INVESTIGATING INNATE IMMUNITY IN AIRWAY EPITHELIAL CELLS

EXPLORING THE ROLE OF ABCF1 IN MUCOSAL IMMUNITY OF HUMAN AIRWAY EPITHELIAL CELLS

By

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LAY ABSTRACT

The human lungs are exposed to over 10,000 litres of air each day from normal respiration, and it is the first point of contact between the outside environment and the internal anatomy. The cells on the surface of the air passages in the lungs are called human airway epithelial cells (HAECs), and they represent a critical defence against inhaled foreign objects that may include air pollution, allergens, bacteria, and viruses. HAECs have a group of proteins called ABC transporters, that are capable of different activities that are essential for maintaining normal lung health. One unique ABC transporter called ABCF1 was found to regulate defences against viral and bacterial infections in non-lung cells. It is unclear whether ABCF1 has the same function and protective capacity in HAECs.

In this Ph.D. thesis, we investigated how ABCF1 functions in HAECs to detect and respond to respiratory infections. By understanding how ABCF1 is involved in protecting the lungs against these infections, novel treatments can be developed to minimize morbidity and mortality in both healthy and vulnerable individuals. In our studies, we evaluated the changes in the response of HAECs that had normal or absent levels of the ABCF1 gene under conditions that resemble an infection. We used computational tools to help us investigate the proteins that interact with ABCF1 and predict their potential function. Our studies have concluded that ABCF1 does have a protective capacity in HAECs, however, we have yet to elucidate how and what other proteins are involved to help it function.

ABSTRACT

Human airway epithelial cells (HAECs) play a pivotal role in creating a mechanical barrier to prevent environmental insults from entering deeper into the lung tissue and in facilitating host defence against pathogens and allergens by producing immune mediators and recruiting inflammatory cells. ABCF1, is a unique member of the ABC transporter family that it is highly expressed in the airway epithelium, however, its function in HAECs is currently not known.

In this thesis, we explored the role of ABCF1 as a dsDNA viral sensor in HAECs. Our findings demonstrated that while ABCF1 is required for an immune response to a double-stranded DNA (dsDNA) viral mimic, VACV-70, our transcriptomic analysis suggested a role in pro-inflammatory responses downstream of toll-like receptors (TLR) 3 and 4 signalling pathways. We followed this outcome by investigating ABCF1 in mediating pro-inflammatory responses to TNF-α and Poly(I:C) through A20, NF-κB and IRF-3 regulated signalling pathways. Our study demonstrated that Poly(I:C) and TNF-α induced IL-8 are regulated by ABCF1 through pathways independent of NF-κB, and IRF-3 activation, although the exact mechanism remains unclear. The next approach was to run a hypothesis-free *in silico* investigation of the ABCF1 protein-protein interaction (PPI) network using publicly available databases and Gene Ontology (GO) term enrichment analysis. Following our *in silico* results of ABCF1 protein interactors, we validated a novel interaction of ABCF1 and SYK in human airway epithelial cells following Poly(I:C) stimulation.

We have demonstrated that silencing ABCF1 under stimulation by VACV-70, TNF-α and Poly(I:C) in HAECs affects the induction of immune mediators, and a candidate protein interaction partner, SYK, is involved in immune signalling, however its exact mechanism is not defined. We propose that further insights into the functions of ABCF1 may aid in understanding how HAECs maintain mucosal immune homeostasis.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

None.

LIST OF ABBREVIATIONS AND SYMBOLS

ABC	ATP-binding cassette
AP-MS	Affinity purification–mass spectrometry
BioGRID	Biological General Repository for Interaction Datasets
BMDM	Bone marrow derived macrophages
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CBL	Casitas B-lineage lymphoma
CC10	Club Cell Protein 10
CCL20	Chemokine ligand 20
CDH1	Cadherin-1
Cdk4	Cyclin-dependent kinase 4
CFTR	Cystic fibrosis transmembrane conductance regulator
cIAP	Cellular Inhibitor of Apoptosis Protein
Co-IP	Co-immunoprecipitation
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptors
dsDNA	Double-stranded DNA
DUB	Deubiquitinating enzyme
EGF	Epidermal growth factor
eIF2	Eukaryotic Initiation Factor 2
ELISA	Enzyme-linked immunosorbent assay
FGF-2	Fibroblast growth factor 2
Flt-3 ligand	Fms-like tyrosine kinase receptor 3 ligand
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GROα (CXCL1)	Chemokine ligand 1

HAEC	Human airway epithelial cells
HBEC	Human bronchial epithelial cells
HMGB2	High-mobility group protein B2
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
IFI16	Interferon Gamma Inducible Protein 16
IFN	Interferon
IKK	IκB kinase
IL	Interleukins
IP-10 (CXCL10)	IFN-gamma-inducible protein 10
IRF	Interferon regulatory factor
ISD	Interferon stimulatory DNA
ISRE	Interferon-sensitive response element
LABA	Long-acting beta-agonists
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharides
LUBAC	Linear ubiquitin chain assembly complex
MCC	Mucociliary Clearance
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MDC (CCL22)	Macrophage-Derived Chemokine
MEFs	Mouse embryonic fibroblasts
Met-tRNA	Methionyl-tRNA
MIG (CXCL9)	Chemokine ligand 9
MIP	Macrophage Inflammatory Protein
MPIDB	Microbial protein interaction database
MRP4	Multidrug resistance protein 4
MyD88	Myeloid differentiation primary response protein 88

NBD	Nucleotide-binding domain
NF-κB	Nuclear factor-κB
NLR	Nod-like receptors
NLRP	Nucleotide binding and leucine-rich-repeat pyrin domain containing
NR1D1	Nuclear receptor subfamily 1 group D member 1
OCC-1	Overexpressed in colon carcinoma-1
OCT4	Octamer-binding transcription factor 4
ORA	Over-Representation Analysis
PAMP	Pathogen-associated molecular pattern molecules
PDGF	Platelet-derived growth factor
PGN-SA	Peptidoglycan - *Staphylococcus aureus*
PIPs	Human Protein-Protein Interaction Prediction
Poly(I:C)	Polyinosinic:polycytidylic acid
PPI	Protein-protein interaction
PRR	Pathogen recognition receptors
RANTES (CCL5)	Regulated upon Activation, Normal T cells Expressed, and Secreted
RLR	Retinoic acid-inducible gene I-like receptors
ROS	Reactive oxygen species
sCD40L	Soluble CD40 ligand
SFK	Src-family tyrosine protein kinases
SOX2	SRY-box 2
STING	Stimulator of interferon genes
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SFK	Src family tyrosine protein kinase
SYK	Spleen tyrosine kinase
TAK1	Mitogen-activated protein kinase kinase kinase 7
TBK1	TANK-binding kinase 1
TGF-α	Transforming growth factor alpha

TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNFAIP3 (A20)	TNF-α-induced protein 3
TNFR1	Tumour necrosis factor receptor 1
TRAF	Tumour necrosis factor receptor-associated factor
TRAM	Translocating chain-associating membrane protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
Ub	Ubiquitin
VEGF-A	Vascular endothelial growth factor A
WebGestalt	WEB-based GeneSeT AnaLysis Toolkit
Y2H	yeast-2-hybrid

DECLARATION OF ACADEMIC ACHIEVEMENT/PREFACE

The Ph.D. thesis is prepared in a "Sandwich" thesis format according to the instructions described in the "Guide for the Preparation of Master's and Doctoral Theses" provided by the School of Graduate Studies at McMaster University. Chapter 1 is an introductory chapter that provides general background information and rationale for the studies that will be discussed in the following chapters. Chapters 2-4 each contain independent studies that all tie together to contribute to characterizing the function of ABCF1 in human airway epithelial cells in the context of innate immunity.

Chapter 1: This chapter provides background information that is essential for understanding the rationale and objectives for each of the studies discussed in Chapters 2-4.

Chapter 2: Quynh T. Cao, Jennifer A. Aguiar, Benjamin J-M. Tremblay, Nadin Abbas, Nicholas Tiessen, Spencer Revill, Nima Makhdami, Anmar Ayoub, Gerard Cox, Kjetil Ask, Andrew C. Doxey and Jeremy A. Hirota. ABCF1 Regulates dsDNA-induced Immune Responses in Human Airway Epithelial Cells. *Frontiers in Cellular and Infection Microbiology* 2020; **10**: 1-17.

In this chapter, we focused on investigating the role of ABCF1 as a dsDNA viral mimic sensor and antiviral responses in human airway epithelial cells. This work was completed between December 2018 to January 2020 during my master's before I transferred to the Ph.D. stream. As the primary author, I was responsible for the development of the hypothesis, experimental design, majority of the experimentation, data analysis and interpretation. I received experimental

assistance from NA and NT. The bioinformatics analyses were completed by Dr. AD, Dr. JA, and BT from the University of Waterloo. The histology, digital scanning and microscopy were done by SR, Dr. AA and Dr. KA at the Molecular Phenotyping and Imaging Core Facility (MPIC). The primary epithelial cells were generously provided by NM and Dr. GC. Dr. JH overlooked the project and drafted the manuscript.

Chapter 3: Quynh T. Cao, Mira Ishak, Israel Shpilman, and Jeremy A. Hirota. TNF-α and Poly(I:C) induction of A20 and activation of NF-κB signalling are independent of ABCF1 in human airway epithelial cells. *Scientific Reports* 2023; **13**: 14745.

The objective of this chapter was to investigate the impact of ABCF1 on A20 activity following TNF-α and Poly(I:C) stimulation. This work was completed between September 2021 to August 2023. As the primary author, I was responsible for the development of the hypothesis, experimental design, experimentation, data analysis, and interpretation, and drafted the manuscript. Experimental assistance was provided by the undergraduate students, MI, and IS. Dr. JH overlooked the project, critically appraised the work, and edited the manuscript.

Chapter 4: Quynh T. Cao and Jeremy A. Hirota. Bioinformatic Exploration and in vitro Validation of ABCF1-Syk Protein-Protein Interaction in Human Airway Epithelial Cells. Manuscript to be submitted.

This work started in September 2023 and was completed in February 2024. As the primary author, I was responsible for conceptualizing the *in silico* work and designing the *in vitro* experiments,

running the experiment, data analysis, interpretation and drafting the manuscript. The protein-protein interaction database idea was provided by Drs AD (University of Waterloo) and JH. Dr. AD (University of Calgary) gave scientific inputs on proteomics. Dr. JH overlooked the project, critically appraised the work, and edited the drafted manuscript.

Chapter 5: Discusses the overall impact of the study presented in this thesis, the significance behind the major findings from each study discussed in Chapters 2-4, their limitations and future direction.

Chapter 1

Introduction

Human airway epithelial cells (HAEC) – Overview

The respiratory system is one of the two systems in the human body that directly interacts with the external environment[1]. The respiratory tract is divided into two zones: the proximal conducting zone and the distal respiratory zone[2]. The proximal conducting zone includes the nasal cavity, trachea, and the bronchi and it prepares the inhaled air for gas exchange by moistening, cleaning, and warming the air[2]. The distal respiratory zone contains the respiratory bronchioles and alveoli, and it allows for gas exchange with the inhaled air and blood[1, 2]. Through normal respiration, the lungs are constantly exposed to various pathogens, toxins, allergens, and foreign particles, therefore the respiratory tract requires immunological surveillance to maintain lung homeostasis and protection[1].

The majority of the respiratory tract is lined with pseudostratified epithelial cells. The epithelium of healthy individuals has 14 types of cells: 12 in the surface epithelium and 2 in the submucosal glands[3]. Most of the cells in the airway are epithelial cells (89.1%) [3]. The rest are stromal cells (4.7%), such as endothelial cells, fibroblasts, smooth muscle cells, and pericytes; immune cells (6.2%), including myeloid and lymphoid cells; and basal cells (30%)[3].

In the tracheobronchial section of the respiratory tract, the pseudostratified airway epithelium is made up of three major cell types: basal cells, ciliated cells, and non-ciliated cells[1]. Basal cells reside within the basement membrane and are the main stem cells of the airways with the ability to self-renew post injury and differentiate into other cell types,

including goblet cells, club cells and ciliated cells[2, 3]. Ciliated cells are columnar and have hair-like structures, called cilia[2]. They are terminally differentiated cells, and they make up the majority of all the epithelial cells in the conducting airways[2]. These cells have an important role in maintaining airway homeostasis by facilitating mucociliary clearance (MCC)[1, 2]. Goblet cells are the main mucus producing cells in the airways and work together with ciliated epithelial cells to assist with MCC[2]. The mucus it produces is made of electrolytes, metabolites, antimicrobial products, and mucin glycoproteins, and is essential for trapping foreign particles in the respiratory tract[2]. Club cells are primarily found in the respiratory bronchioles and functions as stem cells to facilitate epithelial repair like basal cells, as well as secretory cells that secrete CC10 to maintain airway integrity[2, 4]. Less common cells in the airway epithelium include pulmonary neuroendocrine cells which make up approximately 0.5% of all airway epithelial cells and serve as a communicator between the immune system and nervous system; tuft cells are proposed to present antigens to developing thymocytes; hillock cells are involved in epithelial cell differentiation, cellular adhesion, and immunomodulation; and microfold cells initiates immune responses by endocytosis[2].

The pseudostratified airway epithelium functions as a barrier that separates the external environment from the internal milieu[1]. It has three main activities: MCC, functions as a physical mechanical barrier and regulates mucosal immunity. MCC primarily involves mucus and ciliated cells to trap and remove unwanted environmental microorganisms and particulates from the airways[1]. Mucus is produced primarily by secretory cells, such as

goblet cells, as well as submucosal glands[1]. It contains over 200 proteins including mucins, that make up the majority of the barrier to trap potential pathogens and particulates, as well as keeping the airway hydrated[1, 5]. The coordinated beating of the cilia on epithelial cells pushes objects trapped in the mucus from the terminal bronchioles towards the pharynx where they can be removed[1, 6].

Tight and adherens junctions are both located at the apicolateral border of the airway epithelium to create a mechanical barrier between the luminal space and pulmonary parenchyma[1, 6]. Tight junctions are made of several transmembrane proteins, including occludins, claudins, and junctional adhesion proteins to regulate the transport of solutes and ions across the epithelial cells[1]. Adherens junctions are consisted of transmembrane proteins, including E-cadherin and β-catenin, located below the tight junctions and are responsible for connecting adjacent cells in the epithelium[1]. Together, these two groups of proteins form a mechanical barrier that prevents inhaled pathogens and environmental insults from entering the submucosa and injuring the airways, while also serving as a signalling hub to regulate gene expression, cell proliferation and differentiation in the epithelium[1].

Human airway epithelial cells (HAECs) have a crucial role in regulating and maintaining immunological activities in the epithelium. They secrete a variety of antimicrobial products into the mucus barrier upon detecting pathogens, and recruit immune cells to regulate

mucosal immunity[1, 5, 7]. This includes small cationic molecules, such as the β-defensins, LL-37, and CCL20, and larger proteins, such as lysozyme, lactoferrin, and mucin[5].

HAECs express pattern recognition receptors (PRRs) on the cell surface and intracellularly to detect and respond to invading pathogens. They recognize whole viruses, bacteria and fungi, as well as their components called pathogen-associated molecular patterns (PAMPs)[5, 7]. PAMPs can go through the respiratory mucus layer to gain access to the surface or intracellular receptors and stimulate an inflammatory response[5]. Amongst the PRRs are Toll-like receptors (TLRs) that can recognize a large array of pathogenic components. This includes lipoproteins (TLR1, TLR2, and TLR6), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), DNA (TLR9), and RNA (TLR3, TLR7 and TLR8)[5]. Activation of the TLRs will initiate signal transduction in a MyD88-dependent or -independent manner to activate transcription factors, Nuclear factor-κB (NF-κB) and interferon regulatory factors (IRF), to allow for the downstream expression of pro-inflammatory genes including type I and III IFNs, TNF, IL-1β, IL-6 and IL-8[5]. Other PRRs found in HAECs includes Nod-like receptors (NLRs) which activates NF-κB, while retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) activates IRF-3 and IRF-7 upon detection of PAMPs in the cytosol [5, 8].

HAECs express receptors for immune mediators, such as cytokines and chemokines, to signal neighbouring epithelial cells of an infection, and to recruit innate and adaptive immune cells[9]. One of these receptors is CXCR3, which binds to CXCL9, CXCL10, and

CXCL11[5]. These ligands help recruit leukocytes, such as T lymphocytes, to the site of infection or injury and initiate inflammation[5]. Another receptor expressed by HAECs includes TNFR1, a receptor for TNF, a cytokine family that also promotes inflammation[5].

ATP Binding Cassette (ABC) – Overview

ATP Binding Cassette (ABC) is a family of transporter proteins that are conserved in both prokaryotes and eukaryotes[10]. Currently, there are 49 known human ABC transporters that are subdivided into 7 subfamilies from ABCA to ABCG[10]. Most ABC transporters possess a transmembrane domain, and nucleotide-binding domains (NBDs) with highly conserved sequences, including Walker A and Walker B motifs, signature H and Q loops, that are essential for NBD mediated ATP hydrolysis[11–13]. These transporters functions as an importer or exporter depending on the direction of transport. By hydrolyzing ATP, ABC transporters can move substrates such as metal ions, peptides, amino acids, lipids, and sugars, across the lipid membrane in or out of cells[11]. Two ABC transporter subfamilies, ABCE and ABCF, are unique as they lack a transmembrane domain and, therefore are unlikely to function as a transporter[11].

Three known ABC transporters have important roles in normal and pathological lung functions: ABCC4, ABCC7, and ABCA3. In primary HAECs, ABCC4, also known as multi-drug resistance transporter-MRP4, has been demonstrated to have roles in being a pharmacological inhibitor[15]. Previous groups have demonstrated that by inhibiting ABCC4 in chronic lung diseases, it was shown to increase intracellular cAMP levels by

minimizing its extracellular transport activity, therefore potentiating CFTR function, as well as glucocorticoid signalling to manage disease progression and exacerbations[15]. In healthy individuals, the function of ABCC7, also known as cystic fibrosis transmembrane conductance regulator (CFTR), functions as an ion channel that transports chloride and bicarbonate ions across the apical membrane of epithelial cells to maintain ion and fluid homeostasis[15]. However, mutations in the CFTR gene will lead to the development of a chronic lung disease, called cystic fibrosis. The expression of a mutated CFTR can compromise ion transport, therefore causing an increase in mucus viscosity, impaired MCC and modulated immunity[14]. The protein expression of ABCA3 has been demonstrated exclusively in the lungs, specifically in alveolar type II cells[14]. It's been suggested that it functions as a regulator of surfactant production, which is required for lowering surface tension in the alveoli. Individuals with a mutation in the ABCA3 gene have demonstrated surfactant deficiency and severe neonatal lung disease[14].

ABCF1 – Overview

A study by Aguiar et al. investigated the gene expression levels of ABC transporters in healthy HAECs throughout the airway tree generations and demonstrated that ABCF1 is one of the most highly expressed ABC transporters in the lungs[10]. This finding suggests that ABCF1 may have an important role in HAEC physiology[10]. Currently, the protein expression and function of ABCF1 transporter have yet to be characterized in HAECs. However, several groups have investigated their function in different cell types and

demonstrated its roles in immunity, transcription and translation regulation, and embryonic development.

Several studies have demonstrated the role of ABCF1 in innate immune responses. In an *in vitro* study with synoviocytes from rheumatoid arthritis patients, it was observed that TNF-α stimulation led to an increase in ABCF1 mRNA transcript accumulation, suggesting that ABCF1 may have a role in pro-inflammatory responses[16]. A separate group demonstrated that ABCF1 plays a role in sensing cytosolic viral dsDNA in mouse embryonic fibroblasts (MEFs). In this study, they silenced ABCF1 using siRNA, followed by stimulation with a dsDNA viral mimic called interferon stimulatory DNA (ISD), a 45-bp oligomer derived from *Listeria monocytogenes*, to trigger the ISD signalling pathway[17]. This pathway involves the activation of STING, TBK1 and IRF-3 to induce the production of type I interferon[18]. They measured the induction of CXCL10 with an ELISA and demonstrated a decrease in CXCL10 secretion when ABCF1 is knockdown compared to the control cells[17]. They suggested that ABCF1 detects and binds to viral dsDNA in the cytosol of the infected cells and interacts with HMGB2 and IFI16 to stimulate an innate immune response through the activation of IRF-3[19].

Prior studies have also reported the role of ABCF1 as an E2 ubiquitin-conjugating enzyme that regulates LPS-induced TLR4 signalling in murine bone marrow derived macrophages (BMDM)[20]. Here, they demonstrated that under LPS stimulation, ABCF1 had an impact on the activation status of transcription factors NF-κB (MyD88-dependent) and IRF-3

(TRIF-dependent). During LPS stimulation, silencing of ABCF1 led to an increase in phosphorylation of a MyD88-signalling associated kinase (TAK1) and attenuation of a TRIF-signalling associated kinase (TBK1). They also observed a reduced expression of *TNFAIP3* (A20) protein, a negative regulator of MyD88 signalling. However, when they overexpressed ABCF1, there was a shift from MyD88-dependent to TRIF-dependent signalling in BMDM under LPS stimulation. Following these findings, they observed that ABCF1 was targeted for K48-polyubiquitination by cIAP1/2, an E3 ubiquitin ligase, during the early phase of the LPS challenge, however, this shifted to K63 linkages by TRAF6, an E3 ubiquitin ligase, during the late phase of the challenge. They have also demonstrated that ABCF1 associates with and mediates K63-polyubiquitination of SYK as an E2 ubiquitin conjugating enzyme with TRAF6 in an E3-RING enzymatic pathway. ABCF1 was also characterized to polyubiquitinate and interact with TRAF3, along with an unknown E3 ligase during late phase TLR4 signalling, likely in a TRIF-dependent manner. Overall, this study concluded that ABCF1 controls the molecular switch from MyD88- to TRIF-dependent signalling, therefore regulating LPS-induced macrophage polarization from M1 (pro-inflammatory response) to M2 (anti-inflammatory response)[20].

ABCF1 was identified to have an essential role in biogenesis, function and control of translational machinery in eukaryotic cells[21]. A study demonstrated that ABCF1 directly associates with eIF2 to promote the binding of methionyl tRNA (Met-tRNA) to eIF2 and associates with the ribosome in an ATP-dependent manner during mRNA translation initiation[21, 22]. The authors from this study observed that a mutated form of ABCF1

impairs its ability to bind and hydrolyze to ATP, therefore compromising the components involved in start site recognition during the initiation of mRNA translation[23].

ABCF1 expression was also characterized to be essential for embryo development. To study the role of ABCF1 in development and disease, Wilcox et al. created a knockout mouse model. During this study, they observed that heterozygous (*Abcf1*$^{+/-}$) mice development normally, however homozygous (*Abcf1*$^{-/-}$) knockout mice models were lethal[24]. They observed that the *ABCF1* promoter was active in all organs and tissues, and it was highly active throughout mouse embryogenesis, suggesting that ABCF1 knockout can potentially compromise mRNA translation during post-implantation stage in embryo development[24]. To conclude, their findings demonstrated that ABCF1 is essential for blastocyte survival during embryogenesis[24]. A separate group ran a study to investigate the role of ABCF1 in embryonic stem cells. Their findings suggest that ABCF1 functions as a cell specific transcriptional co-activator for the transcription factors in stem cell pluripotency, SOX2 and OCT4; and regulates stem cell pluripotency in response to genome instability[25]. The authors in this study demonstrated that under DNA damage or exposure to pathogen-derived DNAs in embryonic stem cells, ABCF1 binds to the aberrant DNAs accumulating in these cells, causing a loss of interaction of SOX2 and OCT4, and therefore dissociating them from the gene promoters targeted[25].

Overarching Aim and Thesis Objectives

The overarching aim of this Ph.D. thesis is to investigate whether ABCF1 plays a role in innate immune signalling in human airway epithelial cells *in vitro*. The sequence of objectives to approach this aim is as follow:

1. To confirm ABCF1 gene and protein expression in HAECs and localization in human lung tissues (Chapter 2).
2. To investigate whether ABCF1 functions as a dsDNA nucleic acid sensor in HAECs and characterize its roles in antiviral responses (Chapter 2).
3. To observe whether silencing ABCF1 will impact A20 protein expression, thereby increasing pro-inflammatory responses in HAECs under TNF-α and Poly(I:C) challenge (Chapter 3).
4. To generate a list of ABCF1 protein interactors using open-sourced databases and performing a Gene Ontology (GO) enrichment analysis to generate a hypothesis on the potential functions of ABCF1 (Chapter 4).
5. To demonstrate that ABCF1 interacts with SYK under Poly(I:C) challenge (Chapter 4).

Our interest in characterizing the expression and function of ABCF1 in HAECs stemmed from a study by Aguiar et al. In their study, they examined the gene expression levels of ABC transporters in healthy HAECs across all airway generations: small airways, large airways, and the trachea[10]. They observed that one of the ABC transporter genes that was

highly expressed included *ABCF1*, suggesting that it has the potential to play an important role in HAECs[10]. Currently, there are no known studies that have been conducted to characterize the expression and function of ABCF1 in HAECs. We therefore began our investigation to characterize its function in the airway epithelium.

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Chapter 2

ABCF1 Regulates dsDNA-induced Immune Responses in Human Airway Epithelial Cells

Summary and Significance

The human airway epithelium plays a critical role in the human lung by facilitating defence against respiratory viral infections, through the detection of pathogens by the pathogen recognition receptors (PRR) and initiating a downstream innate immune response. A study has demonstrated the role of ABCF1 in sensing cytosolic dsDNA viral mimic in mouse embryonic fibroblast, followed by inducing an antiviral immune response. Currently, there is no evidence on the role of ABCF1 as a cytosolic antiviral sensor in human airway epithelial cells (HAECs). We hypothesize that ABCF1 functions as a dsDNA nucleic acid sensor and has an important role in regulating antiviral responses in HAECs. Our study showed that the *ABCF1* gene and protein is expressed in the HAECs. Under *ABCF1* siRNA-mediated knockdown and dsDNA viral mimic challenge, there was a significant decrease in the induction of CXCL10 compared to the control. However, our transcriptomic analysis demonstrated that there was no global attenuation of genes associated with antiviral immunity. The Gene Ontology (GO) analysis with our transcriptomic data revealed that the genes most impacted by ABCF1 knockdown under viral stimulation were associated with TLR signalling, suggesting that ABCF1 does have a role in innate immunity in the human airway epithelial cells. In summary, our findings suggests that ABCF1 is a candidate cytosolic nucleic acid sensor and modulator of TLR signalling, though its exact mechanism remains to be characterized.

ABCF1 Regulates dsDNA-induced Immune Responses in Human Airway Epithelial Cells

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figure generation. NM: responsible for patient consenting and human tissue acquisition. SR: responsible for histology and imaging. AA: responsible for human ethics protocols, human tissue acquisition, histology, and imaging. GC: responsible for supervision of the clinical research coordinator and human tissue acquisition. KA: responsible for supervision of the trainees, human tissue acquisition, study design, histology, and imaging. AD: responsible for oversight of the entire study & supervision of the trainees and funding. JH: responsible for oversight of the entire study & supervision of the trainees and funding, drafting, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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ABSTRACT

Background: The airway epithelium represents a critical component of the human lung that helps orchestrate defences against respiratory tract viral infections, which are responsible for more than 2.5 million deaths/year globally. Innate immune activities of the airway epithelium rely on Toll-like receptors (TLRs), nucleotide binding and leucine-rich-repeat pyrin domain containing (NLRP) receptors, and cytosolic nucleic acid sensors. ATP Binding Cassette (ABC) transporters are ubiquitous across all three domains of life – Archaea, Bacteria, and Eukarya – and expressed in the human airway epithelium. ABCF1, a unique ABC family member that lacks a transmembrane domain, has been defined as a cytosolic nucleic acid sensor that regulates CXCL10, interferon-β expression, and downstream type I interferon responses. We tested the hypothesis that ABCF1 functions as a dsDNA nucleic acid sensor in human airway epithelial cells important in regulating antiviral responses.

Methods: Expression and localization experiments were performed using *in situ* hybridization and immunohistochemistry in human lung tissue, while confirmatory transcript and protein expression was performed in human airway epithelial cells. Functional experiments were performed with siRNA methods in a human airway epithelial cell line. Complementary transcriptomic analyses were performed to explore the contributions of ABCF1 to gene expression patterns.

Results: Using archived human lung and human airway epithelial cells, we confirm expression of ABCF1 gene and protein expression in these tissue samples, with a role for mediating CXCL10 production in response to dsDNA viral mimic challenge. Although, ABCF1 knockdown was associated with an attenuation of select genes involved in the antiviral responses, Gene Ontology analyses revealed a greater interaction of ABCF1 with TLR signalling suggesting a multifactorial role for ABCF1 in innate immunity in human airway epithelial cells.

Conclusion: ABCF1 is a candidate cytosolic nucleic acid sensor and modulator of TLR signalling that is expressed at gene and protein levels in human airway epithelial cells. The precise level where ABCF1 protein functions to modulate immune responses to pathogens remains to be determined but is anticipated to involve IRF-3 and CXCL10 production.

Key words: ABCF1, airway epithelial cells, virus, CXCL10, innate immunity

INTRODUCTION

The human lung functions at the interface of the external and internal environments and is exposed to over 10,000 litres of air each day from normal respiration. The airway epithelium represents a critical component of the human lung that helps orchestrate defences against inhaled noxious substances that may include air pollution, allergens, bacteria, and viral insults[1-3]. To manage these continuous insults, the airway epithelium has evolved to be a multi-functional barrier tissue with mechanical and immunological impedances, manifested through the mucociliary ladder, protein-protein junctions, and innate immune processes. A dominant exposure important in both healthy individuals and those with underlying chronic respiratory diseases are viral infections. Collectively, respiratory tract viral infections are responsible for more than 2.5 million deaths/year globally and represent an economic burden on health care systems for all demographics[4]. In individuals with underlying chronic airway disease, respiratory tract viral infections increase frequency and severity of disease exacerbations, hospitalizations, and contribute to morbidity and mortality[5-9]. Understanding the mechanisms governing respiratory tract viral infections and host defence is essential for the future development of treatments aimed at minimizing the morbidity and mortality of these pathogens.

Innate immune activities of the airway epithelium rely on accurate sensing of the external environment. The threat posed by viruses that infect the respiratory mucosa is countered by the airway epithelium expressing functional Toll-like receptors (TLRs), nucleotide binding and leucine-rich-repeat pyrin domain containing (NLRP) receptors, and cytosolic nucleic acid sensors that are able to rapidly detect exposures and provide host defence[1-3,10-12]. Antiviral sensing mechanisms in the respiratory mucosa enable responses to influenza A, respiratory syncytial virus,

rhinovirus, and human parainfluenza virus; all single stranded RNA viruses[13]. Double-stranded DNA (dsDNA) viruses are also relevant lung infections, with adenovirus capable of inducing influenza like symptoms in healthy subjects and associated with chronic respiratory disease exacerbations [8,14-16]. Like RNA viruses, adenovirus is able to infect airway epithelium followed by replication, which leads to a variety of innate immune defences able to sense viral nucleic acids and proteins[14,17,18]. Vaccinia virus is another dsDNA virus that is able to infect airway epithelium and has been explored for capacity to genetically engineer the virus for transgene delivery, vaccination strategies, and studying Variola virus infections [19-23]. Exploring how the airway epithelium responds to viruses may provide new strategies for controlling infections, optimizing transgene delivery, and vaccination strategies relevant in lung health and disease.

ATP Binding Cassette (ABC) transporters are ubiquitous across all three domains of life – Archaea, Bacteria, and Eukarya[24]. In humans, the 49 ABC transporters are classified according to structure and function, resulting in 7 families. ABC transporters with clear involvement in lung health and disease include ABCA3 and ABCC7 (better known as cystic fibrosis transmembrane conductance regulator – CFTR), responsible for surfactant production and ion transport, respectively [20,25,26]. The ABCF family members are unique in their structure and function as they lack transmembrane regions and therefore lack capacity for transport of substrates [24,27]. Of the ABCF family members, ABCF1 is most extensively characterized in eukaryotes, with functions ranging from initiation of mRNA translation, immune modulation, and nucleic acid sensing [27-32]. The diverse functions attributed to ABCF1 are physiologically important, as demonstrated by the embryonic lethality of homozygous deletion of ABCF1 in mice[33]. To date,

nucleic acid sensing by ABCF1 has been defined using the dsDNA immunostimulatory DNA (ISD) sequence derived from *Listeria monocytogenes* [34] and a dsDNA HIV sequence, with both nucleic acid motifs inducing CXCL10, interferon-β expression, and downstream type I interferon responses in mouse embryonic fibroblasts[31]. Complementary to dsDNA sensing, immune modulation mediated by ubiquitin-conjugating activities of ABCF1 have been defined in the context of macrophage polarization and immune responses linked to interferon-β production and tolerance important in mouse models of sepsis[32]. In the context of studies using human lung samples, *ABCF1* gene expression has been identified in the human airway epithelium[35], although confirmation of protein and function remains to be determined. The clear *in vivo* demonstration of ABCF1 functions in immune responses in mouse models and the presence of detectable *ABCF1* gene expression the human airways warrant a deeper interrogation into the expression and function of this molecule in human health and disease.

Defining defence mechanisms in airway epithelial cells has important consequences in both lung health and disease, with the potential for interventions that could reduce viral-induced pathologies and exacerbations of chronic respiratory diseases[5-9]. We therefore tested the hypothesis that ABCF1 functions as a dsDNA nucleic acid sensor in human airway epithelial cells important in regulating antiviral responses, using archived human lung samples and human airway epithelial cells. Expression and localization experiments were performed using *in situ* hybridization and immunohistochemistry in human lung tissue, while confirmatory transcript and protein expression was performed in human airway epithelial cells. Functional experiments were performed with siRNA methods as no selective small molecule inhibitors to ABCF1 have been validated to date. Complementary transcriptomic analyses were performed to explore the potential contributions of

ABCF1 beyond dsDNA virus sensing. A focused approach on CXCL10 as a readout was performed based on the discovery of ABCF1 as a dsDNA sensor in mouse embryonic fibroblasts. Further hypothesis-free analyses explored candidate pathways differentially regulated during ABCF1 attenuation. Our results confirm expression of ABCF1 in human airway epithelial cells with a role for mediating CXCL10 production in response to dsDNA viral mimic challenge. Although, reduced expression of ABCF1 was associated with an attenuation of select genes involved in the antiviral responses, Gene Ontology analyses revealed a greater interaction of ABCF1 with TLR signalling suggesting a multifactorial role for ABCF1 in innate immunity in human airway epithelial cells.

METHODS

Human Ethics

All studies using primary human lung material were approved by Hamilton integrated Research Ethics Board (HiREB – 5305-T and 5099-T).

Reagents

In situ hybridization was performed using a custom RNAscope™ probe for ABCF1 (construct targeting 1713-2726 of NM_001025091.1) generated by Advanced Cell Diagnostics (ACD, Newark, California). Negative and positive control probes for quality control of RNA signal in analyzed human tissues were purchased from ACD (data not shown). Protein cell lysates were collected by lysing and scraping cells with RIPA Lysis buffer (VWR, Mississauga, Ontario) mixed with protease inhibitor cocktail (Sigma-Aldrich, Oakville, Ontario). Immunoblots were conducted using Mini-Protean TGX stain-free gels and Transfer-Blot Turbo RTA Transfer Kit reagents (Bio-Rad, Mississauga, Ontario). ABCF1 protein was probed with primary anti-ABCF1 antibody (HPA017578, Sigma-Aldrich, Oakville, Ontario) at 1:100 in 3% Casein in 1X Tris Buffered Saline with TWEEN ® 20 (Sigma-Aldrich, Oakville, Ontario, and Anti-rabbit HRP-linked Antibody (7074S, Cell Signalling Technology, Danvers, MA) at 1:2000. Immunohistochemistry was performed using the same anti-ABCF1 antibody as immunoblotting. ABCF1 and scramble siRNA SMARTpool siGENOME transfection reagents were purchased from Dharmacon (M-008263-01 and D-001206-14, Lafayette, Colorado). The SMARTpool siRNA reagent is a pool of 4 siRNA duplexes all designed to target distinct sites within the specific gene of interest. The 4 different siRNA within the pool were selected by Dharmacon using their design algorithm to have the optimal silencing of the target transcript NM_001025091 and NM_001090, with all siRNAs

targeting within the open reading frame. Cell viability was estimated with the Pierce LDH Cytotoxicity Assay kit (ThermoFisher Scientific, Mississauga, Ontario). RNA samples were lysed with Buffer RLT and purified with Rneasy Mini Kit columns (Qiagen, Toronto, Ontario). The ligands ISD, ISD control, VACV-70, VACV-70 control, and Poly(I:C) were purchased from Invivogen (San Diego, California). Apart from Poly(I:C), where it was applied directly to the cells, the other ligands were complexed with LyoVec transfection reagent (Invivogen, San Diego, California). Human CXCL10 was quantified using a commercial ELISA with ancillary reagent kit (R&D Systems, Oakville, Ontario). The protocol for quantifying CXCL10 was modified with the use of a loading plate for the samples.

Cell Culture

All experiments were performed in submerged monolayer cell culture. An immortalized human airway epithelial cell line (HBEC-6KT) over expressing human telomerase reverse transcriptase (hTERT) and cyclin-dependent kinase 4 (Cdk4) was used as previously described [36-40]. The cell line was obtained from lung biopsies that were not histologically involved with lung cancer from non-smoker donors and it does not have a malignant phenotype[36]. HBEC-6KT were grown in keratinocyte serum free medium (ThermoFisher Scientific, Mississauga, Ontario) supplemented with 0.8 ng/mL epithelial growth factor, 50 µg/mL bovine pituitary extract and 1X penicillin/streptomycin. Calu-3 cells (ATCC HTB-55) were grown in Eagle's Minimum Essential Media supplemented with 10% fetal bovine serum (Wisent, Saint-Jean-Baptiste, QC), 1mM HEPES, and 1X penicillin/streptomycin (Sigma-Aldrich, Oakville, Ontario). Primary human bronchial epithelial cells derived from healthy patient bronchial brushings were grown in PneumaCult ExPlus Medium supplemented with 96 µg/mL hydrocortisone (StemCell

Technologies, Vancouver, BC) and 1X antimicrobial-antimycotics (ThermoFisher Scientific, Mississauga, Ontario). All cells were grown at 37℃ at 5% CO_2. Experiments with primary cells were performed between passages 1 and 4, and experiments with HBEC-6KT and Calu-3 cells were performed within 5 passages.

