LUND
UNIVERSITY

Effects of Insulin and Glucose Stimulation on the Anti-Viral Response in Bronchial Epithelial Cells

Omeyme Naqchi

Master's Degree Project in Molecular Biology, 30 cr
2020

Department of Biology
Lund University

Reset form

Effects of Insulin and Glucose Stimulation on the Anti-Viral Response in Bronchial Epithelial Cells

Omeyme Naqchi

Email: Om2874na-s@student.lu.se

Supervisor Name: Lena Uller

Email: Lena.uller@med.lu.se

Master's Degree Project in Molecular Biology, 30 credits

2020

Faculty of Science
Lund University
Sweden

Abbreviations:

AEC – Airway Epithelial Cell

AHR – Airway Hyperresponsiveness

ATP – Adenosine Triphosphate

BSA – Bovine Serum Albumin

cDNA – Complementary Deoxyribonucleic Acid

Ct – Cross Threshold

DAMPs – Damage Associated Molecular Patterns

DC – Dendritic Cells

dsRNA – Double Stranded RNA

ELISA – Enzyme Linked Immunosorbent Assay

ERK – Extra Cellular Signal Regulated Kinase

FBS – Foetal Bovine Serum

GAPDH – Glyceraldehyde 3-phosphate Dehydrogenase

HBEC – Human Bronchial Epithelial Cell

HDM – House Dust Mite

IAV – Influenza A Virus Th – T Helper

IFNβ – Interferon-beta

Ig – Immunoglobulin

IL – Interleukin

ILCs –Innate Lymphoid Cells

ILC2 –Innate Lymphoid Cell 2

IRFs – Interferon Regulatory Factors

mTOR – The Mammalian Target of Rapamycin

MAPK – Microtubule Associated Protein Kinase

MOI – Multiplicity of infection

MDA5 – Melanoma Differentiation-Associated Protein 5

NKT – Natural Killer Th Cells

NF-κB – Nuclear Factor Kappa-light-chain-enhancer of activated B cells

Poly(I:C) – Polyinosinic: Polycytidylic acid

PAR-2 – Proteinase-Activated Receptor

PAMPs – Pathogen Associated Molecular Patterns

PRRs – Pattern Recognition Receptors

PBS – Phosphate Buffer Solution

PI3K/Akt (PKB) – Phosphoinositide 3-kinases/protein Kinase B(PKB)

RT-qPCR – Real Time Quantitative Polymerase Chain Reaction

RT – Room Temperature

RIG-I – Retinoic Acid-Inducible Gene 1

RV – Rhinovirus

RPMI – Roswell Park Memorial Institute Medium

SEM – Standard Error of the Mean

TLR3 – Toll-like Receptor 3

Th – T Helper Cells

TSLP – Thymic Stromal Lymphopoietin

TNF-α – Tumour Necrosis Factor-alpha

UBC – Ubiquitin CPEST – Penicillin and Streptomycin

Abstract:

Asthma exacerbation is among the leading causes of mortality and morbidity. Bronchial epithelial cells (BECs) are of interest because they represent not only a physical barrier against infections, but also a biological barrier between the inhaled agents, such as allergens, and the immune system. As known, systemic inflammation of the lungs, or dietary factors could have effects on lung disease worsening. Metabolic syndrome is another crucial medical condition that exhibits high levels of glucose (hyperglycaemia), systemic inflammation, obesity, as well as insulin resistance which is a risk factor for asthma development. Insulin resistance also links asthma with metabolic syndrome and obesity. Deficient production of anti-viral interferons (IFNs) may be involved in causing viral-induced asthma exacerbations. Allergens also a risk factor for viral-induced asthma exacerbation. Hence, drugs inducing lung IFN production would be warranted. In the current project, the effects of elevated levels of glucose and insulin on viral-induced IFNβ in BECs and *in-vitro* asthma exacerbation model have been investigated. Although our results are preliminary, we have showed that glucose and insulin might increase viral-induced IFNβ production in BECs and restored house dust mite (HDM)-impaired IFNβ expression in an *in-vitro* asthma exacerbation model. We assume that insulin effects are abrogated in the presence of insulin resistance conditions, which could be a risk factor for asthma exacerbation development in obese and diabetes asthmatics. We have shown that PRRs, including TLR-3, RIG-I, and MDA5, are not involved in the process of the enhancement of IFNβ by insulin and glucose actions.

Keywords: Asthma exacerbation, Allergic Asthma, Rhinovirus, Insulin, Glucose.

1 | Introduction:

Asthma is a chronic inflammatory disorder of the airways, characterized by shortness of breath, airway inflammation, chest tightness and wheezing [1]. It affects approximately 334 million people worldwide with an expected increase to around 400 million [2], with a substantial impact on healthcare costs by 2025 [1, 2]. Different risk factors are implicated in asthma development, including host factors and environmental factors [2, 3]. Asthma is a heterogeneous disease composed of many phenotypes and endotypes. Asthma phenotypes exhibit various visible characteristics (e.g., allergic vs. non-allergic asthma), while endotypes are represented by different molecular mechanisms involved in asthma pathogenesis (e.g., T2-high vs. T2-low asthma) [4]. Although there are some biological markers for asthma, the heterogeneity and complexity of asthma pathogenesis make it problematic [2]. Notably, asthma is associated with the activation of both the innate and adaptive immune systems that are mediated by numerical biological and immunological processes [4]. Various immune cells mediate asthma pathogenesis, such as T helper cells (Th), innate lymphoid cells (ILCs), dendritic cells (DC), natural killer T cells (NKT), macrophages, basophils, eosinophils, mast cells and IgE-producing B cells, in addition to cytokines and chemokines produced by participating cells [2, 4].

Allergic asthma is the most common phenotype with increased morbidity and mortality. Allergic asthma is characterized by the airway hyperresponsiveness (AHR), extreme airway mucus production and airway narrowing [4, 5]. It is usually induced by frequent and subsequent exposures to various environmental allergens, such as pollens, fungal spores, pet's dander and house dust mites (HDM) [4]. Remarkably, allergens influence individuals differently. However, all allergens trigger the inflammation through Th2 activation [4, 6]. Previous studies have indicated that neutrophils are the first recruited cells in allergic immune response [7, 8], which further regulates the immune response by triggering IL-8 (also called CXCL8) and TNF-α release in the airway epithelial cells (AECs), as well as recruiting eosinophils [9, 10]. Also, major cytokines including IL-4, IL-5, IL-13, or IL-9, play essential roles in allergic asthma [4, 10].

Bronchial Epithelial Cells (BECs) constitute the first line of defence in the airways and form both a physical and innate immune barrier to exogenous and endogenous triggers of

inflammation. They are target cells to the inhaled allergens and respiratory pathogens such as rhinovirus (RV) that causes the common cold [2, 11, 12]. Following allergen exposure or RV infection, bronchial epithelial cells start to release cytokines that act as alarmins (IL-25, IL-33 and TSLP) [13], leading to dendritic cell maturation and Innate Lymphoid Cell 2 (ILC2) activation, which in turn produce IL-4 and IL-13. The latter cytokines shown to impair BECs barrier in the human airway [4, 10]. In addition, allergens contributing to asthma pathogenesis, including HDM, exhibit protease activity by which it causes epithelium damage [12, 14]. Damaged epithelium caused by HDM, viral infection, cell injury or stress induces the release of metabolite damage associated molecular patterns (DAMPs) such as ATP and uric acid (UA), and the pro-inflammatory cytokine IL-8 [6]. Released alarmins, ATP and UA, alert the immune system of early damage events [6]. Also, IL-8 and TNF-α are capable of alerting our immune system about cell damage or viral recognition, and especially IL-8, have chemotactic activity for various immune cells [15-17]. DAMPs and Pathogen Associated Molecular Patterns (PAMPs) stimulate the pattern recognition receptors (PRRs) in BECs [18]. PRRs activation leads to downstream of different intracellular signaling pathways, activation of transcriptional

factors and distinct immune response [11]. These innate mechanisms are particularly important in asthma exacerbation.

