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Role of NOX2 and DUOX2 in the antiviral airway responses

par

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Abstract

The mucosal linings of the airways are constantly exposed to an array of microbial pathogens. During the course of respiratory viral infection, Airway epithelial cells (AEC) actively participate in the innate antiviral immune response by limiting the spread of respiratory viruses and by fostering a proinflammatory environment that attracts and activates players of the immune system. A key step in the establishment of the antiviral and proinflammatory state is the activation of Transcription Factors (TFs), such as Nuclear Factor (NF)-κB and Interferon Regulatory Factor 3 (IRF-3), which regulate the expression of antiviral and proinflammatory cytokines.

For the efficient functioning of these events, the signaling pathways involved underlie strict regulatory mechanisms. Recent data suggest that Reactive Oxygen Species (ROS), which are produced upon viral infection, are able to regulate these intracellular signaling pathways. One important source of ROS is the NADPH oxidase (NOX) family of enzymes, which is composed of NOX1-5 and Dual Oxidase (DUOX) 1 and DUOX2. The aim of our study was to identify the NADPH oxidase(s) that regulate(s) antiviral and proinflammatory mechanisms following infection of AEC with Respiratory syncytial virus (RSV), which causes major human lower respiratory tract complications, and Sendai virus (SeV), a non pathogenic virus.

During the course of our studies we identified that NOX2 is a key molecule in the early proinflammatory response to RSV and SeV infection. We demonstrate that NOX2 is necessary for the activation of NF-κB. Consequently, NOX2 impacts on the proinflammatory cytokine secretion upon AEC infection. Further, we observed that expression of the ROS-generating NADPH oxidase DUOX2 is strongly increased following infection of AEC with SeV. We identified that DUOX2 induction requires the synergistic stimulation by IFNβ and TNFα. Importantly, DUOX2 exhibited ROS-dependent antiviral action. We identified that DUOX2 was necessary for sustaining the levels of late antiviral cytokines IFNβ and IFNλ.

When AEC were infected with RSV, DUOX2 expression was barely detectable. Our data reveal that RSV has developed an evasion mechanism to counteract DUOX2 induction likely contributing to RSV pathogenicity.

In conclusion, our work demonstrates for the first time the specific implication of NOX2 and DUOX2 in the antiviral and proinflammatory response to respiratory virus infection.

Key words:

Airways, virus, innate immunity, cytokines, interferons, Reactive Oxygen Species, NADPH oxidase.

Résumé

Les voies respiratoires sont exposées à une panoplie de pathogènes. Lors d'une infection virale respiratoire les cellules qui recouvrent ces voies participent activement à la défense immunitaire contre ces derniers en limitant la propagation du virus et en engendrant une réponse proinflammatoire. Un évènement clef dans ces processus est l'activation des facteurs de transcription, notamment le « Nuclear Factor » (NF)-κB et l'« Interferon Regulatory Factor -3 » (IRF-3), qui régulent l'expression des cytokines antivirales et proinflammatoires.

Des données récentes démontrent que les dérivés actifs de l'oxygène (ROS), produits suite à une infection virale, ont la capacité de réguler les voies de signalisation enclenchées par NF-κB et IRF-3. Une source importante de ROS est la famille de NADPH oxydases (NOX), qui contient les membres NOX1-5 et DUOX1 et 2. L'objectif de notre étude était d'identifier la NOX qui régule les mécanismes antiviraux et proinflammatoires suite à l'infection avec le virus respiratoire syncytial (RSV), qui cause des complications respiratoires majeures, et le virus Sendai (SeV), un modèle viral non-pathogène.

Nos travaux ont permis d'identifier que NOX2 est une molécule clef dans la réponse proinflammatoire suite à l'infection virale. Plus spécifiquement, NOX2 est important pour l'activation de NF-κB et la sécrétion des cytokines régulées par ce dernier. De plus, nous avons observé une forte augmentation de la présence de DUOX2 dans les cellules de voies respiratoires humaines infectées par SeV. Une étude plus approfondie nous a permis de caractériser qu'une synergie entre deux cytokines secrétées lors de l'infection, soit l'interféron (IFN) β et le TNFα, est responsable de l'induction de DUOX2. Nous avons aussi découvert que DUOX2 confère une activité antivirale et est nécessaire pour maintenir les taux des cytokines antivirales tardives IFNβ et IFNλ.

Lors d'une infection avec RSV, l'induction de DUOX2 n'est pas détectable. Nous avons mis en évidence que RSV interfère avec l'expression de DUOX2 ce qui pourrait suggérer sa pathogénicité.

En conclusion, nos travaux démontrent pour la première fois une implication spécifique des NADPH oxydase NOX2 et DUOX suite aux infections virales respiratoires.

Mots clefs :
Voies aériennes, virus, immunité innée, cytokines, interférons, dérives actifs de l'oxygène, NADPH oxydase.

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Abbreviations

ADAM17	A Disintegrin and Metalloproteinase 17
AEC	Airway epithelial cells
AIR	Autoinhibitory region
ALI	Air-liquid interface
ARE	Apical recycling endosome
ASGM1	Asialo-GM1
ASL	Airway surface liquid
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BALF	Bronchoalveolar lavage fluid
c-Flip	Cellular FLICE-like inhibitory protein
CARD	Caspase recruitment domain
CBP	Creb binding protein
cDC	Conventional dendritic cell
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CH	Congenic hypothyroidism
cIAP	Cellular inhibitor of apoptosis
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
DBD	DNA binding domain
DC	Dendritic cell
DCFDA	Dichlorofluorescin diacetate
DD	Death domain
DI	Defective interfering
ds	Double strand
DUOX	Dual oxidase 1
DUOXA	DUOX activator
EGFR	Epidermal growth factor receptor
EGFα	Epidermal growth factor α
EMCV	Encephalomyocarditis virus
ENA-78	Epithelial neutrophil activating protein-78
ER	Endoplasmatic reticulum
Erk	Extracellular signal-regulated kinase
ENaC	Epithelial sodium channel
F	Fusion protein
FAD	Flavin adenine dinucleotide
FADD	FAS-associated death domain-containing protein
FI-RSV	Formalin-inactivated RSV vaccine
G	Glycoprotein
GAS	γ-interferon activated sequence
GCD	Chronic granulomatous disease

GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gro-a	Growth-regulated oncogene α
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HN	Hemagglutinin neuraminidase
HNE	Human neutrophil elastase
IAD	IRF association domain
ICAM	Intercellular adhesion molecule
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
IFN	Interferon
IFN-lR1	IFNλ receptor 1
IFNAR	IFNα receptor
IKK	IκB kinase
IL	Interleukin
IP-10	IFNγ-induced protein
IP3	Inositol triphosphate
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
JAK1	Janus kinase 1
JUNK	C-Jun-NH_2-terminal kinase
kb	kilobases
leRNA	Leader RNA
LGP2	Laboratory of genetics and physiology 2
LOX	Lipoxygenase
LPO	Lactoperoxidase
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
M	Matrix protein
MDA5	Melanoma differentiation-associated gene 5
MeV	Measles virus
MIP-1α	Macrophage inflammatory protein-1a
MMP9	Matrix Metalloprotease 9
mRNA	Messenger ribonucleic acid
MSK-1	Mitogen and stress related kinase 1
Muc	Mucin
MuV	Mumps virus
N	Nucleocapsid protein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NAP1	NAK-associated protein 1
NEBs	Neuroepithelial bodies
NEMO	NF-κB modulator
NK	Natural Killer
NLRX1	Nucleotide-binding oligomerization domain (NOD), leucine

	rich repeat containing X1
NOD	Nucleotide-binding oligomerization domain
NOX	NADPH oxidase
NS	Non structural protein
OAS1	2', 5'-oligoadenylate synthetase
$OSCN^-$	Hypothiocyanate
P	Phosphoprotein
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinosite 3 kinase
PIV	Human parainfluenza virus
PKA	Protein kinase A
PKAc	cAMP dependent protein kinase A
PKC	Protein kinase C
PKR	Protein kinase R
PMA	Phorbol-12-myristate-13-acetate
PRR	Pathogen recognition receptors
PTP1B	Protein tyrosine phosphatase 1B
RANTES	Regulated and normal T cell expressed and secreted
RD	Repressor domain
RHD	Rel homology domain
RNA	Ribonucleic acid
RIG-I	Retinoic acid inducible gene-I
RIP1	Receptor interacting protein 1
RLR	RIG-I-like receptor
RNAi	RNA interference
RNS	Reactive nitrogen species
RSV	Respiratory syncytial virus
RV	Rhinovirus
SCN^-	Thiocyanite
SeV	Sendai virus
SH	Short hydrophobic protein
SH3	C-terminal Src homology 3
SHP-2	Src-homology 2 containing protein phosphatase 2
SOCS	Suppressors of cytokine signaling
SOD	Superoxide dismutase
SP	Surfactant proteins
ss	Single-strand
STAT	Signal transducer and activator of transcription
TAB	TAK1-binding protein
TACE	TNFα-converting enzyme
TAD	Transactivation domain
TAK1	Transforming growth factor β-activated kinase 1
TANK	TRAF family member associated NF-kB activator
TBK1	TANK-binding kinase 1

TCR	T cell receptor
TF	Transcription factor
TGFα	Tumor growth factor α
Th	T helper
TJ	Tight junction
TLR	Toll-like receptor
TM	Transmembrane domain
TNFa	Tumor necrosis factor α
TNFR1	Tumor necrosis factor receptor 1
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF	TNF receptor associated factor
trRNA	Trailer RNA
TSS	Transcription star site
Tyk2	Tyrosine kinase 2
VEGF	Vascular endothelial growth factor
VEGF	Vasoactive Endothelial Growth Factor
vRNAP	Viral RNA-dependent RNA polymerase
WHO	World health organization
α	Alpha
β	Beta
γ	Gamma
ε	Epsilon
κ	Kappa
λ	Lambda
ω	Omega

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Man sieht nur mit dem Herzen gut,
das Wesentliche ist für die Augen unsichtbar.
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1 Introduction

1.1 The *Paramyxoviridae* – Respiratory viruses of etiologic importance

The *Paramyxoviridae* family contains some of the most severe respiratory viruses, whose infection might lead to respiratory tract disease. One of these is Respiratory Syncytial Virus (RSV). The first chapter of this doctoral thesis aims to introduce notions of the *Paramyxoviridae* family of viruses in terms of their family organization, virion structure, genomic organization and replication cycle. The focus will be on characteristics related to the two relevant viruses used in this study, RSV and Sendai virus (SeV).

1.1.1 The *Paramyxoviridae* family

Paramyxoviridae viruses are enveloped, negative-sense, single-stranded RNA viruses of the Mononegavirales order. The family is subdivided into two subfamilies, the Paramyxovirinae and the Pneumovirinae. The Paramyxovirinae subfamily divides itself into seven genera: Respirovirus, Rubulavirus, Morbillivirus, Avulavirus, Henipavirus, Acquaparamyxovirus and Ferlavirus (Knipe et al., 2001) (Table 1). Some important etiologic agents of this subfamily include different strains of Human Parainfluenza Virus (PIV; Respirovirus and Rubulavirus genera), Mumps virus (MuV; Rubulavirus genus), and Measles virus (MeV; Morbilivirus genus). Sendai virus (SeV), a member of the Respirovirus genus, is a murine parainfluenza virus type 1. It infects rodents and is believed to be the leading cause of pneumonia in mice (Faisca and Desmecht, 2007). In the laboratory, SeV is a prominent model in the study of the airway response to respiratory infections. The second subfamily, termed Pneumovirinae, is divided into two genera, Pneumovirus and Metapneumovirus, among which Respiratory Syncytial Virus (RSV; genus Pneumovirus) is an important etiologic agent (Table 1). RSV causes about 60% of all lower respiratory tract

infections in infants and is additionally a major cause for severe respiratory morbidity and mortality in elderly and immunocompromised individuals (Hall, 2001). It is also becoming increasingly acknowledged that RSV infection can lead to respiratory complications in healthy adults (Walsh, 2011). The World Health Organization (WHO) estimates that RSV is responsible for 64 million clinical infections and 160,000 deaths annually worldwide (Falsey et al., 2005). In the United States, RSV is the most frequent cause for hospitalization in infants (Leader and Kohlhase, 2002).

Table I: Examples of members of the *Paramyxoviridae* family

Family *Paramyxoviridae*	
Subfamily Paramyxovirinae	
Genus Rubulavirus	Mumps virus (MuV)
	Human parainfluenza virus type 2, type 4a and 4b
Genus Avulavirus	Newcastle disease virus (NDV)
Genus Respirovirus	Sendai Virus (SeV)
	Human parainfluenza virus type 1 and type 3 (hPIV1/3)
Genus Henipavirus	Hendra virus (HeV)
	Nipah virus (NiV)
Genus Morbillivirus	Measles virus (MeV)
Subfamily Pneumovirinae	
Genus Pneumovirus	Human respiratory syncytial virus (hRSV)
Genus Metapneumovirus	Human metapneumovirus (hMPV)

1.1.2 RSV and SeV virion structure

Paramyxoviridae are pleiomorphic in structure – both spherical and filamentous forms of the virus have been observed (Figure 1) (Bachi and Howe, 1973). *Paramyxoviridae* contain a lipid bilayer envelope, which is derived from the plasma membrane of the host cell during viral budding (Harrison et al., 2010). Inserted into this membrane envelope are two surface proteins: Glycoprotein (G) and Fusion protein (F) for RSV; and Hemagglutinin Neuraminidase (HN) and F protein for SeV (Figure 2). These membrane proteins serve two principal functions: first, viral attachment, and second, viral entry (Chang and Dutch, 2012). It is noteworthy to mention that RSV possesses a third membrane protein, the short hydrophobic protein (SH), which does not have a function in attachment or fusion. Inside the virion lies the nucleocapsid core, which contains the RNA genome surrounded by the Nucleocapsid protein (N), complexed to the Phosphoprotein (P) and the Large protein (L), the latter two forming the viral RNA-dependent RNA polymerase (vRNAP). Between the envelope and core, just below the inner membrane layer, lies the viral Matrix (M) protein, which is vital in determining virion architecture, and which is released from the core during virus entry. In addition, the viral M protein plays a central role in viral budding (Knipe et al., 2001).

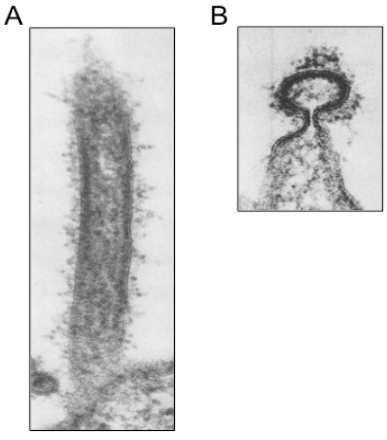

Figure 1: RSV pleiomorphic forms.
Electron microscopy images of filamentous (A) and spherical (B) RSV virions budding from Vero cells. From (Bachi and Howe, 1973), with permission from the American Society for Microbiology.

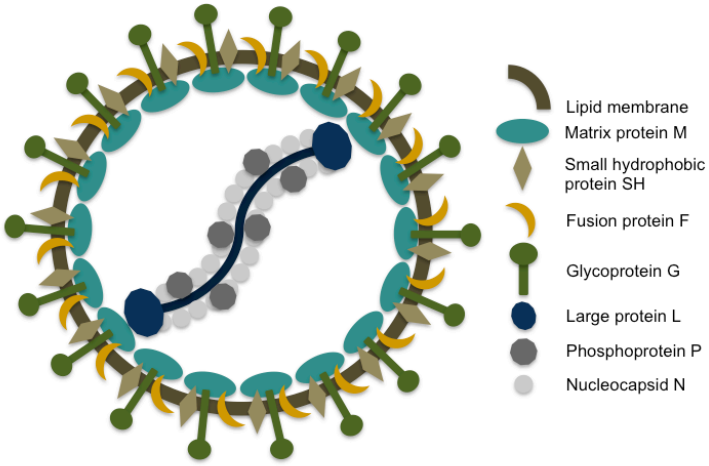

Figure 2: RSV virion structure.

Schematic representation of the RSV virion structure. The viral envelope is made up of the plasma membrane into which viral proteins F, G and SH are inserted. Beneath the membrane layer lies the M protein. The nucleocapsid is made up of the viral genome that is tightly encapsidated by N. N is complexed to P and L proteins that make up the vRNAP. Adapted from (Knipe et al., 2001).

1.1.3 RSV and SeV genome organization

The *Paramyxoviridae* genome is a negative-sense, single-strand genome of 15-19 kb that encodes 6 and 10 viral genes for SeV and RSV, respectively (Figure 3). The genome generally possesses a 3'-extracistronic region of approximately 50 bp, called the "Leader" region, and a 5'-"Trailer" region of 50-161 bp. Between genes, intergenic sequences are found that serve as termination and initiation sequences in mRNA synthesis (Knipe et al., 2001). The SeV genome encodes for the following proteins in a 3'-5' order: 3'-N-P/C/V-M-F-HN-L-5'. P, C and V proteins are encoded from one P gene whose mRNA has several open reading frames. In contrast, the RSV genome encodes for 11 proteins in the following 3'-5' order: 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'. The M2 gene is transcribed into an mRNA with two overlapping reading frames

resulting in the synthesis of two proteins, M2-1 and M2-2 (Knipe et al., 2001). RSV and SeV also code for accessory proteins that are not present in the infecting virion but have important roles as inhibitors of the host antiviral defense. Notably, NS1 and NS2 proteins of RSV, and V and C proteins of SeV can foster evasion of the antiviral response (Gotoh et al., 2001).

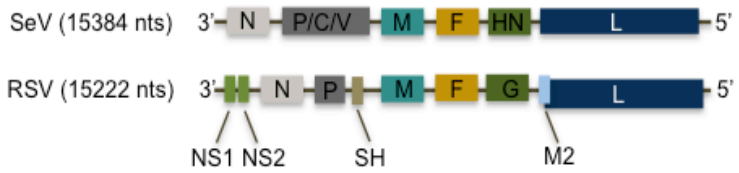

Figure 3: SeV and RSV genome.
Schematic representation of the SeV and RSV genome. For explanation, see RSV and SeV genome organization. Adapted from (Knipe et al., 2001).

1.1.4 RSV and SeV replication cycle

1.1.4.1 Virus attachment and entry

Before infection of the host cell can occur, virus particles have to attach to their target cells, which brings the virion and host cell into close proximity. Then *Paramyxoviridae* entry into the host cell is mediated by fusion of the virus with the cellular membrane (Figure 4). These steps, attachment and entry, are assured by the viral attachment and fusion proteins G and F for RSV, and HN and F for SeV. In order for attachment to occur, certain viruses will attach to cell surface molecules found on the exterior of the host cell. This is the case for SeV, as SeV HN protein attaches to

sialic acid containing cell surface glycoproteins and glycolipids (Villar and Barroso, 2006). RSV, on the other hand, attaches to glycosaminoglycans containing the disaccharide heparan sulfate and chondroitin sulfate via the G protein (Feldman et al., 1999; Krusat and Streckert, 1997). After attachment, the F protein initiates the fusion process, whereby the nucleocapsid is delivered into the cytoplasm. A recent publication has demonstrated that cholesterol-rich lipid raft domains are important for RSV entry into airway epithelial cells (AEC) (San-Juan-Vergara et al., 2012). Their viral entry can occur either through receptor-mediated endocytosis or direct penetration at the plasma membrane. Both processes have been described in the case of SeV and RSV infection (Kolokoltsov et al., 2007; Rasmusson et al., 1998; Srinivasakumar et al., 1991). In the context of RSV fusion with the plasma membrane, the protein nucleolin has recently been decribed as a novel fusion receptor as it interacts with RSV-F at the cell surface and is essential for efficient RSV infection (Tayyari et al., 2011).

1.1.4.2 Viral mRNA synthesis

Once the virion is released into the cytoplasm, the next strategic step in the virus cell cycle is the generation of viral proteins that will provide building material for new virions. Some viral proteins also serve to interfere with the host metabolism in order to increase the potential for efficient viral replication. The *Paramyxoviridae* life cycle is entirely cytoplasmic (Figure 4). The virus brings its own vRNAP, which is a complex consisting of a tetramer of P proteins and one L protein. P proteins serve to recruit L proteins to the viral genome, which is strongly encapsidated by the N protein (Cowton et al., 2006). This polymerase transcribes the incoming negative sense genome into 5'-capped, 2-O-methylated and 3'-polyadenylated mRNAs, an activity that is assured by the L protein (Liuzzi et al., 2005). All viral RNA synthesis begins at the 3' end of the genome, where a cis-acting promoter sequence initiates the synthesis of short, non-coding leader RNAs. At the beginning of the viral cycle, vRNAP is restricted to the production of these leader RNAs and thereafter mRNAs (Cowton et al., 2006). Scrolling down the viral genome, vRNAP terminates and reinitiates at each gene junction, thereby generating an mRNA transcript for each encoded gene. However,

vRNAP does not always reinitiate mRNA synthesis at the next gene junction, as it has a tendency to dissociate from its template at these sites. This in turn leads to a decreasing gradient of mRNA abundance from the genome's 3' end onward. This gradient of mRNA results in defined quantities of the viral N protein, which determine the balance between mRNA synthesis and genome replication. Once a sufficient amount of N protein is present in the cell, viral mRNA synthesis stops and vRNAP engages into the viral replication process (Knipe et al., 2001). It is noteworthy to mention that the RSV accessory M2-1 protein interacts with P and viral RNA. M2-1 has been shown to be an essential transcription elongation factor, because in its absence RSV does not transcribe beyond the NS1 and NS2 genes (Fearns and Collins, 1999). M2-2 is non-essential for viral growth but may be involved in the regulation of balancing viral transcription and viral replication (Bermingham and Collins, 1999).

1.1.4.3 Genome replication

The *Paramyxoviridae* negative-sense genome replicates via an intermediary full-length complementary copy called the antigenome. For antigenome synthesis, vRNAP copies the negative sense genome but ignores all of the junctional signals and editing sites. It synthesizes an exact complementary positive-sense antigenome that becomes immediately bound by N proteins. This antigenome serves as a template for genome replication. The 3' end of the antigenome contains a promoter that is recognized by vRNAP, which initiates synthesis of the genome in a manner similar to antigenome synthesis, i.e. ignoring all gene junctions (Cowton et al., 2006). Under conditions where sufficient unassembled N protein is present, encapsidation of the nascent genome prevents termination and leads to the synthesis of an encapsidated minus-strand genome.

1.1.4.4 Virion assembly and virion release

Once the viral genome has replicated, sufficient building blocks for new virion assembly have been synthesized and nucleocapsid assembly is terminated. Virion

assembly can therefore begin and will culminate in the release of new virions. Nucleocapsids are assembled in two steps: first, free N subunits associate with the genome to form the helical ribonucleoprotein structure of viral genomic RNA and N; second, the RNA:N complex associates with the P and L protein complex. Virion assembly then takes place at the cell surface (Harrison et al., 2010). The viral integral membrane proteins are synthesized in the ER and undergo maturation or glycosylation in the Golgi complex before being transported through the secretory pathway and integrated into the cellular membrane. The viral M protein associates with these transmembrane glycoproteins at locations where budding occurs. The clear mechanism of assembly at the membrane is still unknown but the viral M protein is thought to play a major role in bringing the nucleocapsid to the plasma membrane to finalize the formation of the budding virion (Ghildyal et al., 2006). In this process, viral proteins also interact with host cellular factors and these interactions will drive viral budding. For instance, M protein, as well as SeV C protein, interacts with cellular molecules of the vacuolar sorting pathway to organize the assembly of the nascent virion (Irie et al., 2007; Sakaguchi et al., 2005). For SeV and RSV it has been shown that viral budding preferentially takes place at apical side of AEC (Zhang et al., 2002). In this context, the cellular apical recycling endosome (ARE) plays an important role (Brock et al., 2003).

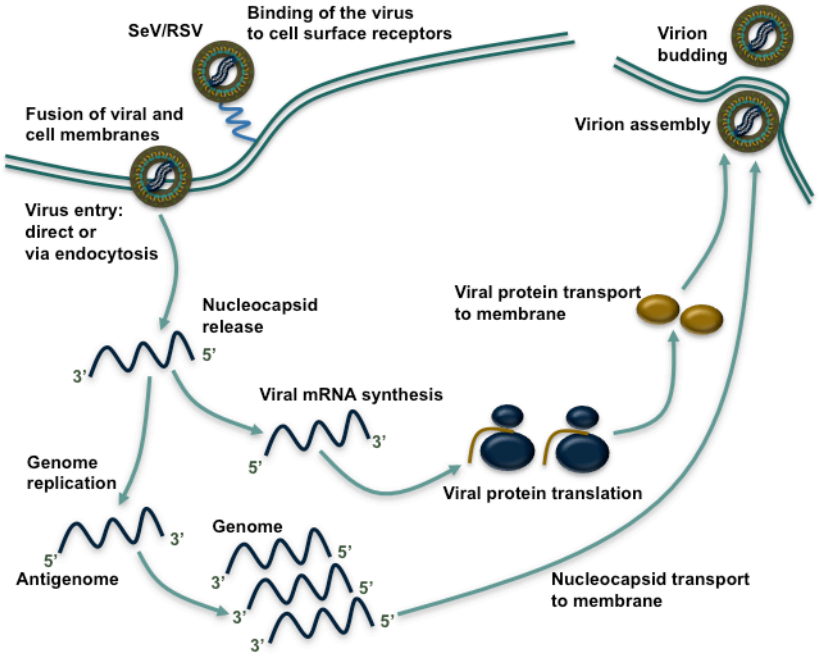

Figure 4: The *Paramyxoviridae* replication cycle.

Following viral entry, the negative-sense viral genome serves as a template for viral mRNA synthesis by the vRNAP. Viral protein synthesis then occurs. When enough building blocks have been generated, viral genome replication begins. The negative-sense viral genome is generated by the vRNAP via a positive-sense antigenome intermediate. Viral genomes are rapidly encapsidated and transported to virion assembly sites at the membrane. Following assembly, virions are released by budding from the plasma membrane.

1.2 The airway response to respiratory virus infection

The airways are a complex barrier that separates the exterior environment with its hazardous components from our body. The following chapter will introduce airway physiology and airway defense strategies that serve to minimize or combat respiratory infections. These mechanisms range from general airway cell defense mechanisms to the specific detection of viral pathogens, leading to the initiation of a more global airway immune response. Lastly, *Paramyxoviridae* evasion of these mechanisms and resulting pathology will be discussed.

1.2.1 Airway physiology at the site of infection

The classical lung anatomy model consists of 24 generations of dichotomously branching airway tubes, where each tube divides into two smaller airways. At each division, the diameter of the airway decreases (Weibel, 1963). From the trachea to the bronchi (generation 0-10), the airways form a moderately tight pseudo-stratified ciliated columnar epithelium, exhibiting Transepithelial Electric Resistance (TEER) measurements of approximately 300 Ωcm^2 (Wijkstrom-Frei et al., 2003). The main purpose of the epithelium in these proximal airways is to filter particles and kill invading microbes. The three main cell types present in this section of the airways are: ciliated cells, goblet cells, and basal cells. The main function of ciliated cells is to propel foreign particles and organisms out of this airway region via the mucociliary clearance mechanism. The goblet cell, which extends from the larger to the smaller bronchi, but is absent in the bronchioles, contains abundant granules for mucus secretion. Basal cells are the stem cell-like progenitor cells of the surface epithelium, and all ciliated and non-ciliated cells in the bronchial mucosa are derived from basal cells. Basal cells can survive mucosal injury, and consequently serve in the reconstruction of the bronchial mucosa (Fischer, 2009). The epithelium is covered with

a layer of airway surface liquid (ASL). The ASL is made up of two phases, the first of which is a periciliar layer of thin fluid film, into which the cilia beat at a rate of 12-16 beats/second (Ng et al., 2004; Rutland et al., 1982; Tomashefski et al., 2008). Glands which are present in the submucosa of the trachea and bronchi, and which contain mucus and serous cells, contribute to the secretion of this mucus layer. The second phase is a high-viscosity upper mucus layer, which originates from submucosal glands, but also from goblet cells. The ASL contains a variety of molecules that serve in airway antimicrobial defense (Ganz, 2002) (Vareille et al., 2011).

The membranous bronchioles are located in the descending airways. Here, the diameter of the airway decreases to about 1 mm (Tomashefski et al., 2008). Membranous bronchioles are lined by ciliated columnar epithelial cells and non-ciliated Clara cells. Clara cells replace the disappearing goblet cells. The Clara cell also contains an abundance of secretory granules that primarily secrete antimicrobial and immunomodulatory surfactant proteins (SP). Due to its function as a reserve and reparatory cell, the Clara cell replaces the basal cell in the bronchioles. The terminal bronchiole leads into the acinus, the functional unit of the lung, which consists of the respiratory bronchiole, the alveolar ducts, the alveolar sac and the alveoli. The alveolar sac and the alveoli are the site of gas exchange. The alveoli are made up of large, thin type I cells, which only account for 40% of the alveolar lining cells, but cover 90% of the alveolar surface; and small cuboidal type II cells, which constitute 60% of the surface cells, but cover only 5% of the alveolar surface (Tomashefski et al., 2008). Besides secreting surfactant, type II cells also function as a reserve cell, as they can mature into type I cells. In the alveolar sac, the epithelium is very tight and has TEER measurements of more than 2,000 Ωcm^2 (Fischer et al., 2007). The alveoli are lined with a very thin fluid film and, as a result, antimicrobial factors secreted in this zone are highly concentrated.

For an efficient gas exchange of oxygen and carbon dioxide between air spaces and red blood cells, the alveolar arrangement is ideal. Alveolar interstitial cells are tightly surrounded by capillaries. The endothelial cell and epithelial alveolar type I cell cytoplasm is spread as thinly as possible, which leads to an air-blood distance of approximately 0.6 µm. At any given moment, about 200 mL of blood can be found

within the capillary network that spreads over 126 m^2, which is equivalent to a 1.6 mL spread(Starosta et al., 2006) over 1 m^2 (Tomashefski et al., 2008). The pulmonary endothelium of the alveolus, which occupies a surface area of more than 140 m^2, is the largest and densest in the human body. Endothelial cells are connected to each other by loose junctions, which readily allow the passage of fluids, macromolecules and immune cells into the interstitial compartment (Tomashefski et al., 2008).