In vitro experiments

All *in vitro* knockdown experiments in HBEC-6KT were done using siRNA transfected with DharmaFECT Transfection Reagent according to the manufacturer's instructions. Cells were transfected with siABCF1 or siCTRL for 24 hours. For non-challenge experiments, cells were immediately collected for outcome measurements. For challenge experiments, after knockdown, cells were transfected with an immunostimulatory ligand for 24h followed by outcome measurements of cell viability (LDH and cell morphology), function (cytokine secretion), protein expression (immunoblot), or gene transcription (microarray). For TNF-α stimulation experiments, 10 ng/ml was incubated for 24h followed by protein collection for immunoblots. For ISD and VACV-70 stimulation experiments, a concentration-response study was performed using 0.316-3.16 μg/ml (ISD) or 0.1-3.16 μg/ml (VACV-70) followed by incubation for 24h. For Poly(I:C) stimulation experiments, 1 μg/ml was incubated for 24h.

Cytokine Analysis

Cell supernatants were collected following *in vitro* experiments and sent to Eve Technologies for a Human Cytokine Array/Chemokine Array 48-Plex (Eve Technologies, Calgary, Alberta). Eve Technologies uses the Bio-Plex ® 200 to detect 49 different cytokines, chemokines and growth factors: sCD40L, EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GROα,

IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-17E/IL-25, IL-17F, IL-18, IL-22, IL-27, IP-10 (CXCL10), MCP-1, MCP-3, M-CSF, MDC (CCL22), MIG, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES (CCL5), TGF-α, TNF-α, TNF-β, VEGF-A.

Histology, digital slide scanning and microscopy

In situ hybridization and immunohistochemistry was performed using a Leica Bond Rx autostainer with instrument and application specific reagent kits (Richmond Hill, Ontario). The human lung tissues selected for analysis were formalin fixed, paraffin embedded, lung samples from archived hospital clinical samples, determined to be free of defined lung pathology, collected during a tumour resection procedure. No history on smoking was available for the samples analyzed. Following selection, four micron thick sections were stained with using RNAscope™ probes (*in situ* hybridization) or antibody (immunohistochemistry) following directions supplied with the Leica Bond reagent kits. For IHC, heat-induced antigen retrieval in citrate buffer was performed at pH 6 with primary antibody diluted at 1:50. Slides underwent digital slide scanning using an Olympus VS120-L100 Virtual Slide System at 40X magnification with VS-ASW-L100 V2.9 software and a VC50 colour camera (Richmond Hill, Ontario). Image acquisition and formatting was performed using Halo Software (Indica Labs, Albuquerque, NM).

Gene Expression Omnibus (GEO) dataset mining

Gene expression patterns of *ABCF1* in human airway epithelial cells was determined relative to markers for immune cells (*CD34*), ABC transporters of known function in airway epithelial cells (*ABCC4*, *ABCC7*), and junctions (*CDH1*) in a dataset containing samples from trachea, large

airways (generation 2^{nd}-3^{rd}), and small airways (generation 10^{th}-12^{th}) from healthy subjects (GSE11906, Affymetrix Human Genome U133 Plus 2 microarray platform)[41]. The following probesets were used to extract gene expression data: *ABCF1* (200045_at), *ABCC4* (203196_at), ABCC7 (*CFTR*; 205043_at), *CDH1* (201131_s_at), and *CD34* (209543_s_at). In cases where more than one probe corresponded to a given gene, the following hierarchy was used to select an individual probe for further use: perfect, unique matches (probes ending in _at or _a_at) were preferred over mismatch or non-unique probes (ending in _s_at or _x_at). GSE11906 included 17 trachea (age – 42 +/- 7), 21 large airway (age – 42 +/- 9), and 35 small airway samples.

Processing of raw microarray data

Raw intensity values from a microarray experiment using the Affymetrix Clariom S Human chip-type were imported into the R statistical language environment (version 3.6.1; R Core Team, 2019). Probe definition files were obtained from the Brainarray database (version 24[42]). The Single Channel Array Normalization (SCAN) method was used to obtain $\log_2$-transformed normalised expression values with the SCAN.UPC R package (version 2.26.0[43]), with annotation data from the Bioconductor project (version 3.9[44]). The microarray data generated from ABCF1 siRNA experiments is deposited in GEO with accession number GSE150541.

Analysis of processed microarray data

From the processed expression values, principal component analyses were performed with the prcomp function (version 0.1.0) from the R statistical language (version 3.6.1; R Core Team, 2019) using default parameters. Determination of statistically significant differential gene expression was performed using the empirical Bayes method via the eBayes function from the limma R

package (version 3.40.0[45]. *P* values were adjusted using the Benjamini & Hochberg method, with a significance cutoff of 0.05. Significantly enriched Gene Ontology (GO) Biological Process Terms (ranked by *p* value) were determined using Enrichr ([46,47]). Scatter plots, PCA plots, and GO term enrichment dot plots were generated using the ggplot2 R package (version 3.2.1). Heat maps were generated using the pheatmap R package (version 1.0.12), with $\log_2$ expression scaled by gene and complete hierarchical clustering using a Euclidean distance measure applied. A GO term enrichment clustergram was modified from Enrichr using Inkscape.

Statistical analyses

All experiments were performed with an $n \geq 3$ unless otherwise noted. Experiments with HBEC-6KT and Calu-3 cells were considered independent when separated by a passage. Statistics were determined by permutation ANOVA with a Bonferonni-corrected post-hoc test comparing selected groups with $p<0.05$ determined to be statistically significant.

RESULTS

ABCF1 gene and protein expression is localized to human airway epithelial cells *in situ* and *in vitro*

Expression and functional studies of ABCF1 have focused on human synoviocytes, mouse embryonic fibroblasts, human embryonic kidney cells, and peripheral blood mononuclear cells[27-30,32]. We have demonstrated gene expression of *ABCF1* in human airway epithelial cells[35]. To date, no *in situ* gene and protein expression data has confirmed ABCF1 expression in human lung tissues.

To address this knowledge gap, we first mined publicly available gene expression data from primary human airway epithelial cells from healthy subjects. *ABCF1* gene expression was observed along the airway generations (trachea, large, and small) at levels relative to *ABCC7/CFTR* and *ABCC4*, two other ABC transporters with reported functions in airway epithelial cells [25,40,48,49] (**Figure 1A**). *CD34* and *CDH1* (encoding E-Cadherin) were used as negative and positive control genes, respectively, for airway epithelial cells to provide contextual expression levels.

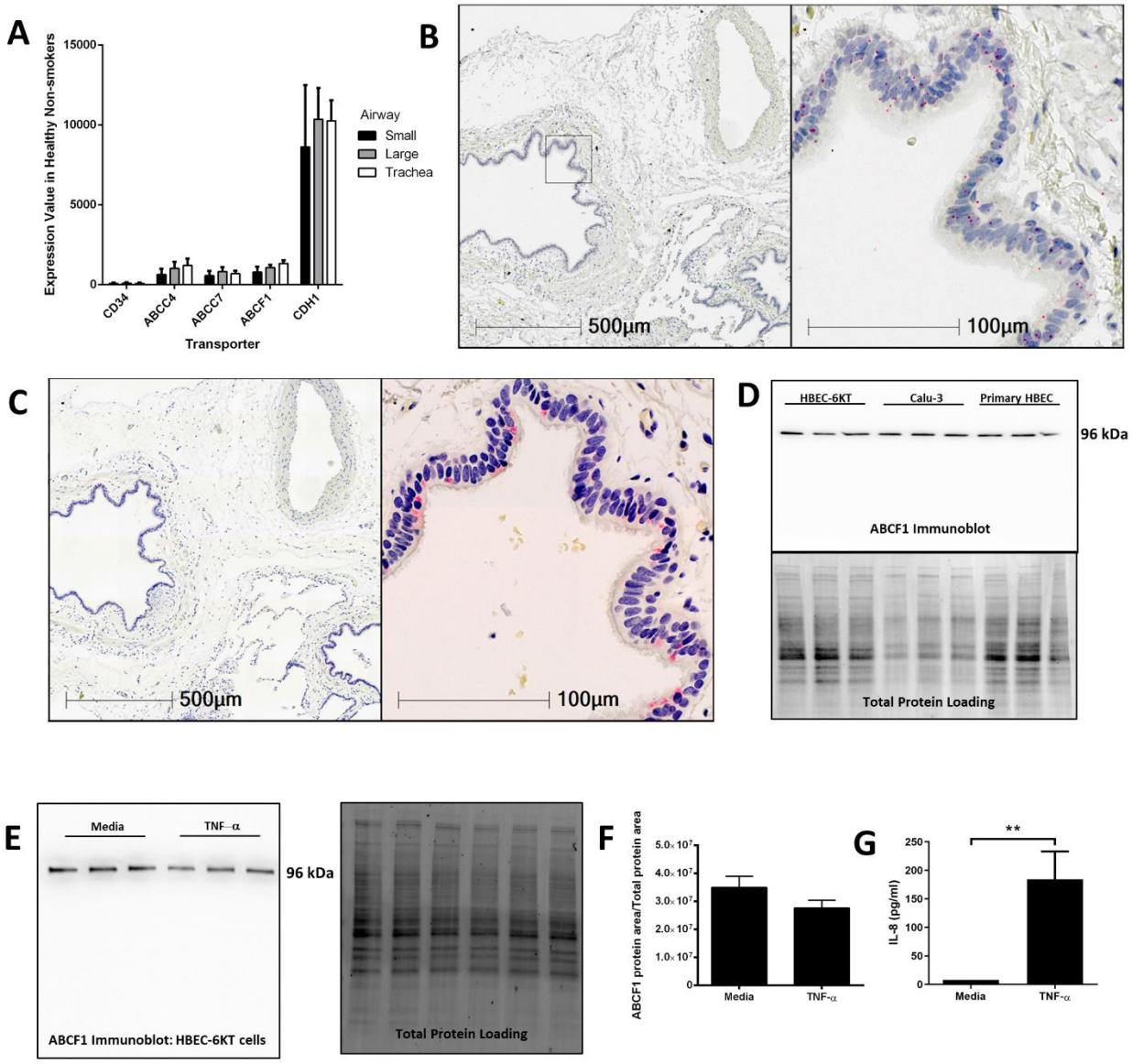
Next, *in situ* localization of *ABCF1* gene transcript was performed using RNAscope™ probes on archived formalin fixed paraffin embedded human lung samples (**Figure 1B**). *ABCF1* gene transcript was observed in small puncta throughout the cytoplasm and nuclear areas of airway epithelial cells. *ABCF1* staining was also observed in submucosal cells with morphology consistent with macrophages.

Protein expression levels were next explored with validation of a commercially available antibody for ABCF1. Positive staining was observed in human airway epithelial cells as shown in a serial section used for *in situ* hybridization (**Figure 1C**) with sparse staining in immune cells with macrophage morphology. Using *in vitro* culture of primary human airway epithelial cells and two distinct airway epithelial cell lines (**Figure 1D**). For each airway epithelial cell type, a single band was observed at the predicted molecular weight of 96 kDa for ABCF1, validating the use of the antibody for *in situ* immunohistochemistry localization.

Lastly, to explore proposed regulatory mechanisms for ABCF1[27], we performed a TNF-α exposure in human airway epithelial cells. Exposure to 10 ng/ml TNF-α for 24h failed to induce a change in ABCF1 protein expression (**Figure 1E-F**), despite inducing an increase in IL-8 (**Figure 1G**).

Collectively our *in vitro* and *in situ* data confirm gene and protein expression of ABCF1 in human airway epithelial cells, warranting downstream characterization and functional studies.

Figure 1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CD34
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Transporter
Airway
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D
HBEC-6KT
Calu-3
Primary HBEC
96 kDa
ABCF1 Immunoblot
Total Protein Loading
E
Media
TNF-α
96 kDa
ABCF1 Immunoblot: HBEC-6KT cells
Total Protein Loading
F
ABCF1 protein area/Total protein area
5.0 x 10^7
4.0 x 10^7
3.0 x 10^7
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Media
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IL-8 (pg/ml)
250
200
150
100
50
0
Media
TNF-α

Figure 1. Validation of ABCF1 gene and protein expression in human airway epithelial cells *in situ* and *in vitro*. **A**: Gene expression analysis of GEO deposited microarray dataset (GSE11906) generated from epithelial cells isolated from trachea, large (2^{nd}-3^{rd} generation), and small airways (10^{th}-12^{th} generation) from healthy subjects (see Methods for details). **B**: In situ hybridization of ABCF1 RNAscope™ probe in human lung under low (10X) and high (40X) magnification. Red puncta are representative of ABCF1 gene transcripts with nuclei counterstained blue. Representative image of n=10. **C**: Immunohistochemistry of ABCF1 in human lung under low (10X) and high (40X) magnification. Representative image of n=10. Pink/red staining is representative of ABCF1 protein with nuclei counterstained blue. **D**: Immunoblot confirmation of ABCF1 protein expression in HBEC-6KT, Calu-3, and primary human airway epithelial cells (each cell type n=3 distinct cell line passages or donors) with a single band observed at predicted molecular weight (96kDa) with total protein loading blot demonstrating equal protein loading for each cell type. **E**: Immunoblot of ABCF1 following TNF-α stimulation of HBEC-6KT cells with corresponding total protein stain. **F**: Quantification of immunoblot of ABCF1 protein expression. **G**: IL-8 secretion from HBEC-6KT cells measured by ELISA as positive control for TNF-αstimulation. All studies n=3 unless otherwise notes. *=$p<0.05$

Reduced expression of ABCF1 under basal conditions *in vitro* has limited impact on HBEC-6KT viability and transcriptional profiles

Functional studies have implicated ABCF1 in translation initiation and have demonstrated that homozygous loss of function results in embryonic lethality[28-30,33]. We therefore first interrogated the basal functions of ABCF1 in our human airway epithelial cells in the context of cell viability and transcriptional profiling.

We performed siRNA experiments to reduce ABCF1 expression levels as no small molecule ABCF1 inhibitor has been described to date. Using siRNA approaches in HBEC-6KT, we confirm that ABCF1 protein levels can be reduced with qualitative (**Figure 2A**) and quantitative measures (**Figure 2B**). Next, we measured LDH levels to measure cell cytotoxicity under ABCF1 silencing. Our findings showed that LDH levels were not elevated when ABCF1 expression was reduced with siRNA when compared to the silencing control **(Figure 2C**). Cell morphology was not different in human airway epithelial cells with reduced ABCF1 expression (**Figure 2D**). Collectively, the quantitative and qualitative data suggest moderate levels of siRNA knockdown are not associated with compromised HBEC-6KT viability under basal conditions.

To interrogate the impact of reduced expression of ABCF1 under basal conditions, a human gene expression microarray analysis was performed. A principal component analysis of ABCF1 reduction and corresponding experimental controls revealed no clustering between experimental replicates for any condition (**Figure 2E**), suggesting that the overall impact of ABCF1 reduction under basal conditions minimally impacted global gene expression patterns. Statistical analysis comparing ABCF1 reduction and siRNA control treated HBEC-6KT confirmed *ABCF1* gene was

down-regulated (**Figure 2F**) which was associated with only one other significantly differentially expressed (up or down) gene, *C12orf75*, which encodes overexpressed in colon carcinoma-1 (OCC-1) protein.

Collectively our *in vitro* studies under basal conditions demonstrate that reduced ABCF1 expression is not associated with changes in viability or significant genome wide changes in transcriptional profiles in HBEC-6KT.

Figure 2

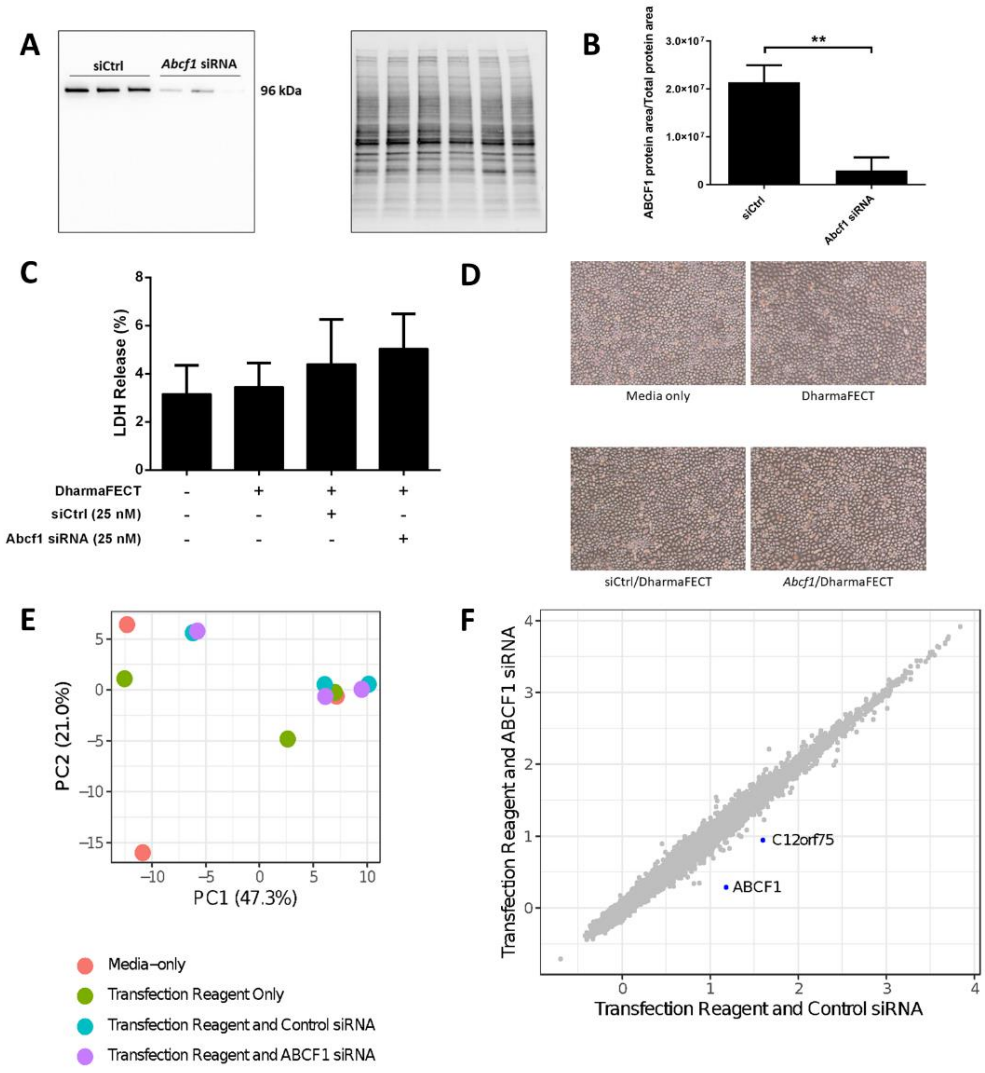

Figure 2. Interrogation of ABCF1 under basal conditions in HBEC-6KT *in vitro*. A: Immunoblot confirming siRNA-mediated knockdown of ABCF1 protein expression in HBEC-6KT cells. **B**: Quantification of ABCF1 protein expression following siRNA treatment. **C**: LDH quantification as a measure of cell viability following siRNA treatment. **D**: Phase-contrast microscopy (4X magnification) of HBEC-6KT following siRNA treatment. **E**: PCA plot of microarray gene expression profiles of HBEC-6KT cells following siRNA treatment. Red circles (media alone), green circles (transfection reagent only), blue circles (transfection reagent and control siRNA), purple circles (transfection reagent and ABCF1 siRNA). **F**: Log_2 expression data for transfection reagent with ABCF1 siRNA compared to transfection reagent with control siRNA. Significantly differently expressed genes are in blue and are down-regulated (*ABCF1* and *C12orf75*). All studies n=3. *=$p<0.05$.

The dsDNA viral mimic VACV-70 induces CXCL10 and an antiviral response in HBEC-6KT *in vitro*

Since reduced expression of ABCF1 under basal conditions resulted in limited impacts on cell viability and gene transcription, we next explored conditions of *challenge* in HBEC-6KT. ABCF1 was described as a dsDNA sensor in mouse embryonic fibroblasts that mediated CXCL10 secretion under challenge conditions with the viral mimic interferon stimulatory DNA (ISD) sequence[31], a 45 bp oligomer shown to activate the STING-TBK1-IRF-3 antiviral sensing axis [34,50].

To determine the response of HBEC-6KT to ISD, we performed a concentration-response study followed by quantification of extracellular CXCL10 secretion as the primary readout for interferon stimulated gene expression and translation as demonstrated in previous literature (**Figure 3A**) [31]. ISD induced an increase in CXCL10 at 1 μg/ml while no increases were observed at lower (0.316 μg/ml) and higher (3.16 μg/ml) concentrations. Importantly, as concentration of ISD increased, the cellular response to the control (ssDNA of the ISD sequence) also increased. These results limited the use of ISD as dsDNA challenge stimulus in HBEC-6KT cells for studying ABCF1 function.

Vaccinia virus is a dsDNA virus that is able to infect airway epithelial cells[19-23]. We therefore determined the response of HBEC-6KT to VACV-70, a 70 bp dsDNA oligonucleotide containing Vaccinia virus motifs[51]. VACV-70 induced a concentration dependent increase in CXCL10 from 0.316 μg/ml to 3.16 μg/ml. In contrast to ISD, no cellular response to the control (ssDNA of the VACV-70 sequence) was observed at any concentration.

To characterize the molecular pathways activated by VACV-70, we performed a transcriptional and pathway analysis of HBEC-6KT following challenge. To interrogate the VACV-70 transcriptional responses a principal component analysis was performed for microarray gene expression data, revealing distinct clustering between stimulation (VACV-70) and control (**Figure 3C**). Statistical analysis revealed 170 up-regulated genes and 42 down-regulated genes with VACV-70 stimulus (**Figure 3D**). VACV-70 up-regulated *CXCL10* gene expression and a curated list of antiviral related interferon stimulated genes (**Figure 3E)**. GO term analysis revealed that the top pathways activated by VACV-70 were associated with type I interferon signalling, viral responses, and cellular responses to viruses (**Figure 3F**).

Collectively our *in vitro* challenge studies confirm that VACV-70, a dsDNA viral mimic, can induce CXCL10 and antiviral transcriptional responses in HBEC-6KT.

Figure 3

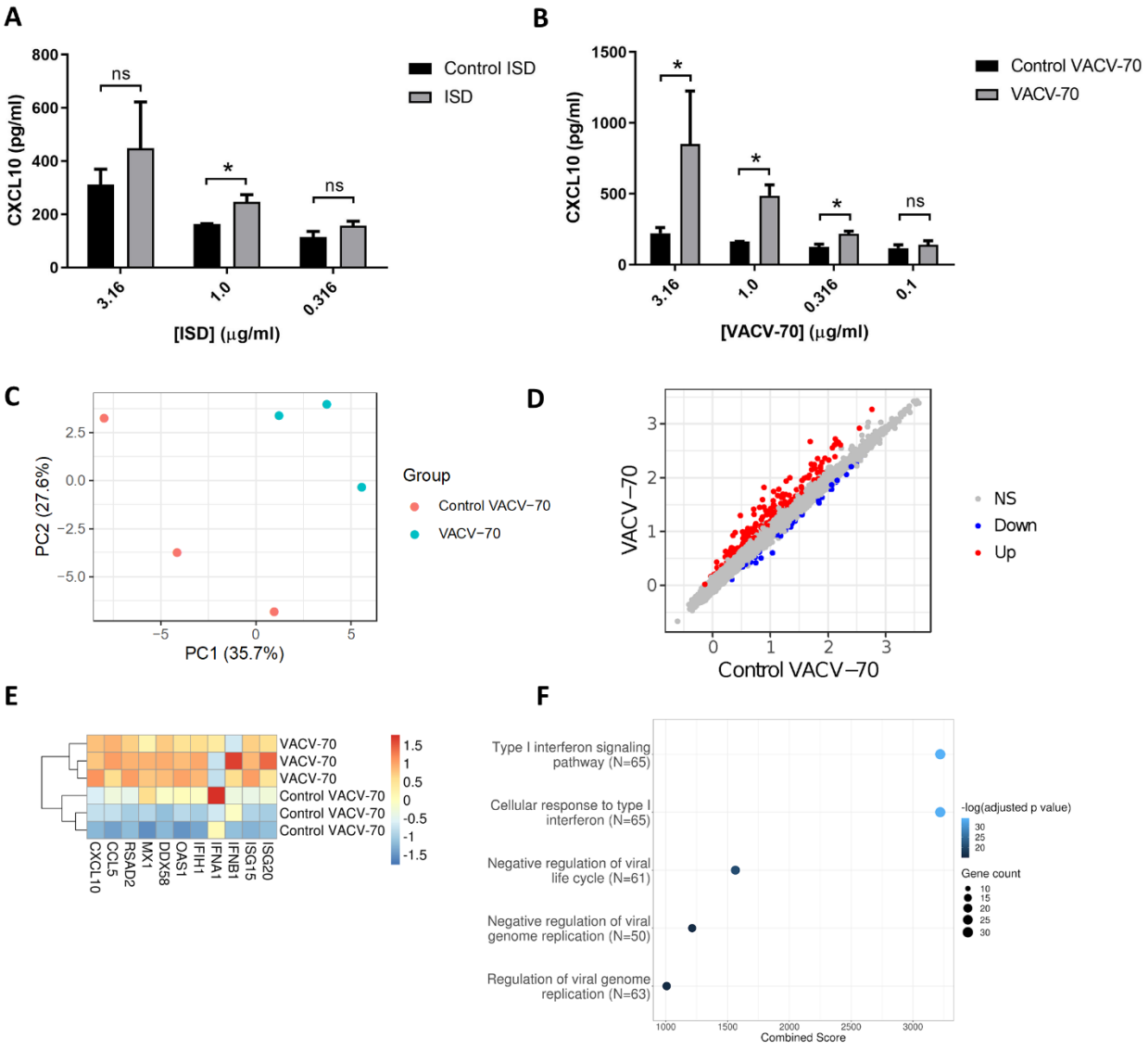
A
CXCL10 (pg/ml)
800
600
400
200
0
ns
*
ns
3.16
1.0
0.316
[ISD] (μg/ml)
Control ISD
ISD
B
CXCL10 (pg/ml)
1500
1000
500
0
*
*
*
ns
3.16
1.0
0.316
0.1
[VACV-70] (μg/ml)
Control VACV-70
VACV-70
C
PC2 (27.6%)
2.5
0.0
−2.5
−5.0
−5
0
5
PC1 (35.7%)
Group
Control VACV−70
VACV−70
D
VACV−70
3
2
1
0
0
1
2
3
Control VACV−70
NS
Down
Up
E
VACV-70
VACV-70
VACV-70
Control VACV-70
Control VACV-70
Control VACV-70
CXCL10
CCL5
RSAD2
MX1
DDX58
OAS1
IFIH1
IFNA1
IFNB1
ISG15
ISG20
1.5
1
0.5
0
-0.5
-1
-1.5
F
Type I interferon signaling pathway (N=65)
Cellular response to type I interferon (N=65)
Negative regulation of viral life cycle (N=61)
Negative regulation of viral genome replication (N=50)
Regulation of viral genome replication (N=63)
1000
1500
2000
2500
3000
Combined Score
-log(adjusted p value)
30
25
20
Gene count
10
15
20
25
30

Figure 3. dsDNA induced antiviral responses in HBEC-6KT *in vitro*. **A**: Concentration-response analysis of ISD-induced CXCL10 protein production for HBEC-6KT cells. Grey bars: ISD, Black bars: control ssDNA generated from ISD. **B**: Concentration-response analysis of VACV-70-induced CXCL10 protein production for HBEC-6KT cells. Grey bars: VACV-70, Black bars: control ssDNA generated from VACV-70. **C**: PCA plot of microarray gene expression profiles of HBEC-6KT cells following transfection with VACV-70 or control. Red circles (control VACV-70), blue circles (VACV-70). **D**: Log_2 expression data for transfection treatment with VACV-70 compared to control VACV-70. Significantly differently expressed genes are identified in blue (down – 42 genes) and red (up – 170 genes). **E**: Heat map of log_2 expression data (scaled by gene) of select known antiviral for VACV-70 and control VACV-70 samples. **F**: Top 5 GO Biological Processes are ranked by increasing $-log_{10}$ adjusted p value, with number (Count) of significantly differentially expressed genes between VACV-70 and control VACV-70 contributing to the total number of genes associated with the given pathway (N) denoted by the size of circle. All studies n=3. *=$p<0.05$.

Reduced expression of ABCF1 under VACV-70 stimulation attenuates CXCL10 secretion with limited impact on HBEC-6KT viability

We have confirmed VACV-70 induction of CXCL10 in HBEC-6KT at the gene **(Figure 3E**) and protein (**Figure 3B**) levels. Furthermore, we have demonstrated siRNA-mediated reduction of ABCF1 expression with no impact on cell viability (**Figure 2A-D**). We therefore performed a VACV-70 stimulation with reduced expression of ABCF1 by siRNA with a primary readout of CXCL10.

ABCF1 reduction was associated with a decrease in CXCL10 protein secretion from HBEC-6KT, with confirmation and quantification of ABCF1 reduction performed by immunoblot (**Figure 4A-C**). Cell viability following VACV-70 stimulation and ABCF1 attenuation was not impacted as assessed by LDH quantification (**Figure 4D**). Qualitative analysis following VACV-70 stimulation and ABCF1 revealed no impact on HBEC-6KT cell morphology (**Figure 4E**).

Collectively our *in vitro* stimulation and functional studies demonstrate that ABCF1 siRNA treatment attenuated VACV-70-induced CXCL10 protein secretion in HBEC-6KT.

Figure 4

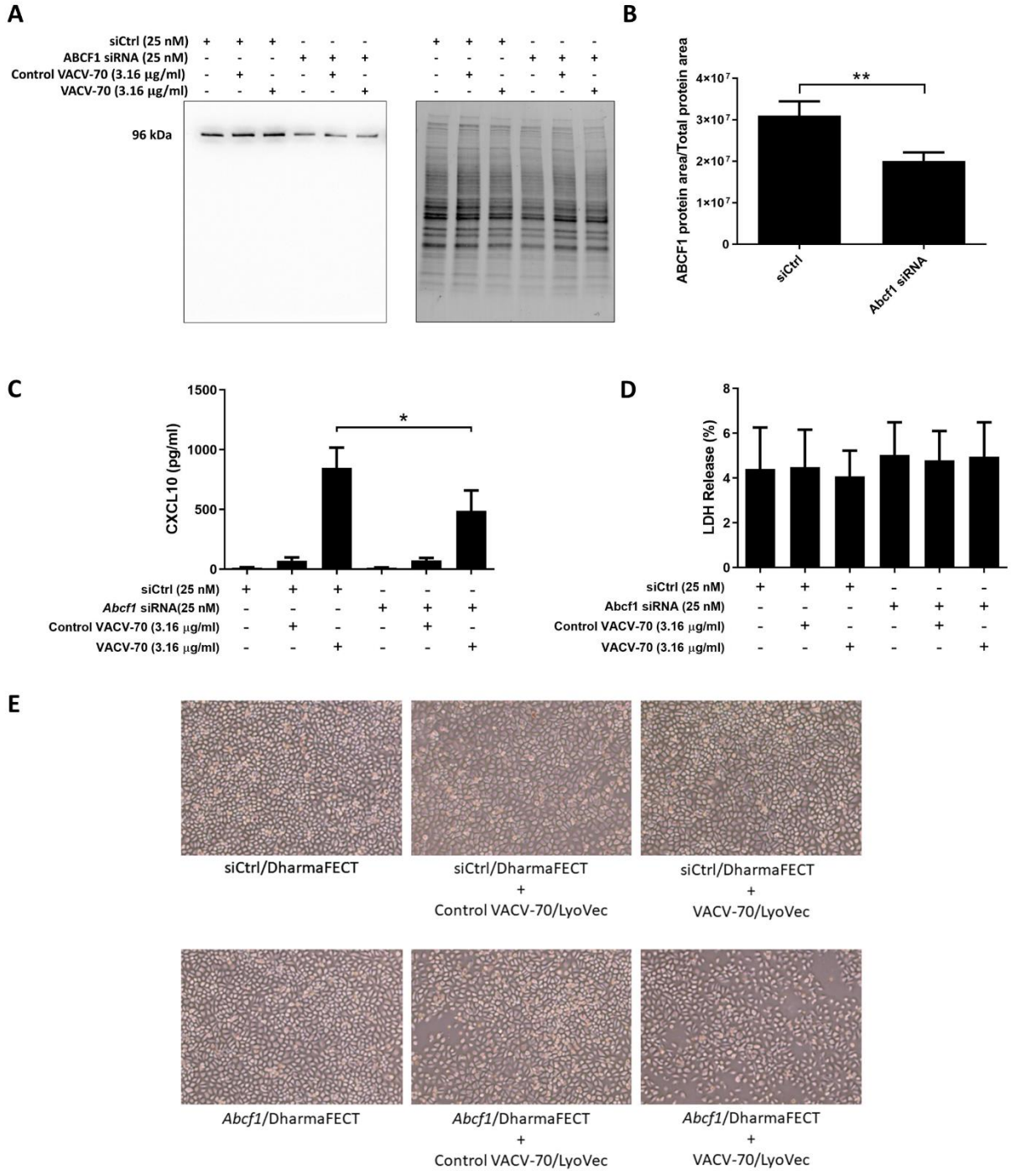
A
siCtrl (25 nM)
ABCF1 siRNA (25 nM)
Control VACV-70 (3.16 μg/ml)
VACV-70 (3.16 μg/ml)
96 kDa
B
ABCF1 protein area/Total protein area
4×10^7
3×10^7
2×10^7
1×10^7
0
**
siCtrl
Abcf1 siRNA
C
CXCL10 (pg/ml)
1500
1000
500
0
*
siCtrl (25 nM)
Abcf1 siRNA(25 nM)
Control VACV-70 (3.16 μg/ml)
VACV-70 (3.16 μg/ml)
D
LDH Release (%)
8
6
4
2
0
siCtrl (25 nM)
Abcf1 siRNA (25 nM)
Control VACV-70 (3.16 μg/ml)
VACV-70 (3.16 μg/ml)
E
siCtrl/DharmaFECT
siCtrl/DharmaFECT + Control VACV-70/LyoVec
siCtrl/DharmaFECT + VACV-70/LyoVec
Abcf1/DharmaFECT
Abcf1/DharmaFECT + Control VACV-70/LyoVec
Abcf1/DharmaFECT + VACV-70/LyoVec

Figure 4. Reduced expression of ABCF1 under VACV-70 stimulated conditions attenuates CXCL10 secretion. A: Immunoblot confirming siRNA-mediated knockdown of ABCF1 protein expression in HBEC-6KT cells under experimental conditions of VACV-70 challenge **B**: Quantification of ABCF1 protein expression following siRNA treatment. **C**: VACV-70 (3.16 μg/ml)-induced CXCL10 protein production for HBEC-6KT cells with ABCF1 siRNA treatment. Black bars: siCtrl treated. Grey bars: ABCF1 siRNA treated. **D:** LDH quantification as a measure of cell viability following VACV-70 and siRNA treatment. **E**: Phase-contrast microscopy (4X magnification) of HBEC-6KT following VACV-70 and siRNA treatment. All studies n=3. *=$p<0.05$.

ABCF1 reduction does not impact VACV-70-induced antiviral transcriptional responses in HBEC-6KT

In parallel to induction of *CXCL10* gene, we have confirmed with GO pathway analysis that VACV-70 induces a dominant antiviral transcriptional signature (**Figure 3E-F**). We therefore next explored how reduced expression of ABCF1 impacts transcriptional responses induced by VACV-70, beyond induction of CXCL10.

A principal component analysis was performed for microarray gene expression data, revealing distinct clustering in samples where ABCF1 expression was reduced relative to control conditions under conditions of VACV-70 stimulation (**Figure 6A – green vs purple)**. Statistical analysis revealed 63 up-regulated genes and 65 down-regulated genes when comparing ABCF1 reduction relative to control under conditions of VACV-70 stimulation (**Figure 6B**). siRNA mediated reduction of ABCF1 was confirmed and associated with attenuation of *CXCL10* gene expression (**Figure 6C,** p=0.06).

To explore a focused transcriptional response of ABCF1 reduction in the context of VACV-70 challenge, a hypothesis-directed approach curated 79 genes from the GO term "Regulation of defence response to virus" and key components of viral sensing for heat map visualization [52](**Figure 6D**). Statistical analysis revealed no global significant difference between ABCF1 reduction and control groups for the expression pattern of this curated list of genes.

To explore the broader transcriptional responses of ABCF1 reduction in the context of VACV-70 challenge, a hypothesis-free directed approach with GO term analysis was performed. Top-ranking

GO pathway terms included *Regulation of toll-like receptor 3-4 signalling pathways*, which were driven by the genes *WDFY1*, *TNFAIP3*, and *NR1D1* (**Figure 6E-F**). Complementary cytokine analysis further revealed impacts of ABCF1 reduction on PDGF-BB, VEGF-A, and to a lesser extent IL-6, IL-8, and IL-1 family members, IL-1a, IL-18, and IL-1RA (**Figure 5**).

As our data suggested that ABCF1 functions in HBEC-6KT may extend beyond sensing of VACV-70 dsDNA viral mimic through regulation of TLR signalling, we explored Poly(I:C), a dsRNA analog and TLR3 ligand that induces interferon responses including CXCL10 production. ABCF1 reduction was associated with a 63% reduction in Poly(I:C)-induced CXCL10 protein ($p=0.07$) secretion but did not impact Poly(I:C)-induced IL-8 protein secretion (**Figure 6G-H**).

Collectively our *in vitro* challenge and functional studies with transcriptional analyses demonstrate a role for ABCF1 in mediating VACV-70 and Poly(I:C) induced CXCL10 secretion and TLR3 related signalling in HBEC-6KT.