Asthma exacerbation is a transient stage, even though it is considered as acute or subacute worsening of asthma symptoms and defect lung function [19, 20]. Various environmental triggers, such as allergens, pollen, air pollutants, and smoke, as well as genetic differences and viral infection, are risk factors of asthma exacerbation [2]. However, RV has emerged as the most prevalent cause of asthma exacerbation [12, 19, 20]. Upon viral infections, RV RNA is amplified, and the host immune response is triggered [12]. During viral replication, a formed double-stranded RNA (dsRNA) intermediate is recognized by toll-like receptor 3 (TLR3), the retinoic acid-inducible gene-I receptor (RIG-I), and melanoma-differentiation associated-gene 5 (MDA5) [21]. Viral-stimulation of the PRRs results in early innate immune responses, including rapid interferon-β (IFNβ) production by the epithelial cells. IFNβ release is regulated through the transcription factors NF-κB and IFN-regulatory factors (IRFs) including IRF3 and IRF7 [22-24]. Interferons (IFNs) are anti-viral proteins that play a fundamental role in limiting viral replication in infected cells and preventing spread to non-infected cells [24]. It is therefore interesting that HDM can directly interfere with IFN production, impaired viral-induced IFNβ

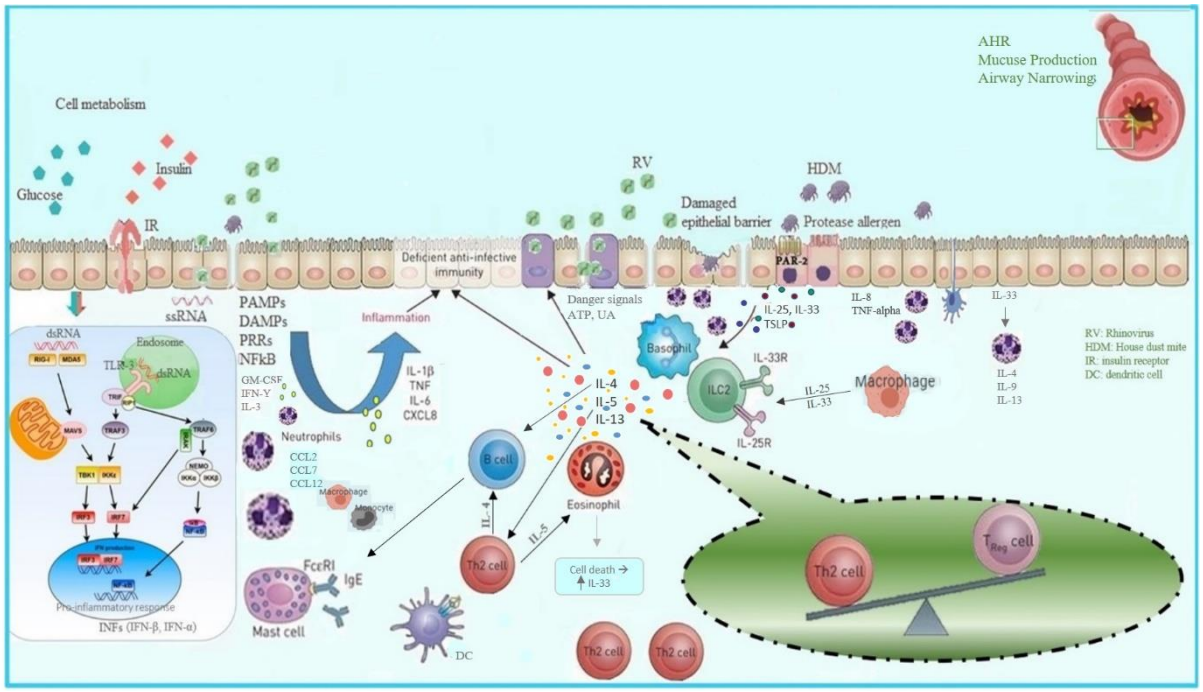

Figure 1 Major mechanism of asthma exacerbation. Major cell types and cytokines involved in asthma exacerbations are depicted. Modified from the original graphic (Addressing Unmet Needs in Understanding Asthma Mechanisms, by Michael R. Edwards1 and Sejal Saglani1, European Respiratory journal, 2017)

and increase the risk of asthma exacerbation [12, 13]. Notably, synthetic dsRNA has been demonstrated to mimic RV infection in BECs not only by inducing cytokines and interferon production, but also reveal pathogenic actions that result from dying cells [25].

Asthma has been epidemiologically and mechanistically linked to obesity and metabolic syndromes, by insulin resistance [26]. Insulin is a pleiotropic hormone, that plays a fundamental role in regulating cell growth and differentiation in the lung [26]. Furthermore, it is implicated in regulating many essential metabolic biological processes, including stimulation of glucose uptake and homeostasis [26]. Additionally, previous studies have indicated that insulin imbalance contributes to inflammatory diseases and play a role in acute and chronic airway inflammation, as it affects various cytokines and inflammatory factors such as IL-8 and TNF-α [27, 28]. Also, insulin stimulates the mammalian target of rapamycin complex 1 (mTORC1), which is essential for IFNβ production [24, 29]. Moreover, it is demonstrated that insulin directly affects TLR3-mediated dsRNA-induced cell apoptosis [30] and mucin production in the respiratory tract through PI3K/Akt and MAPK/ERK pathways [27]. Remarkably, while metabolic syndrome is shown to be associated with asthma independently of obesity, only insulin resistance, which is per se a risk factor of asthma development, is shown to play an important role in the association between asthma and obesity [31]. Altogether, indicate the importance of insulin and glucose in the airway inflammation.

The inflammatory responses must be supported by specific metabolic processes for their energetic requirements [32, 33]. Intriguingly, elevated levels of glucose are associated with the increase of influenza A virus (IAV) as well as the pro-inflammatory and anti-viral cytokines in lung epithelial cell [33]. Moreover, glucose leakage into the airway epithelium has been shown to increase in viral-induced airway inflammation, making patients more susceptible to additional infections [33, 34]. Understanding of the mechanism involved in IFNβ production in BECs and its pharmacological control is an increasingly important field. This is important for discovering novel biological therapies that could limit asthma burden and materialize new treatment strategies that correspond to individual cases that are not responding to conventional therapies. In the current study, we aim to investigate the direct effects of high levels of glucose and insulin on BECs, and to study the effect of glucose and insulin on BECs in asthma exacerbation and severity.

In this research, involving RV16 and poly(I:C)-exposed BEC cell line, that is also challenged with HDM, the direct effects of glucose and insulin on BECs were examined by the

tracking of the gene expression changes of involved chemokines and cytokines both in transcriptional and translational levels. The effects of glucose and insulin on the two central cytokines in asthma exacerbation, TNF-α and IL-8 [25], were examined. In addition to that, ATP release, that mediates the lung inflammation [21] has also been measured. A further comparison was made with high levels of glucose and insulin effects on the anti-viral response, and the participation of endosomal (TLR-3) and cytosolic PRRs (RIG-I & MDA5) in the present of viral-induced cytokine production.

2 | Material and Methods:

Bronchial Epithelial Cell Culturing and Treatment:

Human Bronchial epithelial cell line, BEAS-2B, was cultured in growth medium RPMI-1640 medium (Life Technologies; Stockholm, Sweden) supplement with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PEST) in a 5% CO2 atmosphere at 37°C. BEAS-2B cells were cultured in T75 flasks, and when confluent, they have been passaged and seeded into 12-well plates (Nunc, Life Technologies, Carlsbad, CA, USA). Passage 34 has been used in all experiments. Upon reaching 70-80% confluency, cells were challenged with or without 20 μg/mL of HDM extract (GREER Laboratories, Lenoir, NC, USA) for 24 h. They were then stimulated with 10 μg/mL of viral mimic Poly(I:C) (InvivoGen, San Diego, CA, USA), or infected with the major rhinovirus 0.1 MOI RV16 (Multiplicity of infection (MOI)). Simultaneously, BEAS-2B cells were treated with 30 mM of glucose (Sigma-Aldrich, Stockholm, Sweden) or 30 μg/ml of long-acting insulin (Sigma-Aldrich, Stockholm, Sweden) for 24h. All stimulations were performed in starvation medium (RPMI-1640 medium containing FBS (1%) and 1% penicillin and streptomycin). MOI and concentrations have been used depends on seminal work [6, 11].

Rhinovirus infection was carried out in 350 μL of the starvation medium at room temperature under gentle agitation for 1h. After 1h the cells were washed with the phosphate buffered saline (PBS), and replaced with fresh starvation medium. Cell lysate and supernatant were collected 24 h post RV16 infection.

ATP Measurement:

Released ATP was measured in cell-free supernatant following ATP Kit SL (Biothermal luminescent assay, Handen, Sweden) 1 h post stimulating the cells with glucose or insulin. Briefly, samples were added with Tris-EDTA buffer (0.1 mol/L Tris (hydroxymethyl) aminomethane, 2 mmol/L EDTA, adjusted to 7.75 with acetic acid; Biothermal, Handen, Sweden) in a microplate luminometer, to give a total volume of 160 ul in each well. Then, 40 ul of ATP reagent SL (zlyophilized reagent holding D-luciferin, luciferase, and stabilizers) were added to the wells, and the light emission corresponding to sample Ismp was instantly measured using Clario Star machine. Thereafter, 10 ul of ATP standard diluted 1:5 in Tris-EDTA Buffer were added and followed by Light emission measurement corresponding to sample plus standard ATP Ismp+std. This was followed by the calculation of the sample's ATP concentration by the equation: ATPsmp=10-7x Ismp / (Ismp+std - Ismp). The factor 10-7 is the concentration (mol/L) of ATP standard per well.