Leukocytes are continuously present in the airways, and in normal, non-inflamed lungs the majority of them are macrophages. More specifically, in Bronchoalveolar Lavage Fluid (BALF) of healthy individuals, 94% of all leukocytes were found to be macrophages, followed by lymphocytes (4%), neutrophils (1%), and eosinophils (0.7%). Plasma cells, basophils and mast cells can also be found in these compartments at very low numbers

1.2.2 Airway defense mechanisms

The air we breathe carries many potentially harmful microbes. To protect us against them, the lung harbors an array of defense mechanisms. On the one hand, the composition and tightness of the airways provide anatomic defense mechanisms, due to the presence of tight junction barriers that make it difficult for pathogens to cross into the interstitial space (Bergelson, 2009). The continuous beating of ciliated AECs also contributes to such anatomical defense mechanisms. On the other hand, factors secreted from AECs into the ASL are known to have potent antimicrobial or immunomodulatory functions. These factors include antimicrobial proteins and peptides, which serve to eliminate the invading pathogen (Figure 5) (Tomashefski et al., 2008). Factors like mucins, surfactant proteins, lactoferrin and human β-defensins have been shown to play roles in AEC infection by respiratory viruses, such as RSV (Grover et al., 1997; Kota et al., 2008; LeVine et al., 1999; Vareille et al., 2011). Cytokines and chemokines that in turn attract innate and adaptive immune cells in the fight against an infection are also secreted from AECs. More specifically, via the

secretion of antiviral cytokines, such as type I and III interferons (IFNs), AECs are able to alarm the surrounding cells and signal the presence of a viral pathogen. Via the secretion of proinflammatory cytokines, such as Tumor Necrosis Factor α (TNFα), and chemokines, such as Regulated And Normal T cell Expressed and Secreted (RANTES)/CCL5, into the submucosa, AECs are able to call for "back-up" and recruit and activate players of the innate and adaptive immune response axes, which work together to eliminate infection (Figure 6)(Vareille et al., 2011). The following paragraphs will discuss the nature and function of these cytokines and chemokines in more detail, with an emphasis on their role in respiratory virus infection.

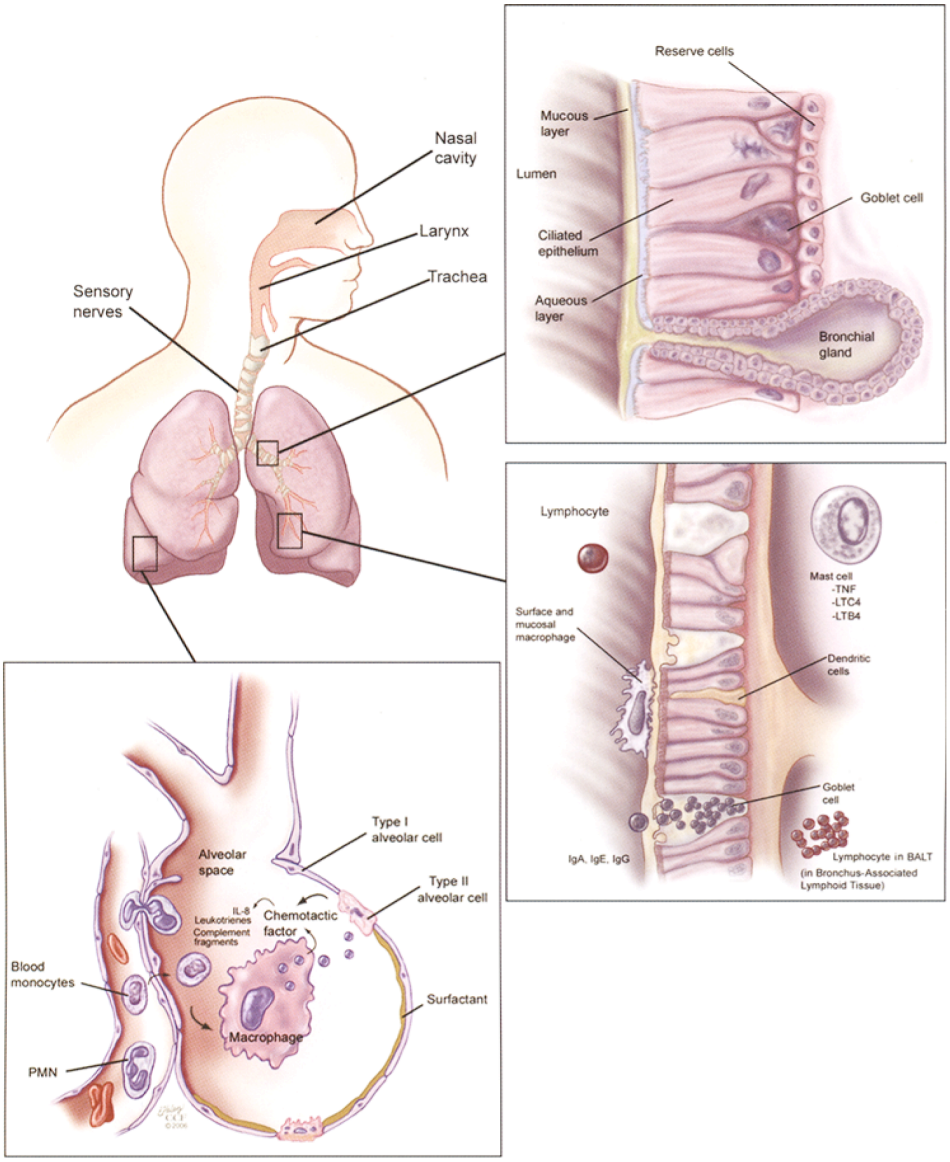

Figure 5: Summary of pulmonary physiology and defenses.

Airway defenses in the trachea and bronchi include cough reflexes and mucociliary clearance. Further, soluble antimicrobial mediators, antibodies as well as innate and adaptive immune players are present. Alveolar defenses rely on antimicrobial properties of surfactant and initial clearance by alveolar macrophages; if needed, additional inflammatory cells are recruited by chemoattractants produced by macrophages and epithelial cells. From (Tomashefski et al., 2008). With permission from Springer Verlag-Gmbh.

Type I and III IFNs

Following respiratory virus infection, AECs secrete considerable amounts of type I IFN, which is composed of 13 different IFNα species, IFNβ, IFNω, IFNκ, and IFNε (Ioannidis et al., 2012; Pestka et al., 2004). Additionally, AECs are considered to be a major source of the newly recognized type III IFNs, which include IFN-λ1/IL29, IFN-λ2/IL-28A and IFN-λ3/IL-28B (Kotenko et al., 2003; Okabayashi et al., 2011; Sheppard et al., 2003). Type I and type III IFNs trigger similar intracellular signaling pathways and biological activities (Zhou et al., 2007). As will be discussed below in more detail, IFNs induce the Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) signaling cascade, which culminates in the transcription of a vast quantity of Interferon Stimulated Genes (ISGs) that are responsible for establishing an antiviral state (Stark and Darnell, 2012). Whereas type I IFNs execute their antiviral functions on diverse subsets of cells due to the ubiquitous presence of IFNα receptor (IFNAR), the primary targets of type III IFNs are epithelial cells of airway mucosal sites since IFNλ recetor 1 (IFN-λR1) chain is primarily expressed on epithelial cells (de Weerd and Nguyen, 2012; Mordstein et al., 2008; Mordstein et al., 2010).

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Proinflammatory cytokines and chemokines

Aside from secreting IFNs, AECs respond to viral infection by releasing a vast number of different proinflammatory cytokines, which are crucial for the activation of the innate and adaptive immune responses.

Neutrophils are the first responders recruited into the infected airways following viral infection and their recruitment is mediated by the secretion of interleukin (IL)-8/CXCL8, Growth-regulated oncogene α (Gro-α)/CXCL11 and Epithelial Neutrophil Activating protein-78 (ENA-78)/CXCL5 from AECs (Message and Johnston, 2004; Vareille et al., 2011). The expression of these cytokines has been observed in RSV- and SeV-infected AECs (Oshansky et al., 2010; Villenave et al., 2010; Zhang et al., 2001a). Neutrophil survival and activation is assured by AEC-secreted granulocyte

colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

RANTES/CCL5, together with IL-5, GM-CSF, eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26, recruits eosinophils to the infected site (Gleich, 2000). As with IL-8, RANTES chemokine levels following RSV infection correlate with disease severity (Hornsleth et al., 2001).

One further AEC-secreted cytokine that has been implicated in the recruitment of innate immune players to the lung is macrophage inflammatory protein-1α (MIP-1a)/CCL3, which can recruit macrophages and Natural Killer (NK) cells (Biron et al., 1999). Macrophages in turn find themselves activated by AEC-secreted IL-1β and TNFα (Message and Johnston, 2004). Conventional dendritic cells (cDCs) are recruited via MIP-3α/CCL20 secretion (Gill et al., 2005). Additionally, type I IFNs drive local DC differentiation and maturation. A second subset of DCs, plasmacytoid DCs (pDCs), is also recruited into the lung upon viral infection. The presence of pDCs is important for viral clearance as they are major IFNα producers (Smit et al., 2006).

T and B lymphocytes constitute the cellular and humoral axes of the adaptive immune response, respectively. Respiratory virus infection generally induces an important cellular immune response of $CD4^+$, $CD8^+$ and γδ-T cells (Braciale et al., 2012). $CD8^+$ cytotoxic T cells play a crucial role in the clearance of viral infection. AECs can induce the migration of the $CD4^+$ T helper 1 (Th1) subset of T cells to the mucosa via production of RANTES/CCL5 and IFNγ-induced protein (IP-10)/CXCL10, and that of $CD4^+$ Th2 subset of T cells via production of IL-1β (Vareille et al., 2011). Th1 cells in turn produce IFNγ, IL-2, IL-12 and TNFα, which all contribute to efficient cellular virus clearance, whereas Th2 cells produce IL-4, IL-10 and IL-13, which play an important role in humoral immunity against viruses (Vareille et al., 2011).

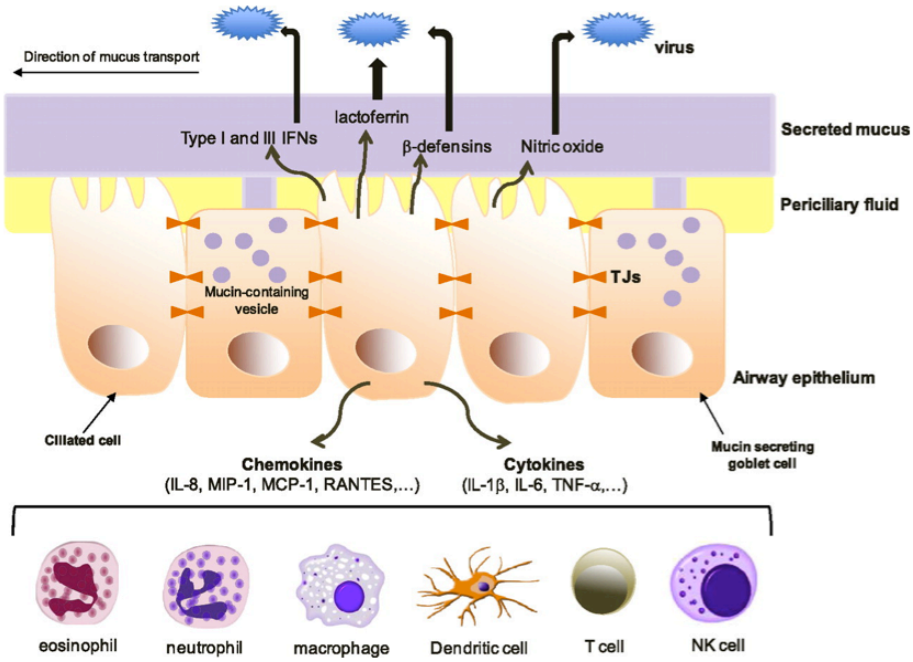

Figure 6: AEC defense mechanisms against respiratory viruses.

AEC act as a barrier against respiratory viruses. The mucociliary apparatus and tight junctions (TJs) add mechanical, biological, and chemical protection. The airway epithelium also regulates both innate and adaptive immune responses, through production of antiviral substances such as IFNs, lactoferrin, and β-defensins in the mucus layer and production of cytokines and chemokines, which recruit and activate immune cells in the submucosa. From (Vareille et al., 2011), with permission from the American Society for Microbiology.

1.2.3 The AEC response to viral infection

The above paragraphs discussed the secretion of cytokines from the airway epithelium. This cytokine secretion is specifically induced upon detection of the invading pathogen by the AEC. The following sections will elaborate the essential events that culminate in this cytokine secretion from virus-infected airway epithelial

cells. These events constitute the core mechanism that drives innate and adaptive immunity – viral detection, which via the activation of specific signal transduction pathways leads to the activation of transcription factors (TFs), such as Interferon Regulatory Factor-3 (IRF-3) and Nuclear Factor-κB (NF-κB). These in turn regulate the gene expression of essential antiviral and proinflammatory cytokines and chemokines. The downstream molecular changes induced by these secreted factors on the airway epithelium and innate immune players will also be discussed. In order to convey only the most relevant knowledge to the understanding of this thesis work, the following section will focus mainly on the events of viral recognition and the antiviral and proinflammatory responses to RSV and SeV infection, since viruses of other families are recognized and signal in quite distinct patterns to induce antiviral and proinflammatory responses that are tailored to defending against them.

1.2.3.1 Viral recognition by Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs)

Recognition of a virus by infected cells requires detection of the viral genome or of its proteins. The intracellular RLR family, comprised of RIG-I, Melanoma differentiation-associated gene 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2) belongs to a series of intracellular Pathogen recognition receptors (PRRs) that detect viral genomes and initiate antiviral responses (Baum and Garcia-Sastre, 2010; Yoneyama et al., 2004). RIG-I has a central DExD/H-box RNA helicase domain, which serves in RNA binding and unwinding, and two N-terminal Caspase recruitment domains (CARDs), which are essential for the initiation of downstream signaling. Additionally, a C-terminal repressor domain (RD) can be found. The domain makeup of MDA5 is similar to that of RIG-I, with the exception that MDA5 does not contain a RD (Yoneyama et al., 2005). LGP2 also possesses helicase domains, but lacks the CARD domains present in RIG-I and MDA5. Several data have suggested that it is a regulator of RLR-signaling triggered by RIG-I or MDA5, although LGP2 has recently been implicated in the antiviral response following encephalomyocarditis virus

(EMCV) infection (Eisenacher and Krug, 2012; Venkataraman et al., 2007). RIG-I signaling activity, unlike that of MDA5, is autoregulated via intramolecular interactions between the CARDs and the RD (Saito et al., 2007). In the absence of an RNA ligand, RIG-I is inactive due to the interaction of these two domains, which free themselves following conformational changes only upon ligand binding. Once in an open confirmation, RIG-I becomes active and the CARDs are free to mediate the association with the downstream adaptor protein Mitochondrial antiviral signaling protein (MAVS) (also known as IFNβ promoter stimulator 1 (IPS-1), Cardif, or Virus-induced signaling adapter (VISA)) (Meylan et al., 2005; Saito et al., 2007; Seth et al., 2005; Xu et al., 2005). MAVS assembles into prion-like aggregates necessary for antiviral signaling (Hou et al., 2011). In this context, MDA5 has been demonstrated to form polar helical filaments on the bound RNA molecules, which nucleates the formation of these MAVS aggregates (Berke et al., 2012).

Generally, RIG-I and MDA5 recognize distinct RNA structures, which leads to the detection of specific viruses (Kato et al., 2006; Loo et al., 2008). However, some viruses can be detected by several different PRRs (Kato et al., 2006; Loo et al., 2008). Recognition by RIG-I is selectively activated by double-stranded (ds)RNA molecules with an uncapped 5'-triphosphate, whereas MDA5 preferentially recognizes longer dsRNA molecules (Hornung et al., 2006; Kato et al., 2008; Pichlmair et al., 2006). Branched higher order dsRNA structures and the absence of 2'O-methylation at the 5'-Cap are also recognized by MDA5 (Pichlmair et al., 2009; Zust et al., 2011). Moreover, sequence composition of the RNA molecule has been shown to be an important determinant in RNA recognition by these sensors. Indeed, PolyU/UC sites, which are polyuridine motifs that contain interspersed C nucleotides, are also important for the activation of RIG-I signaling (Hornung et al., 2006; Kato et al., 2008; Pichlmair et al., 2006). These overall sensing modalities are thought to restrict the detection of nucleid acids by these PRRs of foreign, non-self origin and prevent them from responding to host encoded genes (self). In terms of *Paramyxoviridae* infection, it is generally acknowledged that RIG-I and MDA5 play a role in detecting and initiating the AEC antiviral response to RSV and SeV infection (Gitlin et al., 2010; Kato et al., 2005; Loo et al., 2008; Soucy-Faulkner et al., 2010; Yoboua et al., 2010). However,

which exact RNA motifs are responsible for RIG-I and MDA5 activation in their case remains a matter of debate. Although the study of Rehwinkel *et al.* suggests that SeV genomic RNA could be the RIG-I ligand leading to IFNβ activation, this event is rather unlikely *in vivo* considering the tight encapsidation of the *Paramyxoviridae* genome by the N protein (Gerlier and Lyles, 2011; Rehwinkel et al., 2010). Baum *et al.* have suggested that Defective interfering (DI) genomes, which result during genome replication due to vRNAP jumping from one template to another and thereby generate short genomes with deletions, as well as antigenomes, could be the RIG-I ligand in SeV infection (Baum et al., 2010). Small leader RNAs (leRNA) and trailer RNAs (trRNA) synthetized during viral RNA transcription could also be RIG-I ligands, as these structures possess 5'-triphosphate motifs (Gerlier and Lyles, 2011). If any of these mentioned molecules fail to become encapsidated, they then form secondary structures that are readily recognizable by RIG-I or MDA5, which then activate antiviral signaling as discussed below.

1.2.3.2 IRF-3 and NF-κB activation in RLR signaling lead to antiviral and proinflammatory cytokines production

The production and secretion of type I IFNs, which drives the establishment of the antiviral state, is the culminating event of the RLR signaling cascade. NF-κB, in combination with IRF-3, ATF-2 and c-Jun, the latter two forming the AP-1 transcription factor, and the transcriptional enhancer Creb binding protein (CBP)/p300 form the enhanceasome that directs IFNβ transcription (Panne 2008). Less is known about the activation of AP-1 following viral infection, and this section will thus focus on the events that culminate in the activation of IRF-3 and NF-κB TFs. These TFs are also implicated in the regulation of type III IFN expression (Onoguchi et al., 2007; Osterlund et al., 2007; Thomson et al., 2009). It is generally thought that IFNβ and IFNλ1 are among the first IFN species to be induced upon viral infection, and that they are heavily dependent on IRF-3. The transcription of further IFNα and IFNλ2–3 genes requires the IFN-mediated induction of another member of the IRF family, IRF-7. The

IRF-3/IRF-7 heterodimers formed subsequently regulate the expression of further IFNα and IFNλ2–3 species, thereby amplifying the IFN-induced antiviral response (Figure 7).

Once RIG-I and MDA5 have bound viral RNA, these proteins interact with the downstream adaptor protein MAVS to form a MAVS-signalosome for further triggering of the antiviral signaling pathway (Belgnaoui et al., 2011). MAVS localizes to the mitochondria via a single-spanning Transmembrane domain (TM). Via its CARD domain, which protrudes into the cytoplasm, it interacts with RIG-I or MDA5 (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). RIG-I or MDA5 interaction with MAVS initiates the recruitment of the MAVS-signalosome and approximately 30 MAVS-interacting partners have been described to date (Belgnaoui et al., 2011). Interacting proteins are involved in antiviral and proinflammatory responses, but MAVS also interacts with mitochondrial proteins, as well as with proteins involved in cell death and autophagy. It is noteworthy to mention that, besides being located at the mitochondria, MAVS can also be found at peroxisomes, where it functions in the induction of ISGs independently of type I IFN (Dixit et al., 2010). These two distinct intracellular pools of MAVS have been shown to be necessary for efficient clearance of viral infection.

Once MAVS homo-oligomerizes, it recruits members of the Tumor necrosis factor receptor-associated factor (TRAF) family: TRAF3 and TRAF6. These are E3 ubiquitin ligases that assemble lysine 63-linked polyubiquitin chains, which constitute an important docking site for downstream signaling molecules (Belgnaoui et al., 2011). TRAF3, in complex with NF-κB modulator (NEMO), TRAF family member associated NF-κB activator (TANK) and NAK-associated protein 1 (NAP1), controls the activity of two non-canonical IKK-related kinases, TANK-binding kinase 1 (TBK1) and inducible Inhibitor of κB (IκB) kinase (IKKi/IKKε), which phosphorylate the transcription factor IRF-3 (as will be discussed below) (Guo and Cheng, 2007; Sasai et al., 2006; Zhao et al., 2007). MAVS interaction with TRAF6 forms a complex consisting of Transforming growth factor beta-activated kinase 1 (TAK1) and TAK1-binding protein 2 and 3 (TAB2 and TAB3), which dock onto the by TRAF6 generated

lysine K63-linked polyubiquitin chains. This leads to the activation of the IKK complex and consequent NF-κB activation. In addition, FAS-associated death domain-containing protein (FADD) has been identified in a complex with MAVS, and a FADD/caspase-8-dependent pathway has been proposed to be required for the activation of NF-κB downstream of MAVS (Kawai et al., 2005; Takahashi et al., 2006). Tumor necrosis factor receptor type 1-associated death domain protein (TRADD), an adaptor of the tumor necrosis factor receptor (TNFRI), is further recruited to MAVS and orchestrates the formation of a complex with TRAF3, TANK, FADD and Receptor interacting protein 1 (RIP1), which leads to the activation of IRF3 and NF-κB (Michallet et al., 2008).

IRF-3 activation. IRF-3 is a ubiquitously expressed TF. It is crucial in the early antiviral response due to its function in the regulation of genes such as type I and type III IFNs, but also IFN-independent ISGs as well as proinflammatory cytokines such as RANTES and IL-6 (Grandvaux et al., 2002; Lin et al., 1999; Matsukura et al., 2006). IRF-3 possesses an N-terminal DNA binding domain (DBD) and a C-terminal domain, termed the IRF association domain (IAD), which serves in homo- or heterodimerization with members of the IRF family. Transcriptional activity of IRF-3 is controlled by C-terminal phosphorylation events in three clusters: Ser385 and Ser386 (cluster 1), Ser396 and Ser398 (cluster 2), and Ser402, Thr404, and Ser405 (cluster 3) (Lin et al., 1998). The IKK-related kinases TBK-1 and IKKε have been shown to be responsible for phosphorylation of residues Ser386, Ser396 and Ser402 of IRF-3 (Fitzgerald et al., 2003; Fujii et al., 2010; Mori et al., 2004; Sharma et al., 2003). C-terminal IRF-3 phosphorylation consequently induces a conformational change in IRF-3 that allows homo- or heterodimerization, nuclear localization, binding to IRF-3 or Interferon stimulated response element (ISRE) target sequences, and association with the co-activator CBP/p300 (Lin, Hiscott, MCB 1999) to regulate the expression of IRF-3 target genes. In the context of IRF-3 activation by phosphorylation, it is noteworthy to mention that other key residues have been identified recently. For instance, Ser173, a target for c-Jun-NH_2-terminal kinase (JUNK), and Ser339 have been described in this context (Clement et al., 2008; Zhang et al., 2009a).

NF-κB activation. NF-κB is a homo- or heterodimeric transcription factor, consisting of two of the following subunits: p65/RelA, RelB, cRel, p50 and p52. Of note, in airway epithelial cells, NF-κB is composed preferentially of p65/RelA and p50. Each NF-κB subunit contains an N-terminal Rel homology domain (RHD) that serves in the binding of κB sequences in target gene promoters and a C-terminal Transactivation domain (TAD) that is necessary for activating gene transcription (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). In unstimulated conditions, NF-κB is retained in the cytoplasm via its association with the IκBα inhibitor of NF-κB. Upon phosphorylation of IκBα at Ser32 and Ser36 by the activated IKK complex, composed of NEMO, IKKα and IKKβ and the resulting proteasome-dependent degradation of IκBα, NF-κB translocates to the nucleus to activate transcription of target genes, such as TNFα, IL-8, and RANTES. It is noteworthy to mention that for full transactivation activity, NF-κB subunits have to undergo post-transcriptional modification, such as phosphorylation and acetylation. In this context, it has been shown that depending on the stimuli, phosphorylation of NF-κB at Ser276, Ser311, Ser529, Ser536 and Ser576 can be important for enhancing NF-κB-mediated transcription of target genes (Chen and Greene, 2004). For instance, in the case of RSV infection, Mitogen-and-stress-related kinase 1 (MSK-1)-induced phosphorylation of the p65 subunit at Ser276 has been shown to be essential for NF-κB dependent target gene transcription by ensuring Lys310 acetylation of p65 and efficient transcriptional elongation of mRNA transcripts (Brasier et al., 2011; Jamaluddin et al., 2009).

1.2.3.3 Viral detection by Toll-like receptors (TLRs)

TLRs are PRRs that reside either on the cell surface or in the endosomal compartments of cells. Out of the 13 mammalian TLRs identified to date, 10 are present in humans. Each TLR recognizes a unique Pathogen associated molecular pattern (PAMP), including peptidoglycans (TLR1/2 and 2/6), Lipopolysaccharide (LPS) (TLR4), lipoteichoic acid (TLR1/2 and 2/6), lipoproteins, lipopeptides (both TLR1/2 and 2/6), fungal zymosan (TLR2/6), bacterial flagellin (TLR5), single-

stranded (ss) (TLR7) or dsRNA (TLR3), and CpG DNA (TLR9) (Kawai and Akira, 2008). Considering their specific detection pattern for nucleic acids, it is evident that TLR3 and TLR7 are important candidates for the recognition of RNA viruses (Arpaia and Barton, 2011). With regard to TLR3, it is known that RSV infection upregulates TLR3 in AECs and sensitizes them to further stimulation with nucleic acids (Groskreutz et al., 2006). Further, TLR3 is important for RSV-induced chemokine expression in AEC (Rudd et al., 2005). Additionally, extracellular TLR2, 4 and 6 have been implicated in the immune response against RSV (Kurt-Jones et al., 2000; Murawski et al., 2009; Rudd et al., 2005). However, most of these data demonstrate the importance for TLRs in cells of the immune system, where TLRs are expressed in much higher levels than in AECs. Thus, in the airway epithelium, RIG-I and MDA5 are considered to be the main PRRs responsible for RSV and SeV detection.

1.2.3.4 The IFN-induced JAK-STAT pathway

When type I or III IFNs are secreted, they bind to their cognate receptors to trigger signaling cascades that mediate the induction of an antiviral state. Type I IFNs bind to the IFNα receptor (IFNAR), composed of a heterodimer of IFN-αR1 and IFN-αR2 chains, and induce a JAK-STAT signaling cascade that culminates in the activation of the Interferon-stimulated gene factor (ISGF3) transcription factor complex and transcription of a vast quantity of ISGs (Figure 7) (Stark and Darnell, 2012). Type III IFNs induce a JAK-STAT signaling cascade via their own specific receptor composed of a heterodimer of the IL-10 receptor 2 (IL-10R2) chain and IFN-λR1 chain, which also results in JAK-STAT signaling (Kotenko, 2011). The intracellular domains of the IFNAR1 subunits are associated with Janus protein tyrosine kinases, Tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1). The binding of type I IFNs to their receptor results in cross-activation of these kinases, which then phosphorylate their downstream substrates, STAT1 and STAT2 (Stark and Darnell, 2012). STAT1 and STAT2 heterodimerize and associate with IRF9 to form the ISGF3 transcription factor complex (Stark and Darnell, 2012). This complex then translocates to the nucleus, where it binds to ISRE sequences (Levy et al., 1989; Levy et al., 1988).

This results in the expression of hundreds of ISGs, whose united action defines the antiviral state (Schoggins and Rice, 2011).

In the context of type III IFN stimulation, the binding of IFNλ to its cognate surface receptors results in the activation of STAT1 and STAT2 by yet unknown kinases. These can, as is the case in type I IFN stimulation, participate in the formation of ISGF3 and its binding to target genes with ISRE sequences, or activate the expression of target genes that harbor γ-interferon activated sequences (GAS) in their promoters (Kotenko, 2011).

1.2.3.5 Establishment of the antiviral state by ISGs

The IFN-induced JAK-STAT pathway leads to the expression of hundreds of ISGs (Schoggins and Rice, 2011) that exert diverse functions to limit viral propagation. The list of ISGs is exhaustive and goes far beyond the scope of this thesis. Therefore, only a few classical ISGs, as well as new concepts in this field are discussed here.

As mentioned, ISGs target several steps of the viral replication cycle. Protein kinase R (PKR), for example, shuts down protein translation by phosphorylating the translation initiation factor eIF2α (Toth et al., 2006); interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)/ Interferon stimulated gene 56 (ISG56) also targets translation initiation (Fensterl and Sen, 2011); MxA targets viral transcription by forming spherical structures around the viral nucleoprotein (Haller et al., 2007); and 2', 5'-oligoadenylate synthetase 1 (OAS1), OAS2, OAS3 are capable of activating RNAseL, which functions in genome degradation (Kristiansen et al., 2011). Several ISGs have yet uncharacterized functions such as OASL and MxB to name but a few (Schoggins and Rice, 2011). It is noteworthy to mention that RIG-I and MDA5 are also ISGs. It is the combined action of these and the many other ISGs induced by a given viral infection that will determine the level of inhibition of viral infection. A novel concept in this field is that each virus has a unique, but partly overlapping "ISG profile" – a collection of genes that preferentially inhibits a given virus (Schoggins and Rice, 2011; Schoggins et al., 2011). A subset of ISGs, which includes RIG-I, MDA5,

several IRFs and STAT1 are themselves components of the IFN pathway machinery. This ensures a positive feedback on the antiviral IFN pathway.