Figure 5:

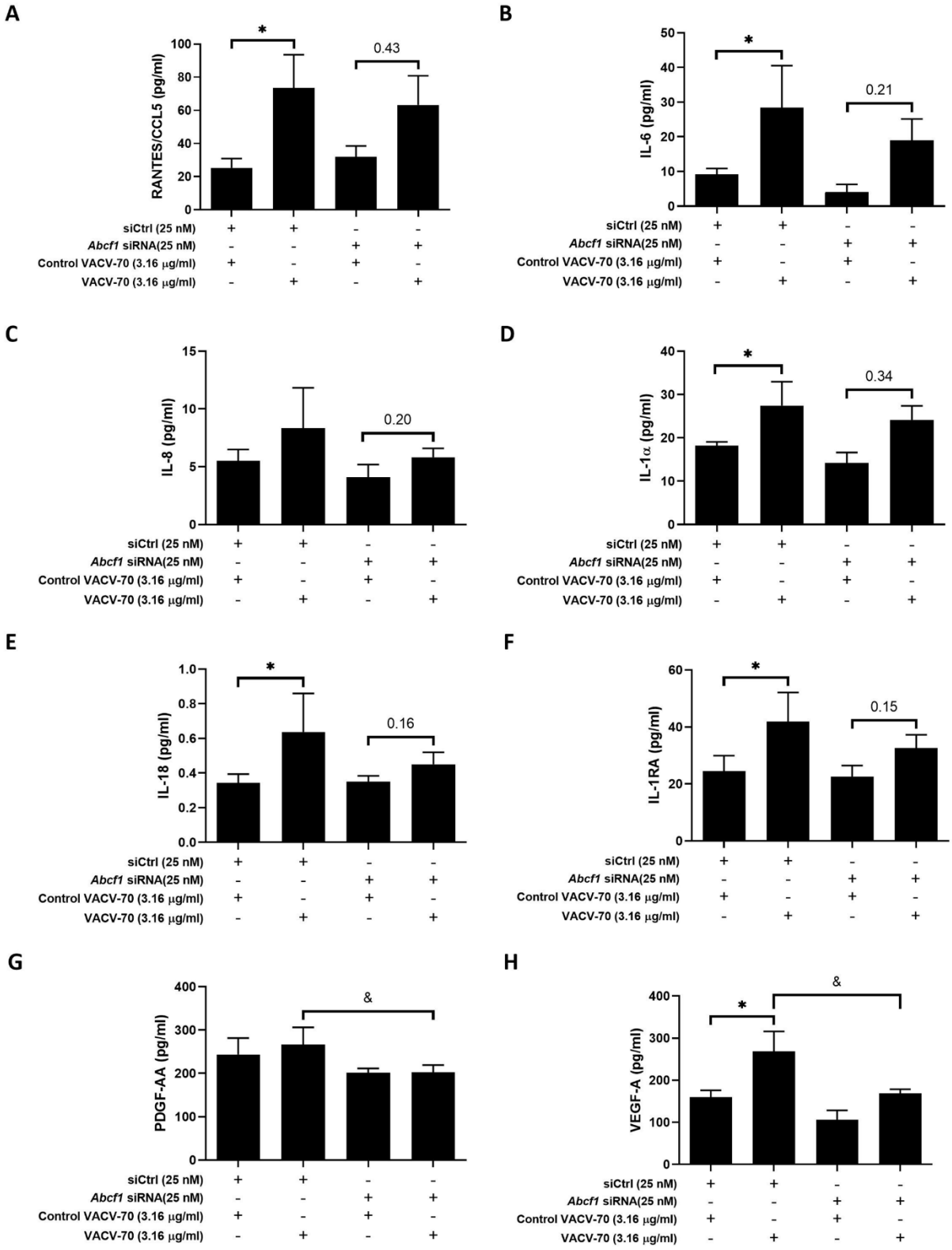
A
RANTES/CCL5 (pg/ml)
*
0.43
B
IL-6 (pg/ml)
*
0.21
C
IL-8 (pg/ml)
0.20
D
IL-1α (pg/ml)
*
0.34
E
IL-18 (pg/ml)
*
0.16
F
IL-1RA (pg/ml)
*
0.15
G
PDGF-AA (pg/ml)
&
H
VEGF-A (pg/ml)
*
&
siCtrl (25 nM)
Abcf1 siRNA(25 nM)
Control VACV-70 (3.16 μg/ml)
VACV-70 (3.16 μg/ml)

Figure 5. Transcriptional interrogation of ABCF1 function during VACV-70 challenge in HBEC-6KT. A: PCA plot of microarray gene expression profiles of HBEC-6KT cells following ABCF1 knockdown and VACV-70 treatment. Red circles (control siRNA and control VACV-70), green circles (control siRNA and VACV-70), blue circles (ABCF1 siRNA and control VACV-70), purple circles (ABCF1 siRNA and VACV-70). **B**: Log_2 expression data for transfection treatment with ABCF1 siRNA and VACV-70 compared to control siRNA and VACV-70. Significantly differently expressed genes are identified in blue (down – 65 genes) and red (up – 63 genes). **C**: Confirmation of ABCF1 and CXCL10 attenuation with ABCF1 siRNA treatment presented as log_2 expression data. Black bars: control siRNA and control VACV-70, light grey bars: control siRNA and VACV-70, dark grey bars: ABCF1 siRNA and control VACV-70, white bars: ABCF1 siRNA and VACV-70. **D:** Heat map of log_2 expression data (scaled by gene) of genes associated with the "regulation of defence response to virus" GO term (n=68) plus the selected known antiviral genes from Figure 3E (n=11) for VACV-70 samples with and without ABCF1 siRNA. **E**: Top 5 GO Biological Processes are ranked by increasing $-log_{10}$ adjusted *p* value, with number (Count) of significantly differentially expressed genes between VACV-70 samples with and without ABCF1 siRNA, contributing to the total number of genes associated with the given pathway (N) denoted by the size of circle. **F**: Significantly differentially expressed genes between VACV-70 samples with and without ABCF1 were submitted to Enrichr for generation of a clustergram defining the gene contribution (Y-axis) to the functional enrichment of the top 5 GO Biological Processes (X axis), with orange squares denoting the association of a differentially expressed gene with a particular GO term. **G:** Poly(I:C) (1.0 μg/ml)-induced CXCL10 and **H:** IL-8 secretion for HBEC-6KT cells with ABCF1 siRNA treatment. All studies n=3. *=$p<0.05$.

Figure 6:

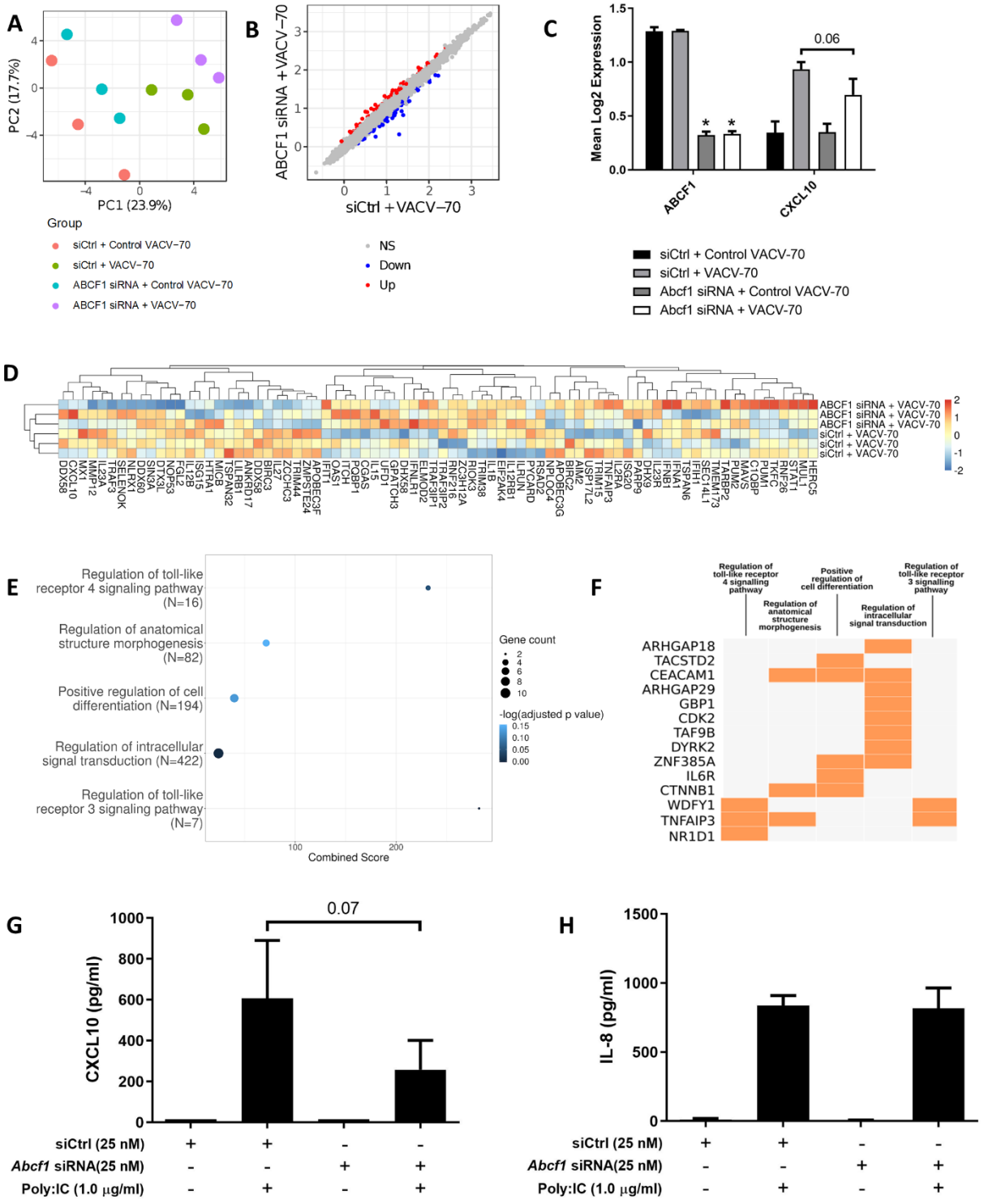
A
PC2 (17.7%)
PC1 (23.9%)
Group
siCtrl + Control VACV-70
siCtrl + VACV-70
ABCF1 siRNA + Control VACV-70
ABCF1 siRNA + VACV-70
B
ABCF1 siRNA + VACV-70
siCtrl + VACV-70
NS
Down
Up
C
Mean Log2 Expression
0.06
ABCF1
CXCL10
siCtrl + Control VACV-70
siCtrl + VACV-70
Abcf1 siRNA + Control VACV-70
Abcf1 siRNA + VACV-70
D
ABCF1 siRNA + VACV-70
ABCF1 siRNA + VACV-70
ABCF1 siRNA + VACV-70
siCtrl + VACV-70
siCtrl + VACV-70
siCtrl + VACV-70
CXCL10 DDX58 MX1 MMP12 IL23A TRAF3 SELENOK DDX60 SIN3A DTX3L NOP53 FGL2 ISG15 RBL2 HTRA1 MICB TSPAN32 LILRB1 ANKRD17 DDX58 BIRC2 IL27 ZC3HC3 TRIM44 ZMPSTE24 APOBEC3F IFIT1 OAS1 ITCH PQBP1 CGAS IL15 UFD1 DHX58 IFNLR1 ELMOD2 TRAF3IP1 TRAF3IP2 RNF216 ZC3H12A ZC3H3 TRIM38 IL1B EIF2AK4 IL12RB1 ILRUN PSMB8-AS1 APOBEC3G NPLOC4 BIRC2 AIM2 USP17L2 TNFAIP3 IL2RA ISG20 PARP9 DHX9 IL23R IFNA1 TSPAN6 IFIH1 SEC14L1 TMEM173 TARBP2 PML C1QBP PUM1 TKFC RNF26 STAT1 MUL1 HERC5
E
Regulation of toll-like receptor 4 signaling pathway (N=16)
Regulation of anatomical structure morphogenesis (N=82)
Positive regulation of cell differentiation (N=194)
Regulation of intracellular signal transduction (N=422)
Regulation of toll-like receptor 3 signaling pathway (N=7)
Gene count
-log(adjusted p value)
Combined Score
F
Regulation of toll-like receptor 4 signalling pathway
Regulation of anatomical structure morphogenesis
Positive regulation of cell differentiation
Regulation of intracellular signal transduction
Regulation of toll-like receptor 3 signalling pathway
ARHGAP18
TACSTD2
CEACAM1
ARHGAP29
GBP1
CDK2
TAF9B
DYRK2
ZNF385A
IL6R
CTNNB1
WDFY1
TNFAIP3
NR1D1
G
CXCL10 (pg/ml)
0.07
siCtrl (25 nM)
Abcf1 siRNA(25 nM)
Poly:IC (1.0 μg/ml)
H
IL-8 (pg/ml)
siCtrl (25 nM)
Abcf1 siRNA(25 nM)
Poly:IC (1.0 μg/ml)

Figure 6. Transcriptional interrogation of ABCF1 function during VACV-70 challenge in HBEC-6KT. A: PCA plot of microarray gene expression profiles of HBEC-6KT cells following ABCF1 knockdown and VACV-70 treatment. Red circles (control siRNA and control VACV-70), green circles (control siRNA and VACV-70), blue circles (ABCF1 siRNA and control VACV-70), purple circles (ABCF1 siRNA and VACV-70). **B**: Log_2 expression data for transfection treatment with ABCF1 siRNA and VACV-70 compared to control siRNA and VACV-70. Significantly differently expressed genes are identified in blue (down – 65 genes) and red (up – 63 genes). **C**: Confirmation of ABCF1 and CXCL10 attenuation with ABCF1 siRNA treatment presented as log_2 expression data. Black bars: control siRNA and control VACV-70, light grey bars: control siRNA and VACV-70, dark grey bars: ABCF1 siRNA and control VACV-70, white bars: ABCF1 siRNA and VACV-70. **D:** Heat map of log_2 expression data (scaled by gene) of genes associated with the "regulation of defense response to virus" GO term (n=68) plus the selected known antiviral genes from Figure 3E (n=11) for VACV-70 samples with and without ABCF1 siRNA. **E**: Top 5 GO Biological Processes are ranked by increasing $-log_{10}$ adjusted *p* value, with number (Count) of significantly differentially expressed genes between VACV-70 samples with and without ABCF1 siRNA, contributing to the total number of genes associated with the given pathway (N) denoted by the size of circle. **F**: Significantly differentially expressed genes between VACV-70 samples with and without ABCF1 were submitted to Enrichr for generation of a clustergram defining the gene contribution (Y-axis) to the functional enrichment of the top 5 GO Biological Processes (X axis), with orange squares denoting the association of a differentially expressed gene with a particular GO term. **G:** Poly I:C (1.0 μg/ml)-induced CXCL10 and **H:** IL-8 secretion for HBEC-6KT cells with ABCF1 siRNA treatment. All studies n=3. *=$p<0.05$.

DISCUSSION

The human airway epithelium expresses a variety of sensors that can detect and initiate an immune response to virus infection. Recognition by these sensors can trigger downstream activation of antiviral responses by inducing the production and release of antiviral and inflammatory cytokines[53]. The recognition sensors include TLRs found at the cell surface such as TLR3, which can detect viral RNA to trigger a type I IFN response by the TRIF signalling pathway. In addition, TLR7 and TLR8 detect viral RNA while TLR9 detects CpG containing viral DNA in the endosomes triggering the same response through the MyD88 signalling pathway[53]. In addition to TLRs, there are several cytosolic receptors including RIG-I-like receptors that recognizes viral RNA, as well as cytosolic DNA sensors such as cyclic GMP-AMP synthase, AIM2-like proteins and DNA-dependent activator of IFN-regulatory factors that produces an IFN response[52]. It is likely that additional candidates are present as redundancy is built into viral sensing mechanisms in host cells[54].

ABCF1, a member of the ATP Binding Cassette family expressed in diverse mammals and different tissue types, has been reported to have diverse functions including initiation of mRNA translation, dsDNA viral sensing, and polarization of immune cell phenotype[27-32]. We have recently reported ABCF1 gene expression levels in human airway epithelium[35], but the function of this molecule remained unexplored. Herein we confirm ABCF1 gene and protein expression *in situ* and *in vitro* in primary human lung tissue and cell lines and explore its function in airway epithelial cells. Under basal conditions, reduced expression of ABCF1 did not lead to quantitative changes in cell viability or qualitative changes in cell morphology associated with cell death. Furthermore, ABCF1 reduction failed to significantly alter basal transcriptional activity in a human

airway epithelial cell line, HBEC-6KT. Under VACV-70 challenge, a model of dsDNA viral exposure, ABCF1 was linked to CXCL10 secretion. Interestingly, despite the demonstrated activation of a viral gene signature by VACV-70, no global change in antiviral gene expression patterns were observed with ABCF1 reduction. In contrast, the gene pathways regulated by ABCF1 under VACV-70 challenge were associated with TLR signalling and intracellular signal transduction. Furthermore, Poly(I:C), a dsRNA analog and TLR3 ligand induced CXCL10 in an ABCF1 sensitive mechanism. Collectively, our findings suggest that ABCF1 may regulate CXCL10 production downstream of dsDNA sensing mechanisms and TLR3 in human airway epithelial cells. It remains possible that ABCF1 can function to complement viral sensing mechanisms mediated by canonical dsRNA viral response machinery (e.g. RIG-I) as there are possible redundancies in viral sensing mechanisms in the cell[54].

ABCF1 (originally called ABC50) was first identified in human synoviocytes at the mRNA level as a transcript regulated by TNF-α exposure[27]. ABCF1 is unique in the mammalian ABC transporter family in that it contains the signature ATP binding LSGGQ amino acid motif and associated Walker A and B motifs for phosphate binding, but lacks a predicted transmembrane region[27,55,56], which is supportive of a cytosolic localization and function. *ABCF1* transcript expression profiling has revealed near ubiquitous expression in human organs including lung, heart, brain, placenta, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes[27]. The expression of ABCF1 has subsequently been identified in the human HeLa cells and embryonic kidney cells and other mammalian cells from rats, rabbits, hamsters, and mice [28-30,32,33]. Highlighting the importance of ABCF1 in normal physiology and development, homozygous deletion of ABCF1 is embryonic

lethal in either C57BL/6 mice or BALB/c mice[33]. As our group recently identified gene expression of *ABCF1* in human airway epithelial cells [35], we set out to first confirm this at the protein level and then determine the function(s) of ABCF1 in human airway epithelial cells. We confirm that *ABCF1* gene expression is present in airway epithelial cells and expressed at levels relative to other known ABC transporters with function in this cell type, *ABCC4* and *ABCC7*/*CFTR*[25,40,48,49]. *In situ* hybridization using RNAscope™ technology demonstrated *ABCF1* transcripts present in the airway epithelial cells in human lung samples, which was consistent with positive immunohistochemical staining of protein in a serial section of the same samples using an antibody validated for specificity. Since the original discovery of ABCF1 was the result of an upregulated transcript resulting from TNF-α stimulation of synoviocytes, we examined if this mechanism was functional in human airway epithelial cells. In contrast to the reported data on synoviocytes, TNF-α stimulation failed to induce ABCF1 protein expression in HBEC-6KT, despite IL-8 induction as a positive control. Interestingly, in a recent report profiling the role of ABCF1 in murine bone-marrow derived macrophages, TNF-α stimulation *suppressed* ABCF1 protein expression[32]. These contrasting observations of ABCF1 expression in HBEC-6KT compared to synoviocytes and bone-marrow derived macrophages could be due to the difference in ontogeny and function of these cells. Collectively our results and those in the literature support gene and protein expression of ABCF1 in human airway epithelial cells, and that regulation of this protein is likely to be cell specific.

The first description of a potential function for ABCF1 in mammalian cells was derived from the experiments on human synoviocytes, suggesting a role in translation due to homology of molecular sequence with yeast proteins that performed this function[27,28]. The embryonic lethality

observed in mice for homozygous ABCF1 deletion and ubiquitous expression across multiple cell and tissue types[33], is consistent with ABCF1 playing a role in a fundamental biological process like protein translation. The observation that proliferating cells including synoviocytes stimulated with TNF-α and T cells stimulated with phorbol myristate acetate and ionomycin, elevate ABCF1 levels is further consistent with a role in translation[27,28]. Subsequent to the discovery of ABCF1 gene expression and homology modeling, biochemical studies implicated the protein in interaction with eukaryotic initiation factor-2 (eIF2), a heterotrimeric protein consisting of α, β, and λ subunits, that is important for translation initiation[28]. A distinguishing feature of ABCF1 relative to other ABC transporters is a N-terminal domain that is able to interact with eIF2α in a process that potentiates binding of methionyl-tRNA and initiation of translation[29]. In addition to eIF2α interactions, ABCF1 associates with ribosomes in a process potentiated by ATP binding to the nucleotide binding domains and inhibited by ADP[28], although the hydrolysis of ATP seems dispensable for ribosome interaction[29]. To explore the potential function of ABCF1 as an initiator of translation in human airway epithelial cells, we undertook a siRNA approach to attenuate gene and protein expression followed by a global assessment of cell viability and transcriptomics. Surprisingly, under basal conditions, reduced expression of ABCF1 at the gene and protein level did not impact HBEC-6KT viability, morphology or transcriptional profile. Importantly, our outcome measurements were performed on HBEC-6KT that were sub-confluent and undergoing proliferation in serum-free media, an experimental condition where ABCF1 function in translation initiation would be relevant. A limitation of our design is that we measured global gene expression under the assumption that this would reflect any global changes in gene translation, an indirect approach which does not allow us to directly implicate ABCF1 expression levels to protein synthesis. Interestingly, our observations of minimal changes in HBEC-6KT may

be consistent with cells of epithelial lineage, as near complete ABCF1 knockdown in HeLa cells was also only associated with a modest attenuation of total protein synthesis[30]. Collectively, our results suggest that ABCF1 may function independent of protein translation functions in HBEC-6KT, as gene and protein attenuation results in no changes in cell viability or global transcriptional profile.

The original discovery that ABCF1 expression was regulated by TNF-α stimulation suggested a link to immune responses, although no differential expression patterns were observed for synoviocytes from healthy individuals or those with rheumatoid arthritis[27]. Subsequently, ABCF1 has been implicated in immune responses via a cytosolic dsDNA viral sensing function using mouse embryonic fibroblasts[31]. Using an integrative bioinformatic and molecular biology approach, a biotinylated ISD sequence was used as a bait and transfected into cells, followed by proteomic interrogation of identified candidates. The ISD bait method was validated by identifying known dsDNA sensors including HMGB1, HMGB2, and HMGB3, components of the AIM2 inflammasome, and the SET complex that plays a role in HIV-1 retroviral detection and infection[57]. Within the pool of unknown dsDNA interacting candidates, ABCF1 was mechanistically linked to ISD induced-CXCL10 production using siRNA methods. The observed ISD induced-CXCL10 converged on IRF-3 signalling, confirmed by showing reduced IRF-3 phosphorylation following ISD treatment under conditions of ABCF1 silencing. In a separate study, ABCF1 has been implicated as a molecular switch downstream of TLR4 signalling in mouse bone-marrow derived macrophages that regulates MyD88 dependent pro-inflammatory and TRIF/TRAM dependent anti-inflammatory processes[32]. Using *in vitro* and *in vivo* model systems, ABCF1 was implicated in polarizing pro-inflammatory macrophages to an anti-

inflammatory/tolerant macrophage phenotype with direct involvement in shifting the systemic inflammatory response syndrome to a endotoxin tolerance phase in sepsis[32]. The mechanism responsible for the ABCF1-mediated polarization of macrophages was identified to be a E2-ubiqutin-conjugating enzyme function. In wild-type macrophages the TRIF-IFN-β pathway is intact with attenuation of the MyD88 pathway, allowing IRF-3 phosphorylation, dimerization, and IFN-β expression. In contrast, heterozygosity for ABCF1 results in attenuation of the TRIF-IFN-β pathway, with reduced IRF-3 activation and IFN-β production. Importantly, these two immunological studies converge on a relationship between ABCF1 and IRF-3, which could involve direct or indirect interactions to facilitate downstream immune responses. Consistent with the potential role for ABCF1 as a dsDNA sensor, we explored immune and transcriptional responses downstream of VACV-70, a dsDNA viral mimic capable of activating STING, TBK1, and IRF-3 independent of TLRs[51]. VACV-70 induced a dominant antiviral signature and pathway activation in HBEC-6KT, consistent with successful transfection and cytosolic sensing. Reduced expression of ABCF1 was associated with a reduction in CXCL10, an antiviral cytokine regulated by IRF-3 activation, independent of any changes in cell viability or morphology. Transcriptomics revealed that although attenuation of CXCL10 was observed with ABCF1 siRNA, a global attenuation of an antiviral signature was not observed. Hypothesis-free GO analysis identified that the key pathways that were significantly impacted by ABCF1 siRNA treatment during VACV-70 challenge were related to TLR signalling. Interestingly, a key gene identified in our VACV-70 challenge and ABCF1 silencing studies is *WDFY1*, which links TLR3/4, TRIF, and IRF-3 signalling[58]. This finding suggested that ABCF1 could potentially be regulating both TLR4 and TLR3/TRIF/IRF-3 signalling[32]. We tested this hypothesis by using Poly(I:C), a dsRNA viral mimic that activates TLR3 and IRF-3[52]. ABCF1 siRNA treatment attenuated

Poly(I:C)-induced CXCL10 production, further demonstrating a link between ABCF1 and TRIF/IRF-3, perhaps through regulation of *WDYF1*. While we have not yet demonstrated the link between ABCF1 and TLR4, a LPS challenge with ABCF1 siRNA treatment would effectively interrogate this. Our exploratory results suggest that ABCF1 is likely to play a complex role in innate immunity in response to cytosolic nucleic acids, with a potential interaction with TRIF/IRF-3 for regulation of CXCL10.

Throughout our study we encountered several technical issues. The absence of pharmacological interventions that could antagonize ABCF1 function required us to pursue molecular approaches with siRNA. siRNA approaches were unable to completely attenuate ABCF1 at concentrations of 25 nM for up to 48h. Longer durations of silencing were not possible as the human airway epithelial cell line used showed changes in morphology with vehicle control transfection reagent beyond 48h of incubation. Our inability to completely attenuate ABCF1 levels was consistent with human embryonic kidney cells[30]. Secondary to addressing ABCF1 expression levels, we sought to explore the functional consequences with the reported dsDNA viral mimic ISD as reported in the literature with mouse embryonic fibroblasts[32]. During our concentration-response studies with ISD, the vehicle control condition resulted in elevations in our primary readout of CXCL10, which suggested an unexplained confounding factor. We therefore opted to use VACV-70 in place of ISD, which limits our ability to directly compare our results to those that have established ABCF1 as a dsDNA sensor with ISD[32]. Importantly, reported findings from the HBEC-6KT cell line will require confirmation using primary human airway epithelial cells under submerged and/or air-liquid interface culture conditions to more accurately model in the *in situ* human respiratory mucosa.

In conclusion, we confirm that ABCF1 is expressed at the gene and protein level *in situ* and *in vitro* in human airway epithelial cells. In HBEC-6KT, ABCF1 has minimal functions for cell viability and transcriptional regulation under basal conditions but is important for mediating immune responses to cytosolic nucleic acids in pathways that involve TLR signalling and CXCL10 production. Our data form the foundation to pursue precisely how ABCF1 is regulated and where it functions in the network of cytosolic nucleic acid sensors and immune responses in human airway epithelial cells.

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Chapter 3

TNF-α and Poly(I:C) induction of A20 and activation of NF-κB signalling are independent of ABCF1 in human airway epithelial cells.

Summary and significance

ABCF1 is the most characterized member of the ABCF family, with proposed functions related to innate immunity in fibroblasts, macrophages, and airway epithelial cells. A study has demonstrated that in murine bone marrow derived macrophages (BMDMs) under the LPS stimulation, ABCF1 negatively regulates MyD88-dependent signalling and positively regulates TRIF-dependent signalling. Our last study showed that ABCF1 mediates an innate immune response in airway epithelial cells under the dsDNA viral mimic stimulation. While the mechanism was unclear, our transcriptomic analysis suggests that ABCF1 has a role in TLR signalling and impacts the gene expression of *Tnfaip3* (A20), a negative regulator of pro-inflammatory responses. Following the findings from our previous study, we hypothesize that silencing ABCF1 in human airway epithelial cells will lead to a reduction of A20 protein expression, which thereby results in a greater pro-inflammatory response mediated by NF-κB signalling. We demonstrated that under TNF-α and Poly(I:C) stimulation with ABCF1 silencing, there was a reduction in the induction of IL-8 and a trend for reduced IL-6, however, the expression levels of A20 and activation status of the transcription factors were unaffected. Our study concluded that ABCF1 regulates the expression levels of the pro-inflammatory cytokines through pathways that are independent of NF-κB and IRF-3 activation. The exact mechanism remains to be characterized.

TNF-α and Poly(I:C) induction of A20 and activation of NF-κB signalling are independent of ABCF1 in human airway epithelial cells.

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ABSTRACT

ABCF1 is the most characterized member of the ABCF family in eukaryotes with proposed functions related to innate immunity in fibroblasts, macrophages, and epithelial cells. Currently, a mechanistic link between ABCF1 and immune responses in human airway epithelial cells (HAECs) remains to be clearly defined. The present study aimed at characterizing the function of ABCF1 in the context of nuclear factor κB (NF-κB) mediated pro-inflammatory responses in an immortalized human airway epithelial cell line, HBEC-6KT. We demonstrated that with ABCF1 silencing under basal conditions, TNF Alpha Induced Protein 3 (TNFAIP3/A20) protein expression and downstream expression and activation of transcription factors, NF-κB and Interferon regulatory factor 3 (IRF-3), were not disrupted. We followed with investigations of ABCF1 function under pro-inflammatory stimuli that are known to be regulated by A20. We demonstrated that under Polyinosinic:polycytidylic acid (Poly(I:C)) and tumour Necrosis Factor-α (TNF-α) stimulation with ABCF1 silencing, there was a significant reduction in secreted levels of interleukin-8 (IL-8) and a trend for reduced IL-6. However, we observed no changes to the expression levels of A20 and the activation status of the transcription factors, NF-κB and IRF-3. Collectively, these studies demonstrate that Poly(I:C) and TNF-α induced IL-8 are regulated by ABCF1 via pathways independent of NF-κB and IRF-3 activation.

Key words: airway, epithelial cells, ABCF1, A20, NF-κB, IRF-3, Poly(I:C), TNF-α, pro-inflammatory, cytokines, innate immunity

INTRODUCTION

Our group have confirmed the expression of ABCF1 in human airway epithelial cells (HAEC) at the gene and protein level both *in situ* and *in vitro* in our previous studies. In addition, we characterized the function of ABCF1 as an important component in modulating innate immune responses in HAECs. We used a hypothesis-free gene ontology (GO) analysis and identified the key pathways that were impacted by ABCF1 siRNA treatment during a cytosolic nucleic acid stimulation[1]. One of these pathways included TLR3/4 signalling and the key gene that was differentially expressed in these pathways was *TNFAIP3*. This gene is expressed as the A20/tumour necrosis factor α-induced protein 3 (TNFAIP3) protein, a ubiquitin ligase and deubiquitinating enzyme that inhibits nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF-3) activation to regulate innate immune responses[2, 3]. To our knowledge, a mechanistic link between ABCF1 expression and A20, NF-κB, and IRF-3 signalling has not been explored in HAECs.

Previously, stimulation of synoviocytes with Tumour Necrosis Factor α (TNF-α) demonstrated a potential role of ABCF1 in promoting inflammatory responses. The study linked pro-inflammatory stimulation *in vitro* with the mRNA transcript of *Abcf1* accumulating in synoviocytes, without mechanistically linking this relationship to functional responses[4]. In macrophages, ABCF1 was shown to be regulating MyD88- and TRIF-dependent pro-inflammatory signalling in response to lipopolysaccharide (LPS) stimulation. It was demonstrated that by silencing of ABCF1, it led to a decrease in protein expression of A20, an increase in NF-κB and a decrease in IRF-3 activation under basal and LPS stimulated conditions. This finding suggested that ABCF1 could be negatively regulating the MyD88-dependent signalling and positively regulate the TRIF-

dependent signalling in response to LPS stimulation[5]. Currently, there are no known implications for lung health and disease associated with ABCF1. Nonetheless, we are intrigued by the possibility of exploring whether ABCF1 plays a functional role in the airway epithelium and whether it has an influence on lung health like certain members of the ABC transporter family of proteins. The two studies mentioned provides an associative and mechanistic links between ABCF1 with immune responses, thereby supporting the need for a more comprehensive exploration ABCF1's characterization in HAECs.

Building upon the conclusions drawn from our earlier study, we put forth the hypothesis that suppressing ABCF1 in HBECT-6KT HAECs will result in a reduction of A20 protein expression, thereby intensifying the pro-inflammatory reactions mediated by NF-κB. In this study, we performed mechanistic studies with ABCF1 silencing under basal and stimulated conditions with readouts of A20, NF-κB, and IRF-3 expression and activation. We demonstrated that under basal unstimulated conditions, ABCF1 knockdown does not impact A20 protein expression or NF-κB and IRF-3 activation. Furthermore, despite the silencing of ABCF1 leading to attenuation of Polyinosinic:polycytidylic acid (Poly(I:C)) and TNF-α induced IL-8, no changes were observed in A20 protein expression or NF-κB and IRF-3 activation. Our results demonstrate that in HBEC-6KT HAECs, ABCF1 regulates IL-8 induction independent of canonical pro-inflammatory pathways and suggests novel regulatory mechanisms for this cytokine.

METHODS

Reagents

ABCF1, A20, NF-κB p65, phosphorylated NF-κB p65 (Ser536), IRF-3 and phosphorylated IRF-3 (Ser386) were probed with anti-ABCF1 primary antibody (HPA017578, Sigma-Aldrich, Burlington, Massachusetts) at 1:500-1:1000, anti-A20 primary antibody (5630T, Cell Signalling Technology, Danvers, Massachusetts) at 1:1000, anti-NF-κB p65 primary antibody (4764T, Cell Signalling Technology) at 1:1000, anti-NF-κB p65 (Ser536) primary antibody (3033T, Cell Signalling Technology) at 1:1000, anti-IRF-3 (ab76493, Abcam, Waltham , Boston) at 1:1000 and anti-IRF-3 (Ser386) (ab68481, Abcam) at 1:1000 in 3% Casein (1706404, Bio-Rad Laboratories, Hercules, California) in 1X Tris Buffered Saline (T5912, Sigma-Aldrich) with TWEEN® 20 (P1379, Sigma-Aldrich) (TBS-T). The secondary antibody used for each protein was anti-rabbit HRP-Linked Antibody from Cell Signalling Technology (7074S, Cell Signalling Technology) at 1:2000 in 3% Casein in TBS-T. ABCF1 (si-ABCF1) and scramble siRNA (siCtrl) SMARTpool siGENOME, as well as the DharmaFECT 1 transfection reagent were purchased from Dharmacon (M-008263-01 and D-001206-14, Lafayette, Colorado). The SMARTpool siRNA reagent is a pool of 4 siRNA duplexes all designed to target distinct sites within the specific gene of interest. The 4 different siRNA within the pool were selected by Dharmacon using their design algorithm to have the optimal silencing of the target transcript NM_001025091 and NM_001090, with all siRNA targeting within the open reading frame. The immunostimulatory ligands, TNF-α was purchased from Peprotech (300-01A, Rocky Hill, New Jersey), Recombinant human IL-17A protein was purchased from R&D Systems (7955-IL-025, Minneapolis, Minnesota); TLR3 Agonist (tlrl-pic), TLR4 Agonist (tlrl-b5lps), and TLR2 Agonist (tlrl-pgns2) were all purchased from Invivogen (San

Diego, California). All immunostimulatory ligands were directly added to the cells with cell culture media without a transfection reagent.

Cell Culture

All experiments were performed in submerged monolayer cell culture using the HBEC-6KT immortalized HAEC line over expressing human telomerase reverse transcriptase (hTERT) and cyclin-dependent kinase 4 (Cdk4)[6–10]. The cell line was obtained from lung biopsies that were not histologically involved with lung cancer from non-smoker donors and it does not have a malignant phenotype[6]. HBEC-6KT were grown in keratinocyte serum free medium (ThermoFisher Scientific, Waltham, Massachusetts) supplemented with 0.8 ng/ml epithelial growth factor, 50 μg/ml bovine pituitary extract and 1 X penicillin/streptomycin (97063-708, VWR, Radnor, Pennsylvania). All cells were grown at 37◦C at 5% CO2.

***In vitro* pro-inflammatory stimulation experiments with siRNA-mediated knockdown of ABCF1**

Pro-inflammatory dose response experiments were performed in HBEC-6KT cells with immunostimulatory ligands for 24 hours (h) at a confluency of approximately 80-90%. Cells were stimulated with TNF-α (10 – 1000 ng/ml), Poly(I:C) (0.01 – 1 μg/ml), PGN-SA (1 – 100 μg/ml), IL-17A (0.1 – 10 ng/ml), and LPS-B5 (0.01 – 1 μg/ml).

All *in vitro* siRNA-mediated knockdown experiments in HBEC-6KT were done using siRNA transfected with DharmaFECT Transfection Reagent according to the manufacturer's instructions. Cells were transfected with si-ABCF1 or siCtrl for 24 h at approximately 70% confluency. After

siRNA-mediated knockdown when the cells are at 80-90% confluency, the cells were stimulated for 24 h with TNF-α and Poly(I:C), followed by outcome measurements of function (cytokine secretion measured by ELISA) and protein expression (immunoblot) (**Figure 1a**). All transcription factors were assessed following 24 h of stimulation. Similar experiments were performed in the absence of any stimulation to investigate baseline ABCF1 function.

Cytokine Analysis

Cell culture media were collected following *in vitro* experiments and centrifuged at 7500 x g for 12 minutes. ELISA assays were run for human IL-6 (DY206, R&D Systems, Minneapolis, Minnesota) and IL-8 (DY208, R&D Systems) according to manufacturer directions with absorbance read using the SpectraMax i3 Multi-Mode Platform (Molecular Devices, Silicon Valley, California) microplate reader. Cytokine analyses were performed with n=5 and analyzed as unpaired Student t-tests.

Western Blotting

HAECs were isolated using a cell scraper (83.395, Sarstedt, Nümbrecht, Germany) and lysed with RIPA lysis buffer (89900, ThermoFisher Scientific) containing protease inhibitor cocktail (P2714-1BTL, Sigma-Aldrich) and phosphatase inhibitor (4906845001, Roche, Mississauga, Ontario). The collected protein was used for western blotting without further sample preparation. Protein from total cell lysate (4 μg) was premixed in 1X Laemmli Sample Buffer (1610747, Bio-Rad Laboratories), then separated on a 4-20% Mini-PROTEAN TGX stain-free precast gels (4568093, Bio-Rad Laboratories) and transferred to a PVDF membrane using the Transfer-Blot Turbo RTA Transfer Kit reagents (1704272, Bio-Rad Laboratories). Membranes were blocked at room

temperature for 1 h using 5% Casein (1706404, Bio-Rad Laboratories) in 1X Tris Buffered Saline with TWEEN® 20. Protein detection was performed using the Clarity Western ECL Substrate (1705061, Bio-Rad Laboratories) and imaged in a ChemiDoc MP Imaging System. All images were acquired using the auto-exposure setting. Signal intensity was normalized to total protein loading from membranes stained for total protein, on ImageLab (Bio-Rad Laboratories), a method that ensures normalization of protein signal is performed in the linear range of detection[11]. Western blot analyses were performed with n=3 and analyzed as unpaired Student t-tests. Although an n=5 was performed for each experiment, an n=3 was used for western blot analyses due to limited sample availability for all the blots required.

