates that the relaxation of muscles following RV or TFF-3 stimulation decreases bronchodilation and relaxation responses. The cADDis results show that TFF-3 stimulation decreases the responsiveness of airway smooth muscle to formoterol, which the addition of Cch augments. These results suggest that TFF-3 may increase an inhibitory signal that Cch treatment enhances, however additional confirmation would be needed.

A major limitation of this study is the lack of understanding regarding how TFF-3 can elicit these types of responses. The specific mechanisms by which RV decreases responsiveness to bronchodilators and the receptors to which RV binds remain unclear. There are gaps in understanding how TFF-3, primarily recognized as a mediator in the gastrointestinal tract, modulates bronchodilation in HASM within the lungs. In future research related to TFF-3 and RV's effects on asthmatic exacerbations, N numbers can be expanded to address response heterogeneity and further support these findings. Although animal models may be a valid choice for experimentation, observations reveal that mouse models have not demonstrated TFF-3 expression or responsiveness to bronchodilators in the same way that human airways do, which may limit the effectiveness of this model.

5 Conclusion

In conclusion, the data identifies potential therapeutic targets that researchers can modulate to decrease the effects of RV on asthma exacerbations. Western blot results indicated that the highest concentration of TFF-3 attenuated the ability of the Iso bronchodilator to reduce Cch-induced pMLC expression. PCLS Assays visually demonstrate the tissue's ability to attenuate cAMP production when treated with RV and TFF-3. The cADDis assay results show that TFF-3 stimulation reduces the airway's responsiveness to bronchodilators, which in turn the addition of Cch can enhance. Gaining a better understanding of how TFF-3 signaling pathways alter bronchomotor tone will improve the efficacy of current treatments within the asthma therapeutic area. Our data suggests that TFF-3 may function as an inhibitory molecule that could serve as a novel therapeutic target to prevent bronchodilator hyporesponsiveness. These results offer hope for improving the current bronchodilators for those exposed to RV and for developing new therapeutics to treat RV-dependent asthma exacerbations. Further studies would need to be conducted to determine the exact mechanisms by which TFF-3 attenuates these responses.

6 References

[1] Bianchi, M. E., & Mezzapelle, R. (2020). The chemokine receptor CXCR4 in cell proliferation and tissue regeneration. Frontiers in Immunology, 11. https://doi.org/10.3389/fimmu.2020.02109

[2] Braga Emidio, N., Brierley, S. M., Schroeder, C. I., & Muttenthaler, M. (2020). Structure, function, and therapeutic potential of the trefoil factor family in the gastrointestinal tract. ACS Pharmacology; Translational Science, 3(4), 583–597. doi:10.1021/acsptsci.0c00023

[3] Caddis: Live cell camp assays. Montana Molecular. (2024, March 8). https://montanamolecular.com/live-cell-camp-assay-caddis/

[4] Fan, M., Song, W., Hao, Z., Zhang, J., Li, Y., & Fu, J.

(2023). Construction of lncrna-MIRNA- mrna regulatory network in severe asthmatic bronchial epithelial cells: A bioinformatics study. Medicine, 102(35). https://doi.org/10.1097/md.0000000000034749

[5] James, J., & Collins, F. (2012). Symptomatic viral infection is associated with impaired response to treatment in children with acute asthma. Pediatrics, 130(Supplement_1). https://doi.org/10.1542/peds.2012-2183rrr

[6] Kennedy, A. J., Sundström, L., Geschwindner, S., Poon, E. K., Jiang, Y., Chen, R., Cooke, R., Johnstone, S., Madin, A., Lim, J., Liu, Q., Lohman, R.-J., Nordqvist, A., Fridén-Saxin, M., Yang, W., Brown, D. G., Fairlie, D. P., & Dekker, N. (2020). Protease-activated receptor-2 ligands reveal orthosteric and allosteric mechanisms of receptor inhibition. Communications Biology, 3(1). https://doi.org/10.1038/s42003-020-01504-0

[7] Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. Current Gastroenterology Reports, 12(5), 319–330. https://doi.org/10.1007/s11894-010-0131-2

[8] Reddel, H. K., Jenkins, C., Quirce, S., Sears, M. R., Bateman, E. D., O'Byrne, P. M., Humbert, M., Buhl, R., Harrison, T., Brusselle, G. G., Thoren, A., Sjobring, U., Peterson, S., Ostlund, O., & Eriksson, G. S. (2011). Effect of different asthma treatments on risk of cold-related exacerbations. European Respiratory Journal, 38(3), 584–593. https://doi.org/10.1183/09031936.00186510

[9] Weste, J., Houben, T., Harder, S., Schlüter, H., Lücke, E., Schreiber, J., & Hoffmann, W. (2022). Different molecular forms of TFF3 in the human respiratory tract: Heterodimerization with IGG FC binding protein (FCGBP) and proteolytic cleavage in bronchial secretions. International Journal of Molecular Sciences, 23(23), 15359. https://doi.org/10.3390/ijms232315359

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