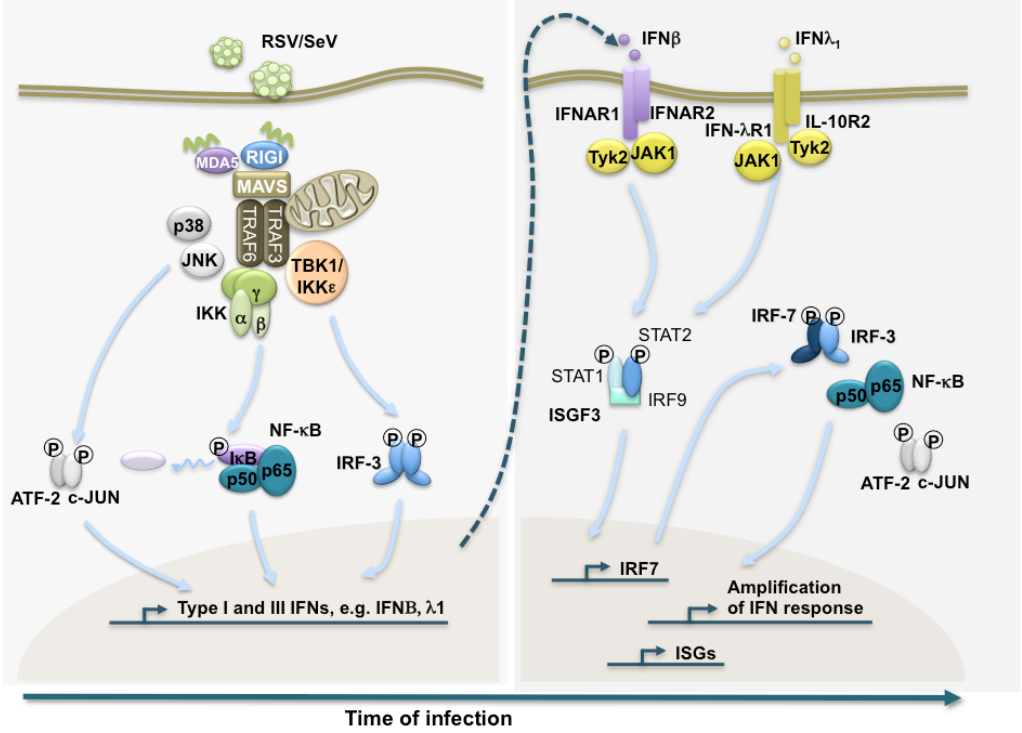

Figure 7: The virus-induced IFN response.

Upon recognition of the viral genome by the RLRs RIG-I and MDA5, a signaling platform is formed at MAVS adaptor protein, which is located at the mitochondria. TRAF6 and TRAF3 recruitment to this complex results in the activation of the IKK complex and its homologues TBK1 and IKKe. The IKK complex phosphorylates IκBα, which thereafter undergoes proteasome-dependent degradation. This culminates in NF-κB liberation and translocation to the nucleus. TBK1 and IKKε phosphorylate IRF-3, which leads to its dimerization and nuclear translocation. NF-κB, together with IRF-3 and the AP-1 transcription factor, which is composed of ATF-2 and c-Jun, regulate the expression of type I and type III IFNs. In the early phase of viral infection, IFNβ, IFNα1 and IFNλ1 are the principally transcribed IFNs. These are then secreted and bind to their cognate receptors. Type I IFNs bind to the IFNAR1 and IFNAR2 chains of the type I IFN receptor, whereas the type III IFN receptor is made up of the IFN-λR1 and the IL-10R2 chains. Binding to these receptors results in the activation of Tyk2 and JAK1, which phosphorylate their downstream targets, STAT1 and STAT2. Upon phosphorylation, STAT1 and STAT2 bind IRF9 to form the ISGF3 transcription factor complex. ISGF3 binds to ISRE consensus elements in its target gene promoters to upregulate the transcription of hundreds of ISGs. IRF-7 is also an ISGF3-regulated gene. In infected cells, IRF-7 heterodimerizes with IRF-3, and in conjunction with NF-κB and AP-1 upregulates the many other isoforms of type I IFN as well as IFNλ2 and IFNλ3. This leads to the amplification of the IFN response and sustained ISG expression.

1.2.3.6 The TNF receptor 1 (TNFR1) signaling pathway

As mentioned above, the activation of the transcription factor NF-κB during virus infection leads to the expression of various target genes. TNFα is a key proinflammatory cytokine whose expression is regulated by NF-κB in this context. Besides its proinflammatory role, TNFα has been attributed an antiviral function (Ito and O'Malley, 1987; Ruby et al., 1997; Wong et al., 1992). TNFα binds to TNFR1, which is expressed on most mammalian cells, whereas the second TNFα-specific receptor, TNFR2, is mainly expressed in oligodendrocytes, astrocytes, T cells, myocytes, thymocytes, endothelial cells and in human mesenchymal stem cells (Cabal-Hierro and Lazo, 2012). One of the main TFs activated by the TNFα-induced signaling pathway is NF-κB itself. In general, early TNFα-induced signaling activates pro-survival mechanisms, which are replaced with a pro-apoptotic program upon prolonged stimulation with TNFα, thereby regulating the survival and cell death by apoptosis of virus-infected cells. This is orchestrated via the consecutive formation of two different TNFR signaling complexes. The first complex (Complex I) controls the expression of anti-apoptotic proteins that prevent cell death, whereas the second complex (Complex II) triggers cell death processes after the internalization of the receptor (Micheau and Tschopp, 2003).

The binding of TNFα to its receptor TNFR1 induces trimerization and the formation of a core complex composed of TRADD, RIP1 and TRAF2 (Cabal-Hierro and Lazo, 2012; Hsu et al., 1996). TRADD recruits TRAF2 via its N-terminal TRAF binding domain, and RIP1 via its C-terminal death domain (DD). Then, cellular Inhibitor of apoptosis 1 (cIAP1) and cIAP2 proteins are recruited to TRAF2 (Mahoney et al., 2008). TNFR1, TRAF2 and RIP1 subsequently undergo ubiquitination in this core complex and become linked to K63-conjugated ubiquitin chains or linear ubiquitin chains (Ea et al., 2006; Wu et al., 2006). TRAF2, cIAP1, and cIAP2 are all E3 ubiquitin ligases and are thought to be important for the generation of K63-linked polyubiquitin chains in this complex. Further, Linear Ubiquitin Chain Assembly Complex (LUBAC), whose recruitment is dependent on cIAP1/2-mediated ubiquitination of the core complex, catalyzes the formation of linear ubiquitin chains

onto this complex (Haas et al., 2009; Ikeda et al., 2011). This complex, while bound to the plasma membrane, triggers cellular pathways that lead to NF-κB and AP-1 activation (Hsu et al., 1996; Mahoney et al., 2008; Micheau and Tschopp, 2003). Regarding NF-κB activation, the ubiquitination of the core complex recruits the downstream complexes NEMO-IKKα-IKKβ via NEMO interactions with the ubiquitin chains and TAK1-TAB2-TAB3 via the action of TAB2 recognizing and binding to ubiquitin chains (Ea et al., 2006; Kovalenko and Wallach, 2006). NEMO and TAK1 are consequently ubiquitinated as well. TAK1 is then activated and its kinase activity executed on the IKK complex results in IKKα/β activation and consequent NF-κB activation, as previously outlined in section 1.2.3.2, via the proteasome-dependent degradation of IκBα. NF-κB then regulates the transcription of proinflammatory, but also anti-apoptotic genes (Wang et al., 1998).

When NF-κB is activated it transcribes the anti-apoptotic Cellular FLICE-like inhibitory protein (c-Flip) that inhibits activation of caspase-8 (Micheau et al., 2001). When NF-κB activation is blocked, c-Flip is not produced and cells enter caspase-8-mediated apoptosis downstream of TNFR1 signaling (Yeh et al., 2000). Under conditions were caspase-8 activity is inhibited such as during vaccinia virus infection, RIP1 and RIP3 kinases become phosphorylated and are recruited to yet a different complex, the necroptosome, which when formed triggers an inflammatory mode of programmed necrotic cell death termed necroptosis that is caspase-independent (Cho et al., 2009; Vandenabeele et al., 2010). Necroptosis is now viewed as a back-up death-inducing cellular defense mechanism that helps to reduce virus persistence and propagation in instances when apoptosis is disabled by viruses (Cho et al., 2009).

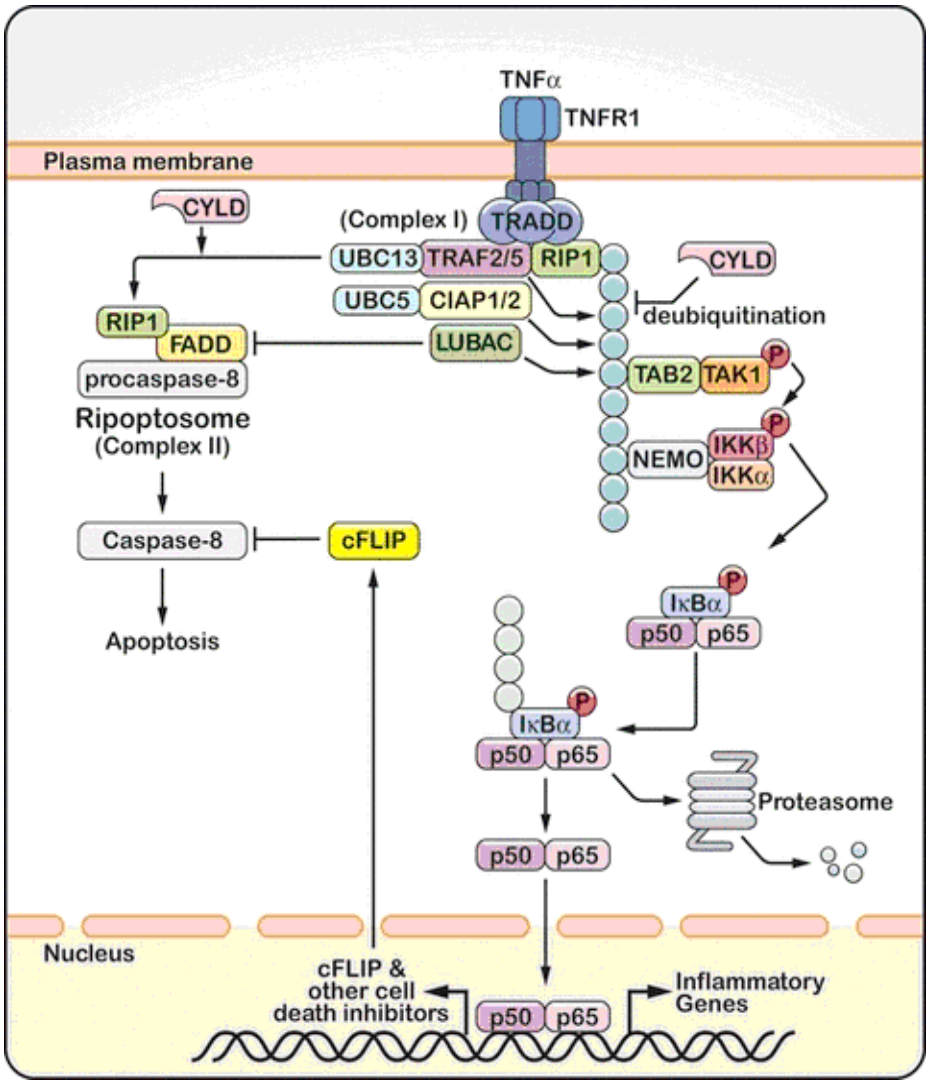

Figure 8: TNF-induced NF-κB activation.

The binding of TNF to TNFR1 induces the trimerization of the receptor and recruitment of a protein complex (complex I) that includes the adapter protein TRADD, the protein kinase RIP1, and ubiquitin E3 ligases TRAF2, cIAP1, cIAP2, and LUBAC. Some or all of these E3 Ubiquitin ligases catalyze polyubiquitination of RIP1, which recruits and activates the TAK1 and IKK complexes. Deubiquitination of RIP1 by CYLD not only inhibits TAK1 and IKK activation but also facilitates the formation of a cytoplasmic complex (complex II or Ripoptosome) consisting of RIP1, FADD, and procaspase-8. Within this complex, pro-caspase 8 auto-cleaves itself to generate mature caspase-8, which then initiates apoptosis. However, caspase-8 is normally inhibited by caspase inhibitors such as c-FLIP, which is induced by NF-κB. Ubiquitin E3 ligases inhibit cell death indirectly by activating NF-κB and/or directly by blocking the formation of Complex II. From (Chen, 2012). With permission from John Wiley and Sons.

1.2.4 Viral modification and evasion of antiviral immunity

In order for viruses to be able to survive, they must constantly co-evolve with the host organism they infect. Host mechanisms exist to counteract viral infection, and viruses have in turn evolved strategies to interfere with these mechanisms. It is now clear that the successful survival of viruses is ensured by interference with host antiviral mechanisms at various levels. The following section will discuss how *Paramyxoviridae* RSV and SeV interfere with early antiviral signaling pathways and the establishment of the antiviral state by AECs. Viral interference mainly targets the recognition of viral RNA by PRRs, and a number of viral molecules contribute to the global interference of innate and adaptive immunity. The combination of all of these events renders the viral infection effective and pathogenic at the same time.

1.2.4.1 General viral evasion mechanisms

The *Paramyxoviridae* family of viruses encodes various genes that are necessary for virus replication biology. Some of these proteins, however, have a negative impact on antiviral immune responses. By means of their designated action during the virus life cycle, they can indirectly foster the evasion from host immunity. One such example is the process of encapsidation of viral RNA by the N protein. Encapsidation of viral RNA prevents the formation of dsRNA molecules that could potentially activate RLR- and PKR-dependent antiviral mechanisms (Gerlier and Lyles, 2011). Additionally, encapsidation has the effect of shielding 5'-triphosphate moieties from recognition by RIG-I (Gerlier and Lyles, 2011). Further, the capping mechanisms of viral mRNAs ensured by the *Paramyxoviridae* L protein allows viral mRNAs to appear as cellular mRNAs, which consequently go unrecognized by the antiviral detection system (Gerlier and Lyles, 2011). An early report by Atreya *et al.* has suggested that one function of the RSV NS1 protein is to place a break on viral transcription and replication to certain levels, so that the ongoing virus life cycle can remain unrecognized by the cell (Atreya et al., 1998). A similar function is attributed to SeV C protein (Curran et al., 1992; Horikami et al., 1997). In the absence of C protein, viral

dsRNA accumulates during the SeV life cycle, which becomes recognized by PKR and leads to the shutdown of cellular translation and consequent viral replication (Takeuchi et al., 2008). RSV N protein has been shown to sequester PKR away from eIF2α, thereby inhibiting the phosphorylation of this translation initiation protein and preventing the shutdown of protein synthesis (Groskreutz et al., 2010).

1.2.4.2 Blocking IFN production

The blocking of initial type I IFN induction is an efficient early viral evasion mechanism to shut down the establishment of antiviral activities. Several key mechanisms of antiviral signaling are targeted in order to achieve this.

RIG-I and MDA5, the two antiviral sensors for *Paramyxoviridae* RNA, are the first targets for viral evasion mechanisms. RSV NS2 protein has been shown to inhibit RIG-I association with MAVS via binding and sequestration of the N-terminal CARD domain of RIG-I, whereas SeV V protein interferes with MDA5 antiviral activity (Childs et al., 2007; Childs et al., 2009; Ling et al., 2009). Consequently, both, NS1 and NS2 inhibit IFNα, IFNβ and IFNλ production in RSV-infected cells (Ramaswamy et al., 2006; Spann et al., 2004). Several other molecules in the RLR signaling pathway are inhibited by NS1 and NS2. For instance, NS1 or NS2 were shown to target TRAF3 and IKKε (Swedan et al., 2009). RSV NS1 protein binds to a multitude of intracellular molecules with diverse function (Wu et al., 2012). In this screen, which attempted to identify interaction partners, NS1 was shown to interact with nucleotide-binding oligomerization domain (NOD), leucine rich repeat containing X1 (NLRX1), an essential regulatory molecule that prevents inductions of the RLR antiviral signaling pathway (Wu et al., 2012). However, the mechanistic consequence of this interaction remains to be fully determined.

1.2.4.3 Evasion of the IFN-mediated induction of the antiviral state

The induction of IFN gene expression is not the only target of viral evasion mechanisms. The downstream IFN-dependent activation of the JAK-STAT pathway,

which leads to the establishment of the antiviral state, is also targeted by paramyxoviruses. SeV C protein, for example, has been shown to inhibit the IFN-mediated induction of the antiviral state (Garcin et al., 1999; Gotoh et al., 1999). It interferes with STAT2 phosphorylation, which could constitute a potential mechanism to explain SeV C inhibition of the IFN pathway (Gotoh et al., 2003). RSV NS1 and NS2 also target this pathway, mainly by interfering with or enhancing the degradation of the STAT1 and STAT2 transcription factors (Elliott et al., 2007; Lo et al., 2005; Ramaswamy et al., 2004). Besides NS1 and NS2, RSV G protein can regulate this antiviral innate immune pathway, since infection with a recombinant RSV lacking the G protein leads to an increase in IFNβ and Interferon Stimulated Gene 15 (ISG15) levels in infected cells. Mechanistically, it has been demonstrated that RSV G upregulates the negative regulators of the JAK-STAT pathway, Suppressors of cytokine signaling 1 (SOCS1) and SOCS3, to inhibit the IFN-induced induction of ISG15 (Moore et al., 2008).

1.2.4.4 Interference with innate and adaptive immune responses

Paramyxoviridae proteins do not only interfere with early antiviral signaling pathways. Events downstream of AEC cytokine secretion and antiviral state induction, which activate the innate and adaptive axes of the immune system, are also viral targets. In this context, interference by RSV results in significant immunoperturbance and RSV-associated pathology.

RSV G protein has been shown to harbor potent immunomodulatory functions. This is based on its structural resemblance to the leukocyte-attractant chemokine fractalkine/CXCL3. As mentioned previously, infected cells not only express G proteins on their surface but also release a form of soluble G protein. G protein mimicry to fractalkine reduces pulmonary influx of immune cells to the site of infection, which can in turn influence the outcome of innate and adaptive anti-RSV responses (Harcourt et al., 2006). Soluble G protein is also associated with the inhibition of TLR2, TLR4 and TLR9 pathways in human monocytes, significantly suppressing the TLR-dependent immune responses of this cell type (Polack et al.,

2005). Secretory G protein can further function as an antigen decoy mechanism that serves to foster the escape of the virus from neutralizing antibodies and reduces antibody-mediated clearance by immune cells (Bukreyev et al., 2012; Bukreyev et al., 2008). RSV NS1 protein has been shown to suppress the activation of $CD8^+$ and Th17 T cells and skew the T cell response towards a Th2 response (Munir et al., 2011). RSV also infects and replicates efficiently in immune cells, such as dendritic cells, macrophages, eosinophils and T cells, where the expression of RSV proteins interferes with immune mechanisms initiated by these cells (Halfhide et al., 2011).

Thus, RSV and SeV efficiently target and evade antiviral host mechanisms on several levels. Some of these mechanisms culminate in the alteration of innate and adaptive antiviral responses. In the case of RSV infection, a possible outcome is the establishment of severe immunopathology, as will be outlined in the following section.

1.3 RSV-induced pathology – a multifaceted syndrome

RSV infects very early in life, with the peak incidence of severe disease before six months of age. Whereas in these young individuals RSV infection is associated with lower respiratory tract complications, such as respiratory wheezing, bronchiolitis, and pneumonia, which often requires hospitalization, healthy adults and older children may also become infected, but will mostly only develop cold-like symptoms. Thus, the clinical symptoms following RSV infection vary strongly depending on the patient's age. Nevertheless, RSV remains a major health threat for pre-term infants, the elderly and immunocompromised individuals (Falsey et al., 2005; Hall et al., 2009).

To date, no specific anti-RSV treatment or vaccine has been identified. High-risk individuals are subjected to prophylactic treatment with anti-RSV monoclonal antibodies, such as palivizumab (Mejias and Ramilo, 2008). Infected individuals receive antiviral therapy with ribavirin in combination with immunoglobulins and corticosteroids, although this treatment is only mildly effective and not recommended for routine treatment (Collins and Melero, 2011). Small anti-RSV molecules, mostly benzimidazole derivatives, are being developed and some of them are now at the stage of clinical trials (DeVincenzo, 2012). RNA interference (RNAi)-based strategies for RSV treatment are also in the testing phase (DeVincenzo, 2012). Vaccine development and testing involving RSV subunit vaccines, live-attenuated vaccines and virus-like particles are also underway (Hurwitz, 2011). It is noteworthy to mention that vaccine development was significantly complicated following a failed vaccination trial in the 1960s in infants and young children using a formalin-inactivated RSV vaccine (FI-RSV). The vaccine was poorly protective and vaccinees who were subsequently infected with RSV demonstrated major airway complications and required hospitalization.

RSV-induced T cell memory is weak, as the virus can readily reinfect during the first few years of life, although reinfection is usually associated with less severe symptoms (Collins and Melero, 2011). Further, RSV infection can drive long-term complications in pulmonary function, such as a possible predisposition to asthma

development (Mailaparambil et al., 2009). RSV-induced complications and disease arise from a combination of various factors: firstly, direct viral damage in the afflicted airways contribute to airway obstruction; secondly, there is a strong immunopathogenic component implicated in RSV-induced disease; thirdly, viral interference with the innate immune system contributes to the development of pathology; lastly, host genetic, social and environmental factors are undoubtedly implicated in the determination of disease outcome (Figure 9) (Mailaparambil et al., 2009). Thus, there is not just one single mechanism that contributes to RSV-induced pathology, but rather an accumulation of diverse mechanisms that potentially drive RSV-induced disease. These multifactorial aspects of RSV-induced immunopathology will be discussed in the following section.

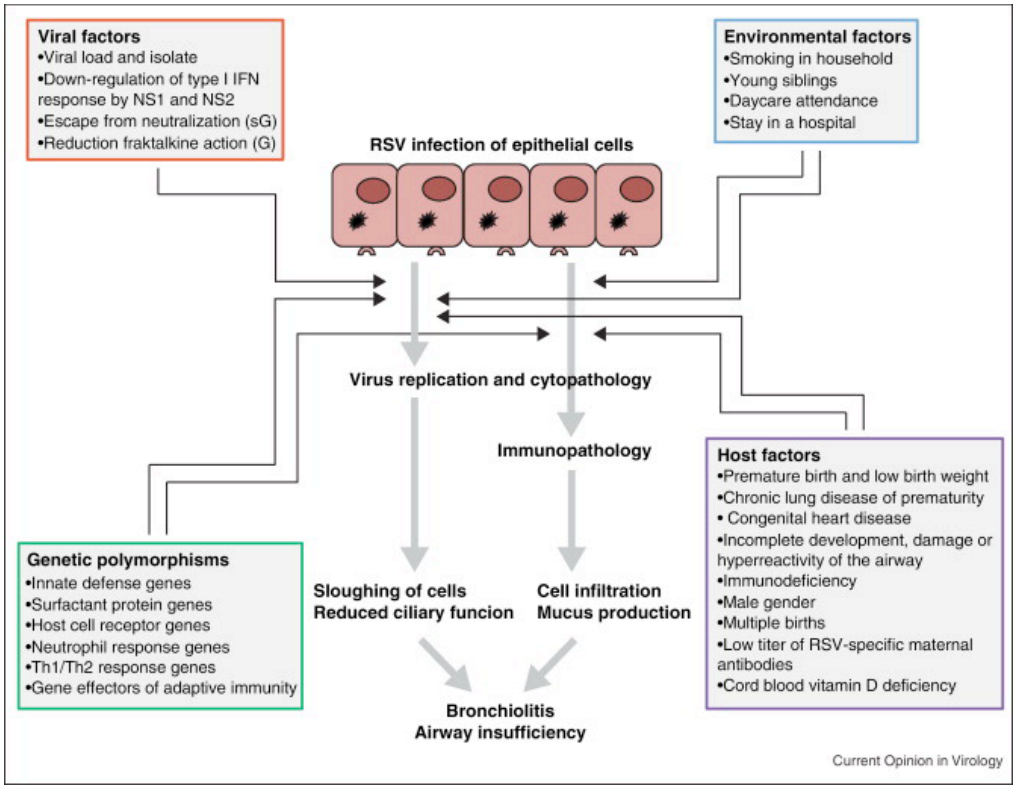

Figure 9: Factors influencing the pathogenesis and clinical disease caused by RSV infection in infants and children. From (van Drunen Littel-van den Hurk and Watkiss, 2012), with permission from Elsevier.

1.3.1 RSV-induced airway obstruction

RSV infection is generally limited to superficial epithelial cells and does not invade underlying cells as RSV preferentially buds from apical cells (Johnson et al., 2007; Wright et al., 2005; Zhang et al., 2002). RSV infection itself, however, is not highly cytopathic; it is known to impair ciliary beating and cause sloughing of infected epithelial cells. This, in combination with peribronchial mononuclear cell infiltration, submucosal edema, basal cell hyperplasia and mucus secretion, contributes significantly to airway obstruction - a common consequence of RSV infection (Johnson et al., 2007; Reed et al., 2008). These symptoms play an even more devastating role in infants who have narrow airways and are still undergoing airway development. Syncytia formation may also be observed in the bronchoalveolar epithelium, but is not common. RSV has been shown to interfere with apoptotic processes, although this finding is not the general consensus (Bem et al., 2010; Groskreutz et al., 2007; Kotelkin et al., 2003; Thomas et al., 2002). However, the blocking of apoptosis might be a means of increasing the pool of infectious virions, which in turn contribute to RSV disease, as levels of replicating RSV correlate with disease severity (El Saleeby et al., 2011).

1.3.2 Inflammation and infiltration of innate immune players

Inflammation as a consequence of RSV infection serves to "[…] destroy, dilute and/or sequester the virus and the tissue that is injured […]". However, much of the problematic of RSV-induced inflammation stems from the "[…] inability to limit and sequester damaged tissue and to proceed onwards to a resolution phase […]" in uncontrolled inflammation, also termed "cytokine storm" (Rosenberg and Domachowske, 2012).

Abundant evidence exists that inflammatory cytokines and chemokines are released into the airways by AECs and macrophages of infants and children with RSV disease, which then recruit and activate granulocytes (Everard et al., 1994). The neutrophil chemoattractant IL-8, TNFα, and the CC chemokines MIP-1α, MCP-1 and RANTES are among those that are detected most frequently in the airways and nasal passages of infected infants and children (McNamara et al., 2005; Mobbs et al., 2002). Upregulation of IL-8 is correlated with the severity of RSV disease, and leads to recruitment of neutrophils, which constitute the majority of infiltrating cells (at least 84%) (Everard et al., 1994; McNamara et al., 2003; Rosenberg and Domachowske, 2012; Smyth et al., 2002). While neutrophils mediate elimination of virus-infected cells, their high numbers, ability to secrete further cytokines and chemokines, and degranulation products may contribute to RSV-induced immunopathogenesis (Lukens et al., 2010). However, it is still unclear to which extend heightened inflammation, or increased neutrophil levels, are pathogenic in RSV infection (Bennett et al., 2007).

RANTES serves to recruit eosinophils and RANTES levels are elevated in lungs of infected individuals (Harrison et al., 1999; Sheeran et al., 1999). Furthermore, polymorphisms in the RANTES promoter have also been shown to correlate with disease severity (Amanatidou et al., 2008). Thus, several data point towards a role of this cytokine in RSV-induced immunopathology. However, in infants, eosinophils present only 1-3% of airway leukocytes in RSV-infected infants (McNamara et al., 2003). This is in strong contrast to the mouse model, where eosinophils constitute a major granulocyte population following RSV infection that contributes significantly to RSV clearance (Phipps et al., 2007; Tekkanat et al., 2002). These facts clearly show that data generated following RSV infection in the mouse model cannot always be transposed to human physiology so easily, an aspect that will be discussed further below. Thus, whether RANTES and eosinophils are major contributors to RSV-induced disease in infants is still not clear. While increased eosinophilia often occurrs in very young patients following RSV infection and can lead to predisposition to asthma development in later years, contradictory studies exist on whether increased primary eosinophila influences the occurrence of wheezing and asthma upon infection at a later age (Castro et al., 2008; Kristjansson et al., 2006; Pifferi et al., 2001). There is

also in mice generated evidence that demonstrates the beneficial role of eosinophils, as this subset of leukocytes has been shown to contribute to innate antiviral immunity and promote clearance of RSV infection (Phipps et al., 2007).

1.3.3 Adaptive immune responses

Besides innate, inflammatory mechanisms, the adaptive immune response was suggested to be implicated in RSV pathology. More specifically, it has been proposed that immunopathology in RSV infection is due to a Th2 bias of $CD4^+$ T cell responses (Collins and Melero, 2011). Excessive mucus production, airway plugging, wheezing, and long-term effects on lung function are all common manifestations of RSV disease that have some similarities with asthma, which involves a Th2 bias. Efficient virus clearance requires Th1-type T cell responses characterized by IFNγ and IL-2 expression of $CD4^+$ T cells, whereas a Th2 response is characterized by IL-4, IL-10 and IL-13 gene expression (Becker, 2006; Bendelja et al., 2000; Roman et al., 1997). In this context, in young infants, where RSV generally strikes in the most severe manner, a Th2 biased response is predominant, as it is a left over from the prenatal period (Adkins et al., 2004; Kristjansson et al., 2005). However, it is noteworthy to mention that not all studies confirm a predominant Th2-biased T cell response (Brandenburg et al., 2000).

Depletion studies in mouse models of RSV infection indicate that $CD4^+$ and $CD8^+$ T cells are important for RSV clearance, but $CD8^+$ T cells may also contribute to RSV-induced immunopathology (Graham et al., 1991). In RSV-infected infants, however, the timing of the $CD8^+$ T cell response is after the peak in clinical symptoms, and the magnitude of the $CD8^+$ T cell response does not correlate with disease severity, making it questionable that this T cell subset plays a dominant role in RSV-immunopathology (Heidema et al., 2007; Lukens et al., 2010).