Statistical Analyses

The statistical analyses of the processed microarray analysis were performed following the same methodology as described in our previous study[1]. Determination of statistically significant differential gene expression was performed using empirical Bayes method via the eBayes function from limma R package. Gene expression analysis was conducted with a sample size of n = 3, and a gene-level p-value of < 0.05 was considered statistically significant.

All subsequent experiments were conducted with a minimum sample size of $n \geq 3$, unless stated otherwise. Each repetition was obtained from a distinct cell culture. Experiments conducted with HBEC-6KT were considered independent when separated by at least one passage, all within a maximum of 5 passages. Statistical analysis was conducted using Ordinary One-Way ANOVA with a Dunnett's test and unpaired Student t-test comparing selected groups with p-value of < 0.05 determined to be statistically significant on GraphPad Prism 9.

RESULTS

ABCF1 Knockdown does not impact A20 protein expression and function under basal conditions.

We have previously performed a transcriptomic profiling with Gene Ontology (GO) term analysis on ABCF1 knockdown in response to VACV-70, dsDNA viral mimic stimulation. Top-ranking GO pathway terms included *Regulation of toll-like receptor 3-4 signalling pathways*, driven by the genes *WDFY1*, *TNFAIP3* (A20) and *NR1D1*. Induction of the TNFR1 and TLR3 signalling pathways can induce an innate immune response through NF-κB signalling pathway in HAEC. The gene *TNFAIP3*, expressed as A20 protein, is a master regulator of NF-κB activity by ubiquitination[2]. To follow up with our previous findings, we first investigated the expression and function of A20 in our model system under basal conditions.

First, we looked at the differential gene expression of A20, NF-κB p65 and NF-κB regulated cytokines and chemokines (**Figure 1b**) under basal unstimulated conditions. Under siRNA-mediated knockdown of ABCF1, we observed a non-significant trend for downregulated gene expression of A20, IL-18, IL-6, IL-8, IL-α, IL-1β and CXCL10; and a non-significant trend for upregulated gene expression of NF-κB p65 and RANTES when compared to the siCtrl.

Next, we wanted to confirm A20, NF-κB p65, phosphorylated NF-κB p65 (p- NF-κB p65), IRF-3 and phosphorylated IRF-3 (p-IRF-3) protein expression with and without ABCF1 knockdown under basal unstimulated conditions using an immunoblot. We demonstrate significant ABCF1

knockdown of approximately 82.4% (**Figure 1c**). Under this level of ABCF1 attenuation, baseline A20 protein expression (**Figure 1d**) was not impacted.

Figure 1

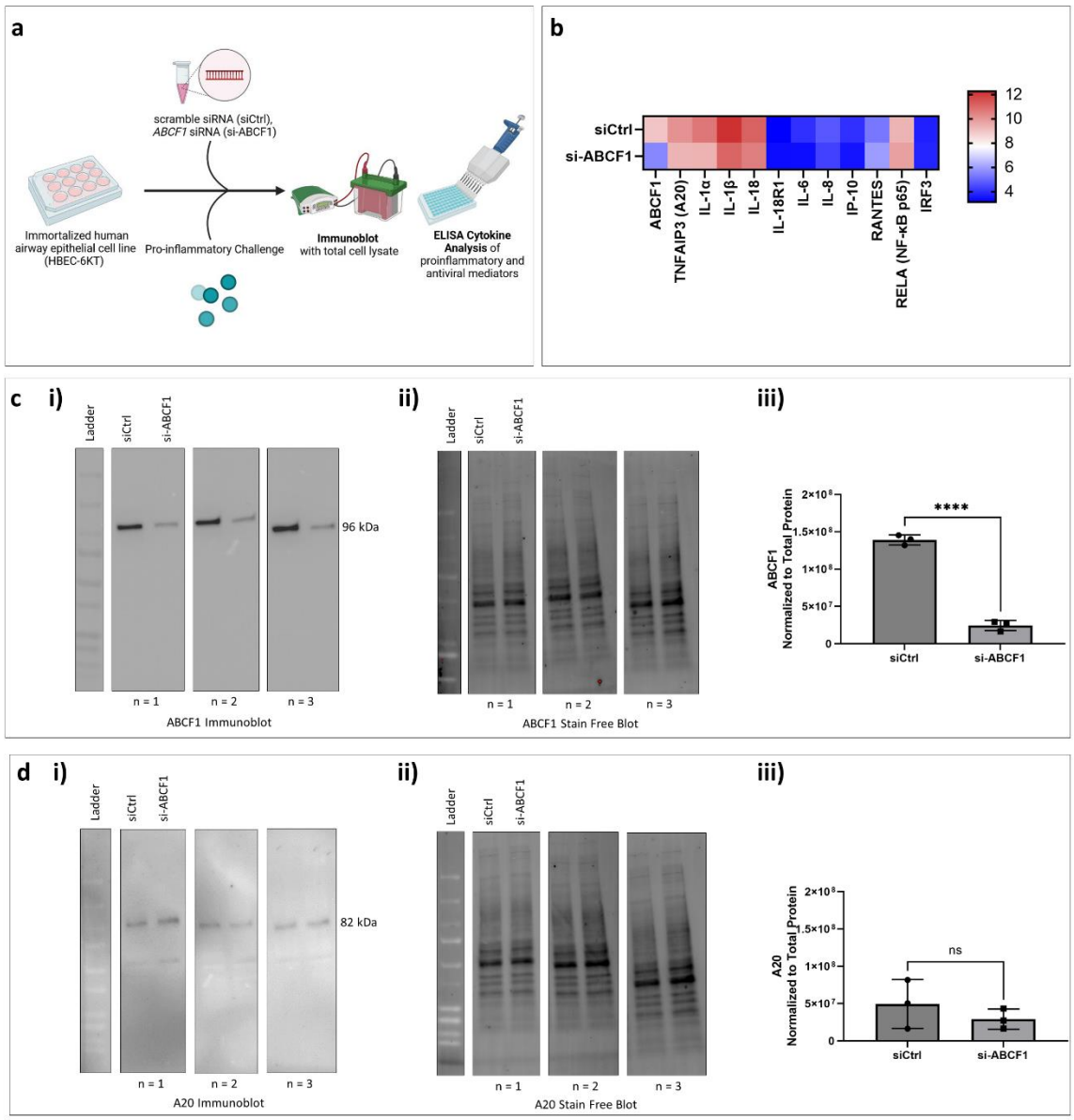

Figure 1 – ABCF1 silencing on A20 protein expression under basal conditions in HBEC-6KT *in vitro*. a) Experimental design schematic. Immortalized human airway epithelial cells grow in cell culture plates, the undergoes ABCF1 siRNA-mediated knockdown followed by pro-inflammatory stimulation. Total cell lysates collected from the cells are used for immunoblotting and cell culture supernatant are used to run ELISAs. **b)** Heat map of expression levels of select

genes associated with antiviral and pro-inflammatory responses for VACV-70 samples with and without si-ABCF1. **c) i)** Immunoblot to confirm siRNA-mediated knockdown of ABCF1 protein expression in HBEC-6KT cells under basal conditions. **ii)** Stain-free blot was used to **iii)** quantify ABCF1 protein expression. **d**) **i)** Immunoblot to confirm siRNA-mediated knockdown of A20 protein expression in HBEC-6KT cells under basal conditions. **ii)** Stain-free blot was used to **iii)** quantify ABCF1 protein expression. All studies n = 3; ****$p < 0.0001$.

Downstream of A20 protein regulation is NF-κB activation[2]. We therefore explored if NF-κB expression and activation were disrupted under basal unstimulated conditions with ABCF1 silencing. The protein expression of total NF-κB p65 (**Figure 2a**) and its phosphorylation form (**Figure 2b**) remained unchanged when compared to the control.

Figure 2

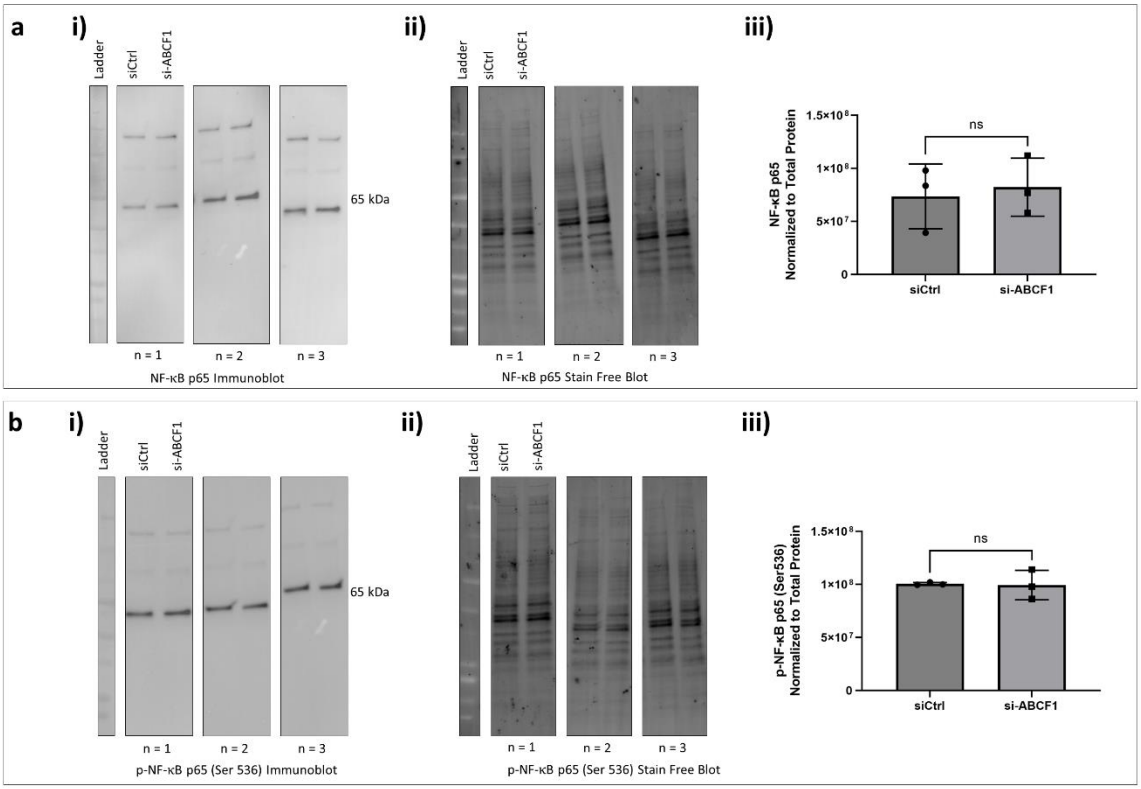

Figure 2 – ABCF1 silencing on NF-κB p65 protein expression and activation under basal unstimulated condition in HBEC-6KT *in vitro*. a) i) Immunoblot of total NF-κB p65 protein expression with and without si-ABCF1 in HBEC-6KT cells under basal conditions. **ii)** Stain-free blot was used to **iii)** quantify NF-κB p65 protein expression. **b) i)** Immunoblot of phosphorylated NF-κB p65 (Ser 536) with and without si-ABCF1 in HBEC-6KT cells under basal conditions. **ii)** Stain-free blot was used to **iii)** quantify NF-κB p65 phosphorylation. All studies n = 3; ns > 0.05.

In addition to A20 regulation, NF-κB signalling is also able to regulate IRF-3 signalling[3]. We therefore explored the protein expression of total IRF-3 and its phosphorylation. Similar to NF-κB, total IRF-3 (**Figure 3a**) and phosphorylated form (**Figure 3b)** were not impacted by ABCF1 knockdown.

Figure 3

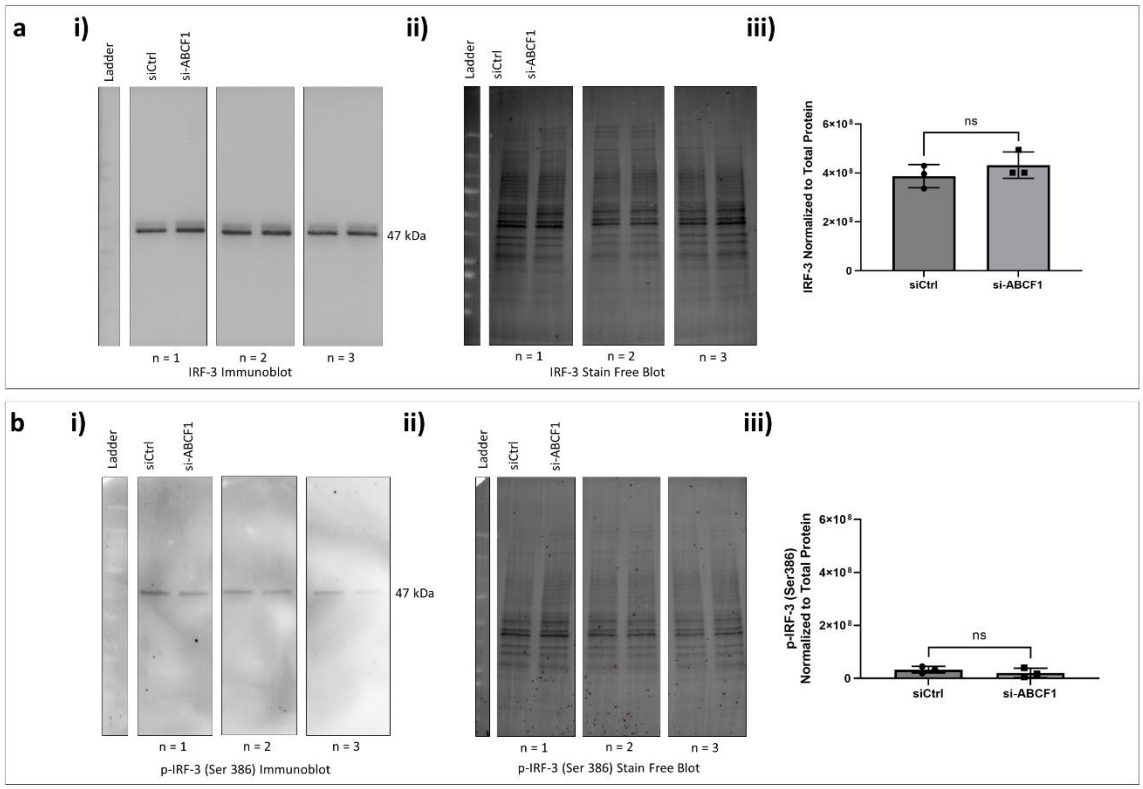

Figure 3 – ABCF1 silencing on IRF-3 protein expression and activation under basal unstimulated condition in HBEC-6KT *in vitro*. a) i) Immunoblot of total IRF-3 protein expression with and without si-ABCF1 in HBEC-6KT cells under basal conditions. **ii)** Stain-free blot was used to **iii)** quantify IRF-3 protein expression. **b)** Immunoblot of phosphorylated IRF-3 (Ser 386) with and without si-ABCF1 in HBEC-6KT cells under basal conditions. **ii)** Stain-free blot was used to **iii)** quantify IRF-3 phosphorylation. All studies n = 3; ns > 0.05.

Collectively, these data suggest that under basal unstimulated conditions, a reduction of ABCF1 greater than 80 % does not impact A20, NF-κB and IRF-3 protein expression and activity in HAECs.

Immunostimulation induction of A20 pro-inflammatory mediated responses in HAECs.

Our experiments with HAECs did not reveal an obvious role for ABCF1 under basal unstimulated conditions. We therefore investigated the impact of ABCF1 on A20, NF-κB, and IRF-3 under pro-inflammatory stimulations.

To select the appropriate stimulant that can induce a pro-inflammatory response in HAECs, we selected five immunostimulatory ligands known to induce A20 mediated IL-6 and IL-8 responses[12–16]. We performed *in vitro* stimulations at log concentrations with TNF-α (10 to 1000 ng/ml), IL-17 (0.1 to 10 ng/ml), LPS-B5 (0.01 to 1 μg/ml), PGN-SA (1 to 100 μg/ml), and Poly(I:C) (0.01 to 1 μg/ml). Following the stimulation, secretion of IL-6 and IL-8 in cell culture supernatant were quantified (**Figure 4**), as well as the protein expression levels of ABCF1 (**Figure 5**) and A20 (**Figure 6**). Of the five stimulants that were tested, TNF-α and Poly(I:C) increased the protein expression of A20 without changing the expression of ABCF1, and strongly induced the secretion of IL-6 and IL-8. LPS, PGN-SA, and IL-17 failed to induce robust IL-6 and IL-8 secretion (**Figure 4**), did not impact ABCF1 expression (**Supplementary Figure 1**), and showed a mild induction of A20 (**Supplementary Figure 2**). Our data validated the selection of TNF-α and Poly(I:C) for downstream analysis of ABCF1 under stimulated conditions.

Figure 4

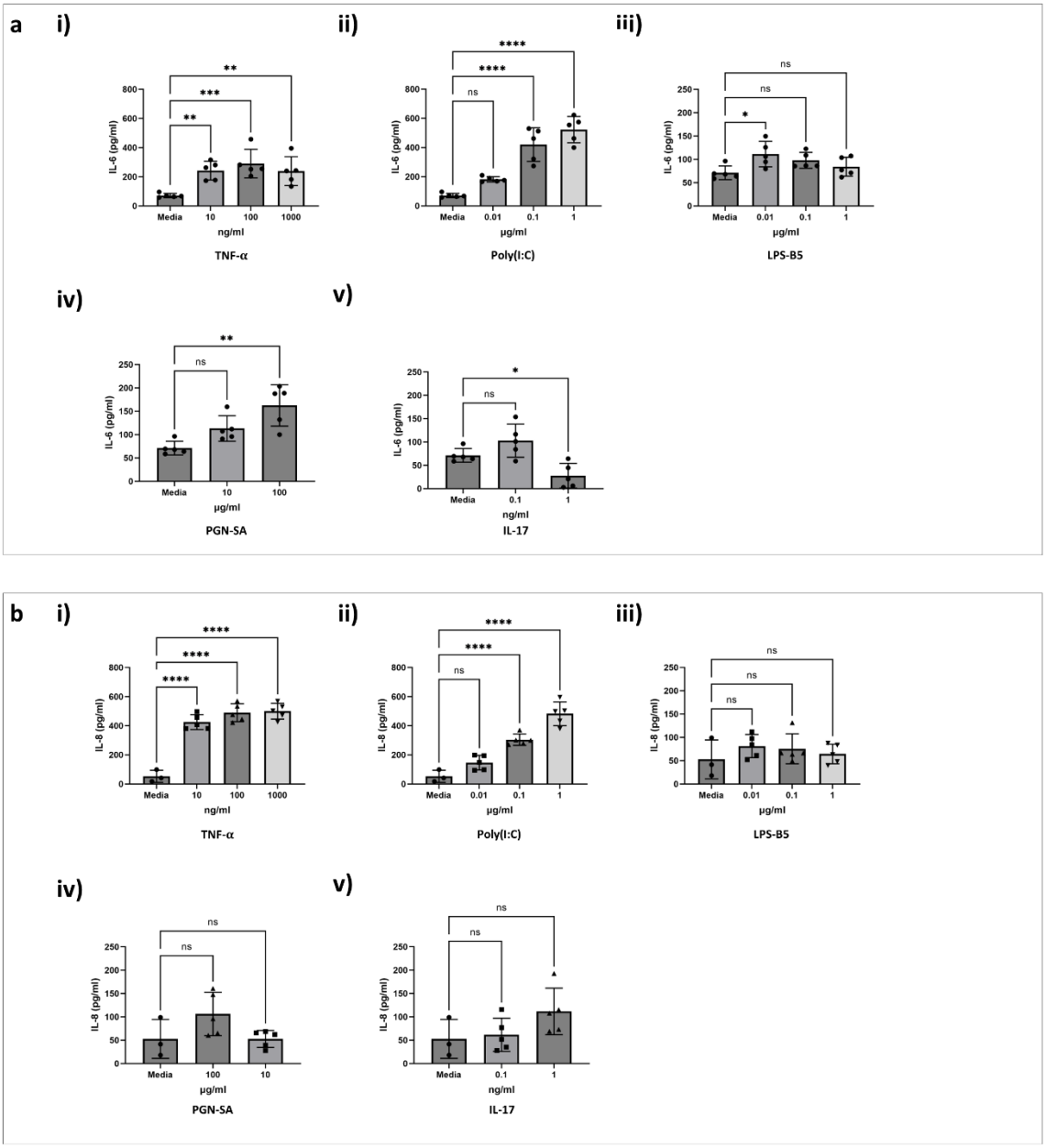

Figure 4 – Responses to pro-inflammatory immunostimulation in HBEC-6KT *in vitro*. a) IL-6 and **b)** IL-8 protein production in HBEC-6KT cells under concentration response induced by **i)** TNF-α, **ii)** Poly(I:C), **iii)** LPS-B5, **iv)** PGN-SA, and **v)** IL-17. All studies n = 3-5; **** $p \leq 0.0001$.

Figure 5

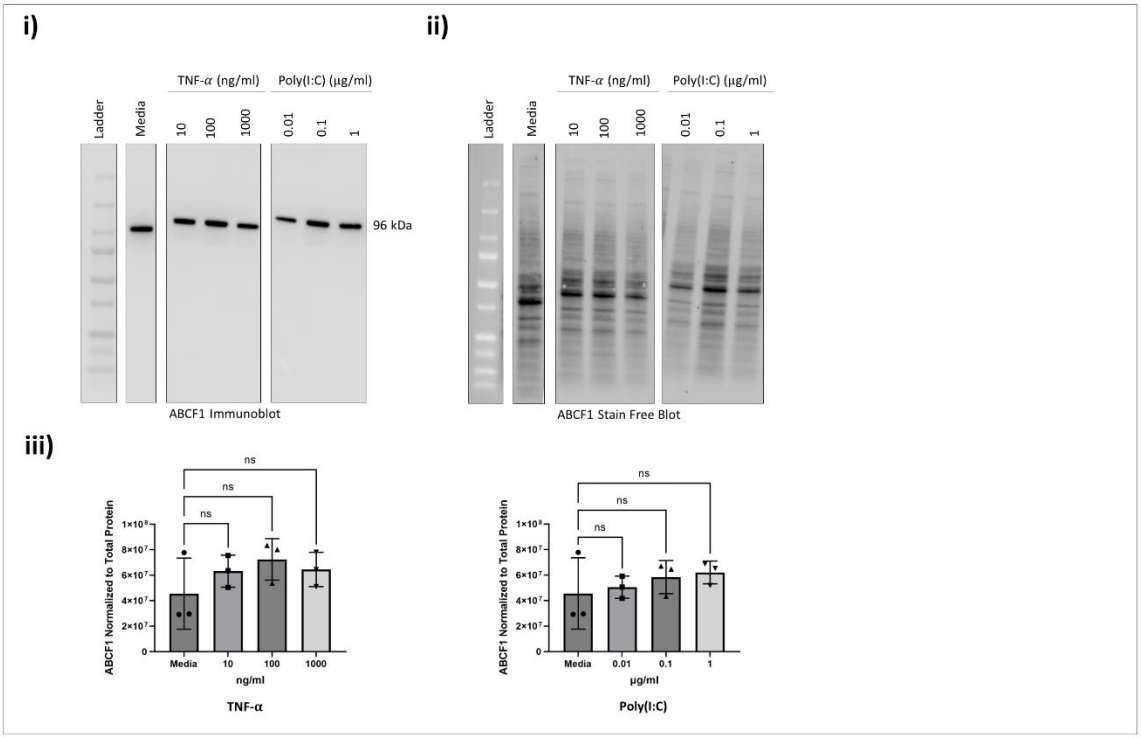

Figure 5 – ABCF1 protein expression in HBEC-6KT *in vitro* under pro-inflammatory immunostimulation. i) Immunoblot of ABCF1 protein expression with TNF-α and Poly(I:C) dosage-based stimulation in HBEC-6KT cells. **i)** Stain-free blot was used to **iii)** quantify ABCF1 protein expression. All studies n = 3; ns > 0.05.

Figure 6

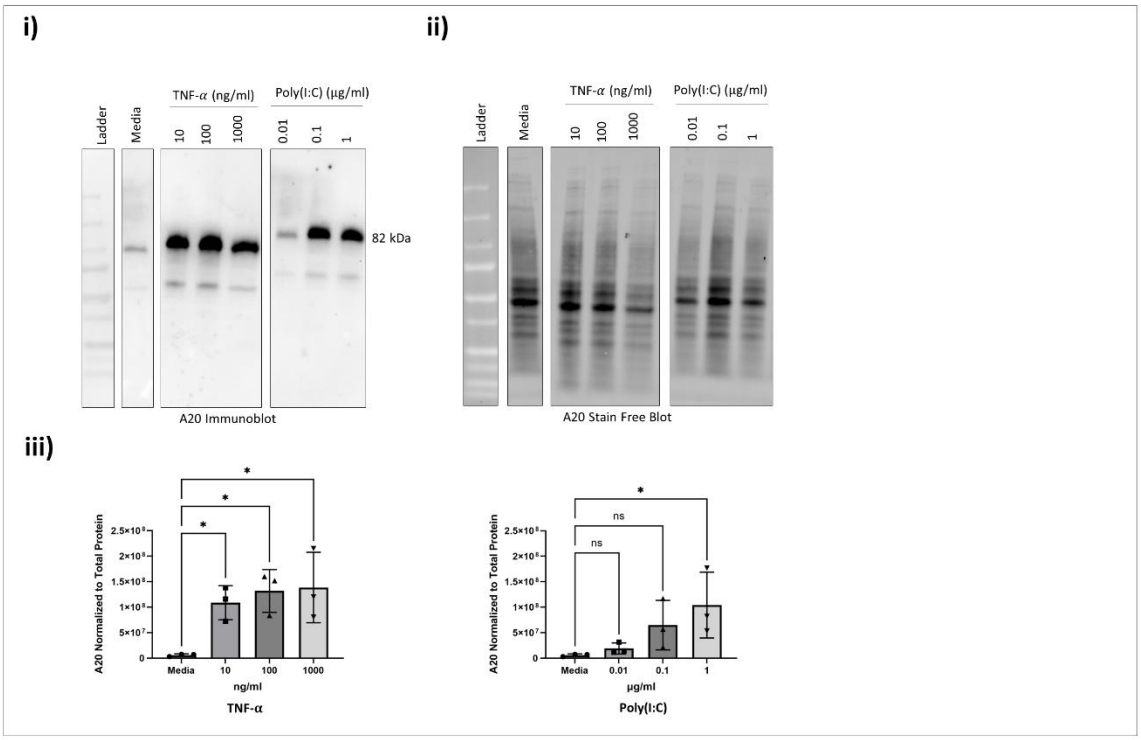

Figure 6 – Pro-inflammatory immunostimulation on A20 protein expression in HBEC-6KT *in vitro*. i) Immunoblot of A20 protein expression with TNF-α and Poly(I:C) dosage-based stimulation in HBEC-6KT cells. **Ii)** Stain-free blot was used to **iii)** quantify A20 protein expression. All studies n = 3; ns > 0.05.

ABCF1 does not regulate A20, NF-*κ*B and IRF-3 mediated inflammatory responses under Poly(I:C) stimulation.

Leveraging our characterization of Poly(I:C) responses in HAECs, we explored the role of ABCF1 with siRNA. ABCF1 knockdown of 81.9% was confirmed in control vehicle and Poly(I:C) stimulated HAECs (**Figure 7b**) and was associated with a significant reduction in IL-8 and a trend for reduced IL-6 (**Figure 7a**). Under these conditions, we observed no changes in A20 protein expression levels (**Figure 7c**).

Figure 7

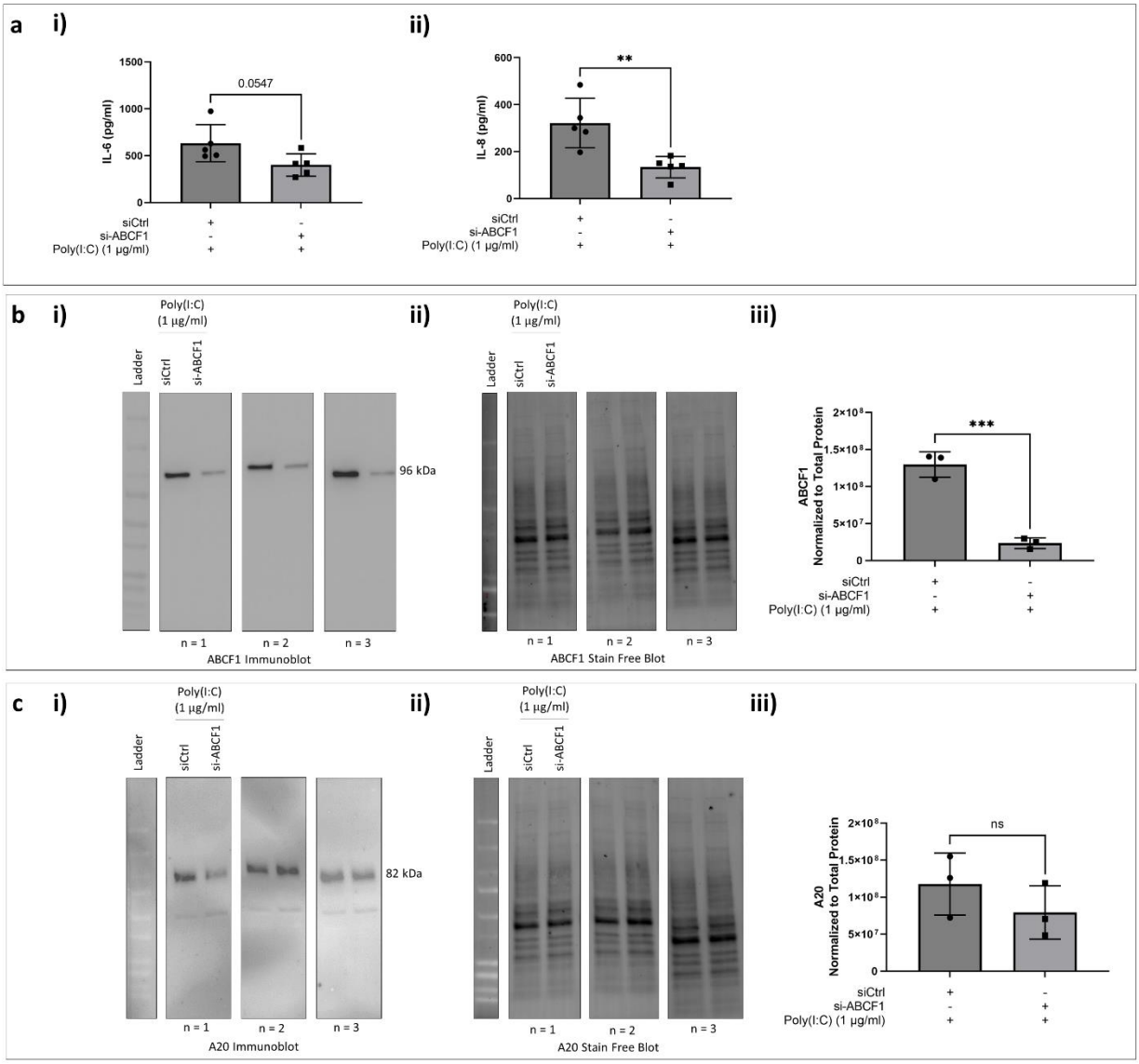

Figure 7 – Poly(I:C) induced pro-inflammatory responses on ABCF1 silenced HBEC-6KT *in vitro*. a) Poly(I:C) (1 µg/ml) induced IL-6 and IL-8 protein production for HBEC-6KT cells with si-ABCF1 treatment. **B)** Immunoblot with quantification on ABCF1 and **C)** A20 protein expression in HBEC-6KT cells under Poly(I:C) (1 µg/ml) stimulation with and without si-ABCF1 treatment. ELISA data n = 5, immunoblot data n = 3; * $p \leq 0.05$.

Since the observed attenuation of IL-8 is not due to changes in A20 protein expression, we investigated whether the silencing of ABCF1 is affecting the protein expression and activation status of the transcription factors, NF-κB and IRF-3. We observed no changes in total NF-κB protein expression levels (**Figure 8a**). Its activation status based on its phosphorylation, also remained unchanged when ABCF1 is silenced under Poly(I:C) stimulation (**Figure 8b**).

Figure 8

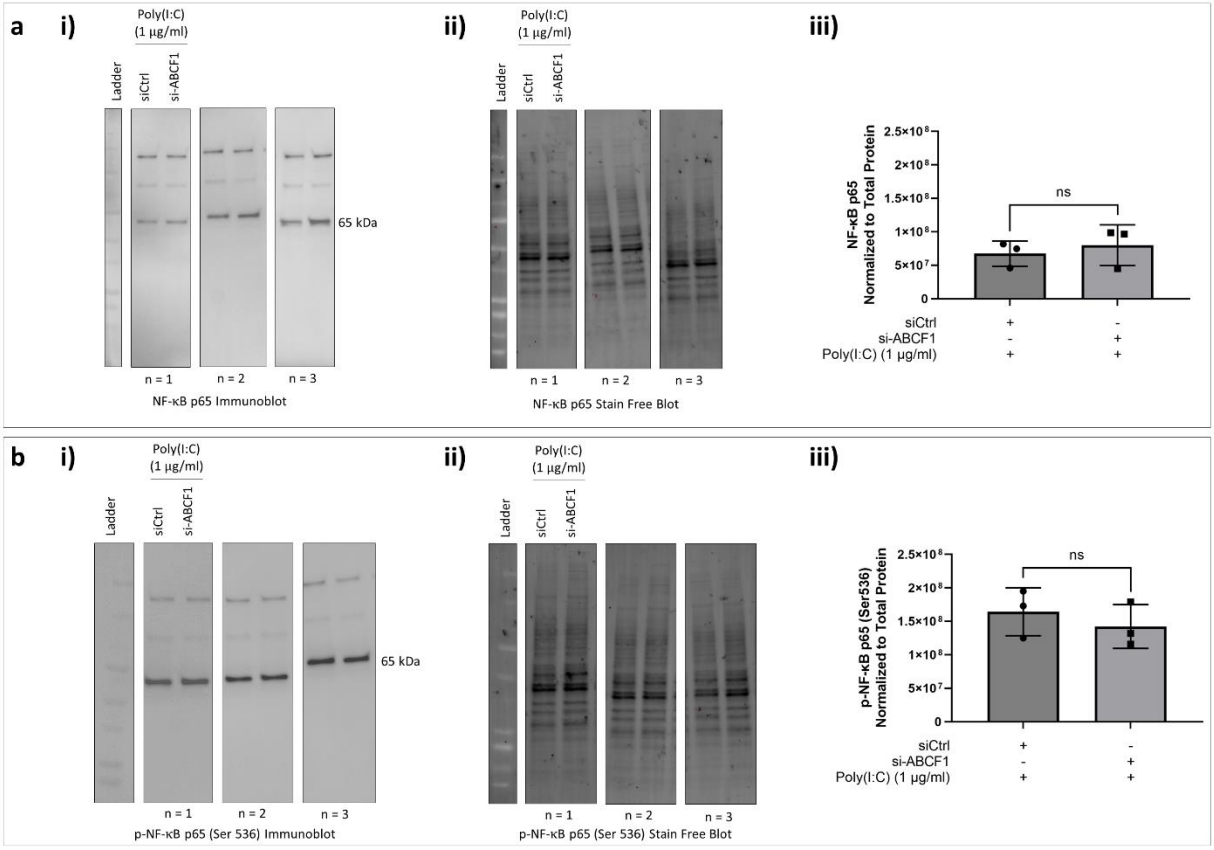

Figure 8 – ABCF1 silencing on NF-κB p65 protein expression and activation under Poly(I:C) stimulation in HBEC-6KT *in vitro*. a) Immunoblot with quantification of total NF-κB p65, and **b)** phosphorylated NF-κB p65 (Ser 536) protein expression in HBEC-6KT cells under Poly(I:C) (1 µg/ml) stimulation with and without si-ABCF1 treatment. All studies n = 3; * $p \leq 0.05$.

Poly(I:C) may signal through IRF-3 and contribute to IL-8 production independent of NF-κB signalling. We therefore analyzed IRF-3 and its phosphorylation. We observed an increase in total IRF-3 protein expression levels (**Figure 9a**), while no changes in level of phosphorylation were observed (**Figure 9b**).

Figure 9

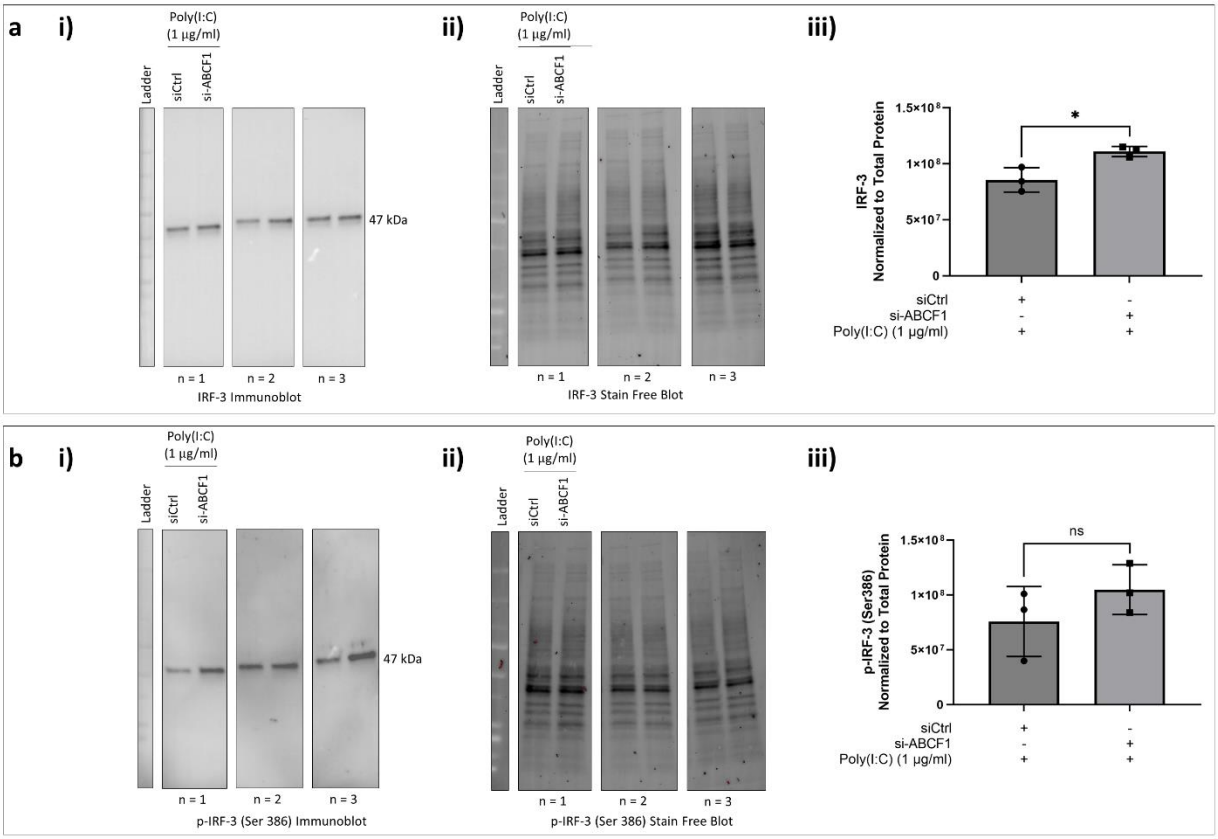

Figure 9 – ABCF1 silencing on IRF-3 protein expression and activation under Poly(I:C) stimulation in HBEC-6KT *in vitro*. a) Immunoblot with quantification of total IRF-3, and **b)** phosphorylated IRF-3 (Ser 386) protein expression in HBEC-6KT cells under Poly(I:C) (1 µg/ml) stimulation with and without si-ABCF1 treatment.