RNA Extraction and Quantification of Gene Expression by Real-Time qPCR:

Total RNA was extracted from BEAS-2B cells using an RNA extraction kit (Nucleospin, RNA I, Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. 1 μg of total RNA was reverse transcribed to cDNA (Precision Nanoscript Reverse Transcription Kit, Pri- merDesign, Southampton, UK) and real-time quantitative PCR was performed using a Mx3005P qPCR system (Stratagene, La Jolla, CA, USA) with standard cycling parameters to perform thermocycling and real-time detection of PCR products. Primers were obtained from Primer Design (Primer Design, Southampton, UK), and the following sequences were used:

Table 1. Primers obtained from Primer Design.

N	Cytokines	F/R	Sequences
1	IFN-β	Forward	TTACTTCATTAACAGACTTACAGGT
		Reverse	TACATAGCCATCGTCACTTAAAC
2	TLR-3	Forward	GTGTGAAAGTATTGCCTGGTTTGT
		Reverse	ATGATAGTGAGGTGGAGTGTTGC
3	MDA5	Forward	CCGTGATTCCACTTTCCTGAA
		Reverse	TTATACATCATCTTCTCTCGGAAATC
4	RIG-I	Forward	TTCTCTTGATGCGTCAGTGATA
		Reverse	CCGTGATTCCACTTTCCTGAA
5	TNFα	Forward	AGGTTCTCTTCCTCTCACATAC
		Reverse	ATCATGCTTTCAGTGCTCATG
6	CXCL8	Forward	CAGAGACAGCAGAGCACAC
		Reverse	AGCTTGGAAGTCATGTTTACAC

Genes of interest were normalized to the geometric means of two reference genes, ubiquitin c (UBC) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the ΔCt method. Within-group comparisons were normalized to an untreated control sample using the ΔΔCt method [35].

Quantification of Protein Expression by Western Blot:

Total protein was extracted from cell lysates using a lysis buffer for western blot consisting of 1% TritonX-100, 10mM Tris-HCl, 50mM NaCl, 5mMEDTA, 30mMNa4P2O7, 50mMNaF, 0.1mMNa3VO4, and 1% phosphatase and protease inhibitors (Sigma-Aldrich, Stockholm, Sweden). Protein concentration was measured using BCA protein assay reagent kit (PIERCE ThermoScientific, Waltham, MA, USA), and an equal amount of protein and Laemmli Buffer were boiled and then loaded and electrophoresed onto a 4-20% TGX stain-free gel (Bio-Rad Laboratories AB, Solna, Sweden). This was followed by blotting on a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories AB, Solna, Sweden) and blocking of the membrane in

5% (w/v) milk in Tris-buffered saline Tween-20 and overnight incubation at 4◦C with primary mAB rabbit antibodies (anti-TLR3, anti-RIG-I, anti-MDA5, anti-PAKT, and anti-GAPDH; Cell Signalling Technology, Leiden, The Netherlands). After that, the membrane was washed and incubated for 1 h with secondary antibodies (anti-Rabbit IgG HRP-linked Ab; Cell Signalling Technology, Leiden, The Netherlands). Chemiluminescent detection was performed using Super Signal West Dura Extended Duration Substance (Bio-Rad Laboratories AB, Solna, Sweden) and immunoblots were visualized by LI-COR Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE, USA) and Image Studio (v3.1.4; LI-COR Biosciences, Lincoln, NE, USA).

Quantification of IL-8 cytokine release by ELISA:

Released CXCL8 were measured in cell-free supernatant 24 h post poly(I:C) stimulation using Luminex immunoassays according to the manufacturer's descriptions (R&D System, Abingdon, UK). The 96 wells plate were coated overnight at room temperature (RT) with 100µl/well of the capture antibody (working concentration for IL-8 4 µg/ml) diluted in the phosphate buffered saline PBS. The next day, wells were washed with wash buffer (0.05% Tween-20/PBS; R&D Systems, Catalog #WA126) using the ELISA plate washer and blocked with 300µl/well Blocking Buffer (1%BSA in PBS; R&D Systems, Catalog #DY995) for 1 h at RT. The plates were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; R&D Systems, Catalog #DY006) before plating out the samples and standards. IL-8 detection limit of the assay is = 31.25 pg/mL. After washing the plate, 100 ul of diluted samples and standards in Reagent Diluent (0.1 % BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl); R&D Systems) were added, and the plate was incubated in the dark and at RT for 2h. The standards were plated out in duplicates 100µl/well, starting with the maximum concentration and diluted by a factor of 1:2 for 7 serial dilutions to reach a final concentration of zero (working concentration 31.2-2000 pg/mL). After two hours incubation at room temperature, the plates were washed with PBS and incubated with 100µl/well of the detection antibody (working concentration 20 ng/ml) diluted in Reagent Diluent for 2 hours. The wells were washed with PBS and incubated with 100µl/ml of Streptavidin-HRP (streptavidin enzyme conjugated to horseradish-peroxidase) diluted 1:40 in reagent Diluent for 20 minutes at room temperature. The plate was washed then, and 100µl/well of substrate solution (1:1 mixture of Color Reagent A(H_2O_2) and Color Reagent B (Tetramethylbenzidine); R&D Systems, Catalog #DY999) was added and incubated for 20 min, where the standard had fully developed observed from the colour change into an appropriate

blue colour. Following that, 50µl of stop solution (2 N H_2SO_4; R&D Systems Catalog #DY994) was added with gentle tapping of the plate. The optical density of each well was measure at a wavelength of 540nm or 570nm using an ELISA microplate reader. Data was acquired on a calibrated and validated Luminex MAGPIX instrument (R&D System, Abingdon, UK), and data were analyzed using Excel.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism software version 7.0 (San Diego, CA, USA), and data are presented as mean ± SEM. Significant variations between unpaired groups were determined using the Kruskall-Wallis test followed by Dunn's multiple comparisons test for comparisons of more than two groups or Mann-Whitney U-test for comparisons of two study groups. P-values <0.05 were considered as statistically significant.

3 | Results:

Glucose and Insulin Induce ATP Release by Bronchial Epithelial Cells.

ATP is released by cells under certain conditions such as stress, pathogenic infections, and tissue injury leading to induction of type-2 cytokines mediated inflammation and Th2 activation [6]. In order to investigate the effect of glucose and insulin on ATP levels, BEAS-2B cells were stimulated with glucose or insulin and ATP levels measured in cell culture supernatants. Figure 1A shows that glucose stimulation leads to significant induction of ATP released by bronchial epithelial cells. Furthermore, stimulation of BEAS-2B cells with insulin also results in a significant increase of ATP levels, as shown in Figure 1B.

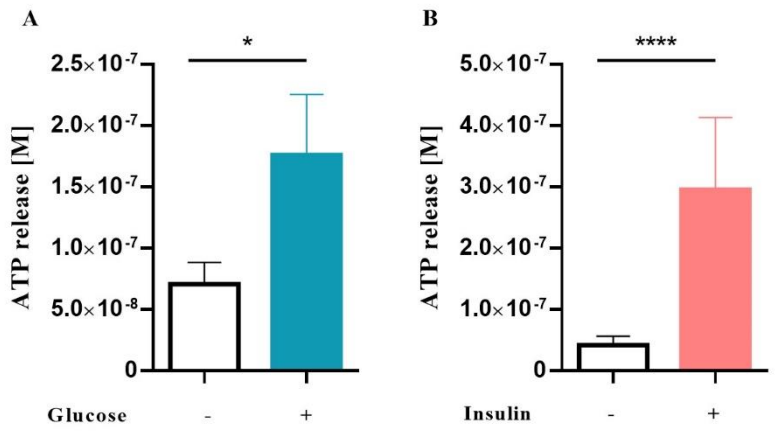

Figure 1: Treatment with glucose and insulin induces ATP release in bronchial epithelial cell. BEAS-2B cells were treated with 30 mM glucose (**A**) and 30 µg/ml of insulin (**B**). ATP levels were measured in cell culture supernatant 1 h post stimulation. Data are presented as mean ± SEM. n= 9 from 9 independent experiments. *P < .05, ****P < .0001 compared to respective control.