It is noteworthy to mention that a panoply of other studies exist in which the mechanisms contributing to RSV-induced pathology have been investigated. These

studies implicate populations such as NK cells and T regulatory cells (Tregs) (Fulton et al., 2010; Lee et al., 2010), however, their discussion would go beyond the scope of this thesis. It is important to stress that most of these results have been generated using the mouse model of RSV infection, and future studies are required to demonstrate if the findings are relevant to RSV-induced human immunopathology. The RSV-mouse model is particularly useful due to the availability of knockout strains and the possibility of immunodepletion experiments. However, mice are not the natural host of RSV and their airway physiology and RSV-induced pathology are quite unlike that of humans. This has pushed the field to start establishing more relevant models to study RSV infection *in vivo*. In this context, the use of newborn lambs has found its way into RSV research. The airway structure and function of newborn lambs are more similar to those of infants compared to mice. Furthermore, the pathology of RSV in lambs is similar to that in human infants, with mild peribronchiolar infiltrates of lymphocytes and plasma cells, and bronchiolitis characterized by degeneration and sloughing of epithelial cells, and major infiltrates of neutrophils (Derscheid and Ackermann, 2012; Sow et al., 2011). It is presently clear that the further characterization of this model will likely be quite useful in the study of the immunopathologic mechanisms that regulate RSV-induced disease.

In the context of RSV infection, certain evidence suggests that Reactive Oxygen Species (ROS) could be a contributor to RSV-induced pathology. As will be mentioned in more detail in the following section, RSV infection leads to increases in ROS production (Casola et al., 2001; Hosakote et al., 2009). Furthermore, a positive correlation exists between the quantity of oxidative stress markers present and severity of bronchiolits in RSV-infected infants (Hosakote et al., 2011). Considering the importance that ROS might play in RSV disease, the following section is thus dedicated to an introduction of ROS, their mechanisms of generation and their action on processes taking place during viral infection.

1.4 ROS and their role in regulation of cellular signaling pathways

ROS have for a long time been considered toxic and harmful to organisms, due to their reaction with essential metabolites such as lipids, proteins, carbohydrates and nucleic acids inside the cell as a mechanism of oxidative stress. The discovery of the oxidative burst in phagocytes and its essential role in pathogen killing was the first example of how ROS could actually be beneficial for a host organism (Baldridge and Gerard, 1932). However, from these discoveries it remained evident that highly ROS-inducing mechanisms, such as the oxidative burst, needed to take place in confined cellular compartments, since damage of cellular macromolecules would be inevitable in such processes. It is only during the last decade of research on ROS that the notion that low levels of ROS, which are specifically produced upon stimulation and can influence cellular signaling pathways via oxidative modification of macromolecules, became accepted (D'Autreaux and Toledano, 2007). This level of ROS production is not considered harmful to the cell, but, on the contrary, is essential for keeping up cellular processes (Droge, 2002). In the following chapter, the nature of ROS and their origin will be discussed. With regard to this thesis, special emphasis will be placed on the Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidase (NOX) family and its mechanism of ROS production. These enzymes are expressed in various tissues and contribute to basal or stimuli-induced ROS production, depending on the context. The mechanisms of how ROS function as subtle redox-regulators of cellular processes will be elucidated below, with an emphasis on events controlling cellular signaling pathways that drive antiviral innate immunity.

1.4.1 The nature of ROS

ROS are chemically reactive molecules containing oxygen that by definition are more reactive than oxygen itself. Their elevated reactivity is partly due to the presence of an unpaired valence shell electron. The principal ROS species are superoxide anion $O_2^{\bullet-}$, hydrogen peroxide H_2O_2, and the hydroxyl radical $HO^{\bullet}$. $O_2^{\bullet-}$ is a byproduct of cellular respiration or other metabolic processes, or can be produced specifically by NADPH oxidases. The spontaneous dismutation of two $O_2^{\bullet-}$ molecules or their dismutation catalyzed by superoxide dismutase (SOD) can lead to the formation of H_2O_2. $HO^{\bullet}$ is mostly generated by an iron-catalyzed Fenton reaction implicating H_2O_2 (Figure 10) (Winterbourn, 2008). These radicals can react with other molecules to form highly oxidizing agents. For instance, $O_2^{\bullet-}$ reacting with nitric oxide (NO) results in the formation of peroxynitrate (NO_3^-), a Reactive Nitrogen Species (RNS) (Winterbourn, 2008). Furthermore, ROS can initiate oxidative chain reactions of unsaturated fatty acids, for example, which results in the creation of reactive fatty acid intermediates.

The specificity of ROS action is determined based on the chemical reactivity, half-life, lipid solubility, and compartmentalization of the ROS produced (D'Autreaux and Toledano, 2007). Additionally, in the context of oxidative protein modification, the environment of the target residue confers specificity to redox modifications. In general, $O_2^{\bullet-}$ is highly unstable, due to a preference for reactions with Fe-S clusters or dismutation to H_2O_2. $O_2^{\bullet-}$ is a highly charged molecule, and thus does not diffuse readily across membranes. However, it has recently been identified that superoxide, generated either at the plasma membrane or on endosomes, can cross the membrane barrier through chloride anion channels (Hawkins et al., 2007; Mumbengegwi et al., 2008). Thus, superoxide might constitute a suitable second messenger in cell areas where these channels are present. Due to its high reactivity, the effects of $O_2^{\bullet-}$ are likely confined to the areas surrounding these channels. The even more reactive $HO^{\bullet}$ has a half-life of only about 10^{-9} seconds and therefore reacts with any kind of macromolecule (Halliwell and Gutteridge, 2007). It is thus implicated in the oxidative modification of macromolecules but considered an unsuitable second messenger. H_2O_2

on the contrary is a less reactive radical and much more stable than $O_2^{\bullet-}$ with a cellular half-life is about 1ms. It readily diffuses across membranes, partly via aquaporin channels, making it fit for the role of a signaling molecule (Bienert et al., 2007). H_2O_2 has a high preference for reactions with Cys and Met residues on target proteins. However, not all Cys residues are equally reactive to H_2O_2. Solvent exposed Cys residues or Cys residues with a pK_a lower than the physiological pH of the cell, stabilized by neighboring positively charged amino acids, readily react with H_2O_2, thereby conferring specificity to the reaction of H_2O_2 with these amino acids (Winterbourn and Hampton, 2008).

1.4.2 The origin of ROS

ROS can be generated during enzymatic reactions, from which they can be produced as byproducts, but also deliberately, in specialized ROS-generating reactions. ROS can also be generated by chemical reactions inside the cell in environments that are high in iron and favor the reduction of O_2. In the following paragraph, the mechanisms of ROS production will be outlined briefly. Of note, the mechanism of specialized ROS production by NADPH oxidases will not be mentioned in this section, but will be discussed in detail in later paragraphs.

As previously mentioned, several cell-intrinsic enzymatic reactions produce ROS as byproducts. One such example is the mitochondrial respiratory chain. It generates ROS as a byproduct at the complex I and III of the mitochondrial respiratory chain (Drose and Brandt, 2012). Recently, complex II has also been attributed an important role in the regulation of mitochondrial ROS production (Ralph et al., 2011). It is noteworthy to mention that the respiratory chain is not the only source of mitochondrial ROS. The activities of the following mitochondrial proteins have been associated with mitochondrial ROS production: p66Shc, amine oxidases, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and glycerol-3-phosphate

dehydrogenase. The process of fatty acid β-oxidation also produces ROS (Brand, 2010; Migliaccio et al., 2006; Starkov et al., 2004).

ROS production also takes place in peroxisomes, organelles specialized in peroxide metabolism, lipid biosynthesis and β-oxidation of fatty acids. The main source of H_2O_2 production in peroxisomes is oxidases that transfer hydrogen from their respective substrates to molecular oxygen. These include Acyl-CoA-oxidases from β-oxidation of fatty acids. Oxidases from amino acid metabolism, as well as amine oxidase and xanthine oxidase. Xanthine oxidase catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid, which contribute to peroxisomal ROS production (Schrader and Fahimi, 2004).

Further enzymatic reactions that produce ROS as byproducts involve the cytochrome P450 enzymes that catalyze the oxidation of hydrophobic organic molecules, cyclooxygenases (COX) and lipoxygenases (LOX), which function in prostaglandin and arachidonic acid metabolism, respectively (Bae et al., 2011).

One chemical, non-enzymatic reaction, that contributes to the formation of the highly reactive hydroxyl radical $HO^{\bullet}$ from previously produced H_2O_2 is the Fenton reaction. During this reaction, ferrous iron Fe^{2+} reacts with H_2O_2 to form ferric iron Fe^{3+} and a hydroxyl radical $HO^{\bullet}$. This radical can oxidize macromolecules and induce various oxidative modifications in the cell. Fe^{2+} can also react with an oxygen molecule to produce a superoxide radical $O_2^{\bullet-}$ and Fe^{3+}. $O_2^{\bullet-}$ then dismutates to H_2O_2, which can in turn feed the Fenton reaction. Thus, by generating the highly reactive hydroxyl radical, the Fenton reaction contributes significantly to an increase in cellular ROS and oxidative damage (Freinbichler et al., 2011).

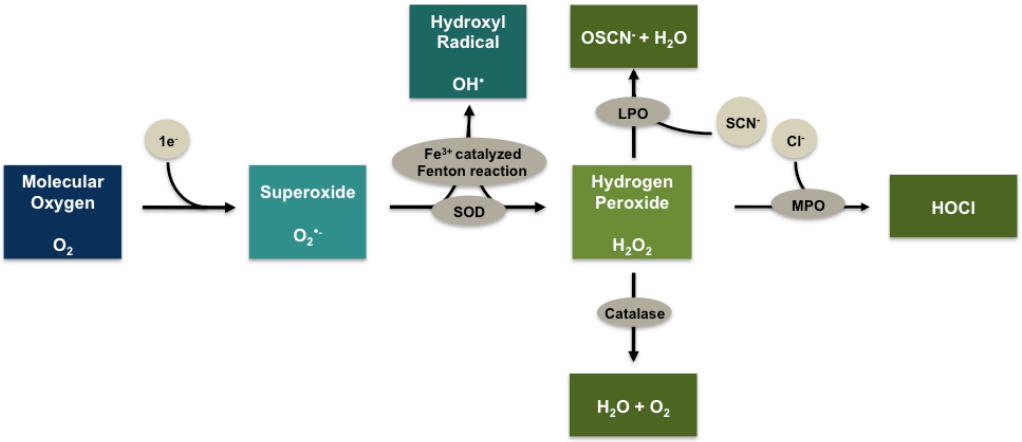

Figure 10: ROS-generating and ROS-involving reactions.

The addition of an electron to molecular oxygen leads to the formation of $O_2^{\bullet-}$. Its dismutation, which can be catalyzed by superoxide dismutase (SOD), generates H_2O_2. H_2O_2 and $O_2^{\bullet-}$ are the origin of the very reactive $^{\bullet}OH$ produced the Fenton reaction. H_2O_2 can be detoxified by catalase. Peroxidase enzymes, such as lactoperoxidase (LPO) or myeloperoxidase (MPO) use H_2O_2 to form microbicidal compounds hypothiocyanate ($OSCN^-$) and hypochlorous acid (HOCl).

1.4.3 The NADPH oxidase (NOX) family of enzymes

NADPH oxidases are a family of enzyme complexes whose primary function is to catalyze the transfer of electrons from NADPH to molecular oxygen, thereby generating $O_2^{\bullet-}$. As will be outlined in more detail, in some cases $O_2^{\bullet-}$ readily dismutates to H_2O_2, making this ROS the primarily detectable ROS. The NADPH oxidase family is made up of seven members, NOX1-5 and Dual oxidase 1 (DUOX1) and DUOX2. The different NOX/DUOX proteins vary in terms of their tissue expression pattern and mode of activation (Table 2) (Lambeth, 2004). Furthermore, they show specific subcellular localization but also generate distinct ROS. NADPH-generated ROS are implicated in the regulation of a great variety of cellular processes.

Briefly, NOX2 is the major NADPH oxidase expressed in phagocytes and is essential for microbial killing in this context. NOX2 and NOX4 also play an important role in the vasculature, as they regulate angiogenesis. NOX1, which is highly expressed in vascular smooth muscle cells and the colon plays a predominant function in these compartments, potentially also in the context of mucosal immunity. NOX3 is expressed in the inner ear, where its function is associated with hearing and balance. NOX5 is highly expressed in the spleen, lymph nodes and testis, but also in cancerous tissues of the prostate and esophagus, where it plays a role in cancer development (Bedard et al., 2012; Bedard and Krause, 2007). DUOX1 and DUOX2 are essential NADPH oxidases in thyroid hormone synthesis, but also regulate antimicrobial defenses of the gastrointestinal and respiratory mucosae (De Deken et al., 2000; Dupuy et al., 1999; Fischer, 2009). More specifically, in polarized AEC cultures, DUOX2 is found at the apical cell pole of ciliated cells (Fischer, 2009).

Table II: NADPH oxidase expression pattern and dependency on regulatory subunits. Adapted from (Bedard and Krause, 2007).

	High levels expression	Intermediate to low level expression	Subunit dependency	Ca^{2+} sensitivity
NOX1	colon	smooth muscle endothelium uterus placenta prostate osteoclasts retinal pericytes	NOXO1 NOXA1 p22phox	
NOX2	phagocytes	B lymphocytes neurons cardiomyocytes skeletal muscle hepatocytes endothelium hematopoietic stem cells smooth muscle	p47phox p67phox p40phox p22phox	
NOX3	inner ear	fetal kidney fetal spleen skull bone brain	p22phox	
NOX4	kidney blood vessels	Osteoclasts Endothelium smooth muscle hematopoietic stem cells fibroblasts keratinocytes melanoma cells neurons	p22phox	
NOX5	lymphoid tissue testis	Endothelium smooth muscle pancreas placenta ovary uterus stomach various fetal tissues		+
DUOX1	thyroid	airway epithelia tongue epithelium cerebellum testis	DUOXA1	+
DUOX2	thyroid	salivary and rectal glands gastrointestinal epithelia airway epithelia uterus gall bladder pancreatic islets	DUOXA2	+

1.4.3.1 NADPH oxidase composition

The NADPH oxidases are multicomponent protein complexes with varying their compositions among the individual members of the family. All NADPH oxidases have a NADPH- and Flavin adenine dinucleotide (FAD)- binding domain in the C-terminal cytosolic region. NOX1-5 enzymes have six transmembrane regions, DUOX1 and DUOX2 have seven. In NOX1-5' third and fifth transmembrane domains, four heme-binding histidines are conserved, and these conserved residues are found in the fourth and sixth transmembrane domain of DUOX1 and DUOX2 (Lambeth, 2004). The composition of the classical NOX2 complex will be outlined below and relevant differences with the other NOX members will be mentioned. NOX2 is made up of a core complex of a membrane-bound flavocytochrome b_{558} complex, composed of the gp91phox and the p22phox subunits. The p22phox subunit is essential for NOX2 ROS production and stability (DeLeo et al., 2000; Dinauer et al., 1990). NOX2 function requires cytosolic factors that, upon activation, assemble with the membrane bound core complex to induce functional ROS production. These subunits include p47phox, p67phox, as well as p40phox and the small GTPase proteins Rac1/2 (Sheppard et al., 2005; Vignais, 2002).

NOX1-4 generally have the same composition with regards to the core enzyme, as all these family members have been shown to depend on p22phox (Bedard and Krause, 2007; Kawahara et al., 2005). However, they differ in the implication and requirement of cytosolic subunits. For instance, NOX1 regulatory subunits were shown to be homologues of p47phox and p67phox, namely Noxo1 and Noxa1, which are constitutively associated with NOX1 and p22phox (Banfi et al., 2003). NOX4, however, does not require a cytosolic regulatory subunit for its activity (Geiszt et al., 2000; Martyn et al., 2006). NOX5, which does not require p22phox or cytosolic regulators for ROS generation, has four intracellular N-terminal EF hand-Ca^{2+}-binding domains, making this protein highly susceptible to Ca^{2+} regulation (Banfi et al., 2001; Banfi et al., 2004; Kawahara et al., 2005). This is also the case for DUOX1 and DUOX2, which contain the same core structure as NOX5, with an additional N-terminal transmembrane domain and an extracellular N-terminal peroxidase domain

whose function remains controversial (Harper et al., 2006; Meitzler and Ortiz de Montellano, 2011). Additionally, DUOX1 and DUOX2 are not dependent on p47phox and p67phox, but proper transport to and expression at the membrane, as well as ROS production depend on activation factors DUOX activator 1 (DUOXA1) and DUOXA2 (Ameziane-El-Hassani et al., 2005; Grasberger and Refetoff, 2006; Morand et al., 2009).

1.4.4 The NOX2 NADPH Oxidase

1.4.4.1 Mechanism of ROS generation by NOX2

NOX2-mediated ROS generation in the phagosome is tightly regulated through the requirement of cytosolic regulatory subunits. In the resting state, p40phox, p47phox and p67phox are found as a trimer in the cytosol. p67phox, through its C-terminal Src homology 3 (SH3) domain, is associated with the C-terminal proline-rich region of p47phox (Lapouge et al., 2002). Further, the SH3 domains of p47phox, which are necessary for its association with the C-terminal proline-rich region of p22phox, bind intramolecularly to the autoinhibitory region (AIR) present in the C-terminal half of p47phox, thereby interrupting binding to p22phox (Groemping et al., 2003). Rac1/2 is bound to GDP (Mizuno et al., 1992). Phagocytosis of bacteria or stimulation with phorbol ester first induces, Rac-GDP exchange to Rac-GTP, whereafter Rac translocates to the NOX2 assembly site; and then, the phosphorylation and conformational change of p47phox, which enables its binding to p22phox (Dusi et al., 1993). The binding of p47phox to membrane-located phosphoinositides via the p47phox phox homology (PX) domain stabilizes p47phox localization to the cytochrome b_{558} complex (Nauseef, 2004). As p47phox relocalizes to the membrane, it brings p67phox and p40phox with it and p67phox consequently interacts with gp91phox and Rac1/2 (Koga et al., 1999). Interaction of p67phox' activation domain with gp91phox results in the activation of electron transfer from NADPH to FAD

(Nisimoto et al., 1999). It is noteworthy to mention that several kinases have been shown to phosphorylate p47phox and p67phox under diverse stimuli conditions. These kinases include members of the Protein kinase C (PKC) family (α, δ, β, ψ, ζ), Protein kinase A (PKA), p21-activated kinase, Extracellular signal-regulated kinases 1 (Erk1) and Erk2, Akt and Phosphoinosite 3 kinase (PI3K) (Lam et al., 2010). The functions of p40phox are much less studied. However, it is believed that p40phox is necessary for p67phox transport to the membrane as its PX domain can interact with phosphoinositides at the membrane, and its C-terminus interacts with p67phox (Ellson et al., 2001; Honbou et al., 2007; Kanai et al., 2001). Although p40phox is basally phosphorylated, it also becomes phosphorylated on Ser315 and Thr154 upon NADPH oxidase activation (Bouin et al., 1998). Once the translocation of cytosolic components to the NOX2 enzyme is activated, electron transfer is initiated. In the first step, electrons are transferred from NADPH to FAD, a process that is regulated by the activation domain of p67phox (Nisimoto et al., 1999). In the second step, a single electron is transferred from the reduced flavin $FADH_2$ to the iron center of the first heme. Then the first heme donates its electron to the second heme. Oxygen bound to the outer heme then accepts the electron and superoxide is produced (Doussiere et al., 1996; Vignais, 2002).

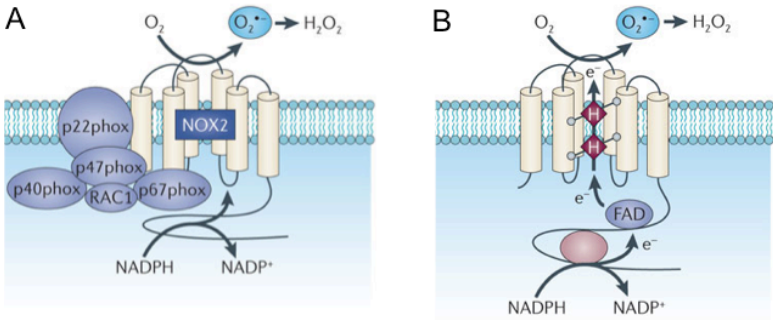

Figure 11: NOX2 structure and NADPH oxidase-dependent ROS production.
In **A**, the structural organization of the NOX2 NADPH oxidase is presented. The enzyme possesses six transmembrane regions and C-terminal binding sites for FAD and NADPH. For proper ROS production, NOX2 requires the membrane-bound p22phox subunit, as well as cytosolic cofactors p47phox, p67phox, Rac1/2 and p40phox. In **B**, the mechanism of ROS generation by NADPH oxidases is presented. Electrons are donated from NADPH and transferred to FAD, reducing it to $FADH_2$. A single electron is then transferred from $FADH_2$ to the first heme center of the NADPH oxidase. This center can only accept one electron. Oxygen is bound to the second heme center and receives the electron from the inner first heme. One-electron reduction of molecular oxygen (O_2) generates superoxide anion ($O_2^{\bullet-}$), which is then dismutated to hydrogen peroxide (H_2O_2). From (Block and Gorin, 2012), with permission from the Nature Publishing Group.

1.4.4.2 Regulation of NOX2 expression

The regulation of NOX2 expression has been studied mainly in myeloid compartments such as monocytes, macrophages and dendritic cells. While NOX2 is expressed constitutively in these cell types, its expression and ROS-producing activity can be augmented by certain stimuli. IFNγ is such a stimulus, and the upregulation of NOX2 expression by IFNγ has been demonstrated by various groups (Anrather et al., 2006; Cassatella et al., 1990; Eklund et al., 1996). Interestingly, IFNγ also positively regulates the expression of p67phox (Eklund and Kakar, 1999). In addition, TNFα can lead to the induction of NOX2, as well as p47phox and p67phox expression in

monocytes (Gauss et al., 2007). Transcription factors that positively regulate the expression of NOX2 in the above-mentioned contexts are IRF-1, IRF-2, Elf-1, PU-1 and NF-κB (Anrather et al., 2006; Gauss et al., 2007; Luo and Skalnik, 1996; Mazzi et al., 2004; Voo and Skalnik, 1999). GATA-1 has also been demonstrated to act as a transcriptional activator of NOX2 expression (Yang et al., 2000). Despite these data, in the context of AEC infection, no data exists to date that documents the regulation of NOX2 expression in this cellular compartment.

1.4.5 The DUOX2 NADPH oxidase

1.4.5.1 Mechanism of ROS generation by DUOX2

Whereas the primary product of NOX2 is $O_2^{\bullet -}$, DUOX2 releases mainly H_2O_2, and this despite its homology with NOX2 as well as fact that all NOX/DUOX enzymes function via a mono-electron transfer (Lambeth, 2004). However, $O_2^{\bullet -}$ formation has been detected during H_2O_2 generation in DUOX-expressing thyroid membrane fractions (Dupuy et al., 1991; Leseney et al., 1999; Nakamura et al., 1991). Thus, it is believed that $O_2^{\bullet -}$ dismutates to H_2O_2 intramolecularly. The DUOXA2 protein may be implicated in the decision of which final ROS species is produced by the DUOX2 enzyme. A recent report by Hoste *et al.* determined that residues in the N-terminal region of DUOXA2 regulate DUOX2 ROS production. In studies with wild-type DUOXA2 sequences, the DUOX2-generated ROS species was H_2O_2, whereas mutation of this region resulted in superoxide leaking from the DUOX2 protein (Hoste et al., 2012). Further studies support the hypothesis that a functional DUOX2-DUOXA2 complex fosters H_2O_2 production, whereas superoxide is formed when the formation of this complex is inhibited due to DUOXA2 retention in the endoplasmatic reticulum (ER) (Luxen et al., 2009; Morand et al., 2009). In this context, the physiological location of the DUOXA2 protein is still under debate. Whereas early findings localized it to the ER, where it functioned in the rapid ER to Golgi transport of correctly folded DUOX2, later studies have shown a necessity for DUOX2/DUOXA2

complexes at the plasma membrane for efficient DUOX2 expression and H_2O_2 production (Grasberger et al., 2007; Grasberger and Refetoff, 2006; Luxen et al., 2009; Morand et al., 2009). Unfortunately, a pitfall of the former mentioned studies is the use of different overexpression constructs that might account for the diverging results. It will thus be necessary to determine the location and function of the DUOXA2 protein at the endogenous level of a specific cell type, before making conclusions about its implication in the ROS-generating activity of DUOX2.

Due to the presence of two EF-hand Ca^{2+} binding motifs, DUOX2 activity is heavily regulated by Ca^{2+}. Ca^{2+}-dependent ROS production has been shown in various studies, especially via the use of agents capable of raising intracellular Ca^{2+} levels such as Adenosine Triphosphate (ATP), ionomycin, or thapsigargin (Ameziane-El-Hassani et al., 2005). Mutation of the EF-hand motifs also abolishes DUOX2 activity (Rigutto et al., 2009). The use of phorbol esters can induce DUOX2 activity in thyroid cells, via a PKC-mediated step that could possible involve direct phosphorylation of DUOX2 (Rigutto et al., 2009).

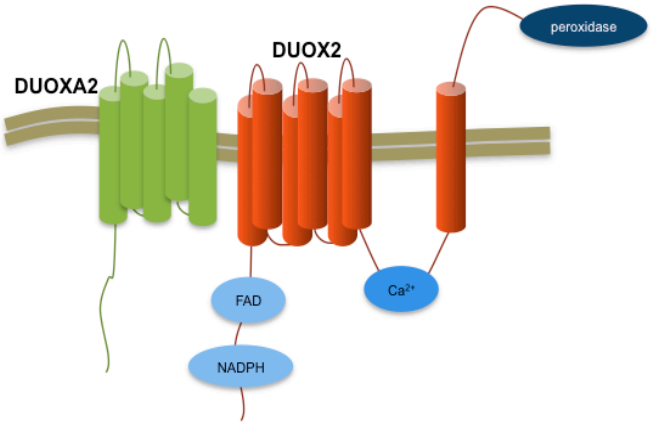

Figure 12: Structural organization of the DUOX2 NADPH oxidase.
DUOX2 possesses 7 transmembrane regions, C-terminal NADPH and FAD binding sites, two intracellular, N-terminal Ca^{2+} binding sites and an N-terminal extracellular peroxidase domain. DUOX2 function requires the presence of DUOXA2, whose location at the plasma membrane or at the ER is still a matter of debate.

1.4.5.2 Regulation of DUOX2 and DUOXA2 expression

The DUOX2 and DUOXA2 genes are located on chromosome 15 in a head to head arrangement (Grasberger and Refetoff, 2006). In the human genome, the transcription start sites (TSS) of DUOX2 and DUOXA2 are only 163bp apart and the two genes are thought to share one bidirectional promoter (Xu et al., 2012). This arrangement implies that DUOX2 and DUOXA2 are regulated in a similar manner, which is supported by the fact that DUOX2 and DUOXA2 are indeed upregulated by similar stimuli, including IFNγ, flagellin and rhinovirus (RV) (Gattas et al., 2009; Joo et al., 2012; Schneider et al., 2010). In addition, both of these genes are repressed in lung carcinomas due to promoter hypermethylation (Luxen et al., 2008). Other stimuli that have been shown to positively regulate DUOX2 expression include TNFα, all-trans retinoic acid (ATRA), IL-4 and IL-13. However, although TNFα induces DUOX2 expression in intestinal epithelial cells, it is not a DUOX2 inducer in pancreatic cancer cell lines or AECs, suggesting a strong cell-type specific expression

pattern of DUOX2 depending on certain stimuli (Harper et al., 2005; Lipinski et al., 2009; Wu et al., 2011). Cell type specific induction of DUOX2 also applies to IL-4/IL-13 stimulations, since these cytokines upregulate DUOX2 in thryocytes and intestinal epithelial cells, but not on AECs (Raad et al., 2012). In the latter model, they have been shown to induce DUOX1 expression (Harper et al., 2005). Cell type specificity of DUOX2 expression is also maintained at the TF level. For instance, whereas STAT1 binding to the DUOX2 promoter has been demonstrated in the IFNγ-mediated induction of DUOX2 expression in pancreatic cancer cells, STAT1 is completely dispensable for DUOX2 regulation in IFNγ-stimulated AEC (Hill et al., 2010; Wu et al., 2011). In the above-mentioned data on IL-4/IL-13 induced DUOX2 induction, a STAT6 implicating JAK-STAT pathway regulates DUOX2 expression. However, aside from these data, the identity of DUOX2-regulating TFs in other contexts, such as respiratory tract infection, remains enigmatic.

1.4.6 Mechanisms of ROS action

The following section will discuss the outcome of ROS production on the cellular environment. ROS can oxidize macromolecules such as lipids, DNA and proteins. Lipid and DNA oxidation are usually associated with oxidative stress and a loss of function of these molecules. Oxidative modification of proteins can have the same result. However, oxidative modification of proteins can also be considered to be a post-translational signal that regulates protein activity. The following paragraphs will focus mainly on oxidative protein modifications in the context of antiviral signaling.

1.4.6.1 Oxidative Protein Modification

Proteins can be modified by ROS in primarly two different ways, causing reversible and irreversible protein oxidations. While protein carbonylation is irreversible, the ROS-mediated attack on thiol groups can generate reversible oxidative

modifications (Antelmann and Helmann, 2011; Curtis et al., 2012). This kind of redox modification plays an important role in redox signaling. Protein carbonylation on the other hand is irreversible and is associated with oxidative damage of proteins.

As concerns the reversible oxidative modification of thiol groups, Cys and Met residues are the main targets. Cys residues are often at the center of the catalytic site of an enzyme and play a crucial role in its function. Due to the surrounding environment in the catalytically active site, which is rich in positively charged aromatic amino acid side chains, redox-sensitive Cys residues have a low pK_a value (D'Autreaux and Toledano, 2007). As a consequence of the intracellular physiological pH, these are present in a deprotonated and therefore highly reactive thiolate form (R-S-) (Brandes et al., 2009). Cys oxidation can thus easily occur on these residues. This form of posttranslational modification can therefore be a means of regulating the function of certain proteins.