Collectively, these studies demonstrate that Poly(I:C) induced IL-8 is regulated by ABCF1 via pathways independent of NF-κB and IRF-3 activation.

ABCF1 does not regulate A20 and NF-κB mediated inflammatory responses under TNF-α stimulation.

It remained possible that ABCF1 regulates NF-κB signalling in a context dependent manner that was influenced by the stimulus. We therefore investigated whether ABCF1 regulates TNF-α mediated pro-inflammatory responses in HAEC using identical approaches to our Poly(I:C) experiments. Consistent with Poly(I:C) data, we observed a significant reduction in IL-8 levels and a trend for reduced IL-6 (**Figure 10a**) with ABCF1 knockdown of 82.2% (**Figure 10b**). Again, no changes in A20 protein expression levels were observed with ABCF1 silencing (**Figure 10c**) under TNF-α stimulation conditions we have shown to induce A20 (**Figure 6**).

Figure 10

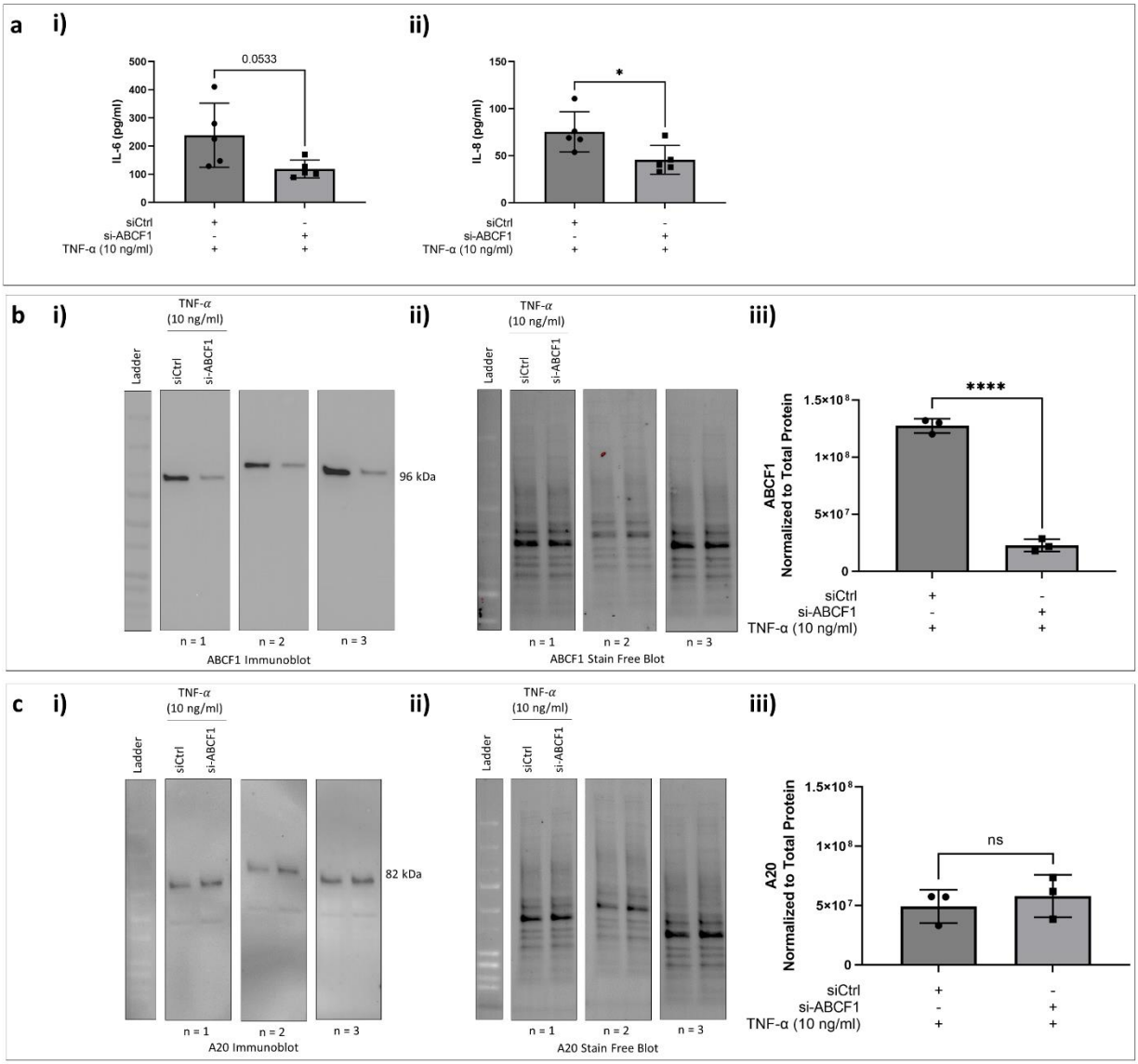

Figure 10 – TNF-α induced pro-inflammatory responses on ABCF1 silenced HBEC-6KT *in vitro*. a) TNF-α (10 ng/ml) induced IL-6 and IL-8 protein production for HBEC-6KT cells with si-ABCF1 treatment. **B)** Immunoblot with quantification on ABCF1 and **c)** A20 protein expression in HBEC-6KT cells under TNF-α (10 ng/ml) stimulation with and without si-ABCF1 treatment. ELISA data n = 5, immunoblot data n = 3; * $p \leq 0.05$.

We next investigated whether silencing of ABCF1 expression under TNF-α stimulation has any impact on NF-κB expression and activity. We observed an increase expression level of total NF-κB with ABCF1 silencing under TNF-α stimulation (**Figure 11a**) although the phosphorylation status of NF-κB, was not changed under these conditions (**Figure 11b**).

Figure 11

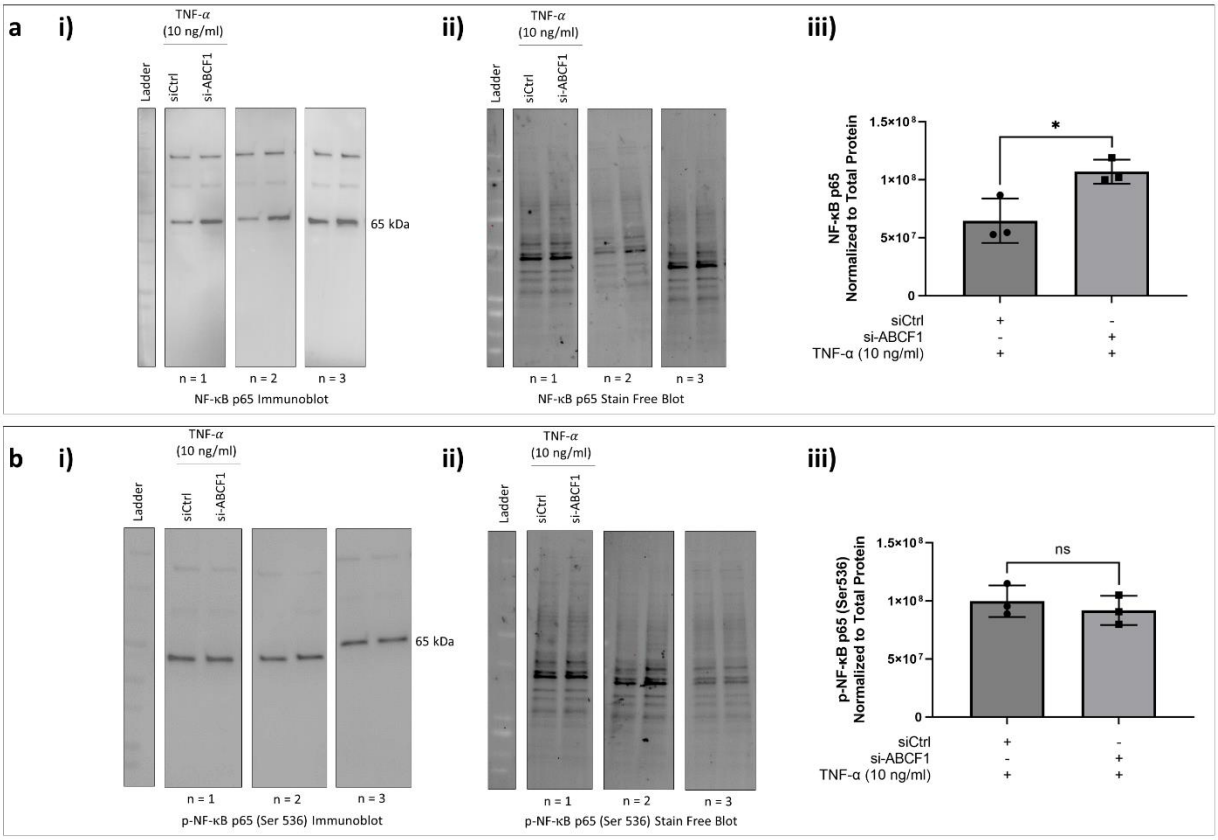

Figure 11 – ABCF1 silencing on NF-κB p65 protein expression and activation under TNF-α stimulation in HBEC-6KT *in vitro*. a) Immunoblot with quantification of total NF-κB p65, and **c)** phosphorylated NF-κB p65 (Ser 536) protein expression in HBEC-6KT cells under TNF-α (10 ng/ml) stimulation with and without si-ABCF1 treatment. All studies n = 3; * $p \leq 0.05$.

Collectively, these studies demonstrate that TNF-α induced IL-8 is regulated by ABCF1 via pathways independent of NF-κB activation.

DISCUSSION

Explorations into the role ABCF1 has in modulating respiratory mucosal immune responses are grounded in reports that this protein has a diverse range of activities including translation initiation, viral sensing in the cytosol, and polarization of immune cell phenotype[5, 12, 13]. Our group previously confirmed the expression of ABCF1 at the gene and protein level in the airway epithelium. Using siRNA-mediated knockdown on ABCF1 followed by a viral cytosolic nucleic acid stimulation in airway epithelial cells, our findings suggested that ABCF1 was not involved in regulating antiviral responses but instead mediates an immune response involving TLR3/4 signalling pathways. One of the genes that was significantly differentially expressed in these signalling pathways under ABCF1 silencing and viral stimulation included *TNFAIP3*, expressed as A20 protein, a ubiquitin ligase and deubiquitinating enzyme that supresses the NF-κB transcription factor activation[2]. The present manuscript explored the regulatory and functional relationship between ABCF1 and A20 in HAECs under basal unstimulated and pro-inflammatory stimulated conditions.

For this study, we investigated the function of ABCF1 in an immortalized human bronchial epithelial cell line called HBEC-6KT. This cell line was developed using retroviral expression of Cdk4 and hTERT to promote cell proliferation and extend their lifespan. This was developed in cells that were obtained from bronchial biopsies from areas of the lungs that were histologically not involved with cancer. Unlike other cell lines that are immortalized by viral oncogenes, the

Cdk4/hTERT cells are phenotypically normal, and they do not exhibit malignant characteristics. Karyotyping analyses have been conducted in similar HBEC lines and demonstrated that the approach of over-expressing Cdk4/hTERT results in cells that are genetically more like parental cells than HPV-immortalized cell lines and distinct from lung cancerous cell lines such as BEAS-2B[6]. It remains possible that the HBEC-6KT cell line used has interruptions in genomic regions that regulate ABCF1 expression and could lead to haploinsufficiency. Routine karyotyping of cell lines has been suggested in the past[14]. The rationale for choosing HBEC-6KT was to ensure continuity with our earlier research that has demonstrated that the expression of ABCF1 protein is conserved in both primary human airway epithelial cells and HBEC-6KT HAECs with functional links to innate immune responses[1].

As no selective pharmacological inhibitor for ABCF1 has been reported and evidence that homozygous deletion of ABCF1 is fatal in mice, we have pursued ABCF1 siRNA approaches in this paper and past reports[1]. Previously, our group showed that with 35% ABCF1 silencing, there was no observed changes in HAEC secreted IL-8 under Poly(I:C) stimulation *in vitro*[1]. In contrast to our previous findings, increased efficiency in gene silencing that produces approximately 80% ABCF1 knockdown led to a decreasing trend in Poly(I:C) induced secretion of IL-8 when compared to silencing control. Compared to our gene knockdown efficiency, Hsu *et al.* demonstrated that with 50-70% knockdown of ORMDL3 gene expression using siRNA in A549 and 1HAE airway epithelial cell lines, they observed no changes to the levels of NF-κB-induced IL-6 and IL-8 under TNF-α and LPS stimulations[15]. However, in our study we were able to achieve sufficient knockdown of ABCF1 to observe changes in the secretion of these cytokines. This was done by ensuring our cell lines were treated with siRNA at an appropriate

confluency of 70% and at a concentration of 25 nM for no more than 24 h to avoid cell death, potentially due to off target effects that accompany this approach[16]. For the purpose of investigating our previous findings, we carried forward with the use of subconfluent HAECs in this study, maintaining continuity with our earlier research. However, future approaches with conditional deletion of ABCF1 in human cell lines using CRISPR-Cas9 knockout via lentiviral transduction and single-cell cloning would strengthen the evidence to support ABCF1's role in immunity. Additionally, exploring novel selective inhibitors of ABCF1 and culturing cells grown at air liquid interface (ALI) would also provide valuable insights on the function of ABCF1[17–19].

The expression and function of A20 in HAECs has been defined in the context of pro-inflammatory responses and long-acting β2-adrenoreceptor agonists (LABAs)/glucocorticoid combination treatments[14, 20]. While characterizing the differences between the pro-inflammatory responses in HAECs and alveolar macrophages, it has been proposed that the response to TLR3 and TLR4 agonists differs between these two cell types. They first demonstrated that under basal conditions, gene expression of A20 was unchanged in both HAECs and alveolar macrophages. Under Poly(I:C) stimulation, they showed that compared to alveolar macrophages, HAECs had a stronger pro-inflammatory response and higher A20 protein expression. While under LPS stimulation, alveolar macrophages had a stronger pro-inflammatory response but A20 levels was not impacted[21]. A separate group proposed that combination treatment in HAECs can augment A20 protein expression to repress NF-κB mediated pro-inflammatory responses. Using HAECs, they demonstrated that LABAs augments A20 expression to negatively regulate NF-κB and consequently improve the anti-inflammatory properties by glucocorticoids[20]. Our findings

confirm the protein expression of A20 in HAECs under basal conditions and demonstrate that ABCF1 silencing by 80 % fails to change A20 protein expression levels. This contrasts to previous research where a group observed a reduced expression of A20 in macrophages with ABCF1 silencing under basal conditions[5]. The contrasting results may owe to the cell type selected, as HAECs and macrophages have shown different A20 expression levels and therefore may be regulated by different mechanisms[21]. The regulation of A20 is intertwined with the transcription factors NF-κB and IRF-3, through negative feedback loops that enable shutting off pro-inflammatory signalling by A20[22]. As ABCF1 silencing failed to regulate A20 expression under basal conditions, we sought to deeper characterize this pathway and define NF-κB and IRF-3 expression and activation. With an immunoblot on total NF-κB p65, a subunit of NF-κB, and its activated phosphorylated form, we showed that the knockdown of ABCF1 led to no changes to the protein expression of total NF-κB p65, and its activation when compared to the silencing control under basal unstimulated conditions. The protein expression of total IRF-3 and its activated phosphorylated form was also unchanged. Collectively, our results do not demonstrate any relationship between ABCF1 and A20 expression and NF-κB and IRF-3 activated signalling pathways in HAECs under basal unstimulated conditions.

It is possible that baseline A20 expression levels are not regulated by ABCF1, while dynamic upregulation of A20 may be dependent on ABCF1, owing to the latter protein's proposed roles in translation initiation and modulation of immune responses[5, 12]. We therefore investigated a variety of pro-inflammatory stimulations that are known to be regulated by A20, including TNFR, TLR2, TLR3, TLR4, and IL-17R induced signalling pathways[2, 23–25]. Under viral stimulation, downstream of TLR3 signalling, A20 directly interacts with IRF-3 kinases, NF-κB-activating

kinase/Traf family member-associated NF-κB activator-binding kinase 1 (NAK/TBK1) and IKK-ι/IKK-ε to inhibit IRF-3 phosphorylation, dimerization, translocation to the nucleus and downstream expression of interferon stimulation response element (ISRE) transcripts[3, 26]. Consistent with literature, TNF-α and Poly(I:C) were able to induce IL-6 and IL-8 cytokine secretion and an associated upregulation in A20 expression in a concentration-dependent manner in HAECs[21, 27–29]. In contrast, those stimuli (LPS, IL-17, PGN-SA) that showed more modest induction of IL-6 and IL-8 failed to induce A20 expression. Prior research showed that ABCF1 silencing in macrophages led to a reduction in IRF-3 dimerization and phosphorylation with and without LPS stimulation[5]. In our HAEC model, *in vitro* LPS stimulation was unable to induce a strong pro-inflammatory response which is consistent with previous reports with primary HAECs[21]. The data suggest that there may be cell specific responses to LPS that lead to A20 regulation. Taken together, our findings describe a relationship where stimuli that induce IL-6 and IL-8 are accompanied by a concomitant expression of A20, a negative regulator of pro-inflammatory responses. These experimental conditions enable mechanistic studies aimed at defining a role for ABCF1 in regulating A20 expression and function.

Since no changes were observed for protein expression levels of A20 under 24 h of stimulation with TNF-α and Poly(I:C), we investigated whether there were changes to the expression levels of the transcription factors NF-κB and IRF-3 at this time point. A study by Son, D et al. have demonstrated that under TNF-α stimulation for 24 h in immortalized intestinal epithelial cells, Caco-2, they observed an increase in NF-κB transcriptional activity associated with IL-8 promoter activity measured with the luciferase assay[30]. In a separate study by Hirata, Y et al., the authors used HT-29 intestinal epithelial cells to show that following Poly(I:C) stimulation for 24 h, they

observed IRF-3 activation[31]. Given the findings from previous studies, we wanted to observe the protein expression levels of the transcription factors following 24 h of stimulation. In our study, we found that the expression of total NF-κB and its activation was unchanged, however, there was an increased expression of total IRF-3, suggesting that increased activation could be possible. Although, the level of IRF-3 phosphorylation remained unchanged when compared to the control, suggesting that while there was a potential for increase IRF-3 activity, its activation was not affected by ABCF1 silencing under Poly(I:C) stimulation.

Downstream of TNFR signalling with TNF-α stimulation, A20 can negatively regulate the activation of NF-*κ*B by inhibiting IκB kinase (IKK) phosphorylation through its protein ubiquitin ligase and deubiquitinatinase activities. This consequently prevents NF-κB from translocating to the nucleus for gene transcription and prevents Protein Kinase A (PKA) from phosphorylating NF-κB subunits, including p65[2, 32]. Phosphorylation of p65, is known to be essential for the stability, degradation, and transcriptional activity of NF-*κ*B with factors involved in gene transcription, including CBP/p300[32]. Early studies characterizing ABCF1 used TNF-α stimulation on fibroblast-like synoviocytes, where an increase in *Abcf1* gene expression was observed and led to the suggestion that it was involved in regulating inflammation[4]. Our study demonstrated that with ABCF1 silencing under TNF-α stimulation *in vitro,* there was a reduction in secreted IL-8 and a more modest trend for reduced IL-6. While there were no changes to the protein expression levels of A20, we observed an increase expression level of total NF-κB. This suggests that there is a potential for increased NF-κB activation and downstream activities. However, the phosphorylation of NF-κB with ABCF1 silencing under TNF-α stimulation was unchanged when compared to the silencing control. This finding suggests that while there is an

increase potential for NF-κB activation, this was not affected by ABCF1 silencing under the TNF-α stimulation.

In conclusion, our study demonstrated the roles of ABCF1 in regulating innate immune responses under pro-inflammatory stimulations. We demonstrated that under basal conditions, ABCF1 silencing led to no changes to the protein expressions of A20 and the transcription factors, NF-κB and IRF-3, and its activation. However, we did observed changes in levels of secreted pro-inflammatory cytokines, IL-8 and IL-6, as well as increased protein expression of total NF-κB and IRF-3 under the stimulations. These findings suggest ABCF1 may have a role in regulating pro-inflammatory responses in HBEC-6KT HAECs, but its exact function will require further investigation. We propose that ABCF1 protein interactome may consist of interactors that are known to be involved in intracellular signalling pathways to regulate innate immune responses. Our findings support future investigations into the functional role of ABCF1 using CRISPR-Cas9 KO cells with an unbiased protein-protein interaction experiments to gain insights into its function in HAECs.

ACKNOWLEGEMENTS

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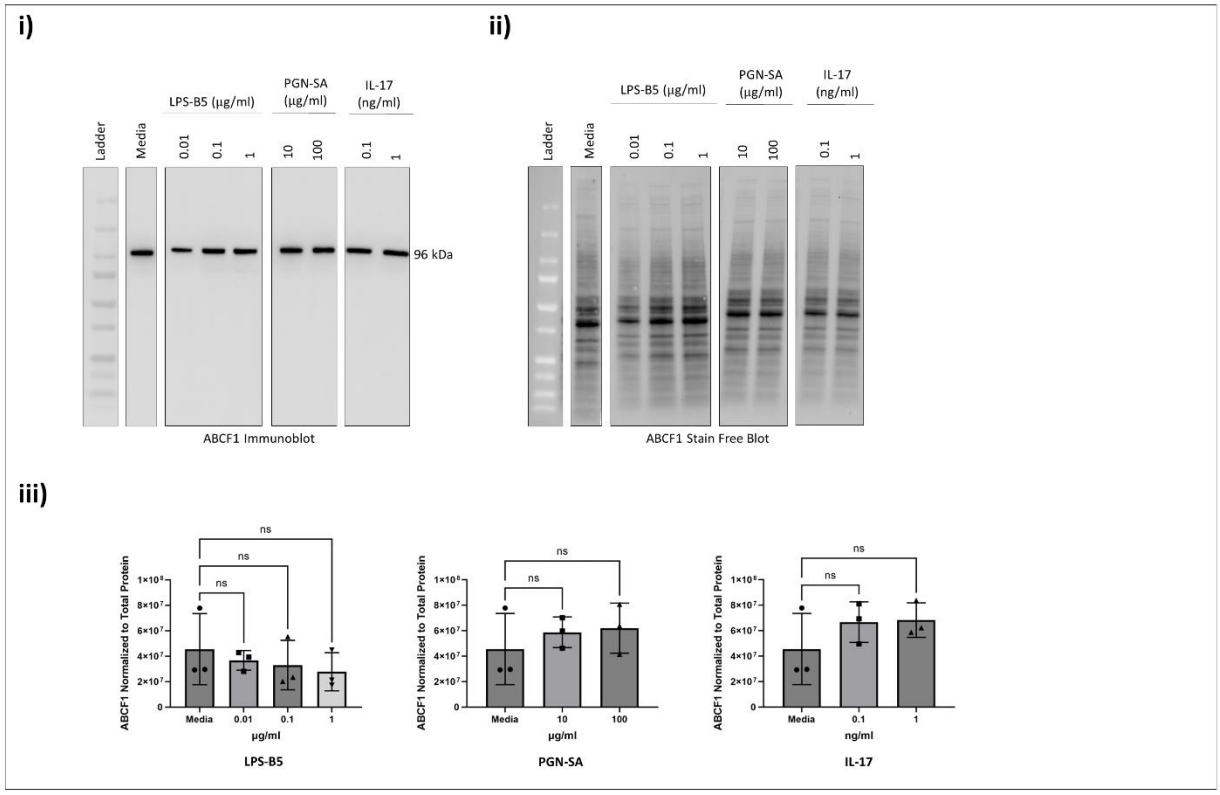
SUPPLEMENTARY FIGURES

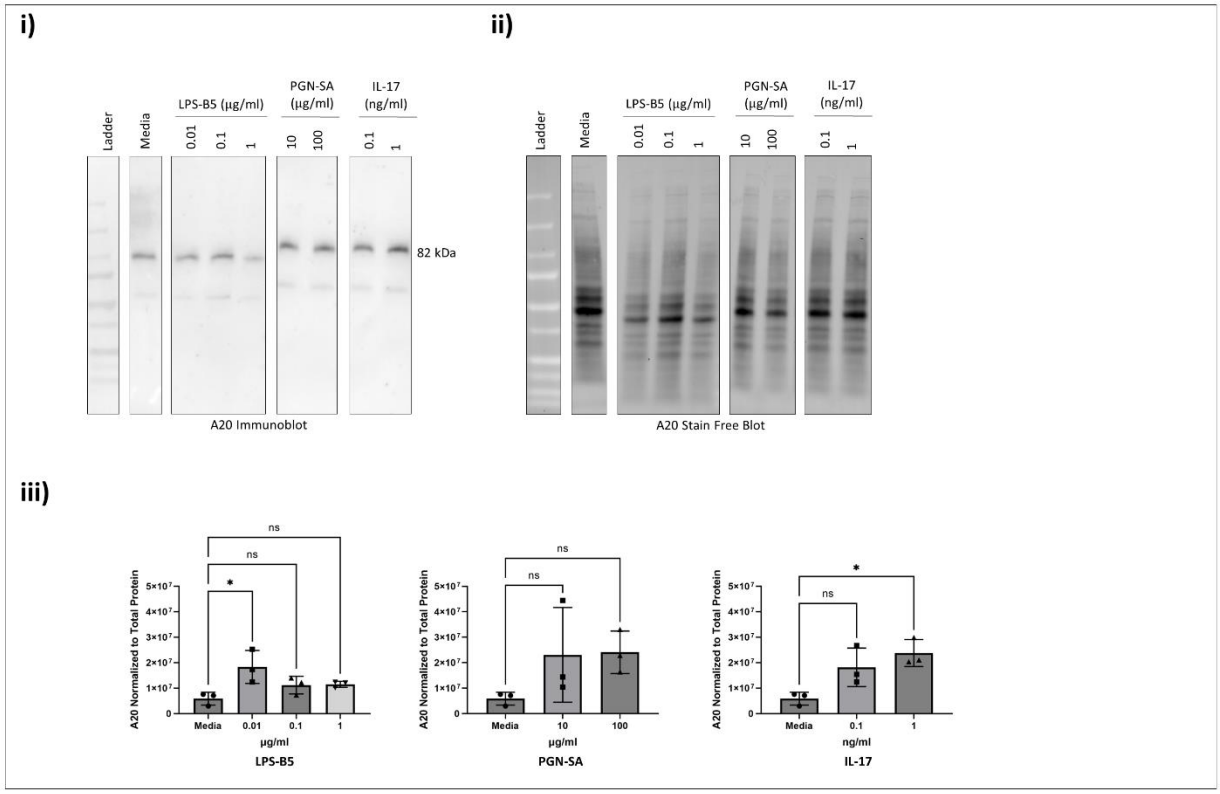
Supplementary 1



Supplementary Figure 1 – Immunostimulationon ABCF1 expression in HBEC-6KT *in vitro*.

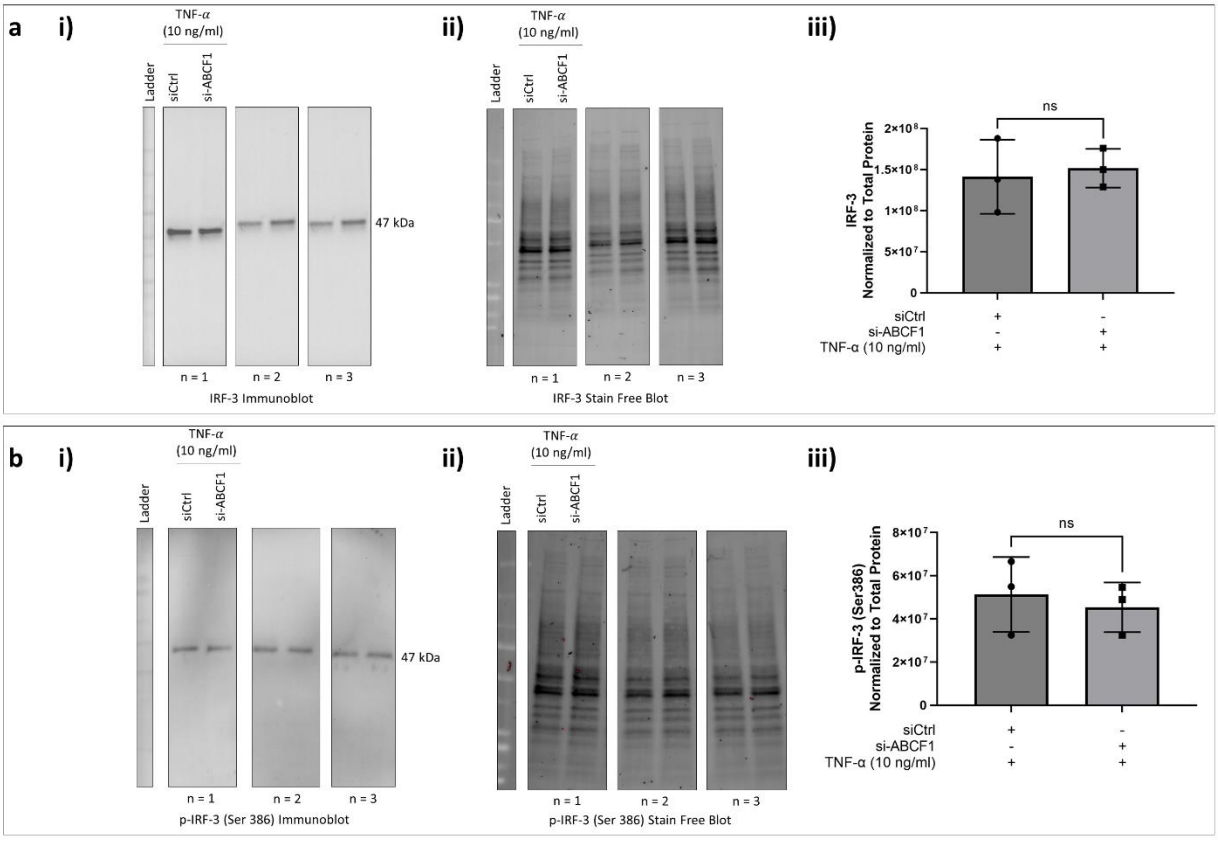
i) Immunoblot of ABCF1 protein expression with IL-17, LPS-B5, and PGN-SA dosage-based stimulation in HBEC-6KT cells. **ii)** Stain-free blot was used to **iii)** quantify ABCF1 protein expression. All studies n = 3; ns > 0.05.

Supplementary 2



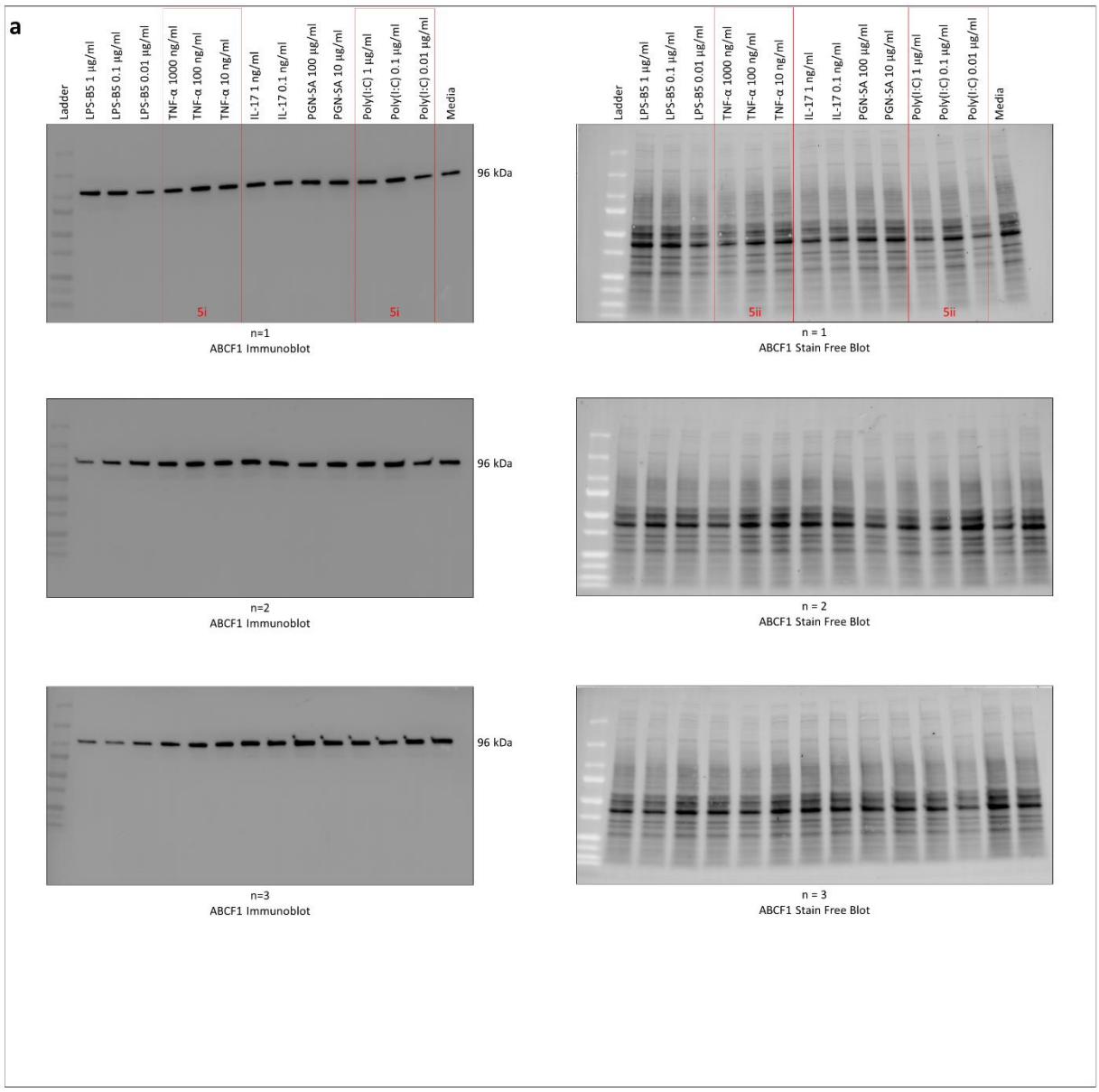
Supplementary Figure 2 – Pro-inflammatory immunostimulation on A20 protein expression in HBEC-6KT *in vitro*. i) Immunoblot of A20 protein expression with IL-17, LPS-B5, and PGN-SA dosage-based stimulation in HBEC-6KT cells. **ii)** Stain-free blot was used to **iii)** quantify A20 protein expression. All studies n = 3; ns > 0.05.

Supplementary 3

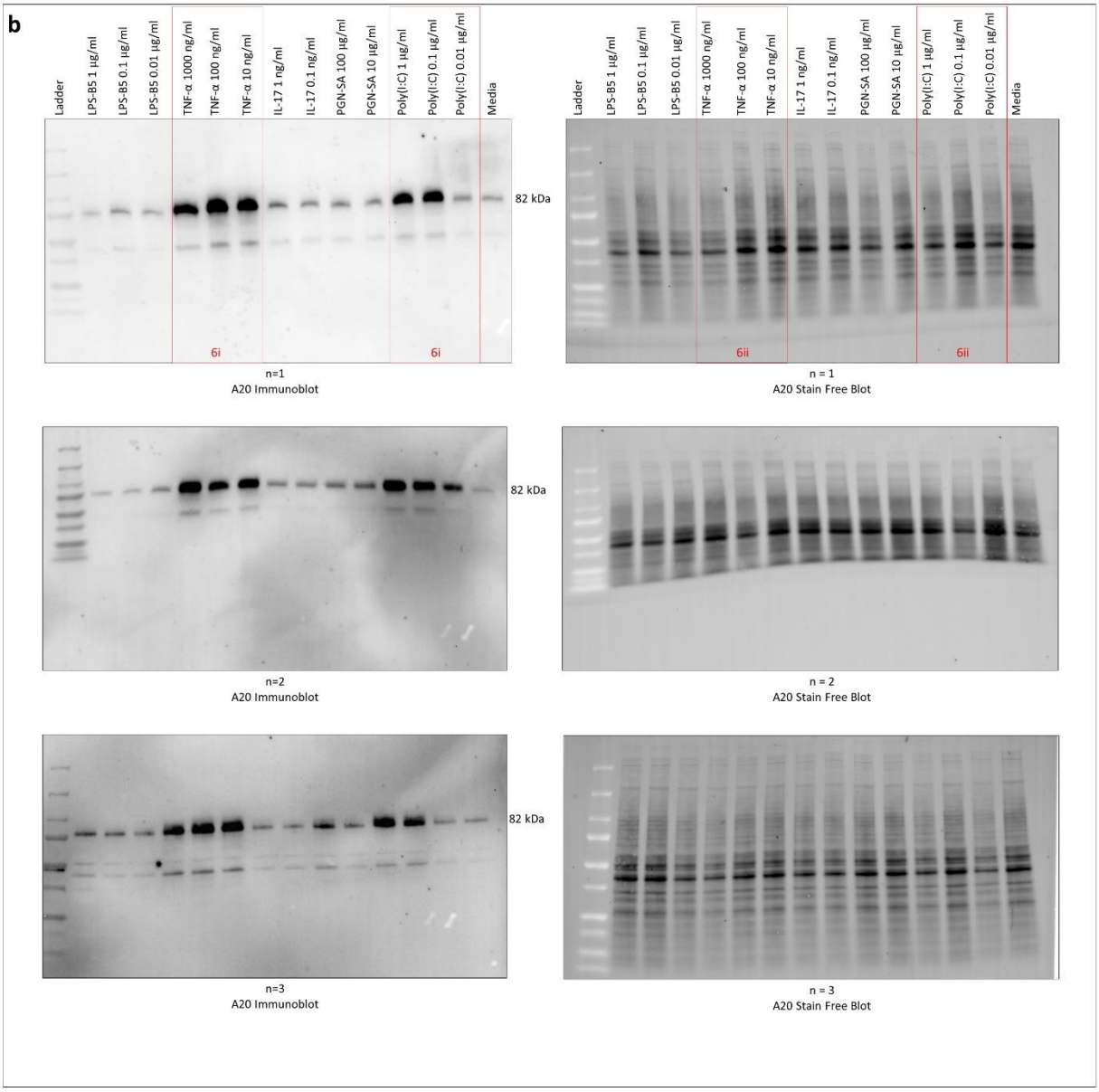


Supplementary Figure 3 – ABCF1 silencing on IRF-3 protein expression and activation under TNF-α stimulation in HBEC-6KT *in vitro*. a) Immunoblot with quantification of total IRF-3, and **b)** phosphorylated IRF-3 (Ser 386) protein expression in HBEC-6KT cells under TNF-α (10 ng/ml) stimulation with and without si-ABCF1 treatment. All studies n = 3; * $p \leq 0.05$.