Gene Expression of the Pro-Inflammatory Cytokine IL-8 Appear to be Decreased by Glucose and Insulin in Bronchial Epithelium

It has been previously demonstrated that TNF-α and IL-8 can function as pro-inflammatory cytokines by promoting inflammation and amplifying the immune response through recruitment of various immune cells to the site of inflammation [17]. IL-8 can also be released by human bronchial epithelial cells (HBECs) after allergen exposure [6, 36]. We investigated if glucose and insulin can induce gene expression of IL-8 and TNF-α in BEAS-2B cells. Poly(I:C) alone, a TLR3 agonist, significantly induces expression of IL-8 (Figure 2 A, B). However, the poly(I:C)-induced IL-8 expression appear to be decreased by the addition of glucose and insulin (Figure 2 A, B). Further stimulation with glucose seems to increase the protein level of poly(I:C)-induced IL-8 in the supernatant, but further studies are needed (Figure 2 C). Same results have been shown when co-stimulating the cells with insulin in combination with poly(I:C) (Figure 2 D). Although co-stimulation of BECs with glucose did not change the expression of TNF-α (Figure 2 E), the stimulation with insulin in combination with poly(I:C) tends to increase TNF-α expression compared to poly(I:C) alone (Figure 2 F).

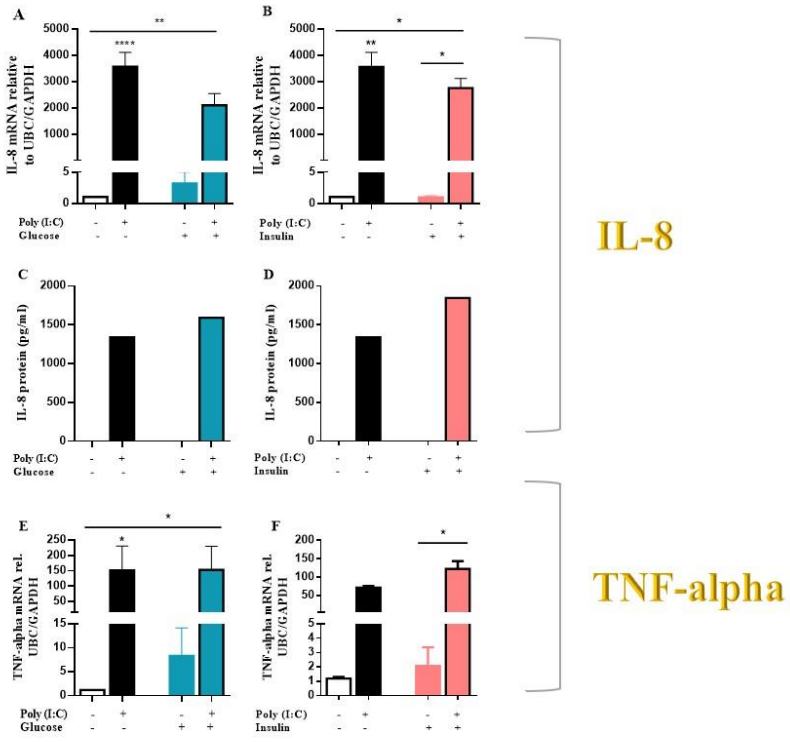

Figure 2: Effects of glucose and insulin on poly (I:C) induced pro-inflammatory cytokine response in bronchial epithelial cells. BEAS-2B cells were treated with 10 µg/ml Poly(I:C), 30 mM glucose or 30 µg/ml insulin alone, or with Poly(I:C) in combination with either glucose or insulin. Cell lysate and supernatant were collected after 24 h stimulation. Gene expression levels of the pro-inflammatory cytokines IL-8 **(A, B)** and TNF-α **(E, F)** were measured by real-time qPCR, and protein expression levels of IL-8 **(C, D)** were measured by ELISA. Data is presented as mean ± SEM fold change of control relative to UBC/GAPDH expression. A, B; E & F n = 7 from 7 independent experiments. For C&D n=2 from 1 experiment. *P < .05, **P < .01, ****P < .0001 compared to respective control.

Glucose and Insulin Appear to Increase Poly(I:C)-Induced Anti-Viral Response in Bronchial Epithelium

Previous studies have shown that people with insulin resistance, such as people suffering from obesity and Type II Diabetes, have higher odds of developing asthma [31]. We, therefore, wanted to study the effect of insulin and diabetes on asthma exacerbations in an in-vitro model of asthma exacerbation. BEAS-2B cells were stimulated with Poly(I:C) alone or poly(I:C) in combination with high levels of glucose or insulin. Figure 3 shows that poly(I:C) significantly induced gene expression of IFNβ (Figure 3 A and E) and PRRs (Figure 3 B-D, F-H) at 24 hrs. Further stimulation with glucose appears to increase poly(I:C)-induced IFNβ (Figure 3 A), MDA5 and RIG-I gene expression (Figure 3 C and D). Similar to glucose, co-stimulation with insulin and poly(I:C) seems to further increase poly(I:C)-induced IFNβ expression (Figure 3 E), while it has no impact on poly (I:C)-induced PRRs (Figure 3 F-H). Neither treatment with glucose or insulin alone stimulate IFNβ gene expression (Figure 3 A and E), nor poly(I:C)-induced protein level of the TLR-3, RIG-I and MDA5 by glucose or insulin (Figure 3 I).

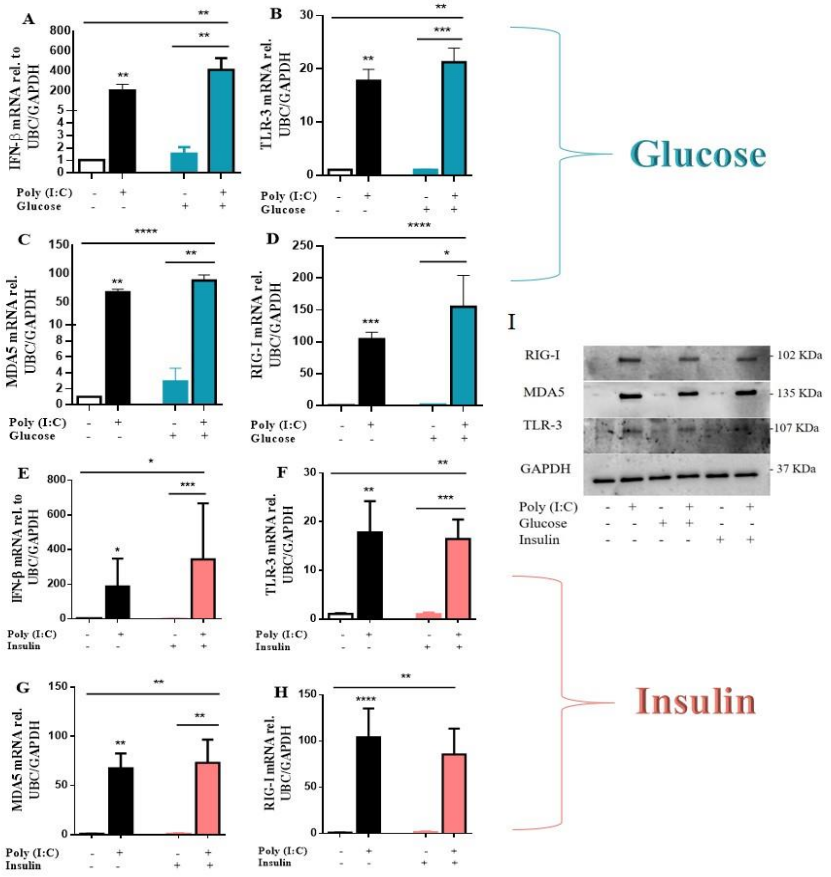

Figure 3: Stimulation with glucose and insulin increase poly(I:C)-induced anti-viral response in bronchial epithelial cells. Beas-2B cells were treated with 10 µg/ml poly(I:C), 30 mM glucose or 30 µg/ml insulin alone, or poly(I:C) in combination with either glucose or insulin. Cells were harvested after 24 h stimulation. Thereafter, gene expression levels of the anti-viral cytokine IFNβ (**A, E**) and Patter Recognition Receptors (PRRs) TLR-3 (**B, F**), MDA5 (**C, G**) and RIG-I (**D, H**) were measured by real-time qPCR. A representative Western Blot image of TLR-3, MDA5, and RIG-I protein expression (**I**). Data is presented as mean ± SEM fold change of unstimulated control relative to UBC/GAPDH expression. n = 7-9 from 9 independent experiments. *P < .05, **P < .01, ***P < .001 ****P < .0001 compared to respective control.