Several possible oxidative thiol modifications have been described so far: S-thiolation (R-SS-R) by formation of intramolecular or intermolecular disulfide bonds or reaction with low molecular weight thiol-containing compounds, S-nitrosation (R-SNO), S-glutathionylation (R-S-GSH), as well as the formation of sulfenic (R-SOH), sulfinic ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$). The latter two are irreversible oxidative modifications (Finkel, 2011).

As regards irreversible protein carbonylation, the amino acid side chains of Pro, Arg, Lys and Thr are particularly susceptible (Curtis et al., 2012). Protein carbonylation results in semialdehyde formation on the amino acid side chain, which may change the tertiary structure of the protein and render the protein more prone to degradation. Further, protein carbonylation on the protein backbone via α-amidation can lead to peptide bond cleavage. Thus, protein carbonylation is generally thought to inactivate modified proteins (Dalle-Donne et al., 2006).

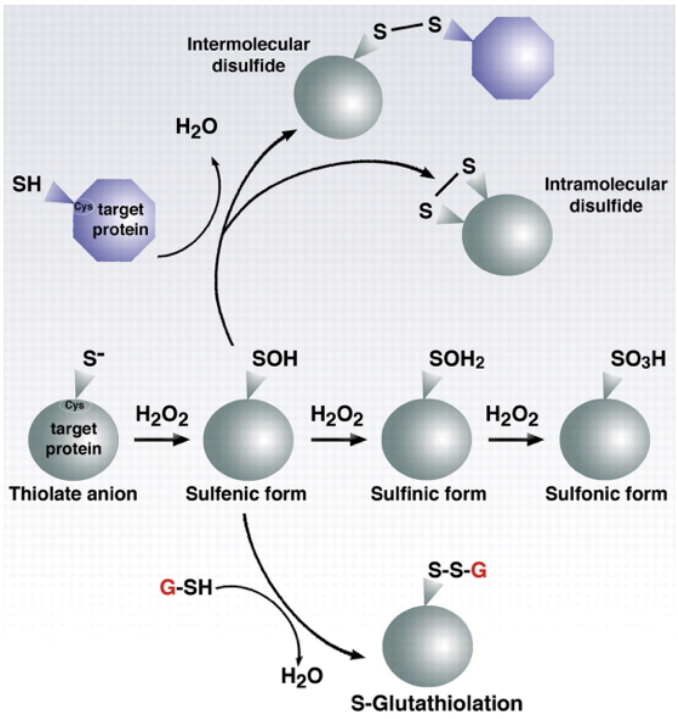

Figure 13: Redox modification of cysteine residues.

Specific reactive Cys residues in the form of a thiolate anion within target proteins can be the subject of covalent redox modifications. Oxidation leads to the formation of sulfenic, sulfinic or sulfonic forms, where the latter two are irreversible. The thiolate anion can also be subject to intra- and intermolecular disulfide bond formation. Further, it can react with other low molecular weight thiols such as glutathione in the process of S-glutathionylation. From (Finkel, 2011).

1.4.7 Evidence of ROS production in virus-infected cells

Before going into the details of regulation of antiviral signaling pathways by ROS, it is necessary to discuss ROS production in the setting of viral infections. Increased ROS production has been documented in the context of various viral infections, including Human Immunodeficiency Virus (HIV) and Hepatitis C virus (HCV) infection (Schwarz, 1996; Stehbens, 2004). In the respiratory context, an increase in ROS production has also been reported in the setting of respiratory virus

infection, such as RSV, RV and influenza (Akaike et al., 1990; Casola et al., 2001; Comstock et al., 2011).

It is important to note that an increase in cellular ROS levels and oxidative stress is not only generated by an increase in ROS production. An imbalance between ROS producing and antioxidant systems can also contribute to an increase in ROS levels. The latter phenomenon has indeed been observed in several viral infections. For instance, in RSV infection, the expression and/or activity levels of superoxide dismutase (SOD), catalase and Glutathione-S-transferase have been shown to be altered (Hosakote et al., 2009). A similar disruption of the antioxidant system has been reported in influenza infection (Hennet et al., 1992; Yamada et al., 2012).

At this point it is necessary to mention that a multitude of assays exist to determine changes in ROS production in a given context. These are often fluorescence-based techniques, some of which can discriminate between intra- and extracellular ROS production based on their capacity to enter cells. Some fluorescence-based techniques are specific for $O_2^{\bullet-}$, whereas others detect H_2O_2. However, this specificity is still a matter of debate (Wardman, 2007). For instance, Dichlorofluorescin diacetate (DCFDA), which is widely used as a probe for intracellular H_2O_2 in the above-mentioned examples, can also react with many other ROS and Reactive Nitrogen Species (RNS) (Chen et al., 2010). Another difficulty in using these probes comes from the many interferants that can influence the ROS signal. For instance, cellular antioxidant systems have been shown to interfere with oxidized fluorescent dyes (Chen et al., 2010; Winterbourn, 2008). Additionally, ROS probes are pH sensitive and changes in intracellular pH, which are observed during viral infection, can potentially falsifye the signal (Ciriolo et al., 1997). Thus, the in literature available data on ROS production must be critically analyzed.

1.4.8 Regulation of antiviral signaling pathways by ROS

As mentioned beforehand, the recognition of virus entry by PRRs engages into signaling cascades that will ultimately lead to the activation of IRF-3 and NF-κB,

which are essential TFs for gene transcription of type I and III interferons. In the following section we outline the current knowledge on redox regulation of antiviral signaling pathways.

1.4.8.1 Redox regulation of the NF-κB signaling pathway

The function of NF-κB has long been known to be highly sensitive to the cellular redox status in various physiological contexts (Morgan and Liu, 2011). In the context of virus infection, data exist that suggest NF-κB is subject to regulation in a redox-sensitive manner. For instance, antioxidant treatment affected levels of NF-κB activation in RSV infection, strongly suggesting a redox-control of NF-κB in this setting (Hosakote et al., 2012; Jamaluddin et al., 2009; Mata et al., 2011). In these contexts, ROS have been associated with an increase in NF-κB activity. Further, although not demonstrated in the context of viral infection, oxidative modification of NF-κB has been studied in various other contexts, such as cytokine stimulation. In those settings, ROS have been associated with both - activation and inhibition - of either NF-κB directly or components of the NF-κB signaling pathway.

Regarding direct oxidation of NF-κB by ROS it is generally accepted that the reducing environment found in the nucleus is important for the efficient activation of NF-κB target genes, whereas an oxidative environment ensures proper signaling in the cytosol (Kabe et al., 2005). In this context, it has been shown that NF-κB binding to DNA requires the reduced state of specific Cys residues in its N-terminal DNA recognition loop. More specifically, the Cys62 residue of the p50 subunit of NF-κB plays an important role. Cys62 is found oxidized in the cytoplasm, but reduced in the nucleus, which is necessary for DNA binding of NF-κB (Nishi et al., 2002). Furthermore, S-glutathionylation has been described for this residue (Pineda-Molina et al., 2001). These data demonstrate that Cys62 of p50 is a redox-sensitive residue that could also come into play in the context of viral infections. Besides the p50 subunit, the p65 subunit of NF-κB also possesses a redox-sensitive Cys at position 38. Alkylation of this residue has been demonstrated in an *in vitro* setting, however, the

physiological significance of this residue in the redox-regulation of NF-κB remains to be demonstrated (Garcia-Pineres et al., 2004). In a different study, this residue, as well as two other Cys residues in p65, Cys160 and Cys216, were targets for redox-regulation following TNFα treatment in combination with a ROS-inducing prostaglandin. S-glutathionylation of p65 in this context abolished TNFα-induced p65 nuclear translocation and Intercellular adhesion molecule 1 (ICAM1) expression (Lin et al., 2012). Thus, several evidence point to redox-sensitive residues in NF-κB that could come into play during the course of viral infection.

A central step in the pathway to NF-κB activation is the degradation of IκBα. ROS have previously been shown to control this event. H_2O_2 treatment affects the phosphorylation of IκBα at Tyr42 and leads to IκBα proteasome-independent degradation, thereby supporting an alternative NF-κB activation pathway (Schoonbroodt et al., 2000). Thus, IκBα is also a potential redox-target in the NF-κB activation cascade.

NF-κB activity is strongly under the control of post-translational modifications, for instance phosphorylation. The phosphorylation of Ser276 of p65 is necessary for the transcription of a subset of NF-κB target genes and for the interaction of p65 with the CBP/p300 activator. Upon stimulation with RSV it has been shown that this phosphorylation, which in this specific context is effectuated by cAMP dependent protein kinase A (PKAc), underlies a redox-sensitive mechanism (Jamaluddin et al., 2009). However, the mechanistic details implicated in the redox regulation of this post-translational modification remain to be explored.

One reason for the existence of redox-sensitive phosphorylation sites in NF-κB or IκBα is a potential for redox-regulation of kinases that act upstream in the signaling pathway. For instance, the activity of the IKK complex is central in mediating NF-κB activation. As mentioned, NF-κB activation depends on IKKβ phosphorylation of IκBα at Ser32 and Ser36, which leads to its proteasome-dependent degradation and liberation of NF-κB for translocation to the nucleus. H_2O_2 has a potential for activating IKKs in some cell types. For instance, dimerization of NEMO was fostered by H_2O_2 treatment through formation of disulfide bonds between Cys54 and Cys347

(Herscovitch et al., 2008). H_2O_2 has also been associated with IKK activation and potentiating Ser180 and Ser181 phosphorylations in the IKK activation loop (Kamata et al., 2002). Contrasting these reports on IKK activation by ROS, several reports have conversly shown that Cys179 of IKKβ is a target of oxidative modifications such as S-nitrosylation or S-glutathionylation, which consequently shut down IKKβ kinase activity and inhibit NF-κB activation (Kapahi et al., 2000; Reynaert et al., 2004; Reynaert et al., 2006). Thus altogether, although the above-mentioned studies to not demonstrate a clear consensus regarding the activating or inhibiting action of ROS on the NF-κB signaling pathway, these data strongly demonstrate that this pathway represents a potential target for redox changes occurring during viral infection.

At this point a word of caution needs to be mentioned regarding the interpretation of studies investigating NF-κB dependent redox regulation. Many of them relied and still rely on the use of H_2O_2 alone or in combination with NF-κB stimulants, such as cytokines. This led to the publication of partially contradictory data, whose interpretation remains valid but when compared to other literature becomes problematic due to the vast differences in experimental setups applied in the individual studies (H_2O_2 concentration and time of stimulation, method of H_2O_2 delivery, cell type, cell number, costimulation with NF-κB stimulants, etc.). Further, the use of antioxidants to study the importance of ROS in NF-κB signaling can lead to false results, as many of them have side effects that interfere with the correct interpretation of results. For instance, N-acetylcyteine (NAC) and Pyrrolidine dithiocarbamate (PDTC) have been shown to attenuate NF-κB activation independently of their antioxidant function (Hayakawa et al., 2003). This issue has been addressed extensively by Oliveira-Marques *et al.* and is likely the reason for the existence of discrepancies in the studies addressing the role or ROS in NF-κB activation (Oliveira-Marques et al., 2009).

1.4.8.2 Redox regulation of the interferon response

In the early antiviral signaling cascade, the TRAF family of proteins plays an important role in mediating the activation of kinases downstream of PRRs, which are in turn essential for TF activation. More precisely, TRAF3 and TRAF6 have been attributed important functions in this context. A recent publication by Gonzalez-Dosal *et al.* has demonstrated that TRAF3 and TRAF6 are subject to S-glutathionylation of a conserved Cys residue in the context of Herpes Simplex Virus 2 (HSV-2) infection in macrophages (Gonzalez-Dosal et al., 2011). S-glutathionylation of TRAF3/6 was important for the activation of IRF-3 and NF-κB during the course of viral infection. This example shows the importance and potential that oxidative modification of thiol residues has in the context of antiviral signaling.

In terms of redox regulation of the IRF-3 antiviral transcription factor, existing data so far are scarce and partly contradictory. The study of Prinarakis *et al.* demonstrates that IRF-3, in the non-infected cell, is S-glutathionylated at a basal level. Upon SeV infection, IRF-3 becomes deglutathionylated by Glutaredoxin-1. The authors show that this process does not affect IRF-3 phosphorylation or nuclear accumulation, but association of IRF-3 with the CBP/p300 coactivator (Prinarakis et al., 2008). With regards to IRF-3's redox sensitive Cys residues, Zucchini *et al*. attempted to analyze their importance in the context of SeV infection. While IRF-3 Cys289 was shown to be potentially redox-regulated, the significance of this residue and its redox-modification could not be confirmed during *in vivo* infection studies. However, alkylating agents still had an effect on IRF-3 activation *in vivo*, thereby suggesting that a still uncharacterized redox-sensitive thiol in a protein found upstream in the IRF-3 activation pathway might play an important role in antiviral signaling (Zucchini et al., 2012). This last study also shows the importance of the experimental setup in studies investigating redox regulation, as there might be a high discrepancy between results generated during *in vitro* reactions of proteins and alkylating agents aimed at identifying potential redox-sensitive residues and their actual biological significance in an *in vivo* setting.

Redox-sensitive mechanisms do not only regulate the early phase of the antiviral

response but equally the IFN-induced JAK-STAT signaling cascade. For instance, the JAK-STAT pathway can be activated by ROS (Simon et al., 1998). STAT phosphorylation is increased upon stimulation with H_2O_2 and one mechanism that could explain this phenomenon is the oxidative inactivation of tyrosine phosphatases implicated in the negative regulation of the JAK-STAT pathway. Protein phosphatases are highly prone to regulation by ROS, as they have a redox-sensitive Cys residue in their catalytic site (Ostman et al., 2011). Indeed, in the context of RSV infection, such a ROS-dependent inhibition of protein tyrosine phosphatases has been observed previously (Liu et al., 2004). One of the tyrosine phosphatases known to regulate the JAK-STAT pathway is Src-homology 2 containing protein phosphatase 2 (SHP-2) (Xu and Qu, 2008; You et al., 1999). Indeed, SHP-2 can become inactivated in the context of ROS exposure and a potential for reversible Cys oxidation has been demonstrated for this phosphatase (Boivin and Tonks, 2010; Kwon et al., 2005). Additionally, protein tyrosine phosphatase 1B (PTP1B) recognizes and dephosphorylates Tyk2 involved in type I IFN signaling (Myers et al., 2001). Several studies have reported the oxidative regulation of this phosphatase in its active site indicating that also this phosphatase could be regulated by differential redox-statuses emerging during virus-induced type I IFN signaling (Haque et al., 2011; Hirakawa et al., 2011). Last, STAT proteins themselves could be targets of redox-regulation. In this context, STAT3 contains redox-reactive Cys that can become oxidized, thereby changing significantly the promoter binding proteins of this protein (Buettner et al., 2011; Li and Shaw, 2004).

1.4.9 NADPH oxidase-dependent regulation of NF-κB

Considering, firstly, the key role NF-κB plays in the regulation of antiviral and inflammatory processes following an infection, and, secondly, its redox-sensitive regulation, as outlined in the previous paragraphs, it is now necessary to discuss evidence linking NADPH oxidases to NF-κB activation. The data describing redox-regulation of this transcription factor by NADPH oxidases is vast, as it is crucial in

processes as diverse as inflammation, immunity, cell proliferation and apoptosis. Adding to complexity is the fact that a given NADPH oxidase is activated in a stimulus-specific and cell-type specific manner. To remain within the scope of this thesis, the discussion in the following paragraphs will limit itself mainly on data related to a pulmonary infection/inflammation setting. As will be outlined, in most cases NADPH oxidases exerce an activating function on NF-κB, although in a few esamples, NADPH oxidase action is associated with NF-κB inhibition. A special emphasis is given to data obtained in NADPH knockout models or by RNAi-mediated decrease of NADPH oxidase expression rather than data solely obtained from the use of non-specific NADPH oxidase inhibitors or antioxidants.

In the contexts of *Pseudomonas aeruginosa* or LPS stimulation p47phox knockout mice have proven useful. In both contexts, the absence of the 47phox protein ablated NF-κB activation in the lung (Koay et al., 2001; Sadikot et al., 2004). It is noteworthy to mention that two recent publications do not support this data on NADPH oxidase-mediated NF-κB activation in the context of LPS stimulation. In a similar model, NADPH oxidase deficiency was associated with a decrease in NF-κB activation (Han et al., 2013; Segal et al., 2010). Although this discrepancy is rather intriguing, especially since all the data were obtained from the same laboratory, differences in the LPS instillation methods (intraperitoneal vs. intratracheal) could account for it. In this line, another independent group demonstrated that NADPH oxidase were not involved in the regulation of NF-κB in heart, lung, liver or kidney following intraperitoneal LPS stimulation of p47phox and gp91phox knockout mice (Zhang et al., 2009b). Interestingly, in a follow-up study from the same group, applying TNFα to p47phox and and gp91phox knockout mice, only NF-κB of the lung tissue was activated in a NADPH oxidase-dependent manner (Zhang et al., 2011). Interestingly, in Peripheral blood monoclear cells (PBMCs) from patients suffering from Chronic Granulomatous Disease (CGD), a disease where genetic mutations in the subunits of NADPH oxidase components render this enzyme inactive (CGD will be discussed in more detail in the following chapter), major steps in the NF-κB activation pathway were functional (Bylund et al., 2007). Thus, from these data it is evident that the dependency of NF-κB

activation on NADPH oxidase needs to be evaluated on a context-specific basis, carefully taking into account the experimental differences used among different research groups.

In a different context, the NOX2 NADPH oxidase has been implicated in the establishment of a cell-to-cell crosstalk between PMNs and lung vascular endothelial cells. NOX2-derived ROS from PMNs were necessary for NF-κB activation and consequent TLR2 and ICAM-1 expression in neighbouring lung endothelial cells (Fan et al., 2003). These data not only present yet another example of NOX2-dependent NF-κB activation, they suggest that NOX2-generated ROS do not only exerce their function in the cell type where they are produced but are able to diffuse to affect cellular signaling pathways in secondary cell types.

Although the above-mentioned data give clear examples of NOX2 function in NF-κB regulation in the lung tissue, the other NADPH oxidases in the lung have also been associated with regulation of NF-κB activity. For instance, DUOX1 influences NF-κB activation downstream of Epidermal Growth Factor Receptor (EGFR) signaling (Boots et al., 2009). Following TNFα stimulation of bronchial epithelial cells, lipid raft resident DUOX1 was found to be essential for NF-κB activation (Wang et al., 2011). Further, in human nasal epithelial cells, DUOX2 was implicated in the activation of NF-κB following stimulation with bacterial flagellin (Joo et al., 2012).

Altogether, this presents clear evidence for a potential of redox regulation of NF-κB by NADPH oxidases.

In conclusion, as the previous paragraphs demonstrate, the alteration of the redox-balance observed in the context of viral infection has the potential to modulate antiviral signaling pathways via reversible oxidative modification of proteins. NF-κB is a redox sensitive transcription factor and its activation status is strongly influenced by NADPH oxidases in various contexts. The following last chapter will make an attempt to introduce specifically the role of the NADPH oxidases as ROS producers and regulators of antiviral defenses of the lung.

1.5 NADPH oxidases in antimicrobial lung defenses

Airway epithelial cells are the very first cells exposed to airborne pathogens. As has been mentioned in the previous chapters, they are specialized in shielding off infection. However, once infection occurs, they are equally equipped for detecting pathogens and mounting defense mechanisms that will alarm surrounding cells as well as players of the innate immune system to clear the infection. This chapter will discuss the present knowledge about the implication of NADPH oxidases in the airway defense. The role of DUOX proteins in the antimicrobial defense as well as in inflammatory mechanisms at airway epithelial surfaces will be mentioned. The role of phagocyte NADPH oxidase NOX2 in diverse contexts of AEC defense will also be treated.

1.5.1 The many roles of DUOXs in the airway epithelial immunity

1.5.1.1 DUOXs contribute to airway antimicrobial defense

The most prevalent NADPH oxidases found in the airway linings are DUOX1 and DUOX2 (Forteza et al., 2005; Geiszt et al., 2003; Shao and Nadel, 2005). There, they exert diverse functions in the antimicrobial defense. First, a large number of evidence points to a major role of DUOX1/2 enzymes as part of an extracellular airway antimicrobial defense system involving DUOX-generated H_2O_2, lactoperoxidase (LPO) enzyme and the compound thiocyanite (SCN^-). It was previously acknowledged that H_2O_2 is constitutively secreted into the airways, and later studies have associated the DUOX1/2 enzymes with this ROS-generating activity (Forteza et al., 2005; Gattas et al., 2009; Geiszt et al., 2003; Moskwa et al., 2007). As for LPO, airway gland cells also secrete this enzyme into the airway lumen (Gerson et al., 2000; Wijkstrom-Frei et al., 2003) and LPO utilizes DUOX-generated H_2O_2 and SCN^-, which is able to pass trough ion channels, such as the Cystic fibrosis transmembrane conductance regulator (CFTR), to form the microbicidal compound hypothiocyanate ($OSCN^-$) (Gerson et al.,

2000; Wijkstrom-Frei et al., 2003). Several studies have reported decreased bacterial burden in cell cultures upon the activation of a functioning DUOX2/LPO/$OSCN^-$ system (Gattas et al., 2009; Gerson et al., 2000; Moskwa et al., 2007). This antibacterial system is defective in diseases like cystic fibrosis, due to a defect in SCN^- transport into the lumen of the airways but potentially also due to a decrease in DUOX2 expression in the lungs of Cystic Fibrosis (CF) patients (Moskwa et al., 2007; Wright et al., 2006). Further, it is targeted by *Pseudomonas aeruginosa*, an important etiologic agent in CF, via the expression of the redox-active virulence factor Pyocyanin. The latter inhibits DUOX upregulation but also DUOX activity by depleting intracellular NADPH levels since it causes intracellular $O_2^{\bullet-}$ production. Pyocyanin is thus able to subvert *P. aeruginosa* killing by the DUOX/SCN^-/LPO system (Rada et al., 2008; Rada and Leto, 2009).

Interestingly, in cell free assays, $OSCN^-$ has also been associated with virucidal activities (Mikola et al., 1995). A recent study however contradicted these early results and suggested that despite the involvement of DUOX-generated H_2O_2 in antiviral defense, the virucidal action was exerted by hypoiodous acid rather than $OSCN^-$ (Fischer et al., 2011). Nevertheless, a role for DUOX enzymes was affirmed, making it clear that these NADPH oxidase members could indeed play a role in airway antiviral defenses.

Besides the DUOX-based contribution to the formation of antibacterial $OSCN^-$, other reports suggest that DUOX-based antimicrobial action relies on different mechanisms. Botteaux *et al*. postulate that DUOX2-produced H_2O_2 might act as a bacterial repellent holding off invasive bacteria from the cell surface and thereby leading to decreased cytoinvasion (Botteaux et al., 2009). Further, DUOX activity might contribute to H^+ secretion into the airways and acidification of the airway lumen, which likely contributes indirectly to the LPO system, since LPO activity and antibacterial action are elevated at a pH around 6 (Schwarzer et al., 2004). Lastly, Harper *et al.* have proposed that the DUOX2 heme peroxidase domain is active, and could potentially contribute to antimicrobial mechanisms in a similar fashion as LPO (Harper et al., 2006). However, such a function has been contested by others who have

found that the mammalian peroxidase domain of DUOX was inactive (Meitzler and Ortiz de Montellano, 2011).

Regarding the antimicrobial function of DUOX1/2 it is noteworthy to mention that further studies, which were mostly conducted in the gut, exist that link the action of DUOX to antimicrobial defenses. For instance, in *Drosophila* it was shown that dDuox-lacking *Drosophila* are severely susceptible to microbe-contaminated food. It was later revealed that DUOX is not only important in fostering *Drosophila* antimicrobial activity but also for regulating homeostasis to gut-resident commensal bacteria (Ha et al., 2009b; Ha et al., 2005). Additionally, Kumar *et al.* demonstrated a role for *Anopheles gambiae* DUOX in maintaining homeostasis with midgut bacteria by establishing tyrosin cross-links in the mucin layer of the midgut, which serve to shield off immune elicitors from gut-resident bacteria (Kumar et al., 2010). That host DUOX generated H_2O_2 can exert its action as far as to the invading pathogen, thereby changing the microbe's virulence, was shown by Corcionivochi *et al*. In their interesting study they demonstrated that DUOX2-generated ROS regulate gut *Campylobacter jejuni* immunity by inactivating a bacterial kinase necessary for capsule formation (Corcionivoschi et al., 2012). Thus, the DUOX2-mediated antimicrobial action was conveyed by rendering a *C. jejuni* virulence factor less active.

Altogether, these data strongly suggest an important role for DUOX-dependent antimicrobial action in the airways. Data generated in different organisms show that this antimicrobial action is of great importance and well conserved. Data generated in the intestinal model hint at possible mechanistic features on how DUOX might foster microbial balance also in the lung environment.

Although all the above-mentioned data attribute a "positive" function for DUOX enzymes in the airway antimicrobial defense, a study published from Chattoraj *et al.* demonstrates that DUOX-derived H_2O_2 can also lead to complications associated with bacterial infections. The authors demonstrated that during RV infection DUOX2-derived H_2O_2 permitted the spread of *P. aeruginosa* planktonic bacteria from biofilms, thereby causing an exacerbated inflammatory cytokine production and increased bacterial transmigration across the airway epithelium (Chattoraj et al., 2011). This

could explain RV-induced bacterial exacerbations of CF patients chronically infected with *P. aeruginosa*.

1.5.1.2 DUOXs and airway inflammation

Besides the above-mentioned role of DUOXs in antimicrobial defenses, DUOX enzymes control several aspects of pathogen-induced inflammation. One mechanism of DUOX-mediated control of inflammation acts via the regulation of A Disintegrin and Metalloproteinase 17 (ADAM17), also called TNFα-converting enzyme (TACE). TACE is a membrane-bound enzyme that cleaves cell surface proteins, such as cytokines or their respective receptors. Via activities such as TNFα-, TNFR-, IL-6 or Epidermal growth factor α (EGFα) cleavage TACE is able to either inhibit or promote inflammatory processes (Scheller et al., 2011). In this context, a study of Yu *et al.* showed that DUOX2 was necessary for TNFR1 shedding upon TLR3 stimulation with poly I:C in AEC (Yu et al., 2011). This could constitute an important immunomodulatory mechanism in the context of viral infections. How exactly DUOX2 or ROS in general might regulate TACE activity is still under debate, but could involve oxidation of the inhibitory prodomain of TACE or oxidative regulation of TACE-activity promoting intracellular signaling pathways (Scott et al., 2011; Zhang et al., 2001b). The DUOX2 homologue DUOX1 has also been attributed an important role in TACE activation following phorbol-12-myristate-13-acetate (PMA) and Human Neutrophil Elastase (HNE) stimulation of AEC. Shao *et al.* demonstrated that DUOX1-produced ROS were necessary for release of Tumor Growth Factor α (TGFα), which culminated into Mucin 5AC (Muc5AC) expression (Shao and Nadel, 2005). Later, Boots *et al.* demonstrated a similar involvement of DUOX1 in LPS and asialo-GM1 (ASGM1) receptor-mediated IL-8 production (Boots et al., 2009). The mechanism of DUOX1-dependent IL-8 and Vascular Endothelial Growth Factor (VEGF) production via a TACE-TGFα-Epidermal Growth Factor Receptor (EGFR) signaling axis was confirmed by other groups following stimulation of TLR2, TLR3, TLR4, TLR5 and TLR6 as well as PMA (Koff et al., 2008; Nakanaga et al., 2007). Thus, from these studies it is evident that the DUOX1/2 enzymes are important regulators of TACE-

mediated shedding of diverse cell surface molecules and that by these mechanisms they might be implicated in the most diverse action of airway inflammation.

A recent study has placed the NADPH oxidase DUOX2 into the NF-κB activation pathway of flagellin-induced TLR5 signaling in normal human nasal epithelial cells. Joo and collegues demonstrated that DUOX2 positively influences flagellin-induced IL-8 and Muc5A levels, necessary for neutrophil recruitment and airway surface defense, via the regulation of NF-κB. More detailed experiments suggest that TLR5 and DUOX2 colocalize, implying that the proximity of TLR5 and DUOX2 is important in this system. However, no mechanism on how DUOX2 fosters NF-κB activation was presented. On the other hand, their work included the very first data obtained in DUOX2 knockout mice challenged with flagellin, which showed that DUOX2 is able to influence the expression of various cytokines and chemokines as well as IFN-related genes culminating in a decreased neutrophil infiltration in DUOX2 knockout mice (Joo et al., 2012). Although from the latter studies on knockout models it is difficult to dissect the importance of DUOX2 in the hematopoietic vs. non-hematopoietic compartment, it is clear that this NADPH oxidase has an important role in regulating airway epithelial defense mechanisms.

1.5.1.3 NOX/DUOX enzymes as regulators of airway epithelial integrity

The respiratory epithelium serves as an important barrier that protects the organism from invading pathogens. To conserve this function during stress, infection and injury, repair mechanisms must function properly to ensure the continuity of airway epithelial integrity. Wound repair is equally important for the resolution of infection and inflammation in airway tissues. The NADPH oxidase DUOX1 has a major implication in the process of wound healing. It is known that DUOX1-produced H_2O_2 mediates wound closure (Gorissen et al., 2013). This involves the above-mentioned EGFR signaling pathway of EGFR leading to the activation of Erk and Matrix metalloprotease 9 (MMP9) expression (Wesley et al., 2007). In this line of evidence, *Pseudomonas* LPS also generates a DUOX1-dependent wound repair process

in the airway epithelium. LPS led to PKC-dependent DUOX1 activation that culminated in a TACE-TGFα-EGFR signaling cascade to initiate cellular repair mechanisms (Koff et al., 2006). In this context, a more recent publication suggests that this involves DUOX1-dependent oxidation of Src and TACE (Sham et al., 2013). An interesting study conducted in the zebrafish model links DUOX-generated H_2O_2 production at the site of the wound to leukocyte recruitment, a necessary event for wound repair. Wounded epidermal tissue might thus use DUOX-generated H_2O_2 not only to activate its own repair program but H_2O_2 could serve as an essential extracellular second messenger for communication of wounded cells with the hematopoietic compartment thereby contributing to the activation of further repair mechanisms.