Supplementary 4

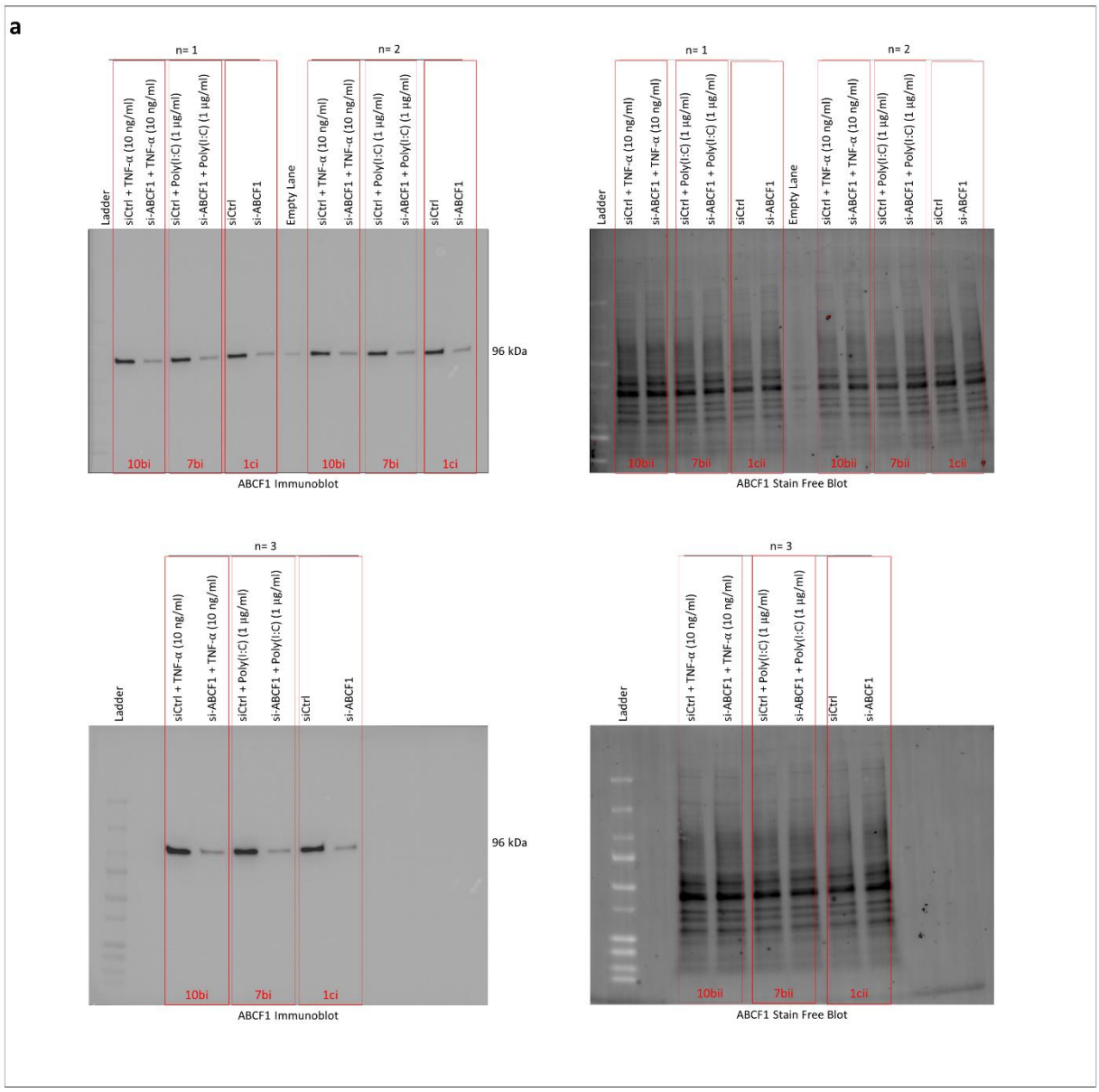


Supplementary 4



Supplementary Figure 4 – Full length immunoblots of **a)** ABCF1 and **b)** A20 in HBEC-6KT under immunostimulatory stimulations *in vitro*.

Supplementary 5



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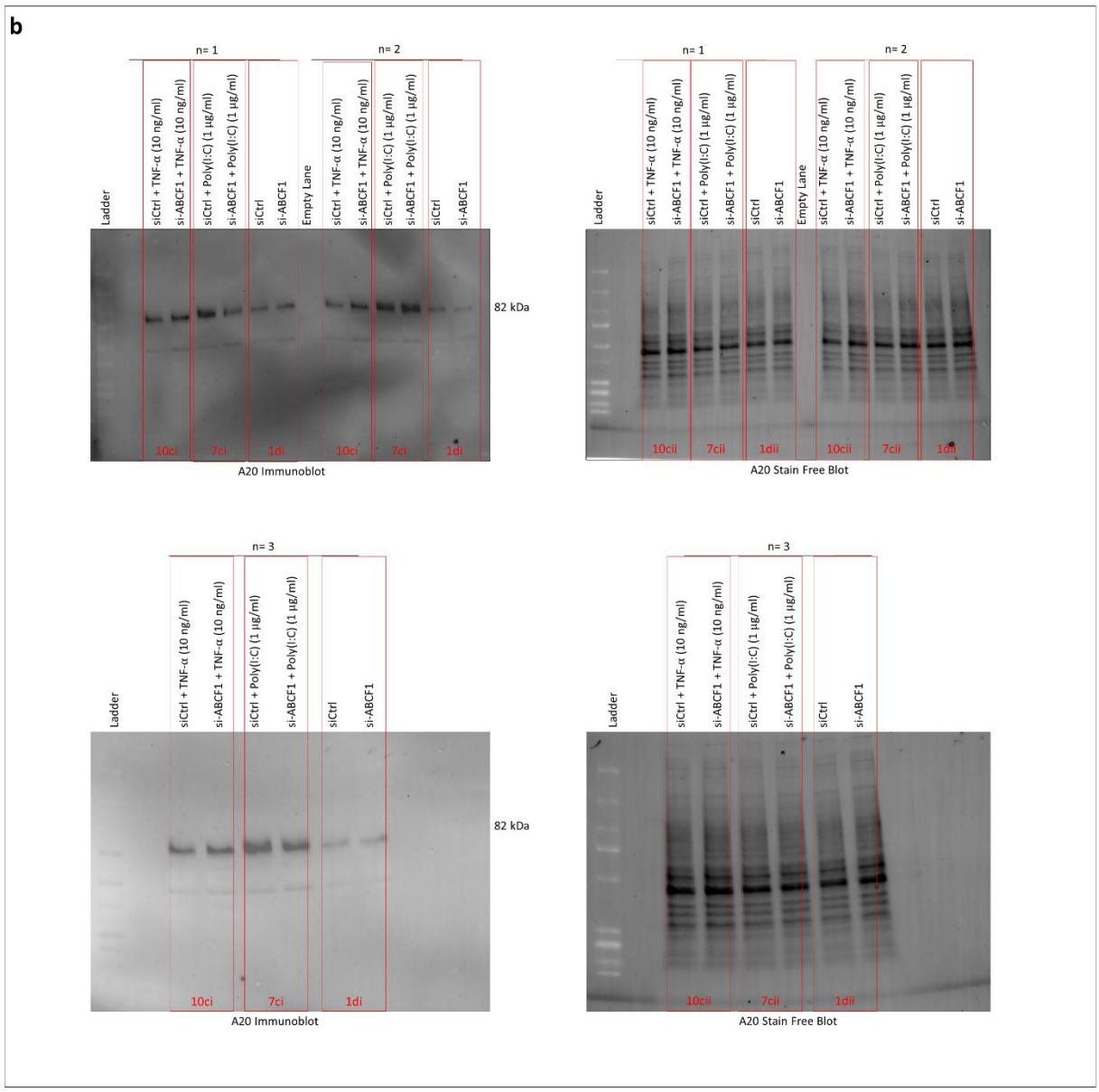

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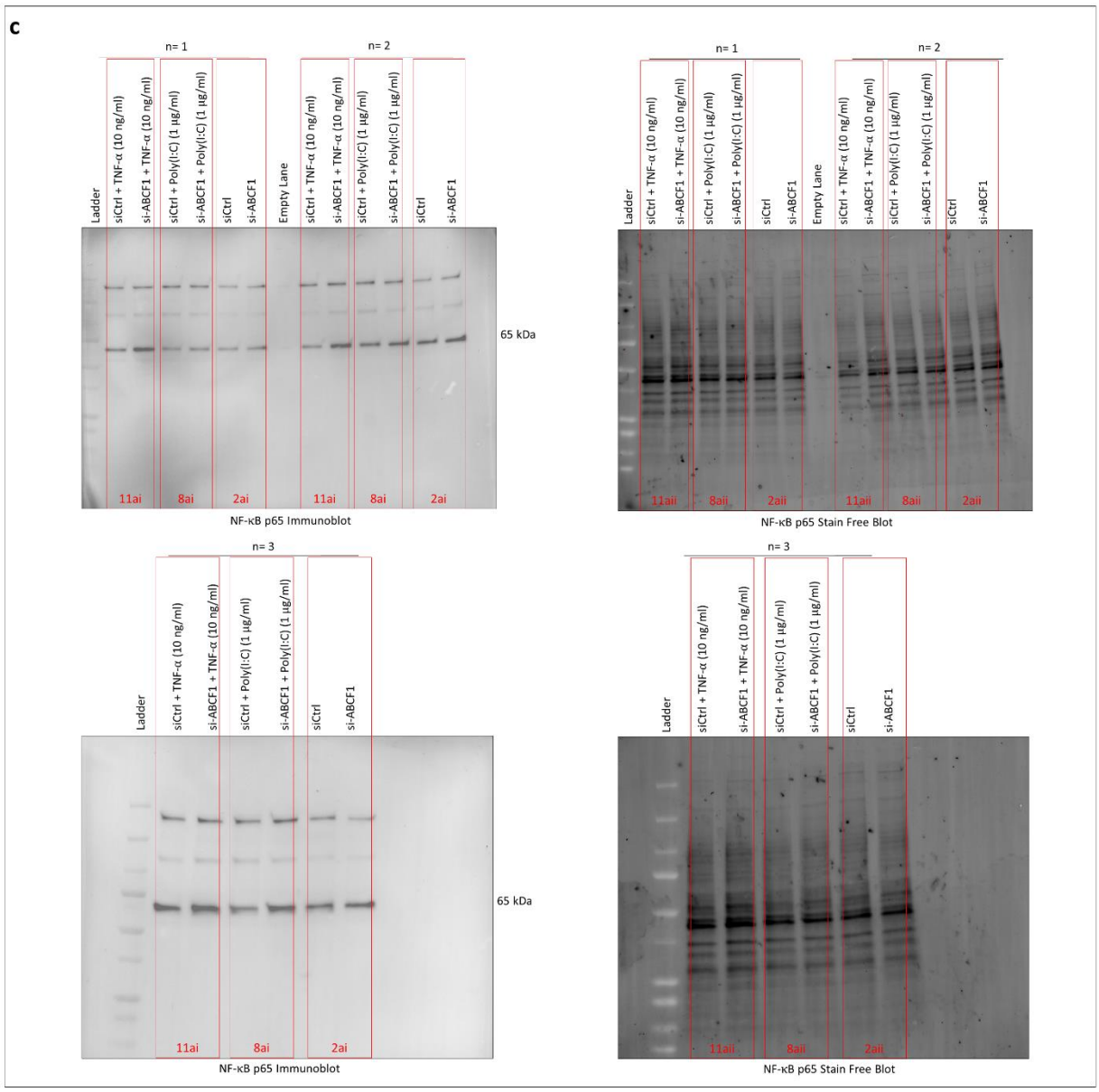

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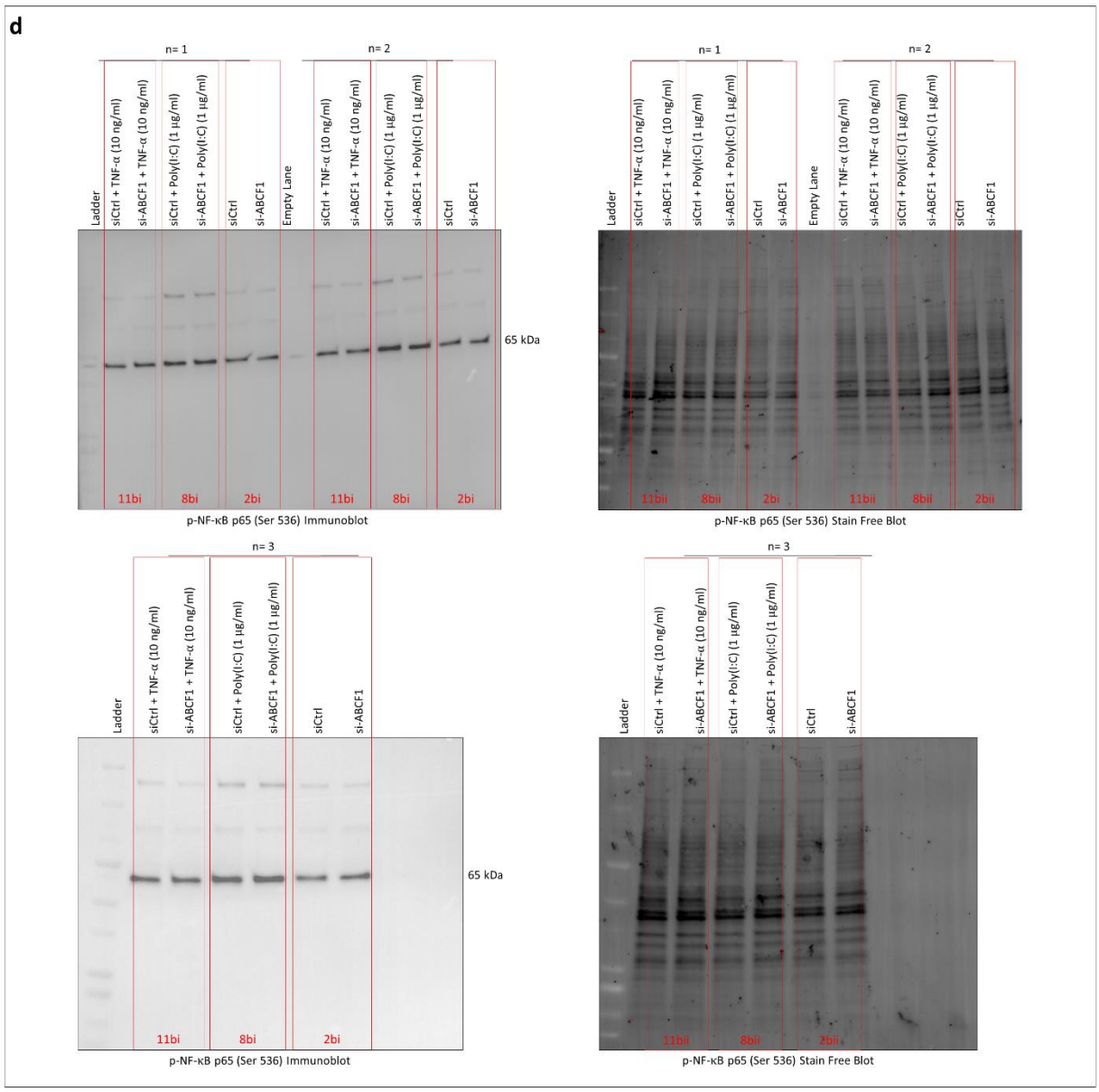

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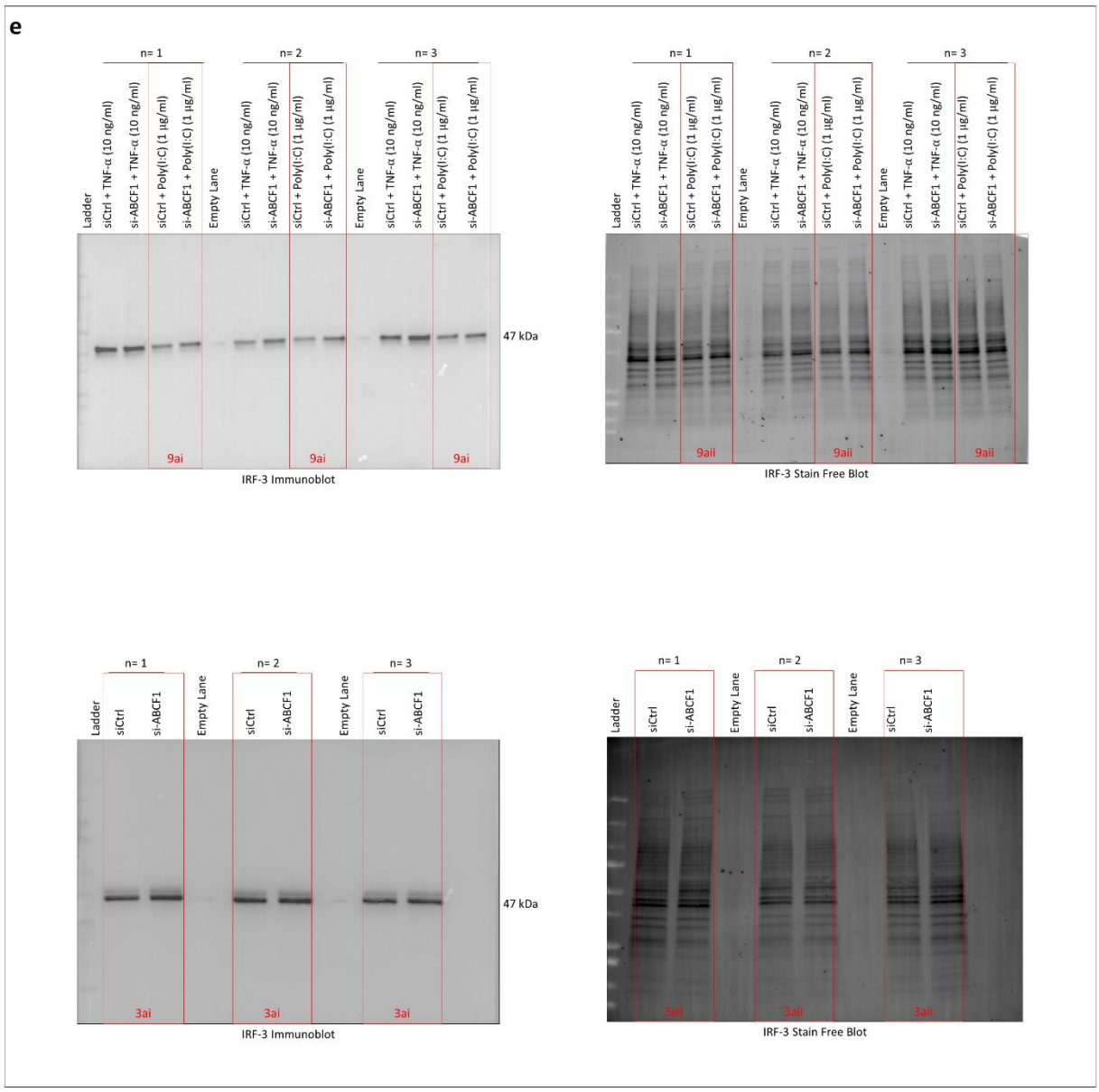
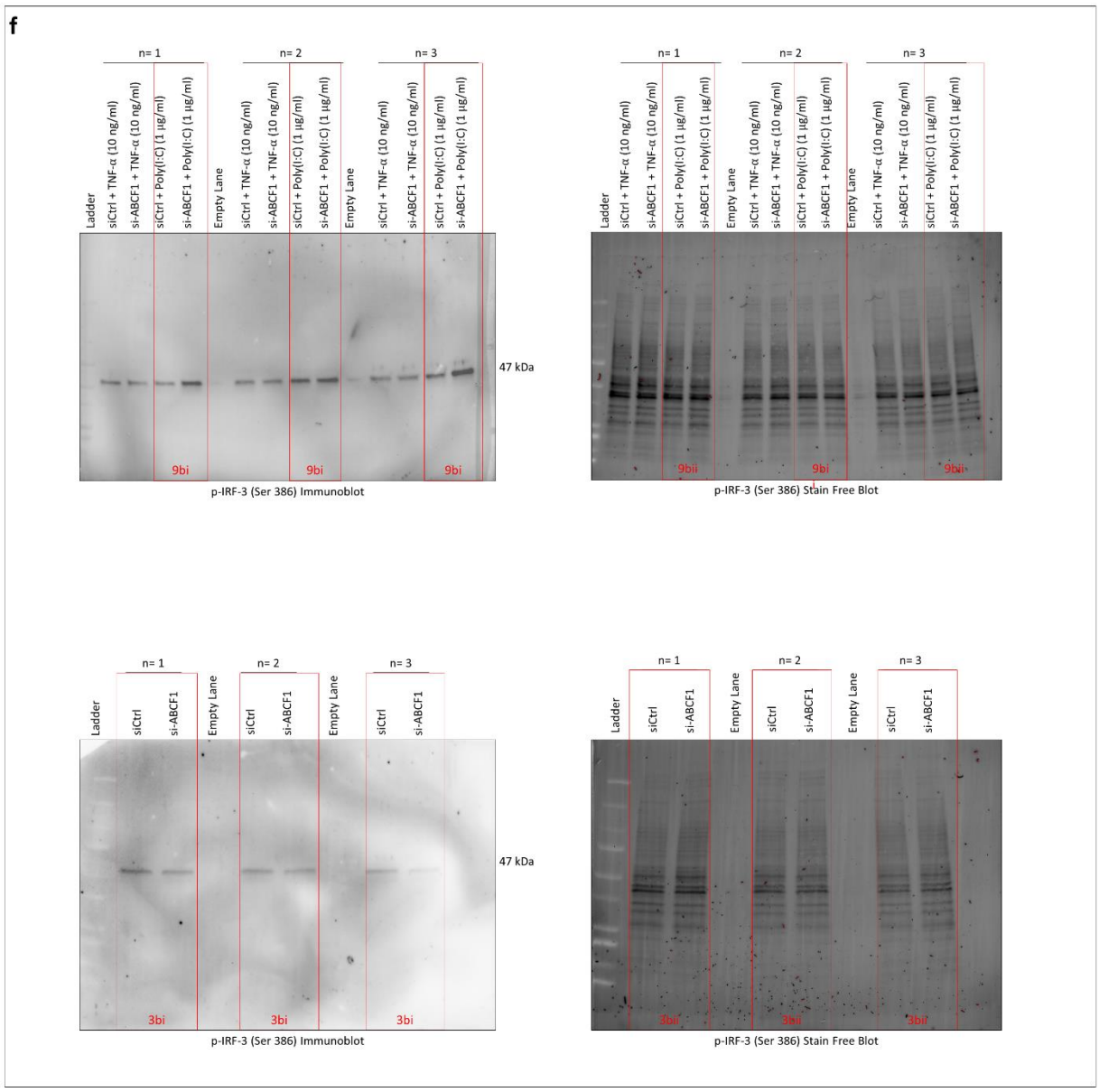


Supplementary 5



Supplementary Figure 5 – Full length immunoblots of **a)** ABCF1, **b)** A20, **c)** NF-κB p65, **d)** Phosphorylated NF-κB p65 (Ser 536), **e)** IRF-3, and **f)** Phosphorylated IRF-3 (Ser 386) in HBEC-6KT under immunostimulatory stimulations with siCtrl and si-ABCF1 treatment *in vitro*.

Chapter 4

Bioinformatic Exploration and *in vitro* Validation of ABCF1-SYK Protein-Protein Interaction in Human Airway Epithelial Cells

Summary and significance

Proteins interact with other molecules, including proteins, nucleic acids, lipids, substrates, and small molecules such as metal ions, to carry out a biological function. By exploring the how proteins interact with other proteins, the protein-protein interactions (PPIs), we can predict the function of an uncharacterized protein of interest based on its interaction with proteins with known functions. We have previously demonstrated that ABCF1 has the potential to positively regulate innate immune responses in human airway epithelial cells under pro-inflammatory conditions. Our previous study showed that ABCF1 positively regulates the induction of pro-inflammatory cytokines through pathways that are independent of NF-κB and IRF-3 activation, yet the exact mechanism remains to be characterized. In this study, we first took a hypothesis-free approach by investigating the protein interactome of ABCF1 using publicly available protein interaction databases and ran a Gene Ontology enrichment analysis to further investigate the function of ABCF1 in the context of innate immunity. Our findings suggest that ABCF1 interacts with SYK, which is known to be involved in innate immunity. We then validated our exploratory analysis and showed that ABCF1 interacts with SYK under Poly(I:C) stimulation, but not under resting conditions. We provide the first evidence in human airway epithelial cells that ABCF1 physically interacts with SYK, warranting deeper investigation into how this complex may regulate respiratory mucosal immunity.

Bioinformatic Exploration and *in vitro* Validation of ABCF1-SYK Protein-Protein Interaction in Human Airway Epithelial Cells

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Status of work: Manuscript to be submitted.

Author contributions: QC: Designed, performed, and analyzed *in vitro* experiments, figure generation, drafting and editing of the manuscript. JH: Responsible for the oversight of the entire study (funding, drafting, and editing of the manuscript), and supervision of the trainees. All authors contributed to the paper.

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ABSTRACT

ABCF1 is the most characterized member of the ABCF family in eukaryotes with proposed functions related to innate immunity in fibroblasts, macrophages, and epithelial cells. Our previous work has demonstrated that ABCF1 has a role in innate immune responses in human airway epithelial cells during viral and pro-inflammatory conditions. However, a detailed mechanistic understanding of ABCF1 and its roles in immune responses in the airway remains to be defined. This study aims to provide insights into the roles of ABCF1 in human airway epithelial cells (HAECs) within the framework of innate immunity. We first explored the interactome of ABCF1, to identify key protein interactors with BioGRID, IntAct, and STRING databases. Furthermore, we characterized the functional activities of these interactions with Gene Ontology (GO) term enrichment. Next, we performed an *in vitro* validation on the candidate interactor under resting and a pro-inflammatory stimulated condition (Poly I:C). Our *in silico* results revealed that ABCF1's interactome is enriched with terms related to transcription regulation, stimulus response, and signalling pathways. Notably, some of these interactors, including SYK, have been previously implicated in regulating innate immune responses to pro-inflammatory stimuli. Our *in vitro* validation studies in HAECs demonstrated the interaction between ABCF1 and SYK under Poly(I:C) stimulation, but not under resting conditions. It remains unclear how ABCF1 regulates SYK and the downstream pro-inflammatory responses following Poly(I:C) exposure or other immune stimuli, warranting further investigation on the impact of their interaction. Overall, these findings support the idea that ABCF1 and its associated protein partners play a role in responding to immunostimulatory signals and modulating signalling pathways in HAECs.

Keywords: airway epithelial cells, ABCF1, Protein-protein interaction, enrichment analysis, BioGRID, IntAct, STRING, gene ontology, WebGestalt, Poly(I:C), SYK, pro-inflammatory, TRAF, ubiquitination

INTRODUCTION

Proteins can carry out a wide range of biological function through direct interactions with a range of molecules, including proteins, nucleic acids, lipids, substrates and small molecules such as metal ions[1]. The protein interactome is defined as a large network of protein-protein interactions (PPIs) that take place within a living cell, and it has major roles in both physiological and pathological processes[2, 3]. Some proteins can function independently, such as antibodies which can recognize and neutralize a foreign antigen without relying on other host proteins[4]. However, most proteins interact with other proteins and form complexes to carry out a biological function[5]. PPI implies that the interaction interfaces between the interacting proteins are intentional and not accidental, as well as non-generic[6]. Some examples of effects from PPIs include altering the kinetic properties of proteins such as substrate binding and catalysis, substrate channelling, formation of new binding sites, and activation or inactivation of a protein[7]. Protein interactions can form homo-complexes (association between identical subunits) or hetero-complexes (association between different protein subunits); be obligate (protomers that are structurally unstable and must form a permanent complex with another protein to be functional) or non-obligate (protomers that are independently stable and can form either transient or permanent complexes); and can differ based on their stability and persistence with their protein partners[5, 8, 9]. Studying PPIs can predict the function of an unidentified or uncharacterized protein based on its interaction with proteins with known functions, detect proteins involved in a disease pathway, and identify potential targets for drug development to treat human diseases[2, 5].

PPIs can be identified by *in vitro* techniques that are either high-throughput such as affinity purification mass spectrometry (AP-MS), or low-throughput such as co-immunoprecipitation.

PPIs can also be identified by *in silico* methods such as computational predictions based on sequence-based or structure-based approaches[3, 5]. *In silico* PPI methods are grounded in data collected experimentally or computationally and can then be accessed via publicly available interaction databases [3]. Multiple databases exist, with each database having different sources for data collection. Some collect data exclusively from peer-reviewed publications (e.g., BioGRID, IntAct, MatrixDB), or computationally predicted interactions with no experimental evidence (e.g., STRING, PIPs, MPIDB)[3]. Each database has the PPIs annotated with an identifier (e.g., NCBI Gene Identifier, UniProt Identifier), source (e.g., PubMed Identifier, Digital Object Identifier), interaction type (e.g., physical, association, proximity), and detection method. PPIs can be presented as a network where the nodes represent the interaction partners (proteins or other molecules such as nucleic acids or chemical drugs) and the edges connecting the nodes represents the interaction[3].

Researchers often run a Gene Ontology (GO) Enrichment analysis with the list of PPIs collected from the databases to get additional information on their biological function. The GO consortium annotates gene products with a term to describe their functional properties using the three ontologies that cover three independent biological domains: cellular components, biological processes, and molecular function[3, 10]. A directed acyclic graph is used to present the GO annotations, where each term is represented by a node and the relationship between the terms is represented by an edge that connects the nodes in a hierarchical way[10]. The GO annotations is often followed by an enrichment analysis for each biological domain to identify which GO terms were over- or under-represented based on the protein list of interest[3]. This tool helps the

researcher predict or interpret the function of the protein of interest by highlighting the terms related to its interactors, such as their location, biological events, and functional activities[3].

Previously, our group confirmed the expression of ABCF1 in human airway epithelial cells (HAECs) at the gene and protein level both *in situ* and *in vitro*[11]. Using an *in vitro* siRNA knockdown experiment in an airway epithelial cell line, HBEC-6KT, we have characterized the function of ABCF1 as an important component in innate immune responses in HAECs under dsDNA viral mimic, VACV-70, stimulation[11]. We also ran a microarray using Affymetrix Clariom S Human chip to observe differential gene expression and ran a hypothesis-free GO enrichment analysis to identify the pathways that were impacted by *ABCF1* siRNA treatment [11]. We showed that one of these pathways included TLR3 and TLR4 signalling[11]. The key gene that was differentially expressed in these pathways was *TNFAIP3*. This gene is expressed as the A20/tumour necrosis factor α-induced protein 3 (TNFAIP3) protein, a ubiquitin ligase and deubiquitinating enzyme that inhibits nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF-3) activation to regulate innate immune responses[12, 13].

Next, we investigated whether there was a link between ABCF1 expression and A20, NF-κB, and IRF-3 signalling in HAECs under pro-inflammatory stimulations. Similar to our study above, we ran an *in vitro* siRNA knockdown experiment in HBEC-6KT, and stimulated the cells with Poly(I:C) and TNF-α[14]. We demonstrated that under these stimulations with *ABCF1* silencing, there was a significant reduction in induced levels of IL-8 and a trend for reduced IL-6[14]. However, we observed no changes in the expression levels of A20 and the activation status of the transcription factors, NF-κB and IRF-3[14]. This study demonstrated that Poly(I:C) and TNF-α-

induced IL-8 are regulated by ABCF1 under 24 hours (h) of stimulation through pathways independent of NF-κB, and IRF-3 activation. However, the exact mechanism of how ABCF1 regulates IL-8 induction remains unclear.

Ubiquitination is a post-translational modification involved in protein degradation, directing protein localization, and regulating protein interaction activities[15]. This process involves attaching a conserved ubiquitin (Ub) molecule to target proteins in the form of a monomer or polymer (Ub chains) through a three-step enzymatic pathway with Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and Ub-ligating enzymes (E3)[15]. Different types of ubiquitin chains serve different purposes, where Lysine (K)48-linked polyubiquitin chains target proteins for proteasomal degradation, and K63-linked or linear polyubiquitin chains stabilize PPIs for downstream signalling molecules[12]. Ubiquitination is a dynamic and reversible process where Ub chains can be removed or modified by deubiquitylases or deubiquitinases (DUBs)[15]. An example of DUBs includes A20, which functions as both a K48 ubiquitin ligase and K63 deubiquitinase to negatively regulate NF-κB mediated signalling pathways[12].

Spleen tyrosine kinase (SYK) is a non-receptor tyrosine kinase that is involved in the recognition of pathogen associated molecular patterns (PAMPs) through pathogen recognition receptors (PRRs), including toll-like receptors (TLRs) [16]. Src-family tyrosine protein kinase (SFK) phosphorylate and activate SYK, whereby it then triggers reactive oxygen species (ROS) production following PRR stimulation and activates transcription factors, such as NF-κB and IRF-3, to trigger an immune response[17]. While SYK has a crucial role in immune receptor signalling, it is also involved in cellular adhesion, osteoclast maturation, platelet activation and vascular

development[16]. The function of SYK is negatively regulated by phosphatases such as SHP-1 or targeted for degradation by ubiquitination by E3 ligases, including Casitas B-lineage lymphoma (CBL)[16].

Previous studies have demonstrated the role of ABCF1 in ubiquitination in murine bone marrow derived macrophages (BMDMs). They demonstrated that ABCF1 is targeted for K48-polyubiquitination by cIAP1/2 during the early phase of TLR4 signalling by LPS stimulation, however this shift to K63-polyubiquitination by TRAF6 at late phase[18]. This modification causes ABCF1 to become a molecular switch where it functions as an E2 Ub-conjugating enzyme to transition the macrophage from an M1 pro-inflammatory phenotype (MyD88-dependent signalling) to an M2 anti-inflammatory phenotype (TRIF-dependent signalling) during LPS stimulation[18]. During this transition, ABCF1 was shown to associate with TRAF6 and SYK to form a complex, and K63-polyubiquitinate SYK to mediate TLR4 endocytosis[18]. Additionally, TRAF3, a component essential for the TLR4-TRIF pathway for IRF-3-dependent interferon type 1 production, is targeted for K63-polyubiquitination by ABCF1[18].

To characterize the biological function of ABCF1 in HAECs under immunostimulatory conditions, we propose to run a hypothesis-free *in silico* investigation of the ABCF1 PPI network using publicly available databases and use this network for GO term enrichment. Based on the findings from our *in silico* work, we hypothesize that ABCF1 interacts with SYK to positively regulate innate immune responses in HAECs. We experimentally validated our *in silico* results by performing an immunoprecipitation to investigate ABCF1 interaction with SYK under basal and Poly(I:C) challenge.

METHODS

ABCF1 interactors: BioGRID, IntAct, STRING database

The BioGRID (https://thebiogrid.org/, version 4.4), IntAct (https://www.ebi.ac.uk/intact/, version 1.0.4) and STRING (https://string-db.org/, version 12) databases was queried for ABCF1 (homo sapiens, UniProtID: Q8NE71)[19–21]. First, the list of interactions from BioGRID was downloaded as a TAB 3.0 file (retrieval date: September 15, 2023). Next, the list of interactions from IntAct was downloaded as a JSON file (retrieval data: October 3, 2023). Lastly, the list of interactions from STRING was downloaded as a TSV file (retrieval data: October 3, 2023). All downloaded files were imported into an Excel spreadsheet for analysis. A python script was used for all sets of data to convert the annotated interactors to NCBI/Entrez Gene ID.

We merged the ABCF1 interaction data collected from the three databases in an Excel spreadsheet. Annotations for each interaction included the protein interactor (NCBI/Entrez GeneID), the publication (PubmedID), the interaction type, and the detection method used to identify the interaction. Repeated interactions that were reported from the same publication and non-human interactors were filtered out. Following the filter, this generated the finalized merged dataset for candidate ABCF1 interactors.

We further filtered the list of ABCF1 interactions to include ones that were hetero complexes as they can reveal how ABCF1 participates in a variety of different activities through its interaction with different proteins. Then we annotated them based on the number of publications and detection methods were reported. This annotation method enables us to selectively identify confirmed interactors, rather than relying on chance occurrences. Each interaction was labelled as low,

medium, or high occurrence based on the following criteria. An interaction is labelled as low occurrence when the protein interactor was reported in only one publication or detected by one experimental method; medium occurrence is when the interactor was reported in one publication but had two or more experimental approaches, or was reported in two or more publications but detected by the same experimental technique; and high occurrence when the interactor was reported in two or more publications and had two or more experimental approaches[22]. This new list of 12 ABCF1 interactors is called the ABCF1 interactome.

GO Enrichment Analysis

The WEB-based GeneSeT AnaLysis Toolkit 2019 (WebGestalt) web tool was used to run our GO term enrichment analysis for both the merged dataset, and the list of 12 interactors, ABCF1 interactome[23]. To begin, we ran an Over-Representation Analysis (ORA) with Homo sapiens as our organism of interest and used the GO functional database. The analysis was performed using hypergeometric statistics and a Benjamini-Hochberg adjusted P value. The minimum number of genes was set at 2 and the significance level was set to retrieve the top 10 hits[22, 23]. The report from WebGestalt will display the top 10 categories that were identified as enriched, where each is annotated with their identifiers (GO ID and Entrez Gene ID), the FDR and P-value, and the enrichment ratio. The report will also inform the user how many gene inputs were mapped (the number of genes from the input list that can be recognized by WebGestalt), gene sets (the collection of genes that belong to a certain functional category) and overlapping genes (the number of genes that are in both the input list and in the gene set) were analyzed for each category [23]. We submitted and retrieved the data on November 9th, 2023.