HDM Impaired Anti-Viral Response in BECs Might be Increased by Stimulation with Glucose and Insulin

Poly(I:C)-induced IFNβ gene expression has been found to be reduced following HDM challenge in airway epithelial cells [12]. Previous studies have demonstrated that insulin imbalance contributes to inflammatory diseases and plays a role in acute and chronic airway inflammation [27]. We, therefore, wanted to investigate the effects of glucose and insulin on BECs following allergen challenge, and to confirm whether the PAKT pathway is involved in the PRRs' downstream. We stimulated BEAS-2B cells with Poly(I:C) alone or in combination with either glucose or insulin after 24h HDM challenge. HDM pre-treatment of BECs decreases poly(I:C)-induced IFNβ and PRRs genes expression (Figure 4 A-H). Co-stimulation of glucose and poly(I:C) seems to increase the HDM-impaired IFNβ gene expression (Figure 4 A). While glucose appears to increase HDM-impaired poly(I:C)-induced TLR-3 gene expression (Figure 4 B), it shows no effects on poly(I:C)-induced gene expression of MDA5 and RIG-I (Figure 4 C and D). Similarly to glucose, insulin seems to increase the HDM-impaired poly(I:C)-induced IFNβ (Figure 4 E), and TLR-3 gene expressions (Figure 4 F), but it has no effects on HDM-impaired poly(I:C)-induced MDA5 and RIG-I gene expression (Figure 4 G and H). Insulin induces the PAKT pathway and no effects have been shown by glucose (Figure 4 I).

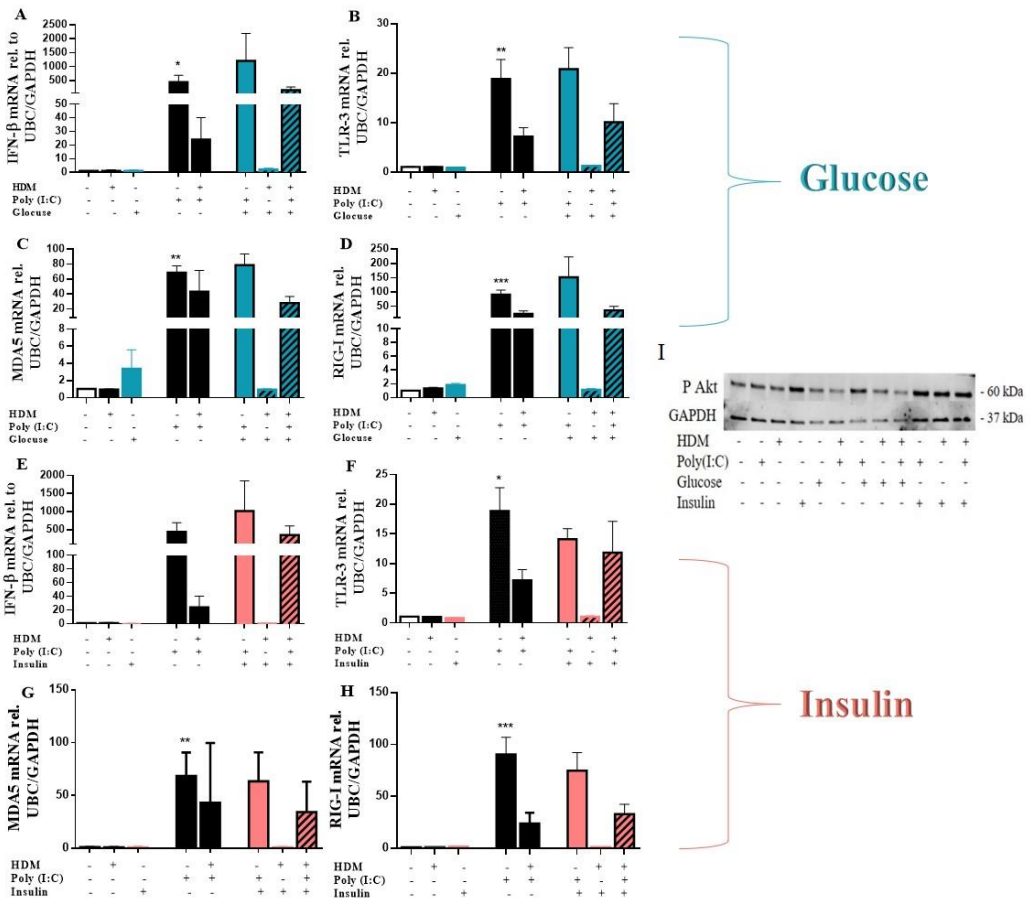

Figure 4: Stimulation with glucose and insulin increase HDM impaired poly (I:C)-induced anti-viral response under allergic background in bronchial epithelial cells. BEAS-2B cells were pre-treated with HDM for 24 h then the cells were stimulated with 10 µg/ml Poly (I:C), 30 mM glucose or 30 µg/ml insulin, or with Poly(I:C) in combination with either glucose or insulin. Cells were harvested for gene expression analysis after 24 h stimulation. Thereafter, gene expression levels of the anti-viral cytokine IFNβ (**A, E**) and PRRs TLR-3 (**B, F**), MDA5 (**C, G**) and RIG-I (**D, H**) were measured by real-time q PCR. A representative Western Blot image of AKT protein is shown (**I**). Data is presented as mean ± SEM fold change of unstimulated control relative to UBC/GAPDH expression. n = 4-6 from 6 independent experiments. *P < .05, **P < .01, ***P < .001.

Glucose and Insulin May Reduce RV16-Induced Pro-inflammatory Response of Bronchial Epithelium.

To determine whether the effects of high levels of glucose and insulin on the pro-inflammatory response to the TLR3 agonist poly(I:C) were similar compared to RV infection, BEAS-2B cells were infected with the major group rhinovirus RV16 alone, or in combination with glucose or insulin. RV16 alone induces IL-8 gene expression (Figure 5 A and B). RV16-induced IL-8 gene expression seems to be decreased following glucose and insulin addition (Figure 5 A and B), correlating to what has previously been shown using Poly(I:C). However, more experiments needed to confirm these results since n number is limited.

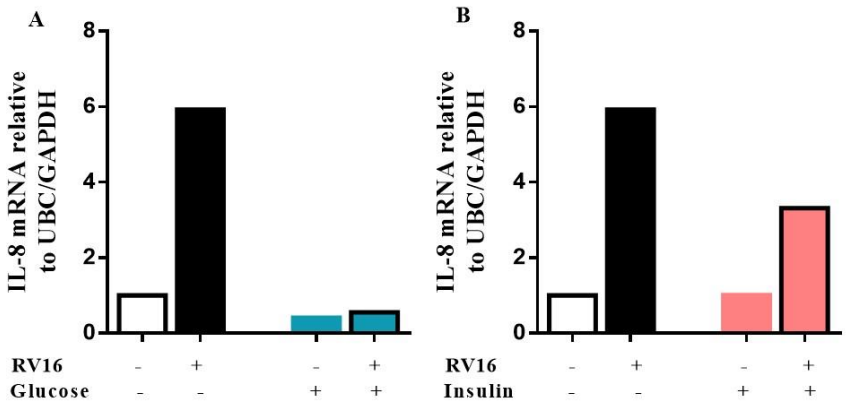

Figure 5: Effects of high levels of glucose and insulin on RV16-induced pro-inflammatory response in bronchial epithelial cells. BEAS-2B cells were infected with 0.1 MOI RV16, or stimulated with 30 mM glucose or 30 µg/ml insulin alone, or with RV16 , or stimulated with 30 mM glucose or 30 µg/ml insulin alone, or with RV16 in combination with either glucose or insulin. Cells were harvested for gene expression analysis after 24 h stimulation, and gene expression levels of the pro-inflammatory cytokine IL-8 (A, B) were measured by real-time qPCR. n = 2 from 2 independent experiments.

RV16 Infectious Appear to Induce PRRs Gene Expression on Bronchial Epithelial cells.

We further investigated whether high levels of glucose and insulin impact the anti-viral response of epithelial cells after RV infection. BEAS-2B cells were again infected with RV16 alone or in combination with glucose or insulin. No effects have been shown on IFNβ gene expression when the cells were infected by RV16 (Figure 6 A and E). Similarly to the results obtained with Poly(I:C) stimulation, RV16 alone appear to induce PRRs gene expression [11] (Figure 6 B-D, F-H) . However, we did not observe any effect on PRRs gene expression when BECs were co-stimulated with RV16 and glucose or insulin (Figure 6 B-D, F-H).