On the contrary to the above-mentioned positive contribution to airway epithelial integrity by epithelium-based DUOX1, another member of the NADPH family, NOX1, might play an adverse effect on epithelial barrier function. Comstock *et al.* showed that loss of airway epithelial barrier function during RV infection is attributable to NOX1-derived ROS. These were found to be responsible for virus-induced breakdown of tight junction complexes (Comstock et al., 2011).

1.5.2 The role of NOX2 in airway immunity

Upon lung infection, lung resident innate immune cells play a major role in the recruitment of further innate and adaptive immune players into the lung. Among these, professional phagocytes, such as macrophages, neutrophils, eosinophils and dendritic cells express NADPH oxidase NOX2 (Babior et al., 1976; Elsen et al., 2004). A major function of NOX2 in airway immunity and immunity in general is the destruction of pathogens during the process of oxidative burst in phagocytes. This mechanism is essential for eliminating bacterial and fungal pathogens that are engulfed during phagocytosis.

The importance of NOX2 is shown in the context of Chronic granulomatous disease (GCD), a disease where genetic mutations in the subunits of NADPH oxidase components render this enzyme inactive. The predominant form of GCD results from mutations in the *CYBB* gene encoding gp91phox (NOX2), which is inherited in a X-linked recessive manner. Other mutations affect p22phox, p40phox, p47phox, and p67phox, which are inherited in an autosomal recessive manner (Bylund et al., 2010). Patients affected by this disease have a deficiency in oxidative burst production and therefore suffer from recurrent infections. Additionally, these patients also show increased inflammation and granuloma formation in organs. These are cellular structures that center around microorganisms or cells containing intracellular microorganisms, and are bordered by a cellular barrier. The increased number of granuloma and the increased inflammatory status in GCD patients imply the implication of NOX2 in processes other than the oxidative burst (Kuijpers and Lutter, 2012). Studies conducted with samples from patients suffering from CGD or studies conducted in mouse GCD models have been valuable in the elucidation of these additional functions of NOX2.

1.5.2.1 NOX2 controls inflammation

In CGD, an increase in the inflammatory environment is a major complication of the disease. This increased inflammation is present at a basal level, as PBMCs from CGD patients show elevated levels in secreted proinflammatory cytokines, even in the absence of microbial infection (Kobayashi et al., 2004). In addition, granulomas found in CGD patients are often sterile, thus not containing microorganisms, which strongly suggests a dysregulation of the proinflammatory setting (Schappi et al., 2008). The exact molecular reason for the increased inflammatory status is not yet clear, but it is evident that the NOX2 complex is implicated in the regulation or dampening of inflammatory processes.

The implication of NOX2 in the regulation of viral infection-related inflammation has been studied by several groups in the context of influenza infection. Infection of NOX2 knockout mice with influenza virus is associated with a reduction

in viral titer (Snelgrove et al., 2006; Vlahos et al., 2011). However, while Vlahos *et al.* documented a reduction of overall inflammation, the number of BALF macrophages, MCP-1 levels and apoptosis, Snelgrove *et al*. described elevated proinflammatory cytokines with a Th1 bias, expanded neutrophils, macrophages and T cell infiltrates, and elevated MCP-1 levels. Thus, although these two studies diverge on the results regarding the inflammatory status, both present reduced lung damage, improved lung function and improved disease outcome in the absence of NOX2. Thus, NOX2 seems to have a negative impact on resolution of viral infection. Whereas Snelgrove *et al.* proposed the reduced virus titers were due to increased macrophage numbers in the airways in the $Nox2^{-/-}$ mice and presumably enhanced clearance of the virus by these cells, the study of Vlahos *et al.* shows a reduction in macrophages in the $Nox2^{-/y}$ lungs, which is thus unlikely to explain the reduction in viral titers observed in their study. It is noteworthy to mention that these divergences as well as those observed related to the inflammatory status could be due to different factors such as genetic background, gender, and viral dose, all of which were not homogenous in the mentioned studies. Regarding the issue of gender, Miller *et al.* previously showed significant gender differences for NOX2 activity in the setting of experimental stroke in mice, and such differences could come into play during influenza infection (Miller et al., 2007). Regarding role of NOX2 in the regulation of virus-induced inflammation, a positive contribution of NOX2 to inflammation has been demonstrated by another group. Decreased inflammation and acute lung injury were observed upon challenge of p47phox knockout mice with a highly virulent H5N1 strain (Imai et al., 2008). The authors of this study elucidated that acute lung injury was caused by the presence of oxidized phospholipids, which in turn can induce a TLR4-TRIF-TRAF6-IL-6 signaling cascade culminating into increased airway inflammation. In the absence of p47phox, these oxidized phospholipids were not detected. Thus, in the context of viral infection, NOX2 appears to promote proinflammatory processes, which in cases of highly virulent infection may lead to major airway complications.

A similar phenomenon is observed in bacterial infections, where NOX2 is associated with increased inflammation as measured by an increase in pulmonary proinflammatory cytokine levels and increased neutrophil infiltration into the affected

lung (Gao et al., 2002; Segal et al., 2010; Zhang et al., 2009b). However, again the underlying mechanisms resulting in increased inflammation and proinflammatory cytokine levels are still unclear. NF-κB, which transcriptionally regulates the expression of proinflammatory cytokines in these settings was previously shown to be either positively, negatively or not at all regulated by components of the NADPH oxidase complex (Sadikot et al., 2004; Segal et al., 2010; Zhang et al., 2009b). Thus, the molecular details by which NOX2 contributes to increased inflammation in viral infections still need to be investigated.

1.5.2.2 NOX2, a pleiotropic regulator of innate immunity

The previous sections discussed the role of NOX2 in driving inflammatory processes during viral and bacterial airway infections. It is noteworthy to mention that NOX2 regulates other immunomodulatory processes that are likely to come into play during the course of viral infections but that have not yet been explored in such a context.

First, NOX2 has been implicated in the mechanism of antigen-crosspresentation in DCs to $CD8^+$ T cells. NOX2-deficient mice and DCs from patients suffering from CGD show decreased cross-presentation of antigenic peptides due to a deregulation of the pH in phagosomic vacuoles. Indeed, for efficient cross-presentation to occur, NOX2 activity must sustain an alkalic pH in the DC phagosomic compartment, which limits the activity of proteases and favors the generation of peptides that have the appropriate length for cross-presentation (Mantegazza et al., 2008; Savina et al., 2006). A similar defect of cross-presentation is observed in p47phox deficient mice exposed to Aspergillus antigens, due to defective DC endosomal alkalization and autophagy (De Luca et al., 2012).

Further, NOX2 has been implicated in the regulation of autophagy by another study. The authors found that NOX2-generated ROS are necessary for LC3 recruitment to phagosomes, a process necessary for the correct formation of the autophagosome and consequent microorganism killing (Huang and Brumell, 2009). It is noteworthy to mention that autophagy, besides being a proposed mechanism for intracellular bacteria

elimination, can also impact on viral infections in pro- or antiviral ways, depending on the virus. In the context of RSV infection, autophagy has been shown to be essential for RSV-induced cytokine stimulation, activation and antigen presentation in dendritic cells (Morris et al., 2011). Thus NOX2 can likely impact airway immunity via autophagy regulation following respiratory virus infection.

1.5.2.3 Polyvalent functions of NOX2 in lung structural cells

The above-mentioned paragraphs discussed the ample functions that NOX2 plays in regulating lung immunity to virus infection. NOX2 is strongly expressed in cells of the immune compartment, which generates a strong bias towards the discussion of NOX2 in cells of the immune compartment. However, NOX2 is also expressed, albeit to lower levels, in lung structural cells, such as the lung epi- and endothelium, as well as in lung fibroblasts. There, it regulates various physiological processes. At this point some of these processes relevant to an infectious setting will be discussed.

Firstly, the lung mucosa hosts so-called pulmonary Neuroepithelial bodies (NEBs), which function in oxygen sensing and mediate responses to hypoxic or hyperoxic conditions. In this context, NOX2 acts as an oxygen sensor and controls the activity of K^+ channels (Buttigieg et al., 2012; Fu et al., 2000). In an infectious context, where changes in oxygenation are likely to occur, NOX2 might thus exerce its oxygen sensing function.

NOX2 is also implicated in the maintenance of lung fluid balance. The latter is essential for proper gas exchange but also for efficient functioning of airway immune mechanisms. The quantity of lung fluid in the luminal space is controlled by the activity of Epithelial sodium channels (ENaCs). This balance can be perturbed in the context of respiratory virus infection (Song et al., 2010). Recent investigations have shown that NOX2 controls ENaC activity and therefore ensures a properly moist luminal space (Goodson et al., 2012; Takemura et al., 2010).

Besides the importance of the lung epithelium, the lung endothelium also plays a vital role in supporting airway defenses. It is strongly implicated in the orchestration of inflammatory processes following viral infection (Teijaro et al., 2011). The NADPH

oxidase NOX2 is implicated in the regulation of endothelial inflammatory processes following LPS stimulation or *P. aeruginosa* infection (Fu et al., 2013; Pendyala et al., 2009). More specifically, following LPS stimulation, endothelial NOX2 regulates the activation of key kinases TAK1 and IKKβ in the NF-κB activation pathway (Menden et al., 2013).

NADPH oxidases also appear to play a role in the regulation of the immune response of lung fibroblasts to rhinovirus infection. However, human lung fibroblasts do not express NOX2, but rather NOX4 (Dhaunsi et al., 2004). Future studies will demonstrate if this NADPH oxidase replaces the polyvalent NOX2 in this subset of lung cells.

In conclusion, NADPH oxidases are pertinent players in the airway microbial defense mechanisms. Not only do they impact pathogen-induced inflammatory responses, as has been shown for DUOX1/2 and NOX2, but these enzymes directly contribute to pathogen elimination in the airway lumen as well as in phagocytes. Many of the data suggest a role for NADPH oxidases in the context of viral infections. We therefore sought to unravel the implication of the NADPH oxidases in *Paramxoviridae* infection of AEC.

1.6 Study hypothesis and strategy

As outlined in the previous sections, AEC are important regulators of airway immune responses following microbial challenge. They are responsible for pathogen detection and the integration of this event to trigger a series of processes that result in the activation of innate and adaptive airway defense mechanisms. Upon AEC viral infection, an antiviral and proinflammatory state is generated that is at the very heart of the viral clearance process. NF-κB is a key transcription factor implicated in the generation of AEC antiviral and proinflammatory responses. To optimally generate these responses, their establishment is tightly regulated at several steps of signaling chain. As we have seen, phosphorylation events are major contributors to the activation of NF-κB. Besides this, the antiviral signaling pathways also come under a different mode of regulation – redox regulation. Importantly, ROS are produced following RSV infection (Casola et al., 2001). Data using chemical inhibitors suggest that NF-κB is a potential target for redox-regulation by ROS in RSV-infection of AEC (Carpenter et al., 2002; Casola et al., 2001; Castro et al., 2006; Roebuck et al., 1999). These data also show that ROS might contribute to the inflammatory process following infection with RSV. NADPH oxidases have previously been demonstrated to regulate NF-κB activation in diverse settings, however, no data so far links them to NF-κB activation following *Paramyxoviridae* infection (Boots et al., 2009; Joo et al., 2012; Sadikot et al., 2004).

Based on this evidence, our study hypothesis was that **NADPH oxidases regulate NF-κB and contribute to NF-κB-mediated proinflammatory mechanisms following *Paramyxoviridae* infection of AEC**. Since the above-mentioned data does not reveal a NADPH oxidase candidate implicated in these processes we aimed to **identify which NOX was responsible for NF-κB regulation following viral infection of AEC**. **Further, we aimed to determine the mechanism by which NOX regulated NF-κB in this context.**

The results generated during the characterization phase of NADPH oxidase expression in our cell model revealed an increased DUOX2 expression in AEC upon SeV infection. The finding of DUOX2 induction of gene expression following virus infection of AEC is supported by other data demonstrating DUOX2 induction in the context of rhinovirus infection (Harper et al., 2005). Hundreds of ISGs are upregulated following viral infection during the antiviral interferon response in order to combat infection, limit viral spread and favor viral clearance (Schoggins and Rice, 2011). The literature demonstrated an essential role for DUOX2 in antimicrobial defenses (Gattas et al., 2009; Moskwa et al., 2007). Based on these data our hypothesis in a second instance was that **DUOX2 is an ISG induced upon viral infection which functions in the antiviral defense**.

In order to adress these questions, we decided to study the importance of the NADPH oxidases in SeV- and RSV-infected AEC. As mentioned, the *Paramyxoviridae* family contains several important etiologic agents of respiratory tract disease, such as RSV. This virus not only interferes with antiviral immunity, it also induces several aspects of immunopathology, among which the so-called "cytokine storm" is believed to be a main contributor to RSV disease. SeV, although not a health threat to humans, readily infects AEC and induces a robust antiviral response. Thus it has become a common model in the study of the antiviral response.

Our main cell model of study consisted of the AEC line A549, which is a type II alveolar cell line. Although this subset of AEC is not the major target of natural RSV infection (which are ciliated bronchial epithelial cells), A549 are commonly used to study the airway antiviral response. Further, it was important for us to stay in a model with which we would be able to compare our data to already existing findings. However, we were well aware of this limitation and we have used other models when avalaible (i.e. other airway cell lines or primary AEC).

2 Results

2.1 Dual role of NOX2 in Respiratory Syncytial virus- and Sendai virus-induced activation of NF-κB in airway epithelial cells

2.1.1 Context and resume of results

The cellular response to viral infection is strongly driven by the activation of intracellular TFs, which regulate the expression of genes that will further drive this response by triggering an antiviral state and activating innate and adaptive immune axes. Among these TFs, NF-κB plays a central role, firstly, via its regulation of proinflammatory cytokines expression, but also via its capacity to regulate the expression of antiviral-state inducing interferons. Redox-regulation of NF-κB has long been described in various physiological settings (Gloire and Piette, 2009; Yao et al., 2007). Further, previous to our study, evidence existed which demonstrated that NF-κB activity underlied similar regulatory mechanisms in the context of viral infections. Indeed, it was shown that in the AEC model, NF-κB activation and the expression of its target genes were regulated in a redox-sensitive manner following infection with RSV (Carpenter et al., 2002; Casola et al., 2001; Mastronarde et al., 1995; Roebuck et al., 1999). Further, AEC infected with RSV elicited increased ROS production (Casola et al., 2001). Importantly, mice challenged with RSV in the presence of antioxidants showed a different inflammatory status, decreased viral burden and an overall decreased disease outcome compared to control mice (Castro et al., 2006). Thus, targeting ROS promised to be beneficial for the outcome of viral infection. However, the source of ROS production was not addressed in the mentioned studies. Work conducted in influenza-infected, NOX2-deficient mice however demonstrated that the NADPH oxidases, and more specifically NOX2, could play an important role in regulating antiviral and proinflammatory responses following viral infection of AEC (Snelgrove et al., 2006). Importantly, these data were since confirmed by a second

group (Vlahos et al., 2011). However, these data gave no idea on the importance of NOX2 in the regulation of antiviral and proinflammatory processes specifically in AEC. Thus, the importance of the NADPH oxidase family members in the setting of AEC viral infection needed to be evaluated. Hence, our study sought to identify the NADPH oxidase family member responsible for NF-κB regulation in a redox-sensitive manner following infection of AEC with *Paramyxoviridae* RSV and SeV. Briefly, using a combination of chemical NADPH oxidase inhibitors and NOX2-targeting RNAi applied to airway epithelial cell lines as well as primary AEC, we were able to demonstrate a role for NOX2 in the regulation of NF-κB. More specifically, we identified that NOX2 was necessary for fostering phosphorylation events of IκBα and the NF-κB subunit p65, cumulating in NF-κB activation and target gene expression. Altogether, our results demonstrate that NOX2 is a key regulator of airway antiviral immunity and inflammation.

2.1.2 Author contributions

I (KF) began working on the data for this article during my Master's studies with Anton-Soucy Faulkner (ASF). Experiments were planned under the supervision of and guidance by my research supervisor Nathalie Grandvaux (NG). My research contributed to seven out of eight figures (for details, see below) of this article. Annick Duval (AD) worked closely with me to finalize experiments, conduct revision experiments and RSV purifications. Alexis Martel (AM) initiated real time PCR experiments and helped me with certain IκBα part of figures. I contributed, in collaboration with Nathalie Grandvaux, to the writing of the article. This article was published in the *Journal of Immunology*, 2008,180(10):6911-22.

Figure 1 : KF/AD/NG
Figure 2 : AD
Figure 3 : KF/AM
Figure 4 : KF/NG et AD (panel E et F)
Figure 5 : AD/KF/AM

2.1.3 Article

Dual role of NOX2 in Respiratory Syncytial Virus- and Sendai Virus-induced activation of NF-κB in Airway Epithelial Cells [1]

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Running title: NOX2-mediated NF-κB regulation in *Paramyxoviridae* infection.

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Abstract

Human Respiratory Syncytial Virus (RSV), a member of the *Paramyxoviridae* family, is the most important viral agent of pediatric respiratory tract disease worldwide. Human Airway Epithelial Cells (AEC) are the primary targets of RSV. AEC are responsible for the secretion of a wide spectrum of cytokines and chemokines that are important mediators of the exacerbated airway inflammation triggered by the host in response to RSV infection. NF-κB is a key transcription factor responsible for the regulation of cytokine and chemokine genes expression and thus represents a potential therapeutic target. In the present study, we sought to delineate the role of RSV-induced reactive oxygen species (ROS) in the regulation of the signaling pathways leading to NF-κB activation. First, we demonstrate that besides the well-characterized IκBα-dependent pathway, phosphorylation of p65 at Ser536 is an essential event regulating NF-κB activation in response to RSV in A549. Using antioxidant and RNA interference strategies, we show that a NOX2-containing NADPH oxidase is an essential regulator of RSV-induced NF-κB activation. Molecular analyses revealed that NOX2 acts upstream of both the phosphorylation of IκBα at Ser32 and of p65 at Ser536 in A549 and Normal Human Bronchial Epithelial Cells (NHBE). Similar results were obtained in the context of infection by Sendai virus (SeV), thus demonstrating that the newly identified NOX2-dependent NF-κB activation pathway is not restricted to RSV amongst the *Paramyxoviridae*. These results illustrate a previously unrecognized dual role of NOX2 in the regulation of NF-κB in response to RSV and SeV in human AEC.

Introduction

Respiratory Syncytial Virus (RSV), an enveloped, negative-sense single-stranded RNA virus of the *Paramyxoviridae* family, is the most important viral agent of pediatric respiratory tract disease worldwide. Clinical manifestations following RSV infection range from rhinitis, otitis or pneumonia to bronchiolitis, an acute lower respiratory tract infection associated with cough and wheezing and substantial morbidity and mortality (reviewed in (1, 2)). Moreover, RSV infection is associated with long-term complications, such as recurrent wheezing and asthma (reviewed in (3)).

The mechanisms underlying RSV-induced airway diseases and long-term consequences are still largely unknown. However, experimental evidence suggests that an excessive inflammatory response triggered by the host plays a major role in the development of the clinical manifestations of RSV infection (4). Airway Epithelial Cells (AEC) of large and small airways are the primary targets of RSV infection and replication. *In vitro* and *in vivo* studies revealed that AEC are mainly responsible for the secretion of a wide spectrum of cytokines and chemokines which include, but are not restricted to, RANTES, MIP-1α, MCP-1, Interleukin (IL)-8 and IP-10, that have profound immune and inflammatory functions, and thus determine the elimination or progression of the infection and/or inflammation of airway mucosa (4-10). Chemokine levels in the lung of RSV-infected mice parallel the intensity of lung cellular inflammation (11). Moreover, IL-8, RANTES, and MIP-1α levels in bronchoalveolar lavages of children infected with RSV correlate with the severity of the disease (12, 13). Thus, chemokines are considered major players in the RSV-induced respiratory pathogenesis.

The Nuclear Factor (NF)-κB transcription factor plays a pivotal role in inflammatory processes triggered by various stimuli through regulation of the expression of genes encoding numerous proinflammatory cytokines and chemokines (reviewed in (14)). Substantial data support a role of NF-κB in the regulation of cytokine and chemokine genes, such as *TNFα*, *RANTES* and *IL-8*, in AEC following RSV infection (4-10). Thus, NF-κB is considered a preferred target in the development

of therapeutic interventions aimed at limiting the inflammatory response. However, the lack of a complete understanding of the pathways leading to its activation is a barrier in achieving this goal.

NF-κB is a ubiquitous family of homo- and heterodimers of Rel proteins, consisting of p65, cRel, RelB, p50 and p52. The Inhibitor of NF-κB (IκB) forms a complex with NF-κB dimers, mainly p65/p50 in epithelial cells, that shuttles between the nucleus and the cytoplasm, with a predominant cytoplasmic localization in unstimulated cells (15). The central event of the classical NF-κB activation cascade following exposure to proinflammatory stimuli is the activation of the IκB kinase (IKK) complex, composed of two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ (16). In turn, IKK phosphorylates IkBα at Ser32 and Ser36 to promote its polyubiquitination and subsequent proteasome-mediated degradation, thereby allowing the freed NF-κB to translocate to the nucleus, bind to kB consensus sequences and transactivate target genes (17). A negative feedback loop involves NF-κB-dependent new synthesis of IκB (15). Recently, additional steps in the activation cascade have been identified, which involve phosphorylation and acetylation of the Rel subunits to fine-tune the control of nuclear localization, DNA-binding affinity, coactivator/corepressor association, and transactivation capacity (reviewed in (18, 19)). Thus far, inducible phosphorylation of cRel in the transactivation domain (TD) and of p65 at Ser residues 276 or 311 in the Rel homology domain or at Ser residues 468, 529, 535 or 536 in the TD have been observed in a stimuli- and cell type-dependent manner (reviewed in (18, 19)). In some conditions, direct phosphorylation of NF-κB subunits was found to define an alternative mechanism of NF-κB activation independently of IκBα degradation. Rather, phosphorylation of NF-κB subunits was found to control NF-κB nuclear accumulation by inducing NF-κB/IκBα complex dissociation (20-23).

RSV infection induces a persistent activation of NF-κB, which likely leads to excessive NF-κB-mediated inflammatory genes expression (24, 25). Activation of the classical NF-κB activation pathway in RSV-infected AEC is supported by *in vitro* and *in vivo* studies that revealed IKK activation, proteolysis of IκB, and p65 DNA-binding (24-32). Interestingly, a temporal dissociation between IκB phosphorylation and NF-κB

DNA-binding activity suggested the existence of an IκB-independent regulation induced by RSV (26). RSV-induced phosphorylation of p65 at Ser276 and Ser536 was previously reported, but its physiological significance in NF-κB activity was not evaluated (33).

In the past decade, the concept that sub-toxic levels of reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, act as cellular switch for signaling cascades leading to regulation of gene expression has emerged. ROS regulate a variety of physiological processes, including cell proliferation, apoptosis and immune and proinflammatory responses (reviewed in (34)). There is now compelling evidence that ROS participate in the regulation of NF-κB activation in a cell type- and stimuli-specific manner (reviewed in (35)). Several data support a role of ROS in the activation of NF-κB in response to RSV infection in AEC. First, RSV infection was shown to trigger ROS production in AEC (36). Moreover, antioxidants, including butylated hydroxyanisol (BHA) or N-acetylcysteine (NAC), blocked *IL-8, RANTES* or *MCP-1* genes expression in human AEC (29, 36-38). Use of NAC suggested that redox-sensitive p65 DNA-binding was responsible for the observed *IL-8*, *RANTES* and *MCP-1* gene expression (29). However, conflicting results proposed that the Activator Protein (AP)-1 transcription factor, but not NF-κB, was responsible for the redox regulation of RSV-induced *IL-8* gene expression in AEC (39).

ROS generating enzymes include the mitochondrial respiratory chain, 5-lipoxygenase, xanthine oxidase and the NADPH oxidases. The most potent source of ROS is the recently discovered family of NADPH oxidase enzymes, which are now known to be functionally expressed in a number of cells, including AEC (reviewed in (40)). In phagocytes, the NADPH oxidase is a multi-subunit complex composed of a membrane bound flavocytochrome b_{558}, consisting of NOX2/gp91phox and p22phox subunits, as well as of the cytosolic regulatory subunits, p47phox and p67phox. NOX2-containing NADPH oxidase complex is widely reputed for its role in bacteria killing during phagocytosis (reviewed in (41)). Six functionally distinct homologues of NOX2, namely NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2, and homologues of the cytoplasmic subunits, NOXO1 and NOXA1, have been identified (reviewed in

(40). Recent functional data have emerged that suggest the involvement of several of these isoforms in the innate host response to invading microorganisms, including innate immune and proinflammatory responses (reviewed in (42)). Surprisingly, while the role of NADPH oxidase enzymes in virus infections, including infection by RSV, is suggested by the use of antioxidants or cells derived from patients with a genetic defect in one of the phagocytic NADPH oxidase subunits (reviewed in (42)), the identity of the NADPH oxidase responsible for ROS production and its functional significance are barely documented. Interestingly, it was recently shown that the absence of NOX2 led to reduced virus titer, increased Th1 cytokines in the airways and a reduced inflammatory infiltrate into the lung parenchyma in Influenza virus-infected mice (43). Similarly, antioxidant treatment was recently found to improve the final outcome of RSV infection in mice by significantly reducing cytokines and chemokines production and recruitment of inflammatory cells, especially neutrophils, to the lung (44), but the source of ROS in this context remains elusive.

Given the observed potential of targeting ROS production to limit the RSV-induced inflammatory response (44), we examined the molecular mechanisms involved in the redox-regulation of cytokine and chemokine genes expression in AEC. This study focuses on the identification of the biological source of ROS and its implication in the regulation of NF-κB. To determine whether this redox regulation was limited to RSV or can be extended to other members of the *Paramyxoviridae* family, we also evaluated the existence of this redox-dependent signaling mechanism in the context of Sendai virus (SeV) infection, which is widely used as a model of *Paramyxoviridae* in gene regulation studies. We demonstrate for the first time that a NOX2-containing NADPH oxidase is essential for RSV- and SeV-induced NF-κB activation in human AEC. NOX2 not only plays a role in the regulation of the classical IκBα-dependent NF-κB signaling pathway, but also regulates p65Ser536 phosphorylation, which appears to be essential for NF-κB activity following RSV and SeV infection. Altogether, this study identifies for the first time NOX2 as a specific source of ROS responsible for the oxidant-dependent regulation of NF-κB observed in RSV and SeV infection, thereby contributing to a better comprehension of NF-κB activation

pathways. Moreover, these results highlight a novel role of NOX2 in host defense in non-phagocytic cells.

Material and Methods

Reagents

Diphenyleneiodonium (DPI), Dimethyl Sulfoxide (DMSO), Tween 20, albumin from bovine serum (BSA) were purchased from Sigma-Aldrich. Human Thyroid Total RNA, Human Fetal Kidney Total RNA, Human Colon Total RNA, and Human Spleen Total RNA were purchased from Clontech. Oligonucleotides used in PCR reactions (Table I) were purchased from Invitrogen. RNAi oligonucleotides (Table II) were purchased from Dharmacon.

Plasmids

pCMV-flag-p65 and pCMV-flag-p65S536A plasmids were obtained from Dr. M. Servant, University of Montreal, Montreal. pRL-null reporter plasmid was from Promega. P2(2X)TK-pGL3 NF-κB luciferase reporter construct was obtained from Dr. J. Hiscott, McGill University, Montreal. Plasmids used for cytokine copy number determination, generated by cloning of the +169 to +872nt fragment of the TNFα transcript (NM_000594) and the +65 to +332nt fragment of the RANTES transcript (NM_002985) into the pCR2.1-TOPO vector using *Eco*RI, were a kind gift of Dr. D. Lamarre, University of Montreal, Montreal.

Virus

Initial stock of RSV A2 strain was obtained from Advanced Biotechnologies Inc. Amplification was performed in HEp-2 cells (ATCC) at a Multiplicity of Infection (MOI) of 0.1 until 50% cytopathic effect was observed. Virus was purified on 30% sucrose cushion after precipitation using polyethyleneglycol (45). Virus titer was determined by methylcellulose plaque assays as previously described (46). Sendai virus (SeV) Cantell strain was obtained from Charles River Laboratories.

Cell culture, plasmid transfection and luciferase assays

HEp-2 cells (ATCC) were cultured in DMEM medium (Gibco) supplemented

with 10% heat-inactivated Fetal Bovine Serum (HI-FBS, Gibco). A549 cells (ATCC), used as a cell line model of human AEC, were cultured in F-12 Nutrient Mixture (Ham) medium (Gibco) supplemented with 10% HI-FBS and 1% L-Glutamine (Gibco). Normal Human Bronchial Epithelial Cells (NHBE) were obtained from Clonetics, cultured in BEGM medium (Clonetics) and used between passage 2 and 4.
Transfection of A549 cells was performed with the *Trans*IT-LT1 Transfection Reagent (Mirus) according to the manufacturer's instructions. For luciferase assays, subconfluent cells in 24-well plates were transfected with 50 ng of pRL-null reporter (renilla luciferase, internal control), 100 ng of P2(2X)TK-pGL3 NF-κB reporter construct (firefly luciferase) and the indicated amounts of expression plasmids. Cells were assayed for reporter gene activities after 24h using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Relative luciferase activities represent the ratio of firefly luciferase normalized to renilla luciferase and are presented as fold over the non-stimulated condition.

Infections

Subconfluent A549 cells were infected with RSV at a MOI of 3 in culture medium containing 2% HI-FBS. SeV infection (40 HAU/10^6 cells) was carried out for 2h in serum-free medium and was further cultured for the indicated time in complete medium. Infection of NHBE cells was performed similarly in BEGM medium. In experiments where DPI was used, the reagent or the corresponding vehicle DMSO was added at the indicated concentration for 1h before infection and maintained at this concentration throughout the infection except in Figure 4F.