Software

The protein interaction data collected from interaction databases was stored and analyzed as Excel spreadsheet (XLSX) files, and annotations were converted using Python 3.11. Graphs were prepared on GraphPad Prism 8 and Cytoscape (version 3.10.1).

Reagents

ABCF1 and SYK were probed with anti-ABCF1 primary antibody (ab190798, Abcam, Cambridge, UK) at 1:1000, and anti-SYK primary antibody (13198T, Cell Signalling Technology, Massachusetts, USA) at 1:1000 in 5% BSA (080-450, Wisent Inc., Quebec, CA) in 1X Tris Buffered Saline (T5912, Sigma-Aldrich, Oakville) with TWEEN® 20 (P1379, Sigma-Aldrich) (TBS-T). The secondary antibody used was an anti-rabbit HRP-Linked Antibody from Cell Signalling Technology (7074S, Cell Signalling Technology) at 1:2000 in 3% Casein (1706404, Bio-Rad Laboratories, California, USA) in TBS-T. Immunoprecipitated proteins used VeriBlot for IP Detection Reagent (HRP) from Abcam (ab131366, Abcam) at 1:500 as a secondary antibody alternative. The immunostimulatory ligand was Poly(I:C) (tlrl-pic, Invivogen, San Diego, USA), which was directly added to the cells with cell culture media without a transfection reagent.

Cell culture

All experiments were performed in submerged monolayer cell culture using the HBEC-6KT immortalized HAEC line over expressing human telomerase reverse transcriptase (hTERT) and cyclin-dependent kinase 4 (Cdk4) as described in our previous study[14].

In vitro immunostimulation and protein collection

Cells are grown to 80-90% confluency, then stimulated with Poly(I:C) (1μg/ml) for 24 h. Similar experiments were performed in the absence of stimulation to investigate baseline ABCF1 function. HAECs were washed with phosphate buffered saline (Corning, 21-040-CM, Corning, USA) collected using a cell scraper (83.395, Sarstedt, Nümbrecht, Germany) and lysed with NP-40 lysis buffer (J60766.AP, ThermoFisher Scientific, Massachusetts, USA) containing protease inhibitor cocktail (P2714-1BTL, Sigma-Aldrich) and phosphatase inhibitor (4906845001, Roche, Mississauga, Canada).

Immunoprecipitation

Immediately after sample collection and lysis, the samples were quantified and used for the indirect co-immunoprecipitation assay. Approximately 500 μg of the samples were pre-cleared with the PureProteomeTM Protein A Magnetic Beads. Next, the sample and capture anti-ABCF1 antibody (sc-377445, Santa Cruz Biotechnology, Dallas, USA), and IgG isotype control (sc-3879, Santa Cruz Biotechnology), underwent continuous mixing overnight. Magnetic beads were then added to the antibody-antigen complex as described in the manufacturer's instructions. With a magnetic stand to engage with the magnetic beads, the beads were washed (1X PBS, 50 mM NaCl and 0.1% Tween-20) five times, and the elution buffer (0.2M Glycin-HCL, pH 2.5) was added to elute the captured target protein and its potential interactor. The eluted protein sample was neutralized with 1M Tris (pH 8.5).

Western Blotting

The collected protein was used for western blotting without further sample preparation. Protein from total cell lysate (4 μg) and immunoprecipitated protein (15μl) was premixed in 1X Laemmli Sample Buffer (1610747, Bio-Rad Laboratories), then separated on a 4-20% Mini-PROTEAN TGX stain-free precast gels (4568093, Bio-Rad Laboratories) and transferred to a PVDF membrane using the Transfer-Blot Turbo RTA Transfer Kit reagents (1704272, Bio-Rad Laboratories). Membranes were blocked and imaged as described in our previous study[14].

Statistics

All experiments were conducted with a sample size of n=3. Each repetition was obtained from a distinct cell culture at a distinct time. Experiments conducted with HBEC-6KT were considered independent when separated by at least one passage, all within a maximum of 5 passages.

RESULTS

ABCF1 protein interactors from publicly available databases

Using publicly available, open-sourced databases that archive and disseminate genetic and protein interaction data, we generated a list of ABCF1 protein interactions derived from primary literature, direct user-submitted data, and/or computational predictions[19, 24]. This interaction dataset was then analyzed to rationalize the findings from existing literature that have described the role of ABCF1 in various biological processes, as well as to infer its potential activities in unexplored areas[22].

BioGRID was queried for ABCF1 (human, DADB-129D20.7) and 184 unfiltered annotations from 110 unique publications or sources were downloaded. IntAct was queried for ABCF1 (human, Q8NE71) from 30 unique publications or sources and 54 unfiltered annotations were downloaded. STRING was queried for ABCF1 (human) and 10 unfiltered annotations from one publication were downloaded. Altogether, we had 15 publications that were identical while the remaining 126 were unique. In the BioGRID and IntAct datasets, 23 annotations were duplicates, therefore we kept one set of these annotations in our finalized merged dataset.

In the finalized merged dataset, there were a total of 141 publications that described 204 hetero-oligomeric protein interactions captured by various detection methods (**Figure 1**). Of these interactions, approximately 53% were detected by affinity capture; 20% were captured by co-fractionation, 10% by co-immunoprecipitation (Co-IP), 8% by proximity labelling, <2% for reconstitution complex, <1% for yeast-2-hybrid (Y2H) and the remaining 6% by other various methods. Of the 204 interactions, 182 were physical, which constitutes most of these interactions.

Figure 1

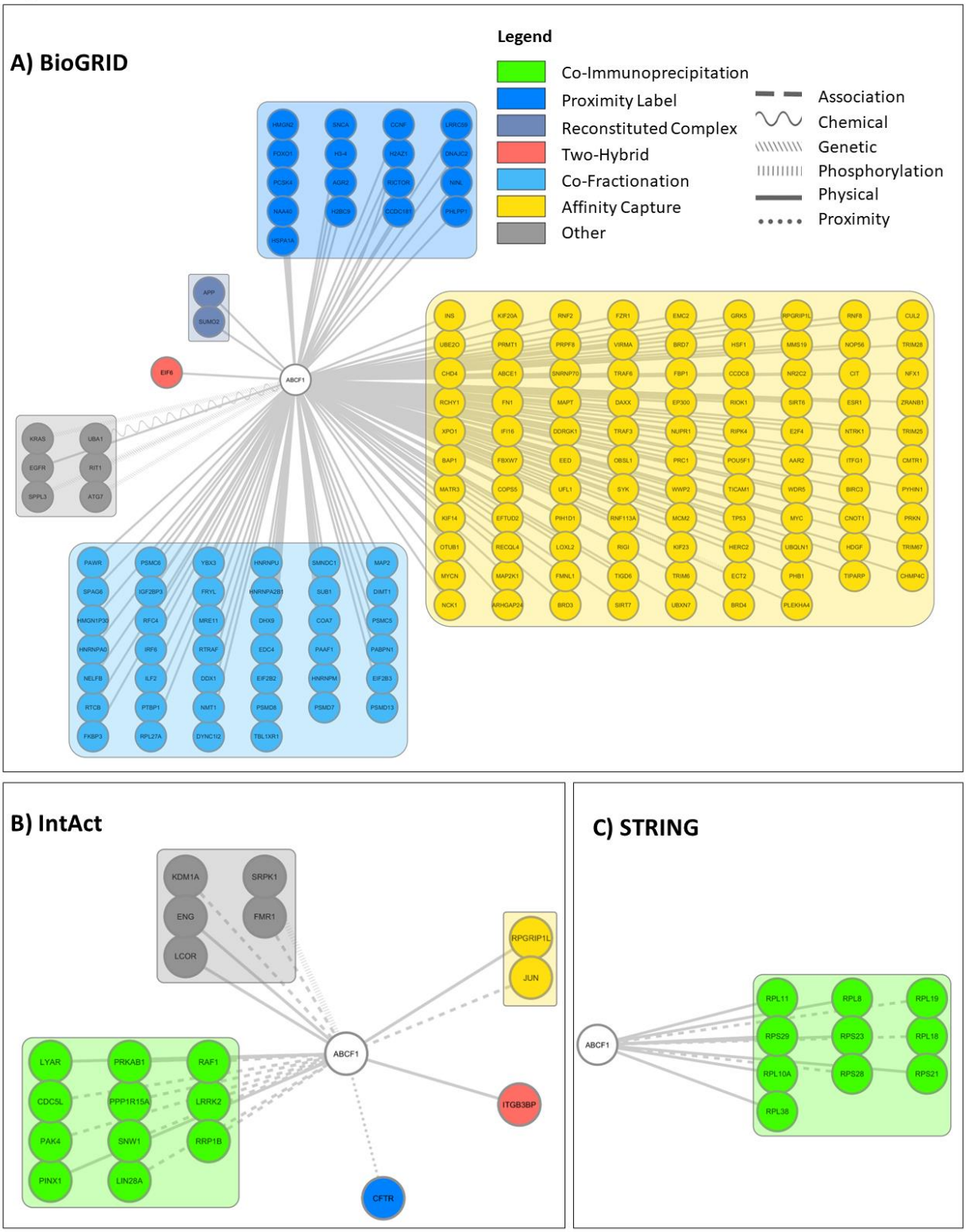
A) BioGRID
Legend
Co-Immunoprecipitation
Proximity Label
Reconstituted Complex
Two-Hybrid
Co-Fractionation
Affinity Capture
Other
Association
Chemical
Genetic
Phosphorylation
Physical
Proximity
ABCF1
B) IntAct
KDM1A
SRPK1
ENG
FMR1
LCOR
RPGRIP1L
JUN
LYAR
PRKAB1
RAF1
CDC5L
PPP1R15A
LRRK2
PAK4
SNW1
RRP1B
PINX1
LIN28A
ITGB3BP
CFTR
C) STRING
ABCF1
RPL11
RPL8
RPL19
RPS29
RPS23
RPL18
RPL10A
RPS28
RPS21
RPL38

Figure 1. Visualization of the ABCF1 protein interaction network as reported in **A)** BioGRID, **B)** IntAct, **C)** STRING. Each node represents a protein, and each line (edge) represents an interaction. The node colour represents which detection method was used to identify the interactor. The edge pattern refers to how the interactors interact with ABCF1. Networks were generated using the Cytoscape tool.

Using the finalized merged dataset, we further condensed the list of interactors and generated a new list of ABCF1 interactors, the ABCF1 interactome, based on the number of publications and detection methods (**Figure 2**). This list consists of 12 interactions that were all labelled as medium occurrences. Of these interactions, 11 were reported in two publications and detected by one experimental approach, whereas one interaction was reported in one publication but was detected by two experimental approaches.

Figure 2

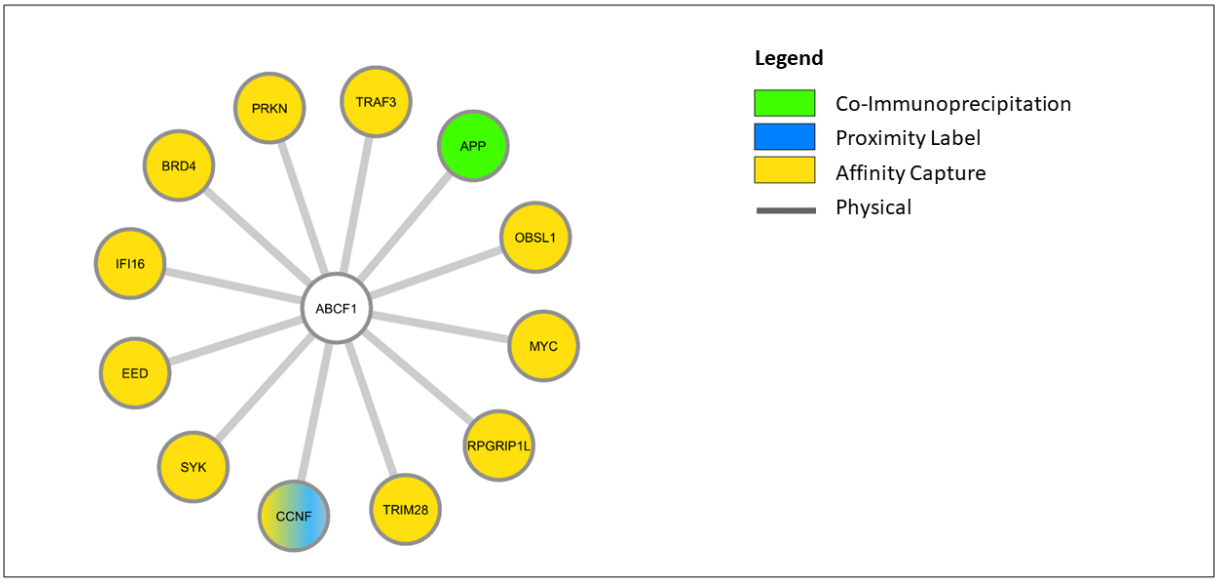

Figure 2. Visualization of the interaction network of ABCF1 interactome. The interactors were selected based on the number of times it has been reported in publications and whether it was detected by different detection methods. Each node represents a protein, and each line (edge) represents an interaction. The node colour represents which detection method was used to identify the interactor. Node colour-coded with multiple colours refers to multiple detection methods used to detect the interaction. The edge pattern refers to how the interactors interact with ABCF1. The network was generated using the Cytoscape tool.

GO term enrichment analysis on ABCF1 protein interactors demonstrated to function in protein ubiquitination and immune responses

With GO term enrichment analysis, we interpreted the function of the ABCF1 based on its set of protein interactors. This provided information on the biological process, molecular function, and cellular components [24].

Among the submitted list of 204 genes from the final merged dataset of ABCF1 interactors, 183 were recognized by WebGestalt. For the analysis of biological processes, molecular function, and cellular components, 176, 171, and 163 IDs were respectively annotated to the selected functional categories. In our biological process analysis, the top 10 categories included cellular responses to stress, regulation of gene expression, regulation of metabolic processes, and regulation of biosynthetic processes (**Table 1 – Biological Process**). In the molecular function analysis, the top 10 categories included ubiquitin protein ligase binding, protein kinase binding, transcription factor binding, and nucleic acid binding (**Table 2 – Molecular Function**). The cellular component analysis showed that the top 10 categories included ribosomes, polysomes, chromatin, nuclear body, and chromosomes (**Table 3 – Cellular Component**).

Table 1. GO Biological Process enriched for all ABCF1 interactors merged from three interaction databases, in WebGestalt. Only the top 10 overrepresented terms are shown with details about the enriched terms: GO term and ID, gene set size associated with that GO term, FDR, P-value adjusted for multiple testing, enrichment ratio and gene overlap which is the number of interactors (genes) from our input list associated with that GO term.

Biological Process GO Term	GO ID	Gene Set Size	FDR	P-value	Enrichment Ratio	Gene count
Regulation of cellular response to stress	GO:0080135	654	<2.2E-16	<2.2E-16	5.4	37
Cellular macromolecule catabolic process	GO:0044265	1108	<2.2E-16	<2.2E-16	4.1	48
Macromolecule catabolic process	GO:0009057	1338	<2.2E-16	<2.2E-16	4.0	56
Negative regulation of gene expression	GO:0010629	1733	<2.2E-16	<2.2E-16	3.9	72
Positive regulation of protein metabolic process	GO:0051247	1640	<2.2E-16	<2.2E-16	3.5	61
Cellular response to stress	GO:0033554	1867	<2.2E-16	<2.2E-16	3.3	66
Positive regulation of macromolecule biosynthetic process	GO:0010557	1826	<2.2E-16	<2.2E-16	3.2	62
Positive regulation of gene expression	GO:0010628	1911	<2.2E-16	<2.2E-16	3.2	64

Positive regulation of biosynthetic process	GO:0009891	1949	<2.2E-16	<2.2E-16	3.1	63

Table 2. GO Molecular Function enriched for all ABCF1 interactors merged from three interaction databases, in WebGestalt. Only the top 10 overrepresented terms are shown with details about the enriched terms: GO term and ID, gene set size associated with that GO term, FDR, P-value adjusted for multiple testing, enrichment ratio and gene overlap which is the number of interactors (genes) from our input list associated with that GO term.

Molecular Function GO Term	GO ID	Gene Set Size	FDR	P-value	Enrichment Ratio	Gene count
Ubiquitin protein ligase binding	GO:0031625	283	5.6E-09	1.2E-11	6.9	20
Ubiquitin-like protein ligase binding	GO:004438	298	2.4E-09	3.8E-12	6.9	21
Chromatin binding	GO:0003682	520	5.2E-10	5.6E-13	5.2	28
Ubiquitin-like protein transferase activity	GO:0019787	416	1.3E-05	5.6E-08	4.5	19
Transcription factor binding	GO:0008134	638	1.4E-07	3.7E-10	4.1	27
RNA binding	GO:0003723	1603	<2.2E-16	<2.2E-16	4.0	65
Protein kinase binding	GO:0019901	631	8.1E-06	3.0E-08	3.7	24
Kinase binding	GO:0019900	711	1.2E-06	3.9E-09	3.7	27
Transcription regulatory region DNA binding	GO:0044212	896	8.7E-05	4.4E-07	2.9E	27
Regulatory region nucleic acid binding	GO:0001067	898	8.7E-05	4.6E-07	2.9	27

Table 3. GO Cellular Component enriched for all ABCF1 interactors merged from three interaction databases, in WebGestalt. Only the top 10 overrepresented terms are shown with details about the enriched terms: GO term and ID, gene set size associated with that GO term, FDR, P-value adjusted for multiple testing, enrichment ratio and gene overlap which is the number of interactors (genes) from our input list associated with that GO term.

Cellular Component GO Term	GO ID	Gene Set Size	FDR	P-value	Enrichment Ratio	Gene count
Polysomal ribosome	GO:0042788	31	1.3E-12	2.2E-15	38.3	11
Polysome	GO:0005844	73	5.0E-10	3.0E-12	17.7	12
Ribosome	GO:0005840	229	4.2E-07	3.6E-09	7.1	15
Ribonucleoprotein complex	GO:1990904	834	<2.2E-16	<2.2E-16	5.4	42
Chromatin	GO:0000785	509	8.2E-09	5.6E-11	5.1	24
Nuclear body	GO:0016604	741	1.2E-11	4.0E-14	4.8	33
Chromosomal part	GO:0044427	886	1.2E-11	3.7E-14	4.4	36
Chromosome	GO:0005694	1014	1.9E-11	8.0E-14	4.0	38
Nucleoplasm part	GO:0044451	1087	2.7E-11	1.4E-13	3.9	39
Catalytic complex	GO:1902494	1346	1.3E-08	1.0E-10	3.1	39

For the condensed list of 12 genes that were curated to form the ABCF1 interactome (**above - Figure 2**), all 12 were recognized by WebGestalt. For the analysis of biological processes, molecular function, and cellular components, all 12 IDs were annotated to each functional category. In the biological process analysis, the top 10 categories included synaptic transmission, regulation of viral replication, and responses to stress (**Table 4 – Biological Process**). The top 10 categories in the molecular function analysis included DNA-binding transcription activity, ubiquitin protein activities, as well as protein kinase activities (**Table 5 – Molecular Function**). In our cellular component analysis, the top 10 categories included ciliary rootlet, linear ubiquitin chain assembly complex (LUBAC), 3M complex, and heterochromatin (**Table 6 – Cellular Component**).

Table 4. GO Biological Process enriched for the ABCF1 interactome in WebGestalt. Only the top 10 overrepresented terms are shown with additional details about the enriched terms: GO term and ID, gene set size associated with that GO term, FDR, P-value adjusted for multiple testing, enrichment ratio and gene overlap which is the number of interactors (genes) from our input list associated with that GO term.

Biological Process GO Term	GO ID	Gene Set Size	FDR	P-value	Enrichment Ratio	Gene count
Regulation of spontaneous synaptic transmission	GO:0150003	5	0.013	4.7E-06	555.5	2
Spontaneous synaptic transmission	GO:0098814	7	0.015	1.0E-05	396.8	2
Negative regulation of viral genome replication	GO:0045071	50	0.013	5.5E-06	83.3	3
Negative regulation of viral life cycle	GO:1903901	74	0.018	1.8E-05	56.3	3
Regulation of viral genome replication	GO:0045069	87	0.021	2.9E-05	47.9	3
Regulation of stress-activated MAPK cascade	GO:0032872	228	0.018	1.6E-05	24.4	4
Regulation of stress-activated protein kinase signalling cascade	GO:0070302	230	0.018	1.6E-05	24.2	4

Regulation of response to stress	GO:0080134	1361	0.006	7.1E-07	8.2	8
Positive regulation of cellular component organization	GO:0051130	1203	0.013	5.8E-06	8.1	7
Regulation of organelle organization	GO:0033043	1245	0.013	7.2E-06	7.8	7

Table 5. GO Molecular Function enriched for the ABCF1 interactome in WebGestalt. Only the top 10 overrepresented terms are shown with additional details about the enriched terms: GO term and ID, gene set size associated with that GO term, FDR, P-value adjusted for multiple testing, enrichment ratio and gene overlap which is the number of interactors (genes) from our input list associated with that GO term.

Molecular Function GO Term	GO ID	Gene Set Size	FDR	P-value	Enrichment Ratio	Gene count
DNA-binding transcription repressor activity, RNA polymerase II-specific	GO:0001227	267	0.23	0.0008	15.6	3
Ubiquitin protein ligase binding	GO:0031625	283	0.23	0.0010	14.7	3
Ubiquitin-protein transferase activity	GO:0004842	390	0.15	0.0001	14.2	4
Ubiquitin-like protein ligase binding	GO:0044389	298	0.23	0.0011	14.0	3
Ubiquitin-like protein transferase activity	GO:0019787	416	0.15	0.0002	13.4	4
Protein kinase binding	GO:0019901	631	0.23	0.0008	8.8	4
Protein kinase activity	GO:0004672	649	0.23	0.0009	8.6	4

Protein domain specific binding	GO:0019904	684	0.23	0.0011	8.1	4
Kinase binding	GO:0019900	711	0.23	0.0012	7.8	4
Sequence-specific DNA binding	GO:0043565	1097	0.23	0.0007	6.3	5

Table 6. GO Cellular Component enriched for the ABCF1 interactome in WebGestalt. Only the top 10 overrepresented terms are shown with additional details about the enriched terms: GO term and ID, gene set size associated with that GO term, FDR, P-value adjusted for multiple testing, enrichment ratio and gene overlap which is the number of interactors (genes) from our input list associated with that GO term.

Cellular Component GO Term	GO ID	Gene Set Size	FDR	P-value	Enrichment Ratio	Gene Overlap
Ciliary rootlet	GO:0035253	10	0.02	1.9E-05	293.2	2
LUBAC complex	GO:0071797	5	0.30	3.4E-03	293.2	1
3M complex	GO:1990393	5	0.30	3.4E-03	293.2	1
Nuclear heterochromatin	GO:0005720	34	0.14	2.4E-04	86.2	2
SCF ubiquitin ligase complex	GO:0019005	56	0.19	6.4E-04	52.4	2
Heterochromatin	GO:0000792	76	0.27	1.2E-03	38.6	2
Nuclear chromatin	GO:0000790	341	0.27	1.4E-03	12.9	3
Receptor complex	GO:0043235	396	0.30	2.1E-03	11.1	3
Nuclear chromosome	GO:0000228	573	0.18	4.5E-04	10.2	4
Cell-cell junction	GO:0005911	441	0.30	2.9E-03	10.0	3

ABCF1 does not impact the protein expression of SYK in HAECs under pro-inflammatory conditions

Expanding upon the findings made in our prior study where we demonstrated the impact of ABCF1 on IL-8 induction under Poly(I:C) challenge, we wanted to investigate the impact ABCF1 silencing has on a candidate immune protein interactor from our *in silico* results, SYK, under resting basal and pro-inflammatory conditions. We investigated the protein expression levels of SYK, under basal, TNF-α, and Poly(I:C) stimulation with and without ABCF1 silencing. We demonstrated that

ABCF1 silencing did not change the expression of SYK under basal and Poly(I:C) (**Figure 3**) or TNF-α stimulation (**Supplementary Figure 1**).

Figure 3

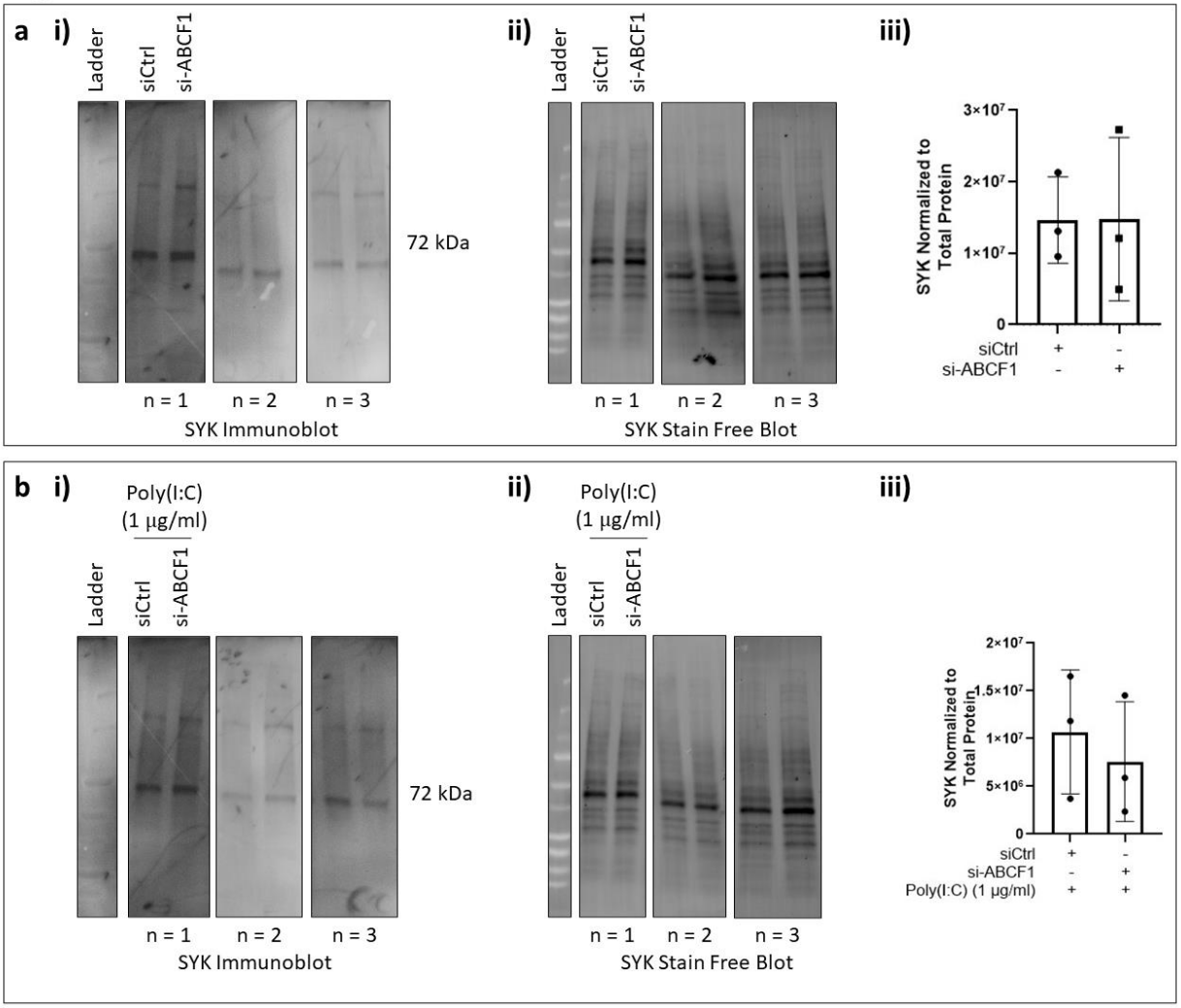

Figure 3 – **ABCF1 silencing on SYK protein expression under Poly(I:C) stimulation in HBEC-6KT *in vitro***. Immunoblot with quantification on SYK protein expression in HBEC-6KT cells under **a)** basal, and **b)** Poly(I:C) (1 µg/ml) stimulation with and without si-ABCF1 treatment. Immunoblot analysis n = 3; * $p \leq 0.05$.

ABCF1 interaction with SYK under basal and Poly(I:C) stimulated conditions in HAECs *in vitro*

A previous study has suggested that ABCF1 interacts with SYK by forming a complex with TRAF6 during TLR4 activation and signalling. Our previous study has demonstrated that HBEC-6KTs do not respond strongly to LPS stimulation, therefore we targeted TLR3 with Poly(I:C) to investigate the interaction between ABCF1 and SYK[14].

We performed a co-immunoprecipitation with ABCF1 as the target bait protein, under basal and Poly(I:C) challenge in HBEC-6KT cells. We demonstrated that ABCF1 can be immunoprecipitated and its expression levels were not impacted by Poly(I:C) stimulation at 1 μg/ml for 24 h when compared to the basal control (**Figure 4**). Next, we showed that under basal conditions, SYK does not interact with ABCF1. However, under Poly(I:C) stimulation at 1 μg/ml for 24 h, we observed that SYK was co-immunoprecipitated with ABCF1 (**Figure 5**).

Figure 4

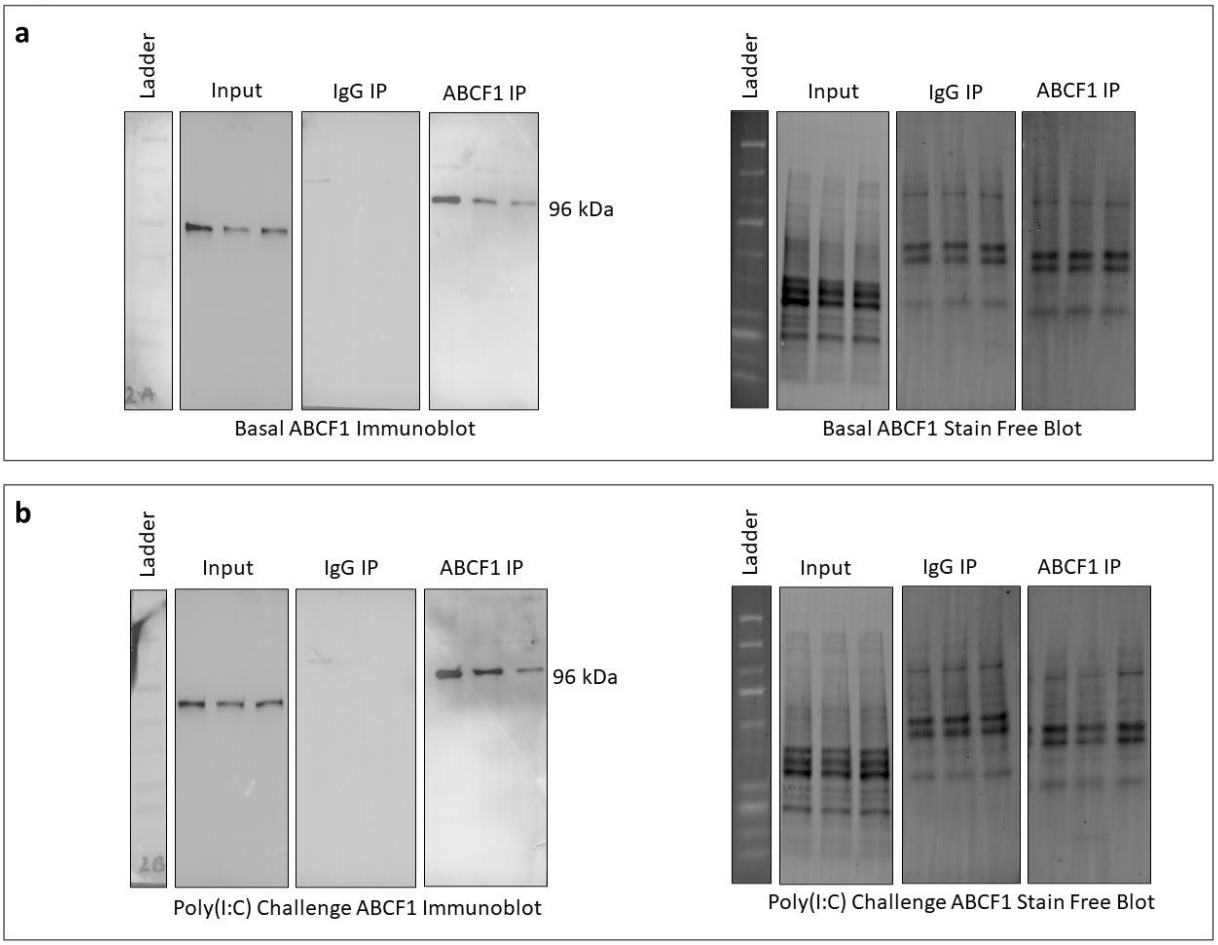

Figure 4 – ABCF1 immunoprecipitation under basal and Poly(I:C) stimulation in HBEC-6KT *in vitro*. Immunoblot on ABCF1 immunoprecipitation in HBEC-6KT cells under a) basal, and b) Poly(I:C) (1 μg/ml) challenge. Immunoblot analysis n = 3.

Figure 5

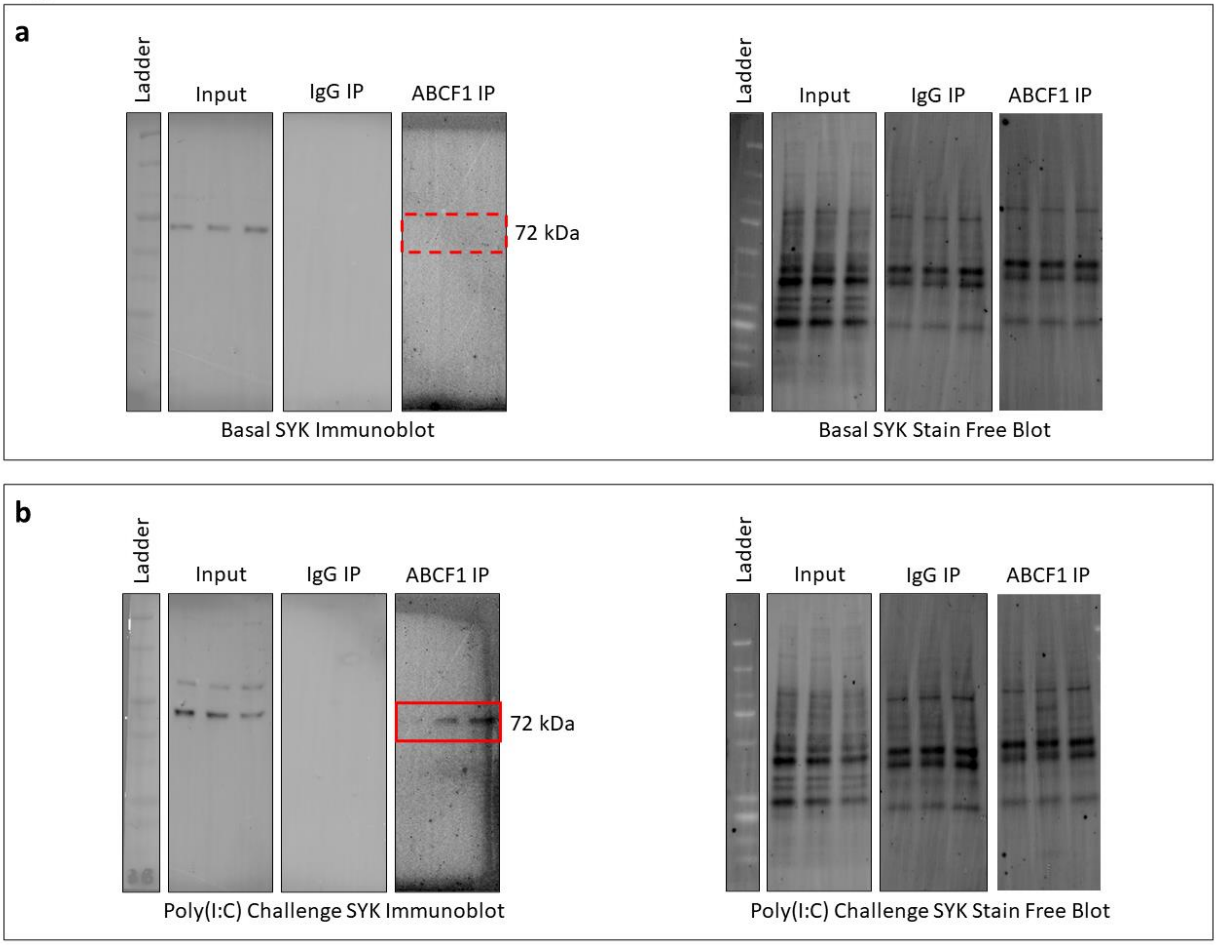

Figure 5 – SYK co-immunoprecipitation with ABCF1 under basal and Poly(I:C) stimulation in HBEC-6KT *in vitro*. Immunoblot on SYK co-immunoprecipitation with ABCF1 in HBEC-6KT cells under **a**) basal, and **b**) Poly(I:C) (1 μg/ml) stimulation. The expected bands for SYK under basal are highlighted in dashed red, while those under Poly(I:C) challenge are highlighted in solid red. Immunoblot analysis n = 3.