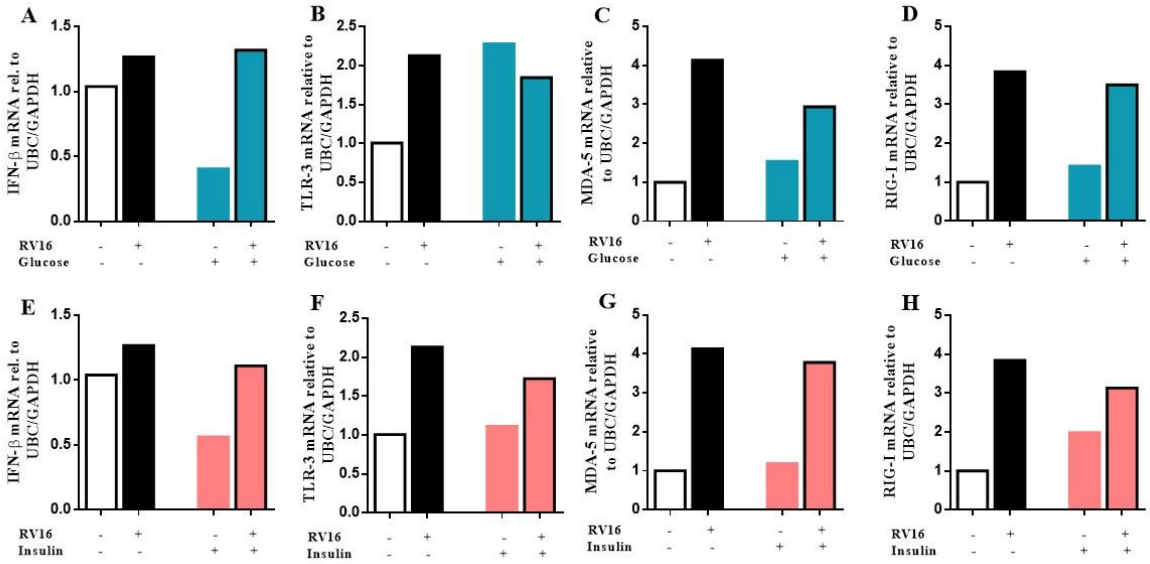

Figure 6: Effects of high levels of glucose and insulin on RV16-induced anti-viral response in bronchial epithelial cells. Beas-2B cells were infected with 0.1 MOI RV16, or stimulated with 30 mM glucose or 30 µg/ml insulin alone, or with RV16 in combination with either glucose or insulin. Cells were harvested for gene expression analysis after 24 h stimulation and gene expression levels of the anti-viral cytokine IFNβ (**A**, **E**) and Patter Recognition Receptors (PRRs) TLR-3 (**B, F**), MDA5 (**C, G**) and RIG-I (**D, H**) were measured by real-time qPCR. n =2 from 2 independent experiments.

Glucose Appear to Restore HDM-Impaired RV16-Induced PRRs Gene Expression in Bronchial Epithelial cells.

In order to investigate the effect of high levels of glucose and insulin in the anti-viral response of epithelial cells under an allergic background, BEAS-2B cells were pre-treated with HDM for 24 hrs. Thereafter, cells were infected with RV16, stimulated with glucose or insulin alone, or co-stimulated with RV16 and glucose or insulin. Our results demonstrated that HDM pre-treatment of BECs decreases RV16-induced IFNβ and PRRs gene expression (Figure 7 A-H), similar to the results found using poly(I:C) stimulation. Co-stimulation of glucose and RV16 appear to restore HDM-impaired RV16-induced PRRs gene expression (Figure 7 B-D), whereas no impact was shown when co-stimulating with insulin and RV16 (Figure 7 F-H).

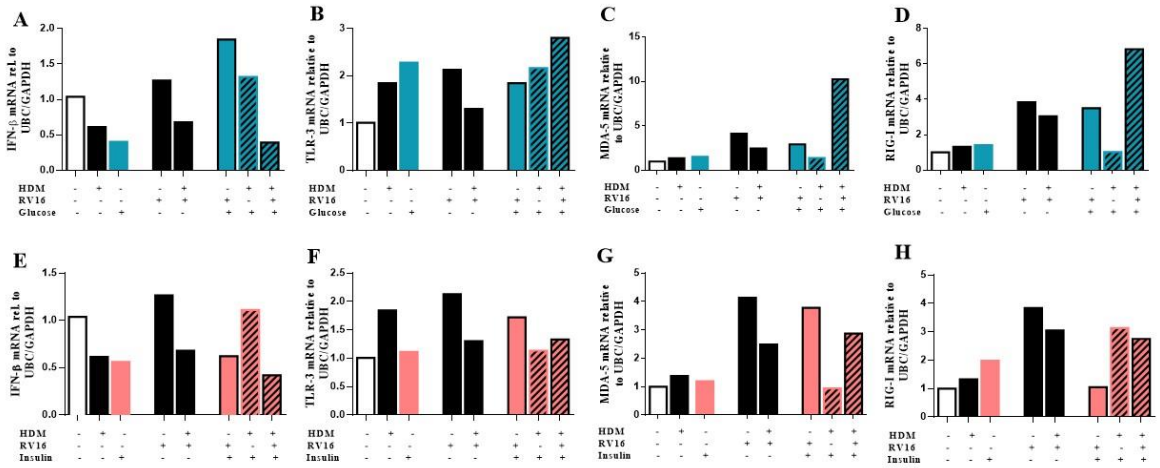

Figure 7: Effect of glucose and insulin on RV16-induced anti-viral response in bronchial epithelial cells in allergic background. BEAS-2B cells were pre-treated with HDM for 24 h then the cells were infected with 0.1 MOI RV16, or stimulated with 30 mM glucose or 30 µg/ml insulin, or with RV16 in combination with either glucose or insulin. Cells were harvested for gene expression analysis after 24 h stimulation, and gene expression levels of the anti-viral cytokine IFNβ (**A, E**) and PRRs TLR-3 (**B, F**), MDA5 (**C, G**) and RIG-I (**D, H**) were measured by real-time qPCR. n = 2 from 2 independent experiments.

4 | Discussion:

Asthma exacerbation is a severe condition and might in worst cases end with mortality [37]. Looking at the fact that asthma exacerbations do not respond well to conventional treatment, there is a need of finding new biological targets for pharmacological intervention. In the current work, we have studied the direct effects of glucose and insulin in modulating viral-induced asthma exacerbation in an *in-vitro* experimental model. Obesity and diabetes may be risk factors for the development of lung infections and the development of asthma exacerbations. Importantly, we found that glucose and insulin stimulation induced the release of alarmins in BECs. Furthermore, glucose and insulin appeared to be involved in poly(I:C)-induced IFNβ expression and the restoration of HDM-impaired IFNβ response in an asthma exacerbation model of BECs. Furthermore, our data rule out the involvement of PRRs in upregulation of IFNβ after stimulation with glucose and insulin. However, further experiments including knockdown of PRRs are needed to confirm these results.

By using a BEC line, we first obtained data on glucose and insulin effects in poly(I:C)-induced IFNβ production and the expression of the main pro-inflammatory cytokines in viral-induced asthma exacerbation, TNF-α and IL-8. Furthermore, we have investigated glucose and insulin-induced ATP release, as well as their effect on the expression of PRRs that engage in the anti-viral response. Thus, we revealed the effect of high levels of glucose and insulin on

viral-induced IFNβ production with and without allergic background. These findings are of interest because they provide us a better understanding of the condition where high levels of glucose and insulin are present in asthmatics individuals, such as obese and diabetics, and its role in asthma severity. Moreover, this might relate to anti-viral drug opportunities targeting the main location for rhinovirus infections, which is the most common risk factor in asthma exacerbation. Provided enhancement of viral-induced IFN production by glucose and insulin required a baseline of diseases that is represented in allergic inflammation in an *in-vitro* model of BECs challenged with HDM.

ATP is released by HBECs post exposure to different protease allergens, including HDM [6]. This process is supposed to occur through protease-activated receptors (PARs) [1]. Interestingly, the mammalian target of rapamycin (mTORC) plays a critical role in regulating cellular metabolic processes such as nutrients and energy production, including glucose and ATP [24]. A previous study has indicated that nutrients excess, high levels of glucose, and insulin activate mTOR [4], which is shown to be associated with asthma onset, and asthma pathogenesis and exacerbation [38]. In this context, mTOR is shown to be highly activated during insulin resistance conditions and obesity [39, 40]. Consistent with that, our results showed an induction of ATP release in the airway epithelium by high levels of glucose and insulin, which might be explained by the previous findings [4, 24].