RNAi oligonucleotides transfections

RNAi oligonucleotides (Table II) transfection was performed as previously described (47) using Oligofectamine reagent (Invitrogen) and pursued for 62h before viral infection as described above. Where RNAi transfection preceded a luciferase reporter gene assay, transfection with reporter plasmids using *Trans*IT-LT1 Transfection Reagent as described above was performed 48h after transfection of the RNAi.

Immunoblot Analysis

Whole cell extracts (WCE) were prepared in Nonidet P-40 lysis buffer as previously described (48) and subjected to SDS-PAGE electrophoresis and immunoblot analysis as performed in (49) using the primary antibodies anti-IκBα-phosphoSer32 (1/1000, Cell Signaling), anti-p65-phosphoSer536 (1/1500, Cell Signaling), anti-actin (1/10000, Chemicon International), anti-IκBα (1/1000, Cell Signaling), anti-p65 (1/400, Santa Cruz Biotechnology), anti-Flag M2 (1mg/ml, Sigma-Aldrich), anti-SeV (1/10000, obtained from Dr. J. Hiscott, McGill University, Montreal) or anti-RSV (1/2000, Chemicon) diluted in PBS containing 0.5% Tween and either 5% nonfat dry milk or 5% BSA for phosphospecific antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence using the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences). In between IκBα-phosphoSer32 and IκBα antibodies, as well as p65-phosphoSer536 and p65 antibodies, the membrane was stripped in 0.2% SDS, 62.5 mM Tris-HCl pH 6.8, 0.1 mM b-mercaptoethanol for 20 minutes at 50°C, washed three times in PBS and blocked in blocking solution.

RNA extraction, RT-PCR and real-time PCR

Total RNA was extracted from A549 using the RNAqueous-4PCR Isolation Kit (Ambion) and treated to remove genomic DNA using either the DNase1 treatment included in the RNAqueous-4PCR Isolation Kit or the reagent included in the QuantiTect Reverse Transcription Kit (Qiagen). Total RNA (1 μg) was subjected to reverse transcription using 0.5 μg of oligo$(dT)_{12\text{-}18}$ primers (Invitrogen) and 200 units M-MLV Reverse Transcriptase (Invitrogen) or using the QuantiTect Reverse Transcription Kit. PCR amplifications were performed using the QuantiTect SYBR Green Kit (Qiagen) in the presence of 2 μM specific primers, except for NOX2 amplification, which was performed using 1 μM specific primers, and β-actin, TNFα and RANTES amplifications, which were performed using 0.4 μM specific primers. $MgCl_2$ concentration in all assays was 2.5 mM, except for NOX1L amplification, which required 5 mM $MgCl_2$. Sequences and annealing temperatures for gene-specific primers are listed in Table I. The absence of genomic DNA contamination was verified

with each of the primer sets by PCR in a reaction without reverse transcriptase. When analyzed on agarose gel, PCR reactions were performed in a T Gradient Cycler (Biometra) for the indicated number of cycles previously established to be in the linear detection range for each gene. For real-time PCR analyses, detection was performed on a Rotor-Gene 3000 Real Time Thermal Cycler (Corbett Research). For *TNFα* and *RANTES* gene expression, standard curves of absolute quantification expressed as copy number and PCR efficiencies were obtained using serial dilutions of pCR2.1-TOPO-TNFα and pCR2.1-TOPO-RANTES plasmids. For *β-actin, NOX1L*, *NOX2 and NOX5* genes expression, standard curves and PCR efficiencies were obtained using serial dilutions of cDNA prepared from cells used as a positive control as described in Figure 5. *TNFα* and *RANTES* data are presented as absolute copy numbers normalized to *β-actin* used as a reference gene. *NOX1L* expression was normalized to *β-actin* and is presented as relative fold expression of *NOX1L* from NOX1-RNAi sample versus CTRL-RNAi sample. Relative *NOX2* and *NOX5* fold expression values were determined applying the $\Delta\Delta C_t$ method (50).

Statistical analyses

All analyses were performed using the SigmaStat 3.5 software.

Results

Phosphorylation of p65 at Ser536 is a key event in the activation of NF-κB triggered by RSV and SeV infection in A549

Activation of the classical NF-κB pathway, involving IKK-mediated phosphorylation of IκBα and its subsequent degradation, is well documented in the context of RSV and SeV infections (49, 51). However, the importance of p65 phosphorylation is still barely documented in these contexts. Thus, we first reevaluated the activation of NF-κB by monitoring not only IκBα but also p65 phosphorylation in RSV- and SeV-infected A549. IκBα phosphorylation at Ser32 and p65 phosphorylation at Ser536 were analyzed by immunoblot using phosphospecific antibodies. As shown in Figure 1, IκBαSer32 and p65Ser536 phosphorylations were both detected with similar kinetic patterns during RSV (Figure 1A and C) or SeV (Figure 1B and D) infection. P65 phosphorylation at Ser276 was neither detected in RSV nor SeV infection (data not shown). Thus, our data reveal that RSV and SeV infection of A549 trigger activation of NF-κB at two levels, namely IκBα and p65Ser536 phosphorylation.

To determine whether p65Ser536 phosphorylation is essential for NF-κB activity in the context of RSV and SeV infection, the effect of Ser536 mutation into Ala (p65S536A), which abrogates phosphorylation at this particular residue, was analyzed by luciferase reporter gene assay. A549 were cotransfected with the NF-κB-responsive P2(2X)TK-pGL3 luciferase reporter gene and the pRL-null renilla internal control plasmids, together with the empty vector, or vector encoding flag-tagged-p65 or -p65S536A and were either left untreated or infected with RSV or SeV. As shown in Figure 2, expression of p65 in non-infected cells resulted in a 53-fold induction of the NF-κB reporter activity, whereas p65S536A only exhibited a minimal effect with a 5-fold induction. RSV and SeV infection of A549 cells resulted in a 14- and 13-fold stimulation of the reporter activity, respectively. These inductions were strongly enhanced by the ectopic expression of p65 to 191- and 179-fold, respectively. On the other hand, RSV and SeV stimulation of the promoter in the presence of p65S536A only reached 43- and 35-fold, respectively. Together, these results demonstrate that

phosphorylation of p65 at Ser536 during RSV and SeV infection in A549 cells is essential to trigger full activation of NF-κB transactivation potential.

DPI inhibits RSV- and SeV-induced NF-κB activation

In a first attempt to determine whether NF-κB activation in the context of infection by RSV and SeV was dependent on ROS production by a NADPH oxidase, we analyzed the effect of Diphenyleneiodonium (DPI), an inhibitor of flavoproteins widely used to target NADPH oxidases. The expression profile of NF-κB target genes, *TNFα* and *RANTES*, was monitored by real-time PCR in A549 cells either left untreated or infected with RSV or SeV for 6h in the absence or presence of 30 mM DPI. As shown in Figure 3A and B, TNFα mRNA levels were strongly induced following RSV and SeV infection of A549 cells. However, pretreatment with DPI reduced *TNFα* expression by 66% and 78% in RSV and SeV infection, respectively. Analysis of *RANTES* gene expression gave similar results, with reduction of RSV and SeV induced RANTES mRNA levels by 4 and 1.6 log, respectively (Figure 3C and D).

As p65Ser536 phosphorylation appears to be essential for NF-κB activation (Figure 2), we next assessed whether abrogation of NF-κB-dependent gene expression by DPI correlated with inhibition of p65Ser536 phosphorylation. A549 cells were infected with RSV for 8h in the presence of increasing doses of DPI. As shown in Figure 4A, RSV-induced p65Ser536 phosphorylation was inhibited by DPI in a dose-dependent manner reaching 72.6 ± 8.2% inhibition at 30mM DPI. Kinetic analysis of p65Ser536 phosphorylation during RSV infection in the absence or presence of 30μM DPI confirmed the inhibition of p65Ser536 phosphorylation by DPI over time (Figure 4B). Similarly, DPI inhibited SeV-induced p65Ser536 phosphorylation in a dose-dependent manner and over the course of infection (Figure 4C and D). Importantly, the effect of DPI is not attributable to an effect on viruses. Indeed, neither RSV nor SeV replication were affected by DPI treatment as demonstrated by the detection of viral protein level by immunoblot (Figure 4E). Moreover, identical effects were observed when DPI was present in the medium during the infection (protocol I, Figure 4F) or when DPI was preincubated with the cells and washed off before infection (protocol II,

Figure 4F). Altogether, these results demonstrate the importance of ROS, most likely from a NADPH oxidase origin, in NF-κB activation through p65Ser536 phosphorylation in RSV- and SeV-infected A549.

Various subunits of NADPH oxidases are expressed in RSV- and SeV-infected A549

Several NADPH oxidase subunits might be responsible for the observed DPI-mediated inhibition of p65 phosphorylation at Ser536 during RSV and SeV infection. As expression of NADPH oxidase subunits appears to be cell type specific, we monitored which of the NOX1-5 and DUOX1-2 catalytic subunits, p22phox and regulatory subunits p47phox, p67phox, NOXO1 and NOXA1 were expressed in A549 during RSV and SeV infection. For this purpose, A549 were infected with RSV and SeV and NOX mRNA expression was analyzed by RT-PCR (Figure 5A) using primers specific for each subunit (Table I). NOX1, NOX2 and NOX5 mRNA were detected in unstimulated A549 and their expression remained steady during virus infection. Using a specific set of primers, the long isoform of NOX1, NOX1L (52, 53), was found to follow the same pattern of expression as total NOX1. On the other hand, the long isoform of NOX5, NOX5L, was not detected in any conditions, thus suggesting that the NOX5 mRNA represent the NOX5S isoform (54). NOX3 and NOX4 mRNA were neither detected in unstimulated nor in virus-infected A549. Noteworthy, DUOX2 mRNA, but not DUOX1 mRNA, was expressed at the basal level and its expression was stimulated by RSV and SeV infection. p22phox mRNA expression was detected at an equal level in all conditions. As shown in Figure 5B, NOXO1, NOXA1, p47phox and p67phox regulatory subunits mRNA were all expressed at the basal level and p67phox mRNA was strongly inducible following RSV and SeV infections. In conclusion, these results demonstrate that NOX1L, NOX2, NOX5S and DUOX2 catalytic subunits of NADPH oxidase family members, p22phox, and regulatory subunits NOXO1, NOXA1, p47phox and p67phox are expressed in A549 during RSV and SeV infection.

RNA interference with NOX2 expression inhibits NF-κB transactivation potential in RSV and SeV infection

Based on the observation that several members of the NOX/DUOX family of enzymes, namely NOX1L, NOX2 and NOX5S, are expressed at the basal level in A549, we hypothesized that one of these isoforms might be responsible for the observed redox-dependent regulation of NF-κB following RSV and SeV infection (Figure 3 and 4). To verify our hypothesis, specific interfering RNA (RNAi) oligonucleotides (Table II) were used in A549 to down-regulate the expression of NOX1, NOX2 and NOX5. As shown in Figure 6A, NOX1-, NOX2- (NOX2(1)) and NOX5- RNAi efficiently inhibited the corresponding NOX expression by 82, 64 and 90%, respectively, as evaluated by real-time PCR. The effect of interference with the expression of each isoform on RSV- and SeV-induced NF-κB transcriptional activity was evaluated using a luciferase reporter gene assay. RNAi-transfected A549 were further transfected with the P2(2X)TK-pGL3 luciferase reporter as well as the pRL-null renilla luciferase (internal reference) constructs and were either left untreated or infected with RSV or SeV. As shown in Figure 6B, interference with NOX1 and NOX5 expression did not affect endogenous NF-κB ability to stimulate gene transcription. However, as shown in Figure 6C and D, when NOX2 expression was reduced by NOX2(1)- RNAi, RSV- and SeV-induced NF-κB transactivation potential was significantly inhibited. RSV-induced NF-κB activity in a time-dependent manner with a 26 ± 1.37 fold stimulation at 16hpi (hours post-infection), while in the presence of NOX2(1)-RNAi, NF-κB stimulation remained lower over time reaching a 9.67 ± 1.62 fold at 16hpi (Figure 6C). Similar results were observed in SeV infection (Figure 6D). Indeed, stimulation of NF-κB activity was of 11.94 ± 1.16 fold in CTRL-RNAi-transfected A549 at 16hpi, whereas it was decreased to 6.68 ± 0.3 fold in NOX2(1)-RNAi transfected cells. Taken together, these results demonstrate that NOX2 is an essential component of the signaling pathway triggering NF-κB activation following RSV and SeV infection in A549.

Interference with NOX2 expression inhibits RSV- and SeV-induced NF-κB activation through diminution of IκBαSer32 and p65Ser536 phosphorylation in A549

Inhibition of NF-κB transactivation potential (Figure 6C and D) in the presence of NOX2-RNAi suggested that upstream events leading to NF-κB activation might be altered by the absence of NOX2 expression. Therefore, RNAi-mediated NOX2 inhibition was further used to investigate the role of NOX2 in NF-κB activation following RSV and SeV infection. A549 were transfected with CTRL- or NOX2-specific RNAi, NOX2(1), and were then left untreated or infected with RSV or SeV for various times. IκBα phosphorylation at Ser32 and p65 phosphorylation at Ser536 were evaluated by phosphospecific immunoblot and the ratio of phospho-IκBα vs total IκBα and phospho-p65 vs total p65 was determined by densitometric quantification (Figure 7). RSV- (Figure 7A) and SeV-induced (Figure 7B) IκBαSer32 and p65Ser536 phosphorylations were significantly inhibited by reduced NOX2 expression. An identical experiment with a second NOX2-specific RNAi, NOX2(2) (Figure 8A), yielded similar results (Figure 8B). To ascertain that the observed effects on NF-κB activation were not resulting from an effect of the RNAi on virus replication, viral proteins expression in various conditions was evaluated by immunoblot using anti-RSV (Figure 7A and 8B) and anti-SeV (Figure 7B) antibodies. Neither NOX2(1)- nor NOX2(2)-RNAi interfered with virus replication. NOX1- and NOX5-RNAi did not alter p65Ser536 phosphorylation (data not shown), thus demonstrating the specific role of NOX2 in this pathway. In conclusion, our results demonstrate that NOX2 plays an essential role in RSV- and SeV-induced NF-κB regulation at two critical steps, namely IκBαSer32 and p65Ser536 phosphorylation in A549.

Dual role of NOX2 in RSV- and SeV-induced NF-κB regulation in NHBE

Although A549 cells are widely considered a cell line model of human AEC, we verified the existence of the newly identified NOX2-dependent pathway of NF-κB regulation in primary NHBE cells. First, NOX2 mRNA expression was analyzed by RT-PCR in NHBE during RSV and SeV infection. Similarly to the observation made in A549, NOX2 mRNA was detected in unstimulated NHBE and its expression remained steady during RSV and SeV infection (Figure 9A). More importantly, interference with NOX2 expression using the NOX2(1) RNAi oligonucleotide (Figure 9B) resulted in significant inhibition of both RSV- and SeV-induced IκBαSer32 and

p65Ser536 phosphorylation as evaluated by phosphospecific immunoblots and phospho-IκBα vs total IκBα and phospho-p65 vs total p65 ratios (Figure 9C and D). These results highlight the important dual role of NOX2 in the regulation of NF-κB during *Paramyxoviridae* infections in the context of normal AEC.

Discussion

NF-κB plays an essential role in the RSV-induced exacerbated inflammatory response through the regulation of cytokines and chemokines expression and thus constitutes an interesting therapeutic target. However, the molecular mechanisms controlling the persistent RSV-induced NF-κB activation in AEC are still far from being deciphered. Although it has long been a matter of debate, cell type- and stimuli-specific redox-regulation of NF-κB is now well documented (reviewed in (35)). Previous studies have shed a light on the possible ROS-dependent regulation of NF-κB following RSV infection in AEC. First, RSV triggers ROS production in AEC (36). Second, RSV-induced expression of genes encoding the chemokines IL-8, RANTES, and MCP-1, which are known NF-κB-target genes, were found to be dependent on a redox sensitive pathway(s) in A549 (29, 36-38). In addition, ROS were found to be involved in the regulation of IκBα degradation, nuclear translocation and *in vitro* DNA-binding activity of p65 in RSV-infected AEC (24, 29). Although these studies support a role of ROS in RSV-mediated NF-κB activation, they did not address two major questions: 1) the biological source of ROS and 2) the steps in the NF-κB signaling pathway that are redox sensitive. Here, we provide the first evidence that RSV triggers NF-κB activation through a NOX2-containing NADPH oxidase in A549 and primary NHBE. This pathway is essential for IκBαSer32 and p65Ser536 phosphorylation events. Furthermore, we demonstrate that this pathway is not restricted to RSV amongst *Paramyxoviridae* as similar results were obtained with SeV. Thus, our results highlight a novel signaling pathway mediating NF-κB activation in AEC infected with RSV or SeV that involves a NOX2-containing NADPH oxidase as a central regulator.

Attempts to identify the origin of RSV-induced ROS in AEC were previously limited to a recent study that suggested the involvement of an uncharacterized NADPH oxidase based on the observation that RSV-induced activation of the Interferon Regulatory Factor (IRF)-3 transcription factor was inhibitable by DPI (55). Although the effect of DPI is widely considered as an indicator of a potential role of a NADPH oxidase, other ROS-generating flavoproteins, including the mitochondrial respiratory chain, could explain the effects observed with DPI (56). Therefore, to definitively

conclude on the involvement of a NADPH oxidase activity, we used selective RNAi against NOX1, NOX2 and NOX5, and provide the first direct proof that a NOX2-containing enzyme specifically participates in the redox-sensitive regulation of RSV- and SeV-induced NF-κB signaling in A549 and NHBE.

Our study shows that transcripts of NOX1, NOX2, NOX5 and DUOX2 together with the small transmembrane subunit p22phox and the various regulatory subunits, p47phox, p67phox, NOXO1 and NOXA1 are expressed in A549 (Figure 5). Previous studies describing the expression of NOX/DUOX isoforms in various AEC cell lines, including Calu-3 submucosal gland cell line, hTE human tracheal surface epithelial cell line and NCI-H292 human pulmonary cell line, highlighted significant discrepancies (57-59). It is noteworthy that NOX4 was not detected in our study (Figure 5), while its basal expression and inducibility following exposure to diesel exhaust particules in A549 was recently reported (60). Expression of alternative NOX4 splice variants was also documented (61). Comparison of our results with the one described by Amara and coll. (60) should be made with caution. Indeed, in their study NOX4 mRNA was only expressed as fold over ubiquitin and not as absolute values and thus the amount of NOX4 might in fact be very low. Here, the use of primers expected to amplify the various potential variants failed to detect NOX4 mRNA in uninfected and infected A549 (Figure 5). Similar results were obtained using a second set of primers (data not shown). These variations in NOX/DUOX detection in cell lines is of particular significance as these cell lines are frequently used as a model of AEC. Here, we focused our functional studies on the role of NOX isoforms and demonstrated a selective role of NOX2. In order to clarify the role of NOX2, it was of importance to verify the expression status of NOX2 in primary AEC. Here, we demonstrate that NOX2 transcripts are expressed in NHBE cells (Figure 9), thus confirming the relevance of the A549 model. Moreover, RNAi experiments allowed us to confirm the role of NOX2 in NF-κB regulation during the course of RSV and SeV infections in NHBE (Figure 9).

With the recent description of the NOX/DUOX family, data are emerging that link NADPH oxidase enzymes activity to NF-κB activation and downstream proinflammatory response. In the context of infectious diseases, different NOX

isoforms, including NOX4 and NOX2, were found to act downstream of Toll-like receptors (TLR) to trigger proinflammatory signals (reviewed in (42)). NOX isoforms were also identified as inflammatory regulators in a few other contexts. Following stimulation of MCF7 cells by IL-1β, NOX2-dependent ROS production in the endosome was shown to be essential for recruitment of the TRAF6 adaptor to trigger an IKK-dependent NF-κB activation (62). In vascular smooth muscle cells (VSMC), NOX4 was shown to orchestrate C-reactive protein-mediated inflammatory activities through regulation of AP-1 and NF-κB (63). Taken together, these data and our study reveal that NOX isoforms are important inflammatory signaling molecules in non-phagocytic cells and that NOX constitute potential anti-inflammatory therapeutic targets.

The majority of studies that report ROS-dependent regulation of NF-κB support a role of ROS in the activation of NF-κB (reviewed in (35)). The use of H_2O_2 as a stimulus revealed that IKK is under redox control in epithelial cells. H_2O_2 was found to trigger IKKa and IKKβ phosphorylation and activation in HeLa cells. More recently, IKK activation in response to H_2O_2 in HeLa cells was shown to be dependent on a Src/PKCd/PKD signaling cascade (64, 65). Conflicting results came from studies performed in C10 immortalized alveolar type II cells, where H_2O_2 inhibited TNFα- or LPS-mediated IKK activation (66). However, our data showing NOX2-dependent IκBα phosphorylation at Ser32 support a ROS-dependent activation of IKK upon RSV or SeV infection in AEC. Although this discrepancy may only reflect stimulus-specificity of the response, one also needs to consider that the use of H_2O_2 does not necessarily reflect physiological concentrations of ROS triggered by physiological stimuli.

The molecular mechanisms regulating NF-κB activation following RSV infection of AEC have not yet been completely elucidated. A temporal dissociation between NF-κB DNA-binding and IκBα degradation (26), suggest that an IκBα-independent mechanism might be involved in early activation of NF-κB. Activation of the non-canonical pathway of NF-κB activation, which involves cleavage of p100 to form the p52 subunit, was recently described following RSV infection (67) and it was proposed that it might be responsible for early activation of NF-κB. Although it might

indeed be relevant for early cytokine genes transcription, it does not explain the early p65 DNA-binding (26). Our data confirm previous reports (31) that degradation of IκBα is only observed between 12 and 24 hours post-infection (hpi) following RSV infection. We now show that p65 Ser536 phosphorylation occurs *in vivo* as early as 3 hpi (Figure 1 and 7) and that it is essential for RSV- and SeV-induced p65 activation (Figure 2). The Ser536 residue of p65 is evolutionary conserved and is phosphorylated in response to numerous stimuli, including DNA-damaging agents, T cell costimulation, Angiotensin (Ang) II and LPS (21, 68-70). Substantial literature supports a function of p65Ser536 phosphorylation in the regulation of p65 affinity to IκBα rather than of its transcriptional activity (reviewed in (18)). A study recently suggested that in fact Ser536 phosphorylated p65 does not interact at all with cytosolic IκBα and regulates a distinct set of target genes (23). Thus, this pathway constitutes a likely candidate to explain the early NF-κB activation prior to IκBα degradation triggered by RSV in AEC. Although redox regulation of this pathway was previously suggested by the observed inhibition of TNFα-induced p65Ser536 phosphorylation by NAC in endothelial cells (71), our data constitute the first identification of the biological source of ROS responsible for its regulation.

Characterization of the signaling molecules targeted by ROS is still barely documented. However, the oxidation of thiol groups in the catalytic sites of kinases and phosphatases appears to be an important posttranslational modification that affects the function of signaling proteins (72). It is thus tempting to hypothesize that NOX2-derived ROS regulate the activation of kinases/phosphatases acting upstream of IκBαSer32 and p65Ser536 in RSV and SeV infections. Oxidation of IKKβ at Cys179 was found involved in negative regulation of NF-κB following anti-inflammatory stimuli (73). Thus, it is more likely that upstream regulators might be targeted by thiol modification to trigger its activation. However, kinases and phosphatases acting upstream of IKK in the context of RSV and SeV infection remain to be identified. Similarly, the signaling pathway leading to p65Ser536 phosphorylation during RSV and SeV infections is still unknown. Five kinases were previously found to phosphorylate p65 at Ser536. IKKα/β phosphorylate p65Ser536 *in vivo* in response to various stimuli, including LPS, Ang II, TNFα or lymphotoxin b receptor signaling (68,

74-76). Additionally, the IKK homologues TBK1 and IKKε, that have recently been found to be involved in the phosphorylation of the IRF-3 and IRF-7 transcription factors following virus infections (47, 77), also phosphorylate p65 at Ser536 in response to TNFα or IL-1 (78, 79). Finally, the RSK-1 kinase was also identified as a p65Ser536 kinase following Ang II stimulation or in response to p53 (21, 80). Which of these kinases is involved in RSV- and SeV-induced p65Ser536 phosphorylation and their redox regulation is currently under investigation.

The importance of the redox regulation of cytokines and chemokines production in RSV pathology was recently illustrated by an *in vivo* study in a mouse model. Indeed, treatment of mice with BHA significantly reduced RSV-induced cytokines and chemokines production and recruitment of inflammatory cells, especially neutrophils, to the lung (44). However, this study used a general antioxidant and did not target the specific source of ROS responsible for NF-κB regulation. Our data now demonstrate that NOX2 represents a potential target to limit the excessive inflammatory response triggered by RSV. During the course of our work, it was shown that absence of NOX2 led to a reduced virus titer, increased Th1 cytokines in the airways and a reduced inflammatory infiltrate into the lung parenchyma in influenza-infected mice (43). These results are in line with the concept raised by our study that inhibiting NOX2 might be a means of controlling the inflammatory response triggered by *Paramyxoviridae* infection.

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Figure Legends

Figure 1. RSV and SeV infection trigger IκBαSer32 and p65Ser536 phosphorylation in A549. A549 cells were left untreated or infected with RSV (MOI=3) (A and C) or SeV (40 HAU/10^6 cells) (B and D) for various times, as indicated. WCE were generated and resolved by SDS-PAGE. After transfer onto nitrocellulose, proteins were immunoblotted (IB) with anti-IκBαSer32 phosphospecific (IκBα-P-Ser32), anti-IκBα (A and B) anti-p65Ser536 phosphospecific (p65-P-Ser536) and anti-p65 antibodies (C and D). Equal loading was verified using anti-actin antibody. The data are representative of at least three experiments. Hpi: hours post-infection.

Figure 2. p65Ser536 phosphorylation is essential to mediate fully active NF-κB transactivation potential in response to RSV and SeV infection of A549. A549 were cotransfected with empty vector or plasmid encoding flag-tagged p65 or flag-tagged p65S536A mutant together with the P2(2X)TK-pGL3 NF-κB reporter firefly luciferase and the pRL-null reporter constructs (renilla luciferase used as an internal control). Cells were either left untreated or infected with RSV and SeV for 16h. A/ Luciferase activity was measured and expressed as fold activation over the non-stimulated cells transfected with empty plasmid after normalization with renilla luciferase activity. Each value represents the mean ± SE of triplicate independent samples. Statistical comparison was performed using a two-way ANOVA-Tukey multiple comparison test between p65Ser536A- and p65-transfected A549 (***, $p< 0.001$). The data are representative of three different experiments. B/ Expression of flag-tagged p65 or flag-tagged p65S536A mutant were analyzed by immunoblot (IB) using anti-flag antibody. Equal loading was verified using anti-actin antibody.

Figure 3. DPI inhibits RSV- and SeV-induced TNFα and RANTES mRNA expression. A549 were left non-stimulated (NS) or infected with RSV (MOI=3) (A and C) or SeV (40 HAU/10^6 cells) (B and D) for 6h in the absence or presence of 30μM DPI. Total RNA was prepared, subjected to reverse transcription and analyzed by

SYBR Green-based real-time PCR using TNFα, RANTES and β-actin-specific primers. Absolute TNFα (A and B) and RANTES (C and D) mRNA copy numbers were quantified using standard curves generated with pCR2.1-TOPO-TNFα and pCR2.1-TOPO-RANTES plasmids. Results are presented as absolute copy numbers of target gene mRNA normalized versus β-actin mRNA used as a reference gene. Data are representative of two experiments performed in independent triplicates. Data are expressed as mean ± SE. Statistical comparison was performed by a t-test using DMSO-treated, RSV-infected A549 as control (***, $p< 0.001$).

Figure 4. DPI inhibits RSV- and SeV-induced p65Ser536 phosphorylation in A549. A549 were pretreated with DMSO (vehicle) or DPI at the indicated concentrations before being left untreated or infected with RSV at a MOI of 3 (A, B, E and F) or SeV at 40 HAU/10^6 cells (C, D, E and F) for the indicated times. In A to E, DPI was kept in the medium during the infection. In F, DPI was either kept in the medium during the infection (protocol I) or washed after 1 hour preincubation before infection (protocol II). WCE were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted (IB) using anti-p65Ser536 phosphospecific (p65-P-Ser536), anti-p65, anti-SeV, anti-RSV and anti-actin antibodies. In anti-SeV and anti-RSV immunoblots, the Nucleocapsid viral protein (N) is shown. In A to D, data were quantified by densitometric analysis using the ImageJ software. Data are representative of at least three independent experiments. Representative immunoblots are shown. Quantification data are expressed as mean ± SE. In A and C, statistical comparison was performed using a one-way ANOVA-post hoc Dunnett's test using DMSO-treated, RSV-infected A549 as control (*, $p<0.05$). In B and D, black bars correspond to DMSO treated cells and white bars correspond to DPI treated cells. Statistical comparison was performed using a two-way ANOVA-Tukey multiple comparison test between NOX2-RNAi- and CTRL-RNAi-transfected A549 (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$). Hpi: hours post-infection.

Figure 5. Expression of NADPH oxidase subunits in A549 during RSV and SeV infection. A549 were either left untreated or infected with RSV (MOI=3) for 24h and SeV (40 HAU/10^6 cells) for 16h. Total RNA was extracted, treated with DNase1 and subjected to reverse transcription as described in Material and Methods. Expression was evaluated by RT-PCR analysis using primers (Table I) specific for each membrane subunit (A) or cytosolic factor (B). Specific positive controls (Ctrl+) were used for each gene: total RNA from colon was used for NOX1, NOX1L, NOXO1 and NOXA1; total RNA from DMSO differentiated HL60 was used for NOX2, p22phox, p47phox and p67phox; total RNA from human fetal kidney was used for NOX3; total RNA from MRC-5 were used for NOX4; total RNA from human spleen was used for NOX5 and NOX5L, total RNA from thyroid was used for DUOX1 and DUOX2. Results were reproduced three times and representative data are shown.