Figure 6

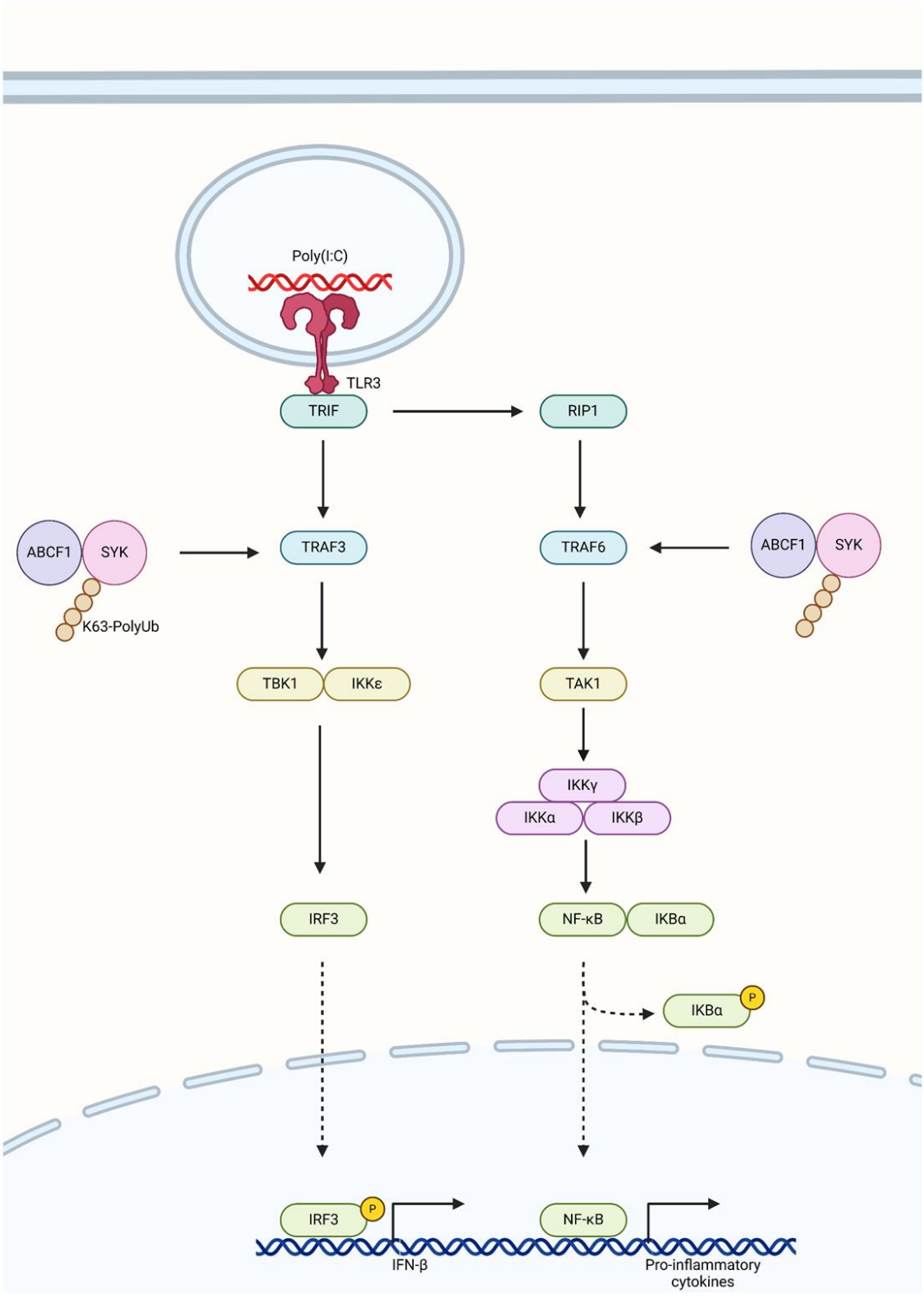
Poly(I:C)
TLR3
TRIF
RIP1
ABCF1
SYK
K63-PolyUb
TRAF3
TRAF6
ABCF1
SYK
TBK1
IKKε
TAK1
IKKγ
IKKα
IKKβ
IRF3
NF-κB
IKBα
P
IKBα
IRF3
P
NF-κB
IFN-β
Pro-inflammatory cytokines

Figure 6 – Putative interaction and function of ABCF1-SYK involved in Poly(I:C) Stimulation in HAECs. Under Poly(I:C) activation of TLR3, ABCF1 interacts with SYK. Previous studies have suggested that ABCF1 functions as an E2 ubiquitin-conjugating enzyme and interacts with SYK to mediate K63-polyubiquitination[18]. ABCF1-SYK complex then associates with TRAF3 and TRAF6 to regulate downstream activation of transcription factors, NF-κB and IRF-3[25]. SYK regulates the shift of NF-κB to IRF-3 by indirectly regulating K63-polyubiquitination of TRAF6 and TRAF3[25].

DISCUSSION

In our early studies, we confirmed the expression of ABCF1 at the gene and protein level in human airway epithelial cells (HAECs) *in situ* and *in vitro*[11]. Using siRNA-mediated knockdown on ABCF1 followed by a viral mimic challenge in HAECs, our findings suggested that it was not involved in regulating antiviral responses but instead mediates an immune response involving TLR3/4 signalling pathways[11]. One of the genes that were significantly differentially expressed in these signalling pathways under ABCF1 silencing and viral mimic challenge included *TNFAIP3*, expressed as A20 protein, a ubiquitin ligase and deubiquitinating enzyme that suppresses the NF-κB and IRF-3 activation[11, 12].

We, therefore, followed up with this finding by investigating the role of ABCF1 on A20 protein expression and function in HAECs under pro-inflammatory and viral mimic challenges. We demonstrated that under basal conditions, ABCF1 silencing led to no changes to the protein expressions of A20 and the transcription factors, NF-*κ*B and IRF-3, and its activation. However, we did observe changes in levels of induced pro-inflammatory mediators, IL-8 and IL-6, as well

as increased protein expression of total NF-κB and IRF-3 under the challenges[14]. These findings suggest ABCF1 may have a role in regulating pro-inflammatory responses to HAECs, but its exact function remained unclear at the end of our second body of work.

In the current study, we combined hypothesis-free, and hypothesis-testing approaches to explore the protein interaction network of ABCF1 and validate any identified candidate(s) *in vitro*. Proteins rarely function independently, but instead interact with one or more proteins to perform various biological functions. By mapping the protein interactions of ABCF1, we can gain a better understanding of its biological function and how it facilitates certain molecular activities[26].

Protein interactions can be broadly classified based on their interaction surface (homo- or hetero-oligomeric), their stability (obligate or non-obligate), and their persistence (transient or permanent)[27]. Over the past two decades, high-throughput methods such as Y2H and affinity purification have been made available to produce large sets of data to map PPI. Y2H identifies protein interacting partners by measuring the transcriptional activation of the reporter gene when two proteins interact, whereas affinity purification relies on immobilizing the bait protein using a tag to purify it along with its interacting partner[28]. These sets of data are accessible through online databases where there is an initiative to actively curate and store interaction information using standardized procedures[24]. Currently, several interaction databases are actively curating interaction data, including IntAct, MINT, BioGRID, DIP, and STRING[24]. In this study, we used BioGRID, IntAct, and STRING databases to explore the ABCF1 protein interactome as their databases have one of the largest interaction data sets[27]. The main source of data from these three databases comes from peer-reviewed publications. However, STRING may also include

computationally predicted interactions with no experimental evidence to increase coverage[24]. While these databases may share the same source of information, they all have a different curation policy, therefore the data we collect from each may not overlap even though they shared the same publications during the curation process, justifying our three-pronged approach[24].

In the ABCF1 protein interactome, most interactions were discovered by affinity capture and annotated as physical. This suggests that ABCF1 interacts closely with their protein partners and forms stable complexes that are essential for various cellular processes[6]. However, physical interaction does not imply that these interactions are static or permanent[6]. It should be noted that not all possible interactions documented in the databases will occur in any cell at any time as interactions are dependent on several factors such as the cell type, cell state, developmental stage, environmental conditions, protein modifications, presence of co-factors or other binding partners[6].

To collect more information on the interaction network, we used the web tool WebGestalt to investigate the three GO terms as it has a user-friendly interface, a broad functional annotation database, multiple testing corrections, and visualization tools[23]. The three ontologies are biological process (series of events accomplished by one or more assemblies of molecular functions); molecular function (activity of the proteins at a molecular level); and cellular component (the location of the proteins within the cell)[24]. This web tool can utilize our list of protein interactors and run an enrichment analysis to annotate each to one or more GO terms[26]. A protein may have more than one annotation if they are involved with different biological processes or functions in different cellular environments[26]. Following the annotations,

WebGestalt runs a statistical test to determine if a particular functional category or pathway is significantly over- or under-represented in the input list compared to what would have been expected by chance alone and provides statistical measures such as P-values and false discovery rates (FDR)[23, 26].

Our WebGestalt analysis from the list of 204 interactors following the merge of three databases contributed towards the enrichment of functional terms related to stress response, catabolic processes, gene expression, and biosynthetic processes. These biological processes were run by ubiquitin protein ligase binding, transcription factor binding, protein kinase binding, and nucleic acid binding in the ribosomes, polysomes, and chromosomes. Previous groups have demonstrated that ABCF1 knockout mouse models are embryonically lethal, and these findings could tie into the essential roles ABCF1 could play during development such as regulating gene expression of essential genes, or biosynthetic processes, as well as responding to DNA-damage induced stress[29, 30].

Meanwhile, our list of 12 interactors from the ABCF1 interactome demonstrated that they were primarily involved in synaptic transmission, regulation of viral replication, and responses to stress. The molecular functions potentially underlying these processes include DNA-binding transcription activity, ubiquitin protein binding, as well as protein kinase binding. The role of ABCF1 as responding to a sensor to detect dsDNA viral mimic has been demonstrated in mouse embryonic fibroblasts and HAECs; transcriptional co-activator in gene transcription has been characterized in embryonic stem cells; and it has been characterized as an E2 ubiquitin-conjugating enzyme in murine bone marrow derived macrophages under LPS challenge[11, 18, 29, 31]. Our

analysis also revealed that most of these activities take place in the ciliary rootlet, LUBAC, 3M complex, and heterochromatin. It is interesting to note that the ciliary rootlet is among the top 10 enriched cellular components, suggesting that ABCF1 may play a significant role in ciliated HAECs.

It should be highlighted that most of these interactions were detected by high-throughput methods and therefore have not been independently confirmed by other methods. This can introduce bias when generating an interactome from the data collected from these databases[24]. Nonetheless, these databases provide valuable sources of information and can guide us in a direction as we further characterize the function and understand the role of ABCF1 in HAECs.

From our list of interactors from the ABCF1 interactome, one of the proteins that is known to be involved in immunity is SYK, a non-receptor type of tyrosine kinase that is expressed in a variety of cell types, including immune cells, epithelial cells, and fibroblasts. It's been shown that TLR ligands and factors, including Poly(I:C), LPS, CpG, lipoprotein, and TNF-α can activate SYK[25]. Once activated, it phosphorylates various intracellular signalling molecules to regulate NF-κB and IRF-3 signalling pathways [25, 32]. SYK has been demonstrated to regulate the ubiquitination of TRAF6 downstream of TLR signalling by inhibiting the K63-polyubiquitination on TRAF6, leading to the inhibition of NF-κB and MAPK signalling[25]. Meanwhile, it promotes K63-polyubiquitination of TRAF3 to enhance the activation of TBK1/IRF-3 signalling[25]. Therefore, SYK can promote or dampen pro-inflammatory responses by inhibiting or activating NF-κB by regulating TAK1/IKK/MAPK signalling cascade[25].

Using our *in silico* results, we hypothesize that ABCF1 interacts with SYK in HAECs. We first confirmed that in HAECs, SYK is expressed in the cells and their expression levels are unaffected by ABCF1 silencing, as well as under TNF-α and Poly(I:C) stimulation. By following our previous findings on the role of ABCF1 on IL-8 induction under Poly(I:C) stimulation, we focused on investigating the interaction between ABCF1 and SYK in HAECs to understand its role in regulating pro- and anti-inflammatory responses.

We validated our *in silico* analysis by challenging HBEC-6KT cells with Poly(I:C) for 24 h to activate SYK and observe whether it interacts with ABCF1 by co-immunoprecipitation. Our findings suggest that under Poly(I:C) challenge, ABCF1 interacts with SYK. This interaction was first identified in a study by Arora et al where they suggested that during late phase TLR4 signalling in BMDMs, ABCF1 associates with SYK through a complex formation with TRAF6[18]. This interaction enables ABCF1 to perform a K63-polyubiquitination on SYK[18]. A study by Coates et al demonstrated that SYK is involved in *Pseudomonas aeruginosa* induced IL-6 and IL-8 in an airway epithelial cell line, BEAS-2B[33]. Their study suggested SYK controls the phosphorylation of p38MAPK and IκBα to induce the release of IL-6 and IL-8[33].

We have previously demonstrated that ABCF1 silencing under Poly(I:C) stimulation led to a loss of IL-8 induction, and here we have shown that ABCF1 and SYK interact. However, it is unclear whether ABCF1 is positively or negatively regulating SYK activity under Poly(I:C) induced signalling, and their impact on TRAF6 and TRAF3 activation (**Figure 6**). We postulate that the interaction between ABCF1 and SYK is involved in regulating downstream signalling to induce IL-8, and ABCF1 silencing will result in the loss of this interaction and pro-inflammatory

response. Further studies will need to be done to bridge the gap between ABCF1 silencing and loss of IL-8 induction under the context of SYK activity.

In conclusion, our analysis of the ABCF1 interactome and the enrichment analysis of the interactome have provided us with potential roles and pathways it's involved in. Using protein interaction databases and enrichment analysis tools has provided us with directionality in investigating the function of ABCF1 in HAECs. We demonstrated in our study that ABCF1 interacts with SYK under Poly(I:C) challenge and not under basal conditions. We propose that ABCF1 interacts with SYK to positively regulate the induction of IL-8 downstream of Poly(I:C) challenge. Our findings support future investigations into the precise mechanism of ABCF1 and SYK under TLR signalling in HAECs to understand ABCF1's role in IL-8 induction.

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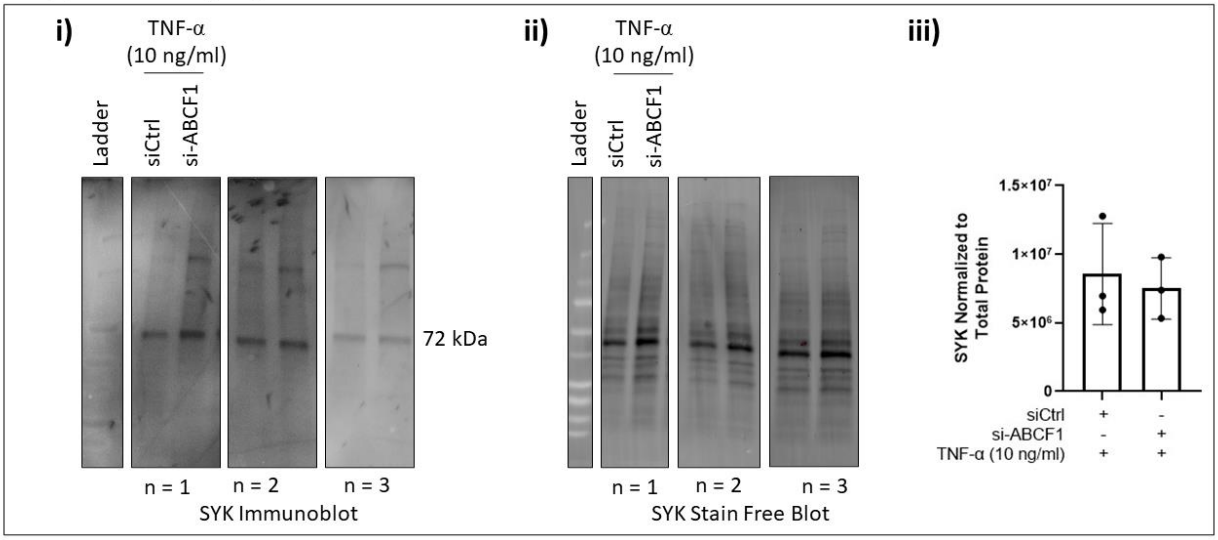
Supplementary Figures

Supplementary Figure 1



Supplementary Figure 1 – ABCF1 silencing on SYK protein expression under TNF-α challenge in HBEC-6KT *in vitro*. Immunoblot with quantification on SYK protein expression in HBEC-6KT cells under TNF-α (10 ng/ml) challenge with and without si-ABCF1 treatment. Immunoblot analysis n = 3; * $p \leq 0.05$.

Chapter 5

Discussion

In this Chapter, studies from Chapters 2-4 will be further discussed under the context of what is known in the current literature and how they tie together. The goal of this section is to discuss:

- The impact of our study on the field.
- Review the relevance of our studies from Chapters 2-4 to the findings in the current literature.
- The limitations of our study.
- Future directions to characterize the function and mechanism of ABCF1 in airway epithelial cells (alternative study approaches we would have pursued).
- Concluding remarks.

Summary of Impact

Our primary research presented in Chapters 2-4 is the first to contribute to our understanding of the function of ABCF1 in respiratory mucosal immunity. In human airway epithelial cells (HAECs), we have characterized ABCF1 as a candidate cytosolic nucleic acid sensor and modulator of TLR signalling that is expressed at the gene and protein levels; demonstrated that Poly(I:C) and TNF-α induced IL-8 is positively regulated by ABCF1 via pathways independent of NF-κB and IRF-3 activation; and lastly, provided the first evidence that ABCF1 physically interacts with SYK following Poly(I:C) stimulation. These novel findings for ABCF1 begin to reveal the function of this previously unexplored protein in HAECs in the context of innate immunity, with the potential to be relevant at other mucosal surfaces throughout the human body.

Interpretation of Primary Research in Context of the State of the Art

The human airway epithelium plays a significant role as a first line of defence against incoming pathogens, allergens, or foreign particles from normal respiration in the respiratory tract[1]. Studies have shown that there are several ABC transporter proteins with significant roles in maintaining normal physiology of the airway epithelium, including ABCC4, ABCC7 and ABCA3. A study by Aguiar et al. investigated the gene expression levels of ABC transporters in healthy HAECs across all generations of the airway tree[2]. In their study, the authors demonstrated that ABCF1 was one of the most highly expressed ABC transporters in HAECs, however, its function and the significance of its gene expression are currently unknown[2]. In this thesis, we aimed to characterize the function and mechanism of ABCF1 under the context of antiviral and pro-inflammatory stimuli in HAECs. Throughout our studies, we used an airway epithelial cell line, HBEC-6KT and performed a siRNA-mediated knockdown on ABCF1 to identify the impact of its

expression under immunostimulatory conditions. The primary readouts in our studies included the induction of antiviral and pro-inflammatory mediators measured by ELISAs, and protein expression was identified by immunoblots. We have conducted a transcriptomic analysis to identify the impact of ABCF1 silencing on genes of interest, as well as an immunoprecipitation assay to validate its protein interacting partners predicted from protein interaction databases.

In Chapter 2, we explored the role of ABCF1 as a dsDNA viral sensor and a mediator in antiviral immune response in HAECs. We hypothesized that ABCF1 can detect cytosolic viral dsDNA and initiate an antiviral immune response through the STING/TBK1/IRF-3 signalling pathway. This hypothesis was driven by the findings from a study by Lee et al. where they characterized the role of ABCF1 as a dsDNA viral sensor[3]. In their study, the authors silenced ABCF1 in mouse embryonic fibroblasts (MEFs) using siRNA, followed by stimulation by a dsDNA viral mimic, ISD, a 45-base pair (bp) non-CpG oligomer from the *Listeria monocytogenes* genome that induces the STING/TBK1/IRF-3 signalling pathway[3]. They measured the induction of CXCL10, a chemokine that is typically produced by leukocytes, epithelial, endothelial, stromal cells, and keratinocytes in response to type I IFNs and IFN-λ[4, 5]. The receptor for CXCL10 is CXCR3, which is predominantly expressed on activated T lymphocytes, dendritic cells, and macrophages, where upon binding promotes chemotaxis of these cells to the site of infection, as well as promoting apoptosis, cell growth inhibition and angiogenesis[6]. Their findings demonstrated that silencing ABCF1 led to a loss of induction in CXCL10 under ISD stimulation[3]. Next, they ran an unbiased mass spectrometry-based approach to identify ABCF1 protein interactors. They identified that both HMGB2 and IFI204 (whose related human protein is IFI16) are involved in the induction of CXCL10 under ISD stimulation[3]. The known function of HMGB2 is to bind to

DNA with no sequence specificity, however, its role in antiviral immunity is unclear[7]. IFI16 is known to recognize viral nucleic acids and induce the STING/TBK1/IRF-3 signalling pathway[8]. Their study concluded that ABCF1 interacts with HMGB2 and IFI204 in a DNA sensing network[3].

Initially, we performed our study by silencing ABCF1 and stimulating HBEC-6KT cells with ISD. Unfortunately, ISD was unable to induce a robust CXCL10 response in our cells therefore we used an alternative dsDNA viral mimic called VACV-70, a 70-bp oligonucleotide containing viral DNA motifs from the Vaccinia virus genome[9]. Under VACV-70 stimulation and ABCF1 silencing in HAECs, we observed a loss of CXCL10 induction[9]. Interestingly, our transcriptomic analysis revealed that ABCF1 silencing did not impact antiviral transcriptional responses at a global scale. Instead, it showed that ABCF1 was involved in TLR3/4 signalling pathways driven by the genes *WDFY1*, *TNFAIP3* (A20), and *NR1D1*[9].

It is worth discussing the model that the authors used in their study where they induced an antiviral response in MEFs with a dsDNA viral mimic. Viral genome analogs, such as ISD and VACV-70, are a convenient and efficient method to activate antiviral immune responses in a variety of different cells[10]. Although the cells can produce a robust response towards these viral mimics, they do not represent the degree and duration of antiviral immunity observed in infection with real viruses and their associated proteins[10]. Additionally, previous studies have demonstrated that MEFs are not permissive to adenovirus replication, a dsDNA respiratory virus[11]. Therefore, the conclusions derived from the study by Lee et al and our study may be conditional based on the experimental model and methodologies used.

The study we performed in Chapter 3 was focused on following the data discussed in Chapter 2 where we concluded that ABCF1 was involved in TLR3/4 signalling pathways. We found that one of the genes impacted by ABCF1 silencing under VACV-70 stimulation was *TNFAIP3* (A20), an inhibitor of NF-κB and IRF-3 signalling. Therefore, we hypothesized that silencing ABCF1 in HAECs would result in a reduction of A20 protein expression, thereby intensifying the pro-inflammatory reactions mediated by NF-κB. This hypothesis was supported by a study run by Arora et al where they concluded that ABCF1 regulates the transition from MyD88- to TRIF-dependent signalling in murine macrophages under LPS stimulation[12]. Using murine bone marrow derived macrophages (BMDMs), the authors demonstrated that ABCF1 silencing with and without LPS stimulation led to decreased A20 protein expression downstream of TLR4 signalling, which led to decreased TRAF6 degradation and increased activation of NF-κB[13]. TRAF6 is an E3 ligase that activates TAK1 and IKK complex to allow for NF-κB activation [14–16]. Typically, downstream of MyD88-dependent signalling, A20 inactivates TRAF6 by removing the K63-polyubiquitination and promoting K48-polyubiquitination for proteasomal degradation[13]. Moreover, they showed that ABCF1 silencing under LPS stimulation had an increase in TAK1 phosphorylation by TAB1[12]. Whereas for TRIF-dependent signalling, they showed a decrease in TBK1 phosphorylation under the same conditions[12]. Importantly, this work was done only in murine macrophages under LPS stimulation, and it remained unknown if similar biology played out in HAECs under other TLR stimulation, such as Poly(I:C).

In our study with HBEC-6KT HAECs, we were unable to induce a strong pro-inflammatory response under LPS stimulation. Previous studies have shown that LPS is a weak stimulus for

inducing IL-8 and IL-6 in HAECs[17]. Therefore, to activate A20 and NF-κB signalling pathways, we selected TNF-α and Poly(I:C) to activate TNFR and TLR3, respectively. Our study showed that ABCF1 silencing led to no changes in the protein expression of A20, IRF-3 and NF-κB[18]. Additionally, the activation status of the transcription factors was unaffected while the induction of IL-8 was decreased significantly, and IL-6 had a decreasing trend when compared to the silencing control[18]. This suggested that ABCF1 is regulating the induction of IL-8 under TNF-α and Poly(I:C) stimulation in a pathway(s) independent of NF-κB and IRF-3 activation[18].

The findings from our study do not replicate the findings from the study by Arora et al[12]. The authors ran their study in murine BMDMs and induced a pro-inflammatory response using LPS while we were interested in characterizing the role of ABCF1 in HAECs. These two studies investigated the function of ABCF1 in different cell types, species, and stimuli. We conclude that the results and interpretations from the study by Arora et al and our study are conditional and influenced heavily by the experimental models used.

Our approaches discussed in Chapters 2 and 3 were hypothesis-driven where we hypothesized the function of ABCF1 in HAECs based on existing literature and deductive reasoning. Unfortunately, our studies have not been consistent with reports in the literature and the function of ABCF1 in HAECs remains to be fully characterized. In Chapter 4, we took a hypothesis-free approach where we explored the ABCF1 protein interactome by identifying its interacting protein partners from publicly available databases and ran a Gene Ontology (GO) enrichment analysis to generate a hypothesis, followed by an experimental *in vitro* validation. We identified a protein interacting candidate of interest, SYK, a non-receptor tyrosine kinase that is known to have a major role in

both positively and negatively regulating downstream pro-inflammatory responses following immunoreceptor activation, including TLRs[19].

In our experimental validation, we demonstrated that SYK and ABCF1 interact following Poly(I:C) stimulation, while this interaction was absent under basal conditions. This interaction is consistent with the findings reported in the study by Arora et al, where the authors observed that ABCF1 interacts with SYK to aid in K63-polyubiquitination, which then promotes the ubiquitination of TRAF3 and TRAF6[12]. This sequence of ubiquitination is essential for shifting a pro-inflammatory MyD88/NF-κB response to the anti-inflammatory TRIF/IRF-3 response in BMDMs[12]. A separate study by Lin et al demonstrated that SYK regulates the ubiquitination of TRAF3 and TRAF6 to inhibit the activation of MyD88/MAPK/NF-κB while promoting the activation of TRIF/TBK1/IRF-3 signalling downstream of TLR stimulation[19].

The interaction between ABCF1 and SYK following Poly(I:C) stimulation could close the gap between ABCF1's function and IL-8 induction discussed in Chapter 3. Since SYK is known to negatively regulate the activation of NF-κB and positively regulate the activation of IRF-3 downstream of TLR signalling, this can suggest that ABCF1 silencing can lead to a loss in SYK activity, therefore affecting the outcome of pro-inflammatory responses under TLR signalling[19]. Our study outlined in Chapter 3 showed that with ABCF1 silencing under Poly(I:C) stimulation, there was a loss of IL-8 induction independent of NF-κB and IRF-3 activation[18]. Therefore, the activity that takes place between ABCF1 and SYK would require further investigation to understand whether ABCF1 is and how it positively or negatively regulates SYK.

Primary Research Study Limitations

As with any experimental approach, the methodologies we have selected for our primary research studies have limitations that must be discussed. First, the study for Chapters 2 and 3 was hypothesis-driven, such that we generated a specific hypothesis and designed experiments to test that hypothesis based on the findings from previous studies by Lee et al and Arora et al[20]. This method has led us to derive conclusions that were not consistent with the literature, and this could be due to the use of different research models (species, cell type, and stimulation conditions) that were not translatable to our model. Therefore, in Chapter 4, we took a hypothesis-free approach where we did not rely on a specific hypothesis but rather on the data gathered from *in silico* tools to generate a hypothesis[20]. This approach allowed us to explore the function of ABCF1 independently of pre-existing bias from other studies' findings.

Secondly, we used an immortalized airway epithelial cell line, HBEC-6KT grown as submerged monolayer conditions in our *in vitro* experimental models. While primary human bronchial epithelial cells grown under air liquid interface (ALI) may possess features closer to an *in situ* airway epithelium, there is also greater heterogeneity due to donor-to-donor variability[21]. Cells grown under ALI conditions are morphologically and histologically more differentiated, and they resemble *in situ* mucosa architecture[22]. However, for our studies, we wanted to use an experimental model that is low cost, has minimal variability between passages, and allows us to generate data that is reproducible and consistent[23, 24]. Future studies with primary HAECs from well defined healthy and diseased donors grown under ALI conditions are useful additions to confirm biological mechanisms identified in more cost-effective and higher throughput cell line model systems. Additionally, while *in vivo* animal studies are valued for their integration of

multiple organ systems at the whole animal level, at present the findings from our studies require further definition before exploration in animal models[25].

Another limitation dominant throughout our studies is the reliance on ABCF1 silencing to mechanistically interrogate biological processes. At present, there are no known small molecule inhibitors of ABCF1 that are available. While we were able to achieve a sufficient knockdown to temporarily silence the gene function and observe a change in outcome measurement, siRNA has some known disadvantages including inducing off-target effects, and having poor stability or pharmacokinetic behaviour [26]. In our studies, we observed stable silencing of ABCF1 that could produce a measurable difference in cytokine releases and protein expression levels in our experimentation. However, the turnover rate of ABCF1 protein is undefined. Therefore, future approaches (discussed more below) with novel ABCF1 inhibitors or genetically modified cell lines may overcome the limitations of using siRNA.

Lastly, there are limitations with running co-immunoprecipitation using solid matrices, such as magnetic beads coated with commercially available antibodies. This includes non-specific binding of proteins to your bait due to low amounts or stringency in washes leading to false positives; low quality antibodies with lack of specificity leading to unwanted background signals or lack of available antibodies for the target protein; low yields of protein purification; imaging interference with both heavy and light chains of IgG during immunoblotting; and limited in immobilizing complexes that are stable for minutes to hours [27, 28]. Additionally, running co-immunoprecipitations followed by immunoblotting only provides static and qualitative outcomes, making it difficult to identify transient protein-protein interactions, quantify the amount of protein

interacting with the immobilized bait, as well as lose information on the kinetics of the interactions between the proteins[28, 29].

Future Directions

Our primary research outcomes have provided a rationale to pursue additional avenues that were beyond the scope of the present thesis. A particular focus in the future should explore the use of live viruses over synthetic viral analogs to activate an antiviral immune response. As previously mentioned, antiviral responses induced by viral analogs have their sets of disadvantages due to the limited immune signalling pathways that can be induced compared to a live virus[30]. For our studies extending Chapter 2, a respiratory dsDNA virus, such as adenovirus, could be explored to activate the STING/TBK1/IRF-3 signalling pathway. As a control, experiments could use a respiratory RNA virus, such as influenza A virus. These proposed additions to our studies would provide a more biologically relevant representation of the antiviral response in the HAECs and any role of ABCF1 in mediating an antiviral response.

We intended to produce a stable ABCF1 knockout cell line using CRISPR-Cas9 delivered by lentiviruses. As mentioned previously, we used siRNA knockdown as an alternative to drug inhibition. However, compared to siRNA which generates a partial gene knockout, full knockout models by CRISPR better represent the loss-of-function phenotype and can generate a homogenous phenotype for the entire population of cells[26]. Due to time and resource limitations, we were unable to generate a stable ABCF1 knockout cell line in HBEC-6KT cells.

We acknowledged the limitations of using a single cell line and have considered including other cell types in our study. By running other cell types in parallel with HAECs, we can account for biological diversity, validate our findings, as well as promote the reliability and reproducibility of our findings [31]. By including an immortalized mouse embryonic fibroblast cell line (e.g., NIH3T3) and an immortalized murine macrophage cell line (e.g., iBMDMs) in our study, we can attempt to reproduce the findings from previous studies with our experimental model, as well as account for mechanistic differences due to different gene expressions and signalling pathways. Additionally, using one cell line can lead to the generalization of the findings, therefore we would also include additional HAECs in future studies that could include Calu-3 cells.

In our study, we ran co-immunoprecipitation (co-IP) to identify protein interactors with ABCF1. However, running co-IP is limited to identifying the interaction between two known proteins in a relevant physiological condition, and the interaction must be confirmed by immunoblotting[32]. We ran our co-IPs using solid matrices, such as magnetic beads, coated with antibodies that specifically recognize ABCF1. While this technique is a simple, cost and time effective method, we have encountered issues where we experienced low protein yields, as well as non-specific binding in our isotype controls[33]. This can be attributed to the lack of availability and quality of the antibody to run our immunoprecipitation.

We have previously considered running BioID which provides a global view of the protein interactors for ABCF1 within living cells. The principle behind this method is that BioID utilizes a mutant form of a prokaryotic biotin ligase, fused to the protein of interest, to catalyze biotinylation on proteins that are near it[34]. Biotinylated proteins will then be purified using

conventional methods, such as co-IP, and analyzed by mass spectrometry[34]. The advantage of running a BioID is its ability to identify weak and/or transient interactions[34]. However, some limitations made us decide not to run it. This includes the plasmid design for the fusion protein where careful consideration must be made in deciding where to incorporate the biotin ligase into the target protein without impacting its stability or function[34]. Additionally, the plasmid must be expressed in a large population of cells that can stably express the fusion protein and produce enough of it to allow for large-scale pulldown[34]. Lastly, protein interactors that are pulldown with the fusion protein could reflect proximity but do not imply that they are directly or indirectly interacting with the target protein of interest, producing false positive results[34].

An alternative method we would have used to purify ABCF1 is to tag it with an affinity tag, followed by running a mass spectrometry to focus on specific protein interactions. While this method is like BioID, the major difference is that it does not involve catalyzing a label to nearby proteins. The advantage of using affinity tags, such as poly-histidine tags, is their availability and specificity for the target protein, as well as the strength and stability of the protein interactions to allow for sufficient protein yield to run mass spectrometry to produce quantitative measures of protein interactions [35, 36]. The limitation of using an affinity tag is that it may miss weak or transient interactions[35]. To achieve our research goals, running an affinity tag purification would be more advantageous than BioID when characterizing the protein interaction partners of ABCF1 as it enables us to study stable protein complexes under different conditions, and closely investigate specific interactions that are relevant to innate immunity based on our *in silico* analysis rather than a global view of interactions[37].

We would be interested in bridging the gap between ABCF1 and IL-8 induction through its interaction with SYK, and the downstream effects on TRAF3 and TRAF6 mediated pro-inflammatory responses. This includes silencing ABCF1 under basal and Poly(I:C) stimulation to investigate whether this negatively or positively regulates SYK and its induction of IL-8. The activity of SYK can then be measured using a peptide-based biosensor assay to detect intracellular SYK kinase activation or inhibition[35]. This assay can be coupled with a luminescent kinase assay to measure the amount of ADP formed from the kinase activity. Then we would measure TRAF activities based on its K63-polyubiquitination patterns to fully characterize the role of ABCF1, and the downstream pro-inflammatory responses mediated by SYK.

Similar to how we investigated the role of ABCF1 and SYK, we would also be interested in characterizing the interaction and activity between ABCF1 and IFI16 under dsDNA viral stimulation. IFI16 is involved in triggering host immunity against viral infection by inducing interferon stimulated genes (ISGs). It recognizes and binds to cytosolic and nuclear viral DNA, then recruits STING to activate TBK1/IRF-3 signalling cascade to induce the production of type I interferons[38]. It's been previously demonstrated that STING negatively controls IFI16 by promoting its ubiquitination for proteasomal degradation, and postulated that ABCF1 interacts with IFI16 in MEFs during an antiviral response[3, 38]. It would be interesting to investigate the role of ABCF1 with each of its interactors, SYK and IFI16, and their impact on downstream immune activities in HAECs.

Concluding Remarks

The research discussed in Chapters 2-4 included: 1) an investigation on the role of ABCF1 in mediating CXCL10 induction and antiviral gene expression to VACV-70 stimulation; 2) an investigation on the role of ABCF1 in IL-6 and IL-8 induction mediated by NF-κB and IRF-3 through TNF-α and Poly(I:C) stimulation; 3) An *in silico* analysis and experimental validation on ABCF1 and its protein interactors involved in innate immunity.

At the outset our studies focused on characterizing the function of ABCF1, few publications defined any role for this protein in innate immunity. Our interest in the role of ABCF1 in the context of immunity started based on the findings discussed by Lee et al., and that became the foundation of explorations. Using HAECs, we were unable to replicate the conclusions derived by the authors from MEFs, and therefore we used the transcriptomic data that directed us to investigate the role of ABCF1 in pro-inflammatory responses. The pro-inflammatory roles of ABCF1 in HAECs were supported in the literature by Arora et al., however, we were unable to make the same observations they made from murine derived macrophages. As a result of challenges reproducing results from existing literature, we decided to take a different approach by utilizing publicly available data on protein interactions and using that data to generate our testable hypotheses and research questions. Ultimately, we end up demonstrating the first protein-protein interaction in HAECs that links ABCF1 with SYK, unquestionably linking ACBF1 to immune responses.

Overall, the findings from each of our studies discussed in this thesis all contribute to further understanding the role of ABCF1 in innate immunity in HAECs, suggesting that it is involved in maintaining mucosal immunity in the airway epithelium. However, its exact function and

mechanism require further research to have a full grasp on the importance of ABCF1 protein expression and its role in the airway epithelium.

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Appendix

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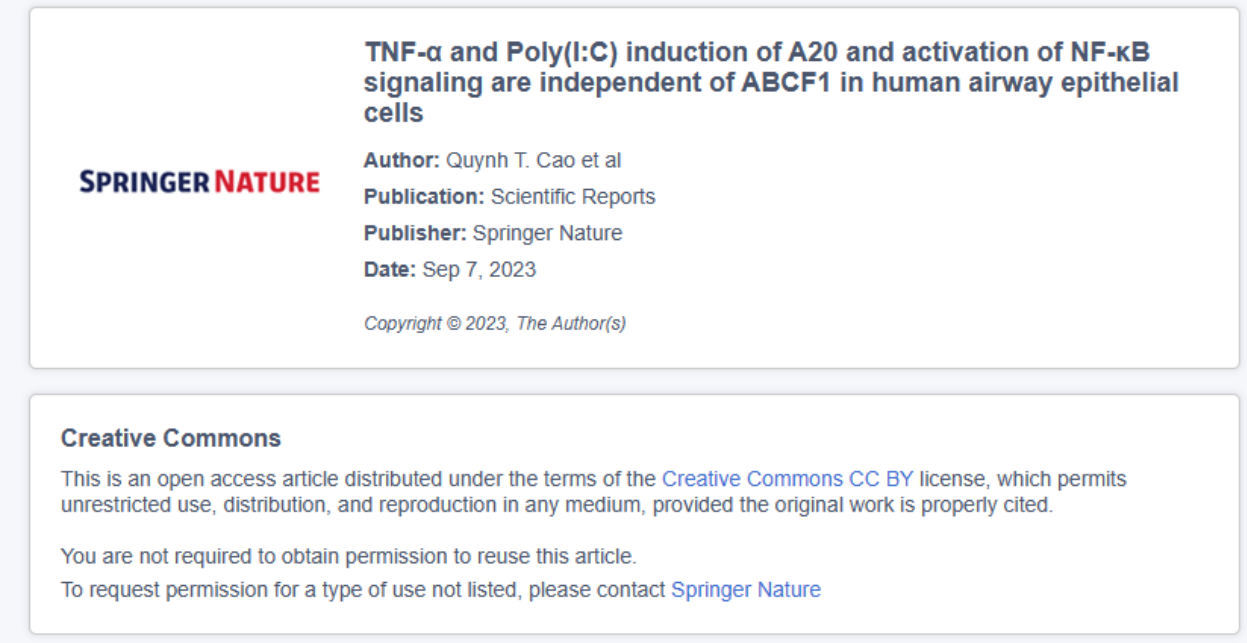

SPRINGER NATURE

TNF-α and Poly(I:C) induction of A20 and activation of NF-κB signaling are independent of ABCF1 in human airway epithelial cells

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