In addition, IL-8 release has been demonstrated in HBECs after HDM challenge [6]. The pro-inflammatory cytokines TNF-α and IL-8 have dual roles. It is beneficial, looking at their participation in maintaining the immune response against viral or bacterial infections, and tumour by the recruitment of the immune cells [16]. However, high levels of TNF-α and IL-8 in the airway are harmful, in which their participation in immune cell recruitment ends with high levels of inflammatory mediators [16]. This leads to an aggravated inflammation reaction, exacerbation of the immune response and airway injury [16]. In our study, we investigated the effects of insulin and glucose on IL-8 and TNF-α expression. There are contradictory findings regarding insulin effects on the expression of the pro-inflammatory cytokines. A previous paper suggests that insulin increases IL-8 levels in airway inflammation [26]. On the other hand, another study has indicated that insulin reduces the expression of IL-8 through the pro-inflammatory receptor, proteinase-activated receptor (PAR-2) in HAECs [28]. Our results showed a reduction trend of IL-8 gene expression after RV16 and Poly (I:C) induction in the presence of high levels of insulin and glucose. In contrast, IL-8 protein levels show a trend to

increase after co-stimulation with poly(I:C) and insulin or glucose. This variation could be explained by the time point since the change of protein level takes longer time to be observed. In addition, poly (I:C)-induced IFNβ production tends to increase by high levels of glucose and insulin. This data can be supported by the previous studies that indicate insulin capacity of enhancing anti-viral response in airway epithelium [26, 28]. The same study suggests that airway inflammation in asthmatics exhibiting insulin resistance could be attributed to the lack of insulin-mediated anti-inflammatory mechanisms [28]. In agreement with our results, a recent study also indicates that glucose metabolism increases anti-viral cytokines through IRF5 and IRF3 transcription factors [33].

A clinical study has shown that exogenous IFNβ administration reduced the viral load and viral-induced asthma exacerbation in asthmatics patient's airways [41]. These findings were supported by a study that indicates the efficiency and safety of using IFNβ as an anti-viral drug to limit virus-induced asthma exacerbation in BECs *in vitro* model, in which RV replication was inhibited [42]. In the present BEC cell line, high levels of glucose, and insulin have augmented viral infection induced IFNβ production. IFNβ expression induced by glucose and insulin participate in viral clearance, which reduces the risk of developing asthma exacerbation. It is previously shown in a mice model that HDM-induced airway inflammation is markedly aggravated by additional challenges with a viral stimulus [43]. For this study, an *in-vitro* asthma exacerbation model has developed by using a baseline of HDM-induced allergic airway inflammation and viral stimulation. TLR-3 agonist poly(I:C) that mimics the biological effects of RV infections has been used. This approach was preferred to ensure successfully induced asthma exacerbation model by HDM-induced inflammation and viral dsRNA. A previous study has demonstrated that HDM impairs anti-viral response in an *in vitro* HBECs and a mouse model [12]. In correspond with that, our data have shown a trend to decrease in IFNβ production in BECs stimulated with Poly (I:C) and RV16 post HDM challenge. Interestingly, the HDM-impaired IFNβ expression induced by Poly (I:C) is restored by high levels of insulin and glucose. Gandhi *et al.* previously suggested that insulin is involved in increasing anti-viral response in AECs [28], which strongly agrees with our results. Also, recent studies have indicated that insulin activation of mTORC1 [29] plays a critical role in the translation and activation of IRFs to maximize IFNβ production in BECs [24]. High levels of glucose and insulin further enhanced IFNβ expression in BECs with established allergic inflammation prior to the poly(I:C) stimulation. Hence, high levels of insulin and glucose were able to increase

IFNβ production in BECs exposed to poly (I:C) and restore a deficient IFNβ response in asthma exacerbation model to levels occurring in non-allergic, viral stimulus challenged cells.

This finding is of interest because rhinovirus infection is a major cause of difficult-to-treat asthma exacerbation, which is efficiently reduced by high levels of insulin and glucose. This way shows positive effects by reducing exacerbation frequency which might be promising in improving the quality of life in asthmatics. However, insulin properties and effects, including glycolytic regulation and insulin anti-viral properties, are abrogated in the presence of insulin resistance conditions including allergic asthma [28]. Therefore, obese and diabetes asthmatics that have insulin resistance might have a greater risk of developing asthma exacerbation that occurs due to decreased insulin receptors on AECs or a lack of insulin signaling.

Poly I:C is a TLR-3 agonist that mimics RV infection. As discussed in the introduction, PRRs including TLR-3, MDA-5, and RIG-I recognize viral dsRNA. Several studies demonstrated that insulin mediates most of its effects by PI3K/Akt and MAPK/ERK signaling pathways in AECs [26-28, 30]. The current study has included the detection of PI3K/AKT pathway involvement in insulin's enhancement of poly(I:C)-induced IFNβ production. Our results have shown that AKT-dependent insulin pathway is not involved in IFNβ production enhancement by glucose and insulin. In addition, PRRs showed no involvement in the glucose's and insulin's IFNβ enhancement in BECs exposed to Poly (I:C) and RV16 after HDM challenge. Although high levels of glucose do not change IFNβ expression, the PRRs (MDA5 and RIG-I) appears to be increased by glucose in RV-induced IFNβ expression in BECs prior challenged with HDM. This data is obtained from two experiments. Increasing the n numbers might be useful for investigating whether PRRs mediate glucose induced IFNβ-production in asthmatics.

Taking in consideration, there were some limitations of this study. This study neither demonstrated the precise effects of glucose and insulin on the anti-viral response, nor involved pathways were identified, due to time limitation. Moreover, more experiments are needed to confirm the gene expression through measurement of the protein levels by ELISA and Western-Blott. Also, additional inflammatory mediators and allergens that are associated with asthma pathogenesis could be checked. Therefore, further experiments are needed to clarify the results of this study, although it provides preliminary results.

In conclusion, despite the need for more confirmation, we have showed that high levels of glucose and insulin might be involved in upregulation of IFNβ expression level that is induced

by poly(I:C) in BEC cell line. The present study importantly included an *in vitro* approach where we firstly showed that BECs with established allergic inflammation exhibited deficient IFNβ production in response to poly(I:C) stimulus. Secondly, that insulin and glucose appeared to restore the IFNβ deficiency in response to poly (I:C) in this cell line. The present data thus refers to that high levels of glucose and insulin may restore deficient lung IFNs production in exacerbating asthma, but it exhibits the opposite role in obese and diabetics asthmatics that have a greater risk of developing asthma exacerbation due to insulin resistance. We suggest that further studies are warranted to explore involved pathways in the action of glucose and insulin in IFNβ enhancement during asthma exacerbation. Investigation of insulin-dependent MAPK/ERK pathway and mTOR pathway in allergic asthma. In addition, investigation of glucose actions in IFNβ production enhancement in an independent insulin manner and its association with PRRs are of interest.

Acknowledgments:

This study was performed under the supervision of Professor Lena Uller and co-supervisors Sangeetha Ramu and Dr Juan José Nieto. I want to thank Sangeetha Ramu and the group members for provided help throughout my master thesis, and special thank goes to Professor Lena Uller and Dr Juan José Nieto for their valuable support and guidance. I also want to thank my family members for continuous faith and love. A special thank goes to my dad Mohamad Taher Alnakshi, and my mum Alma Alnakshi, the heroes of my life.

5 | References:

1. Menzel M, Akbarshahi H, Mahmutovic Persson I, Puthia M, Bjermer L, Uller L: **Caspase-1 deficiency reduces eosinophilia and interleukin-33 in an asthma exacerbation model**. *ERJ Open Res* 2017, **3**(4).
2. Hossain FMA, Choi JY, Uyangaa E, Park SO, Eo SK: **The Interplay between Host Immunity and Respiratory Viral Infection in Asthma Exacerbation**. *Immune Netw* 2019, **19**(5):e31.
3. Menzel M, Akbarshahi H, Tufvesson E, Persson C, Bjermer L, Uller L: **Azithromycin augments rhinovirus-induced IFNbeta via cytosolic MDA5 in experimental models of asthma exacerbation**. *Oncotarget* 2017, **8**(19):31601-31611.
4. Boonpiyathad T, Sozener ZC, Satitsuksanoa P, Akdis CA: **Immunologic mechanisms in asthma**. *Semin Immunol* 2019, **46**:101333.
5. Lan H, Luo L, Chen Y, Wang M, Yu Z, Gong Y: **MIF signaling blocking alleviates airway inflammation and airway epithelial barrier disruption in a HDM-induced asthma model**. *Cell Immunol* 2020, **347**:103965.

6. Ramu S, Menzel M, Bjermer L, Andersson C, Akbarshahi H, Uller L: **Allergens produce serine proteases-dependent distinct release of metabolite DAMPs in human bronchial epithelial cells**. *Clin Exp Allergy* 2018, **48**(2):156-166.
7. Monteseirin J: **Neutrophils and asthma**. *J Investig Allergol Clin Immunol* 2009, **19**(5):340-354.
8. Polak D, Hafner C, Briza P, Kitzmuller C, Elbe-Burger A, Samadi N, Gschwandtner M, Pfutzner W, Zlabinger GJ, Jahn-Schmid B *et al*: **A novel role for neutrophils in IgE-mediated allergy: Evidence for antigen presentation in late-phase reactions**. *J Allergy Clin Immunol* 2019, **143**(3):1143-1152 e1144.
9. Ciepiela O, Ostafin M, Demkow U: **Neutrophils in asthma--a review**. *Respir Physiol Neurobiol* 2015, **209**:13-16.
10. Sun B, Zhu L, Tao Y, Sun HX, Li Y, Wang P, Hou Y, Zhao Y, Zhang X, Zhang L *et al*: **Characterization and allergic role of IL-33-induced neutrophil polarization**. *Cell Mol Immunol* 2018, **15**(8):782-793.
11. Menzel M, Ramu S, Calven J, Olejnicka B, Sverrild A, Porsbjerg C, Tufvesson E, Bjermer L, Akbarshahi H, Uller L: **Oxidative Stress Attenuates TLR3 Responsiveness and Impairs Anti-viral Mechanisms in Bronchial Epithelial Cells From COPD and Asthma Patients**. *Front Immunol* 2019, **10**:2765.
12. Akbarshahi H, Menzel M, Ramu S, Mahmutovic Persson I, Bjermer L, Uller L: **House dust mite impairs antiviral response in asthma exacerbation models through its effects on TLR3**. *Allergy* 2018, **73**(5):1053-1063.
13. Jartti T, Gern JE: **Role of viral infections in the development and exacerbation of asthma in children**. *J Allergy Clin Immunol* 2017, **140**(4):895-906.
14. Matsumura Y: **Role of Allergen Source-Derived Proteases in Sensitization via Airway Epithelial Cells**. *J Allergy (Cairo)* 2012, **2012**:903659.
15. Berry M, Brightling C, Pavord I, Wardlaw A: **TNF-alpha in asthma**. *Curr Opin Pharmacol* 2007, **7**(3):279-282.
16. Liu G, Zhu R, Li B: **TNF-alpha and IL-8 of the patients with allergic asthma**. *J Huazhong Univ Sci Technolog Med Sci* 2005, **25**(3):274-275, 309.
17. McClure R, Massari P: **TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens**. *Front Immunol* 2014, **5**:386.
18. Kvarnhammar AM, Cardell LO: **Pattern-recognition receptors in human eosinophils**. *Immunology* 2012, **136**(1):11-20.
19. Jartti T, Bonnelykke K, Elenius V, Feleszko W: **Role of viruses in asthma**. *Semin Immunopathol* 2020, **42**(1):61-74.
20. Gern JE: **How rhinovirus infections cause exacerbations of asthma**. *Clin Exp Allergy* 2015, **45**(1):32-42.
21. Calven J, Akbarshahi H, Menzel M, Ayata CK, Idzko M, Bjermer L, Uller L: **Rhinoviral stimuli, epithelial factors and ATP signalling contribute to bronchial smooth muscle production of IL-33**. *J Transl Med* 2015, **13**:281.
22. Jefferies CA: **Regulating IRFs in IFN Driven Disease**. *Front Immunol* 2019, **10**:325.
23. Matsukura S, Kokubu F, Kurokawa M, Kawaguchi M, Ieki K, Kuga H, Odaka M, Suzuki S, Watanabe S, Takeuchi H *et al*: **Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-kappaB and/or IRF-3 in airway epithelial cells**. *Clin Exp Allergy* 2006, **36**(8):1049-1062.
24. Fritsch SD, Weichhart T: **Effects of Interferons and Viruses on Metabolism**. *Front Immunol* 2016, **7**:630.
25. Calven J, Yudina Y, Hallgren O, Westergren-Thorsson G, Davies DE, Brandelius A, Uller L: **Viral stimuli trigger exaggerated thymic stromal lymphopoietin expression by chronic obstructive pulmonary disease epithelium: role of endosomal TLR3 and cytosolic RIG-I-like helicases**. *J Innate Immun* 2012, **4**(1):86-99.
26. Singh S, Prakash YS, Linneberg A, Agrawal A: **Insulin and the lung: connecting asthma and metabolic syndrome**. *J Allergy (Cairo)* 2013, **2013**:627384.

27. Na HG, Kim YD, Bae CH, Choi YS, Jin HJ, Shin KC, Song SY: **High Concentration of Insulin Induces MUC5AC Expression via Phosphoinositide 3 Kinase/AKT and Mitogen-activated Protein Kinase Signaling Pathways in Human Airway Epithelial Cells**. *Am J Rhinol Allergy* 2018, **32**(5):350-358.
28. Gandhi VD, Shrestha Palikhe N, Hamza SM, Dyck JRB, Buteau J, Vliagoftis H: **Insulin decreases expression of the proinflammatory receptor proteinase-activated receptor-2 on human airway epithelial cells**. *J Allergy Clin Immunol* 2018, **142**(3):1003-1006 e1008.
29. Weichhart T, Hengstschlager M, Linke M: **Regulation of innate immune cell function by mTOR**. *Nat Rev Immunol* 2015, **15**(10):599-614.
30. Numata T, Araya J, Fujii S, Hara H, Takasaka N, Kojima J, Minagawa S, Yumino Y, Kawaishi M, Hirano J *et al*: **Insulin-dependent phosphatidylinositol 3-kinase/Akt and ERK signaling pathways inhibit TLR3-mediated human bronchial epithelial cell apoptosis**. *J Immunol* 2011, **187**(1):510-519.
31. Cardet JC, Ash S, Kusa T, Camargo CA, Jr., Israel E: **Insulin resistance modifies the association between obesity and current asthma in adults**. *Eur Respir J* 2016, **48**(2):403-410.
32. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, Matos C, Bruss C, Klobuch S, Peter K *et al*: **LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells**. *Cell Metab* 2016, **24**(5):657-671.
33. Qiming Wang PF, Rui He, Mengqi Li, Haisheng Yu, Li Zhoe, Yu Yi, Fubing Wang, Yuan Rong, Yi Zhang, Aidong Chen, Nanfang Peng, Yong Lin, Mengji Lu, Ying Zhu, Guoping Peng, Liqun Rao and Shi Liu: **O-GlcNAc transferase promotes influenza A virus–induced cytokine storm by targeting interferon regulatory factor–5**. *Science Advances* **Vol. 6, no. 16, eaaz7086**.
34. Mallia P, Webber J, Gill SK, Trujillo-Torralbo MB, Calderazzo MA, Finney L, Bakhsoliani E, Farne H, Singanayagam A, Footitt J *et al*: **Role of airway glucose in bacterial infections in patients with chronic obstructive pulmonary disease**. *J Allergy Clin Immunol* 2018, **142**(3):815-823 e816.
35. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.
36. Bossios A, Gourgiotis D, Skevaki CL, Saxoni-Papageorgiou P, Lotvall J, Psarras S, Karpathios T, Constandopoulos AG, Johnston SL, Papadopoulos NG: **Rhinovirus infection and house dust mite exposure synergize in inducing bronchial epithelial cell interleukin-8 release**. *Clin Exp Allergy* 2008, **38**(10):1615-1626.
37. Edwards MR, Saglani S, Schwarze J, Skevaki C, Smith JA, Ainsworth B, Almond M, Andreakos E, Belvisi MG, Chung KF *et al*: **Addressing unmet needs in understanding asthma mechanisms: From the European Asthma Research and Innovation Partnership (EARIP) Work Package (WP)2 collaborators**. *Eur Respir J* 2017, **49**(5).
38. Zhang Y, Jing Y, Qiao J, Luan B, Wang X, Wang L, Song Z: **Activation of the mTOR signaling pathway is required for asthma onset**. *Sci Rep* 2017, **7**(1):4532.
39. Yoon MS: **The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling**. *Nutrients* 2017, **9**(11).
40. Laplante M, Sabatini DM: **mTOR signaling at a glance**. *J Cell Sci* 2009, **122**(Pt 20):3589-3594.
41. Djukanovic R, Harrison T, Johnston SL, Gabbay F, Wark P, Thomson NC, Niven R, Singh D, Reddel HK, Davies DE *et al*: **The effect of inhaled IFN-beta on worsening of asthma symptoms caused by viral infections. A randomized trial**. *Am J Respir Crit Care Med* 2014, **190**(2):145-154.
42. Cakebread JA, Xu Y, Grainge C, Kehagia V, Howarth PH, Holgate ST, Davies DE: **Exogenous IFN-beta has antiviral and anti-inflammatory properties in primary bronchial epithelial cells from asthmatic subjects exposed to rhinovirus**. *J Allergy Clin Immunol* 2011, **127**(5):1148-1154 e1149.
43. Mahmutovic Persson I, Akbarshahi H, Menzel M, Brandelius A, Uller L: **Increased expression of upstream TH2-cytokines in a mouse model of viral-induced asthma exacerbation**. *J Transl Med* 2016, **14**:52.