Figure 6. Interference with NOX2 expression, but not interference with NOX1 and NOX5 expression inhibits RSV- and SeV-induced NF-κB transactivation potential. A549 were transfected with Control (CTRL)-, NOX1-, NOX2 (NOX2(1))- and NOX5-specific RNAi oligonucleotides (Table II) as described in the Material and Methods section. In (A), NOX1, NOX2 and NOX5 mRNA levels were quantified by real-time PCR using specific primers (Table I). NOX1L expression is presented as fold of NOX1-RNAi- vs CTRL-RNAi-transfected cells after normalization to β-actin. NOX2 and NOX5 fold expression values were determined using the $\Delta\Delta C_t$ method (50). In (B), CTRL-, NOX1- and NOX5-RNAi-transfected A549 were further transfected with the P2(2X)TK-pGL3 NF-κB firefly luciferase and the pRL-null renilla luciferase (internal control) reporter constructs and either left unstimulated (NS) or infected with RSV or SeV for 16h. Luciferase activity was measured and expressed as fold over the non-stimulated CTRL-RNAi transfected A549 after normalization with renilla luciferase activity. In C and D, CTRL- and NOX2(1)-RNAi transfected A549 were cotransfected with P2(2X)TK-pGL3 and pRL-null constructs and either left unstimulated or infected with RSV (C) or SeV (D) for the indicated times. Luciferase activity was measured and expressed as fold over the non-stimulated CTRL-RNAi transfected A549 after normalization with renilla luciferase activity. Each value

represents the mean ± SE of triplicate independent samples subjected to different RNAi exposure. Statistical comparison was performed using a two-way ANOVA-Tukey multiple comparison test between NOX2-RNAi- and CTRL-RNAi-transfected A549 (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$).

Figure 7. Interference with NOX2 expression using NOX2(1)-RNAi oligonucleotide inhibits RSV- and SeV-induced IκBαSer32 and p65Ser536 phosphorylation in A549. CTRL- and NOX2(1)-RNAi (A and B) transfected A549 were infected with RSV at a MOI of 3 (A) or SeV at 40 HAU/10^6 cells (B) for the indicated times. WCE were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and proteins were immunoblotted (IB) using anti-IκBαSer32 phosphospecific (IκBα-P-Ser32), anti-IκBα, anti-p65Ser536 phosphospecific (p65-P-Ser536), anti-p65, anti-RSV (A), anti-SeV (B) and anti-actin antibodies. Phosphorylation of IκBα at Ser32 and p65 at Ser536, expressed as a ratio vs the total amount of IκBα and p65, respectively, was quantified by densitometry using the ImageJ software, and expressed as fold over the CTRL-RNAi transfected, unstimulated condition. Data are representative of three independent experiments. Representative immunoblots are shown. Quantification data are expressed as mean ± SE. Statistical comparison was performed using a two-way ANOVA-Tukey multiple comparison test between NOX2-RNAi- and CTRL-RNAi-transfected A549 (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$). Black bars correspond to CTRL-RNAi transfected cells and white bars correspond to NOX2(1)-RNAi transfected cells. Hpi: hours post-infection.

Figure 8. A second NOX2 specific RNAi oligonucleotide (NOX2(2)) inhibits RSV-induced IκBαSer32 and p65Ser536 phosphorylation in A549. A549 were transfected with Control (CTRL)- and NOX2-specific (NOX2(2)) RNAi oligonucleotides (Table II) as described in the Material and Methods section. In (A), NOX2 mRNA levels were quantified by real-time PCR using specific primers (Table I) and expressed as fold expression determined using the $\Delta\Delta C_t$ method (50). The data are representative of three different experiments. In (B), CTRL- and NOX2(2)-RNAi-transfected A549 were

further infected with RSV for the indicated time and analyzed by immunoblot as described in Figure 7. Quantifications were performed using the ImageJ software and expressed as fold over the CTRL-RNAi transfected, unstimulated condition. Representative immunoblots are shown. Quantification data are expressed as mean ± SE. Statistical comparison was performed using a two-way ANOVA-Tukey multiple comparison test between NOX2-RNAi- and CTRL-RNAi-transfected A549 (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$). Black bars correspond to CTRL-RNAi transfected cells and white bars correspond to NOX2(2)-RNAi transfected cells. Hpi: hours post-infection.

Figure 9. Interference with NOX2 expression inhibits RSV- and SeV-induced IκBαSer32 and p65Ser536 phosphorylation in NHBE. In A, NHBE were either left untreated or infected with RSV (MOI=3) or SeV (40 HAU/10^6 cells) for 16h. Total RNA was extracted, treated with DNase1 and subjected to reverse transcription. Expression of NOX2 mRNA was evaluated by RT-PCR analysis using specific primers (Table I). In B, C and D, NHBE were transfected with Control (CTRL)- or NOX2-specific (NOX2(1)) RNAi oligonucleotides (Table II) before being left untreated or infected as described in (A). In B, NOX2 mRNA levels were quantified by real-time PCR and expressed as fold expression. In C and D, WCE were analyzed by immunoblot as described in Figure 7. Representative immunoblots are shown. Quantification data are expressed as mean ± SE of three independent experiments. Statistical comparison was performed using a two-way ANOVA-Tukey multiple comparison test between NOX2-RNAi- and CTRL-RNAi-transfected NHBE (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$). Black bars correspond to CTRL-RNAi transfected cells and white bars correspond to NOX2(1)-RNAi transfected cells. Hpi: hours post-infection.

Table I. *List of primers used to monitor the expression of NADPH oxidase subunits and cytokine genes in RT-PCR or real-time PCR analyses* [a]

Gene		Sequence (5'-3')	T_{hyb} [b]
NOX1	S	gtacaaattccagtgtgcagaccac	62
	AS	cagactggaatatcggtgacagca	
NOX1L	S	tggaggaattaggcaaagtg	63
	AS	caaaggaggttttctgtttcag	
NOX2	S	tgttcagctatgaggtggtga	60
	AS	tcagattggtggcgttattg	
NOX3	S	tcacaaactggtcgcctatg	63
	AS	agggttccttgccagaaaat	
NOX4	S	ctcagcggaatcaatcagctgtg	62
	AS	agaggaacacgacaatcagccttag	
NOX5	S	gtgctacatcgatgggccttatg	65
	AS	ccccgtgatggagtctttcttct	
NOX5L	S	ggaggatgccaggtggctccggt	64
	AS	agccccactaccacgtagccc	
DUOX1	S	cgacattgagactgagttga	62
	AS	ctggaatgacgttaccttct	
DUOX2	S	cgacattgagactgagttga	62
	AS	cagagagcaatgatggtgat	
p22phox	S	cgctggcgtccggcctgatcctca	60
	AS	acgcacagccgccagtaggtagat	
p47phox	S	tgccaactacgagaagacctc	62
	AS	acagaaccaccaaccgctct	
p67phox	S	cggacaagaaggactggaag	62
	AS	acatgcagccaatgttgaag	
NOXO1	S	ttctctgtgcgctggtcaga	62
	AS	tcttgagctgcctgaattcgt	
NOXA1	S	tgggaggtgctacacaatgtg	60
	AS	ttggacatggcctcccttag	
GAPDH	S	accacagtccatgccatcac	58
	AS	tccaccaccctgttgctgta	
Actin	S	acaatgagctgctggtggct	58
	AS	gatgggcacagtgtgggtga	
RANTES	S	taccatgaaggtctccgc	60
	AS	gacaaagacgactgctgg	
TNFα	S	cagagggcctgtacctcatc	55
	AS	ggaagacccctcccagatag	

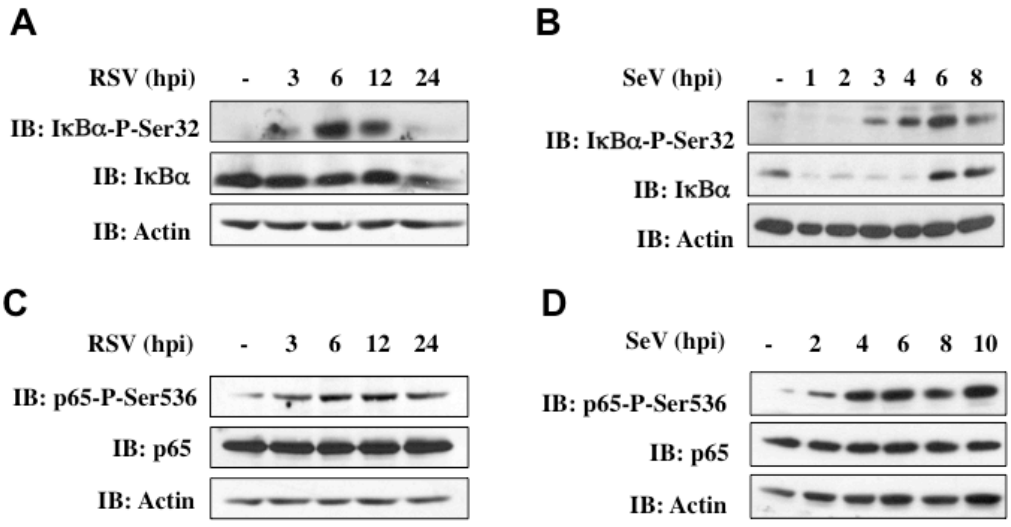
[a] Amplification specificity of primers for the corresponding NADPH oxidase was verified using Amplify3X software.
[b] Hybridization temperature (° C)

Table II. *List of RNAi oligonucleotides used for inhibition of NOX expression*

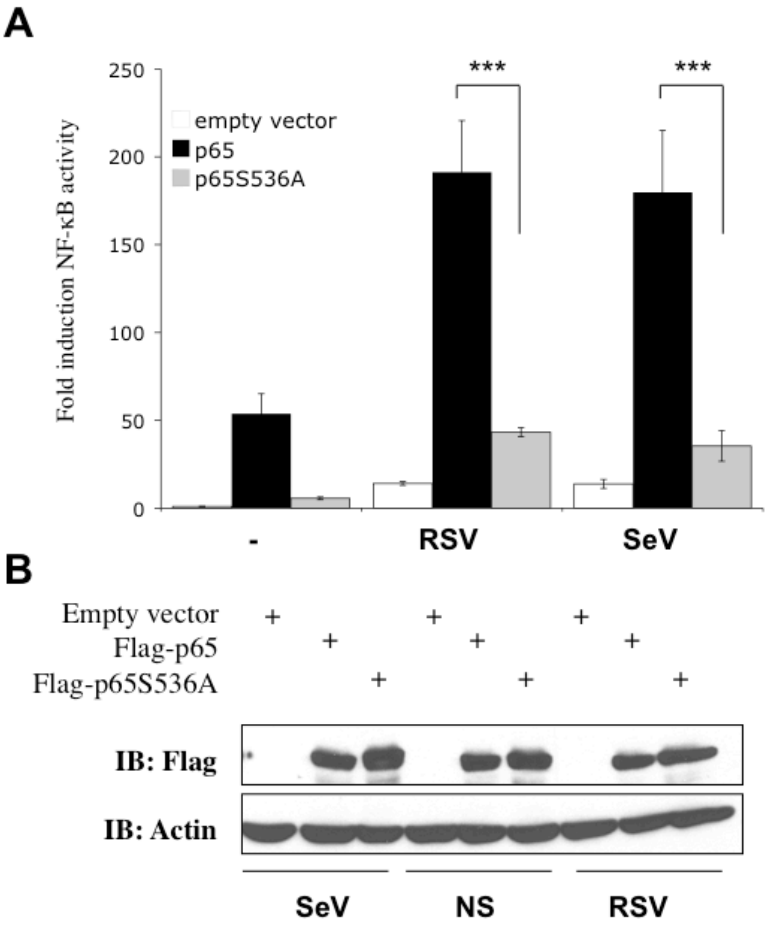
Target genes		Sequences
NOX1[a]	target	5'-nngcacaccuguuuaacuuug-3'
	sense oligonucleotide	5'-gcacaccuguuuaacuuuguu-3'
	antisense oligonucleotide	5'-caaaguuaaacaggugugcuu-3'
NOX2(1)[a]	target	5'-nngaagacaacuggacaggaa-3'
	sense oligonucleotide	5'-gaagacaacuggacaggaauu-3'
	antisense oligonucleotide	5'-uuccuguccaguugucuucuu-3'
NOX2(2)[a]	target	5'-nnguggaugccuuccugaaau-3'
	sense oligonucleotide	5'-guggaugccuuccugaaauuu-3'
	antisense oligonucleotide	5'-auuucaggaaggcauccacuu-3'
NOX5[b]	target	5'-nngguggacuuuaucuggauc-3'
	sense oligonucleotide	5'-gguggacuuuaucuggaucdtdt-3'
	antisense oligonucleotide	5'-gauccagauaaaguccaccdtdt-3'
CTRL[a]	target	5'-nncauagcguccuugaucaca-3'
	sense oligonucleotide	5'-cauagcguccuugaucacauu-3'
	antisense oligonucleotide	5'-ugugaucaaggacgcuauguu-3'

[a] Sequences of NOX1-, NOX2(1)-, NOX2(2)- and CTRL-RNAi were predesigned by Dharmacon.
[b] NOX5 specific RNAi was previously described (81).

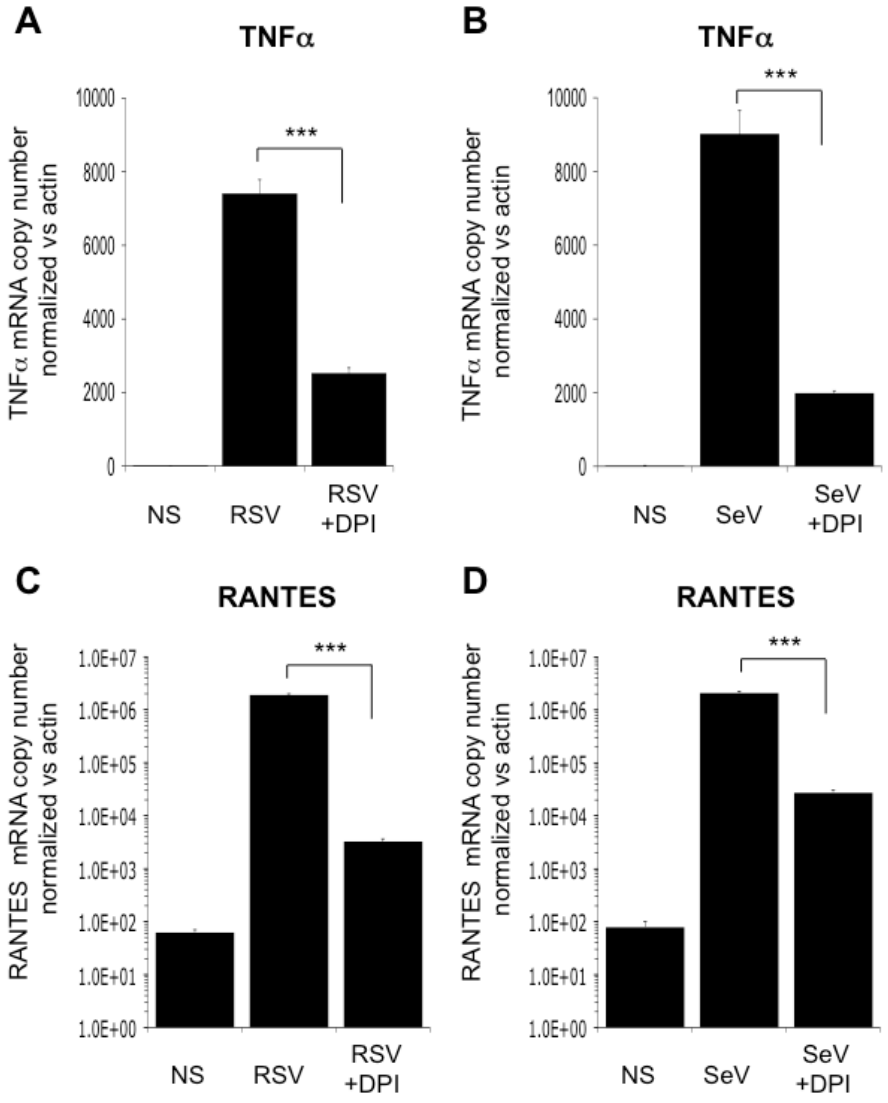
Fink *et al.*, Figure 1



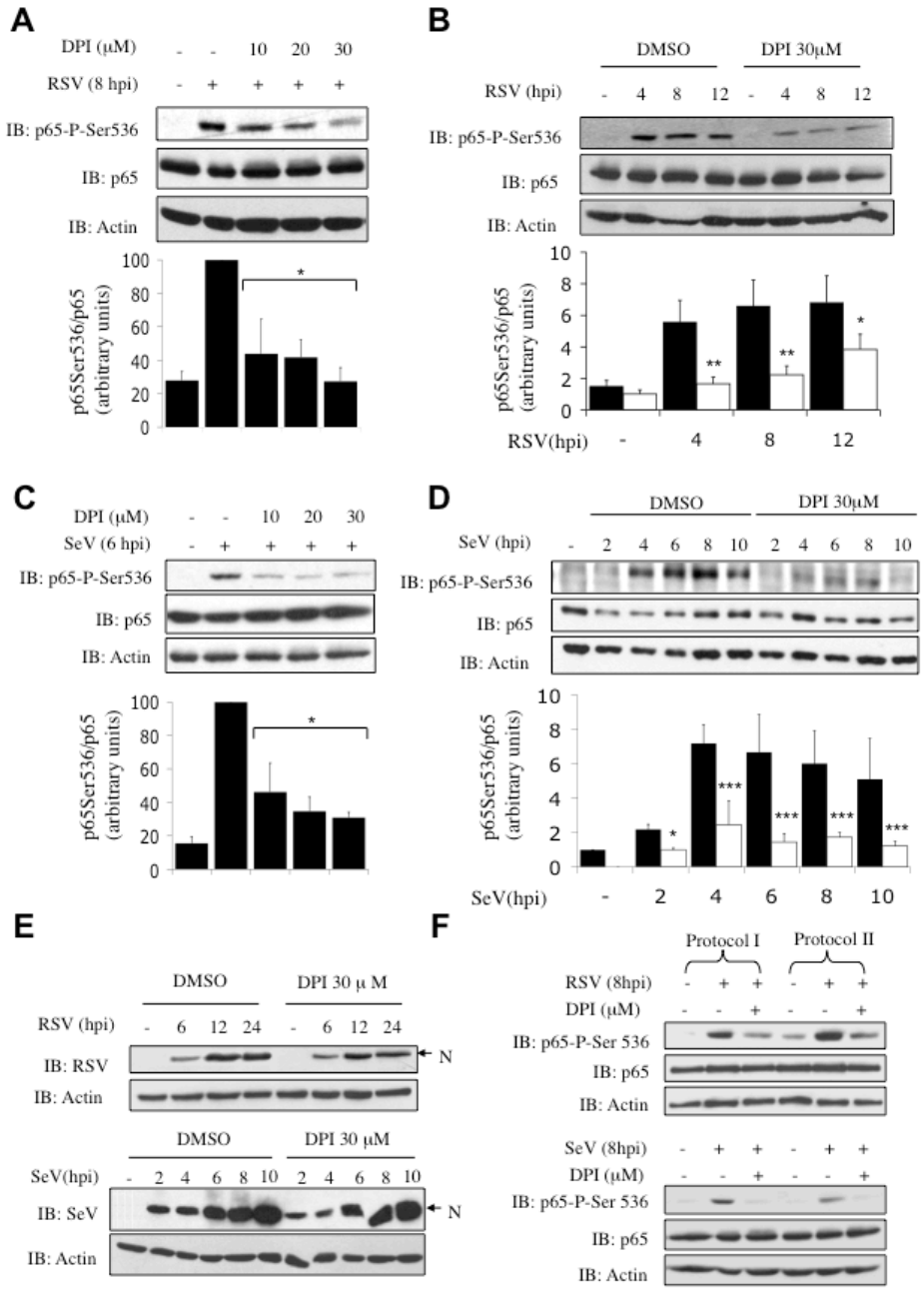
Fink *et al.*, Figure 2



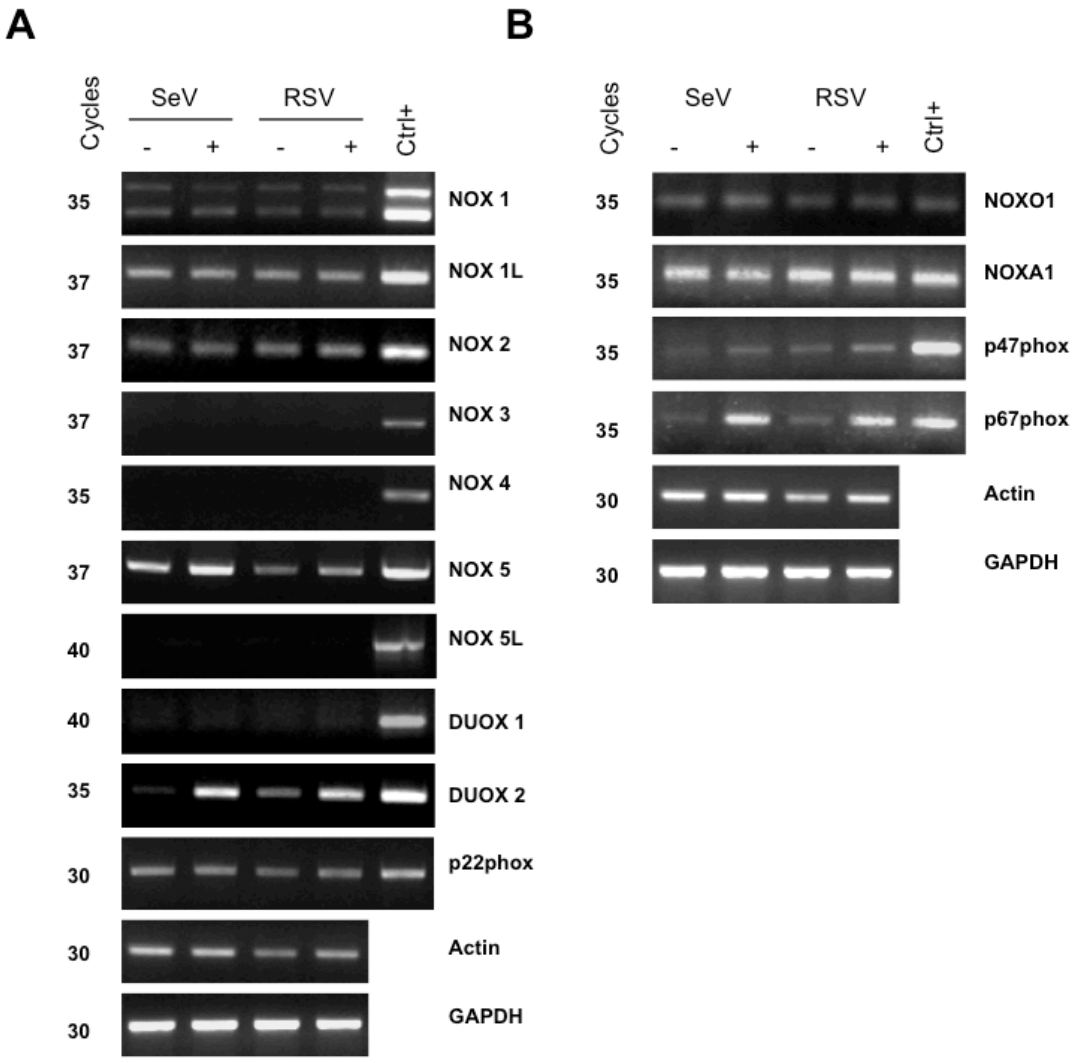
Fink *et al.*, Figure 3



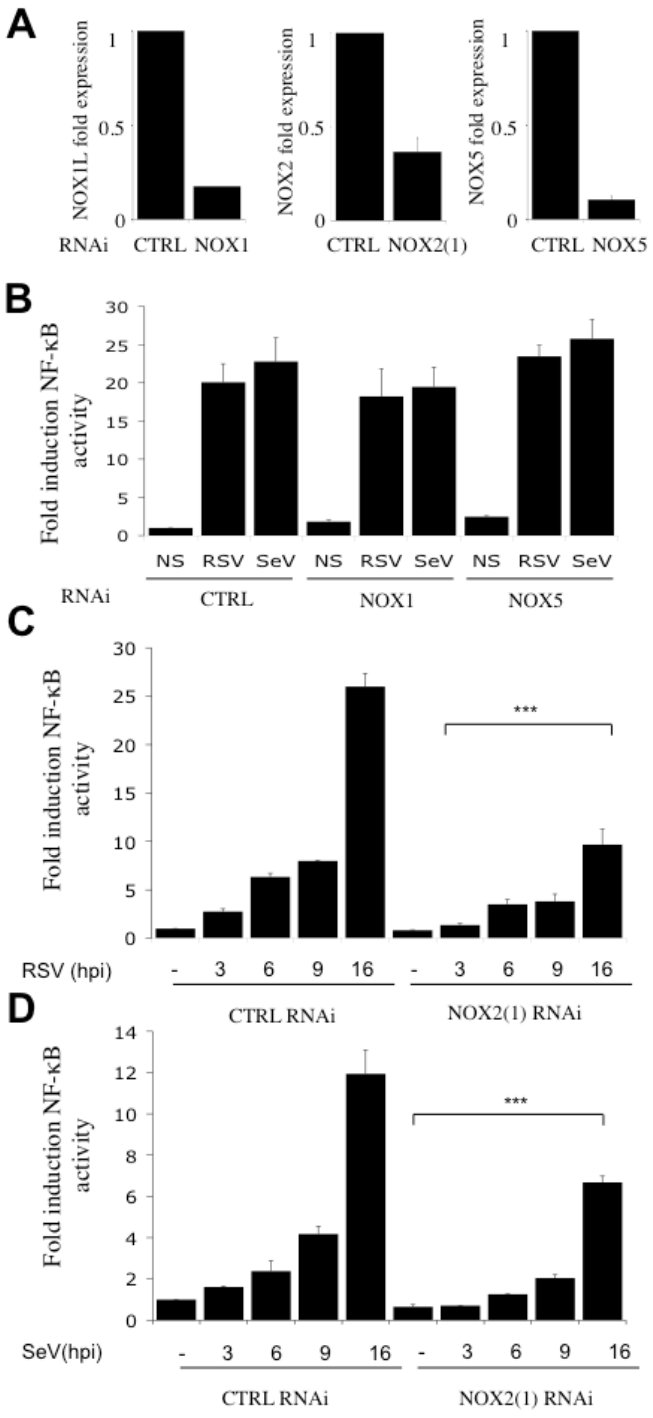
Fink *et al.*, Figure 4



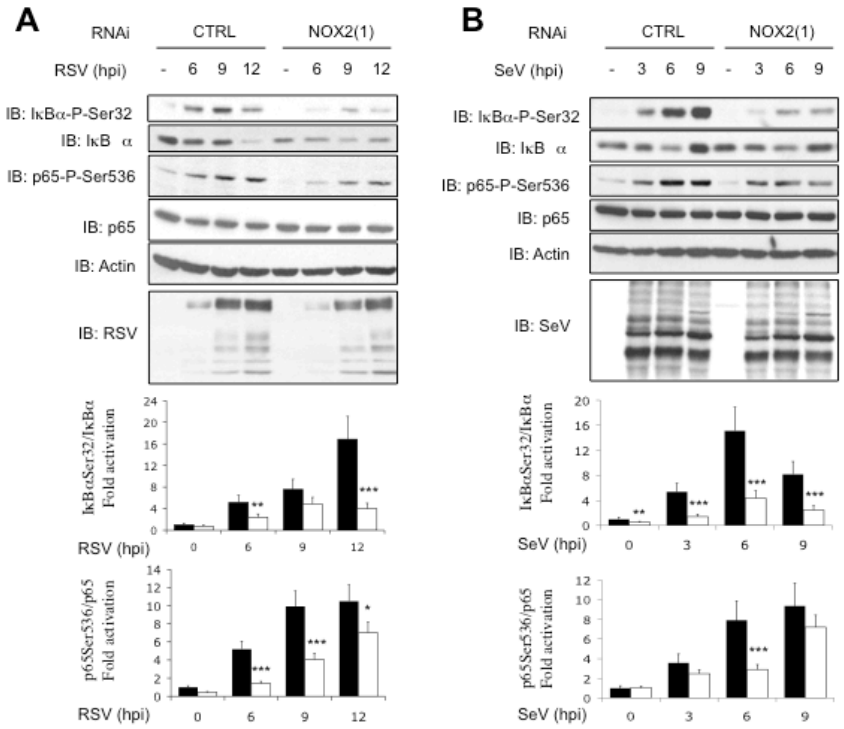
Fink *et al.*, Figure 5



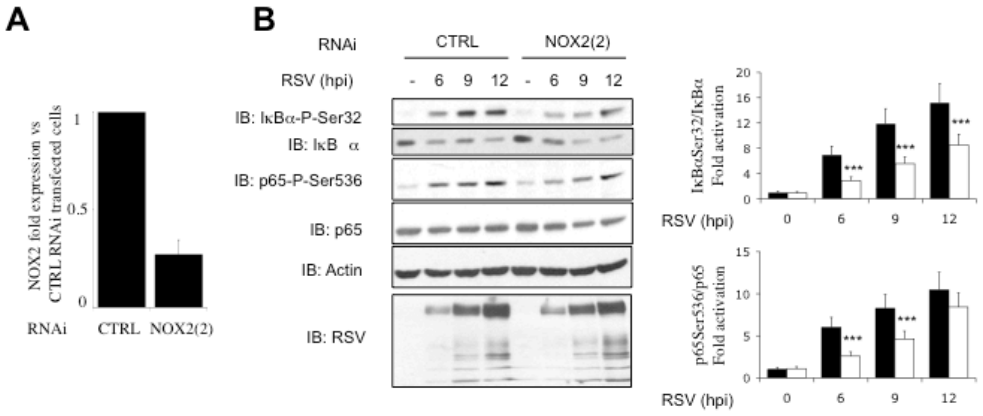
Fink *et al.*, Figure 6



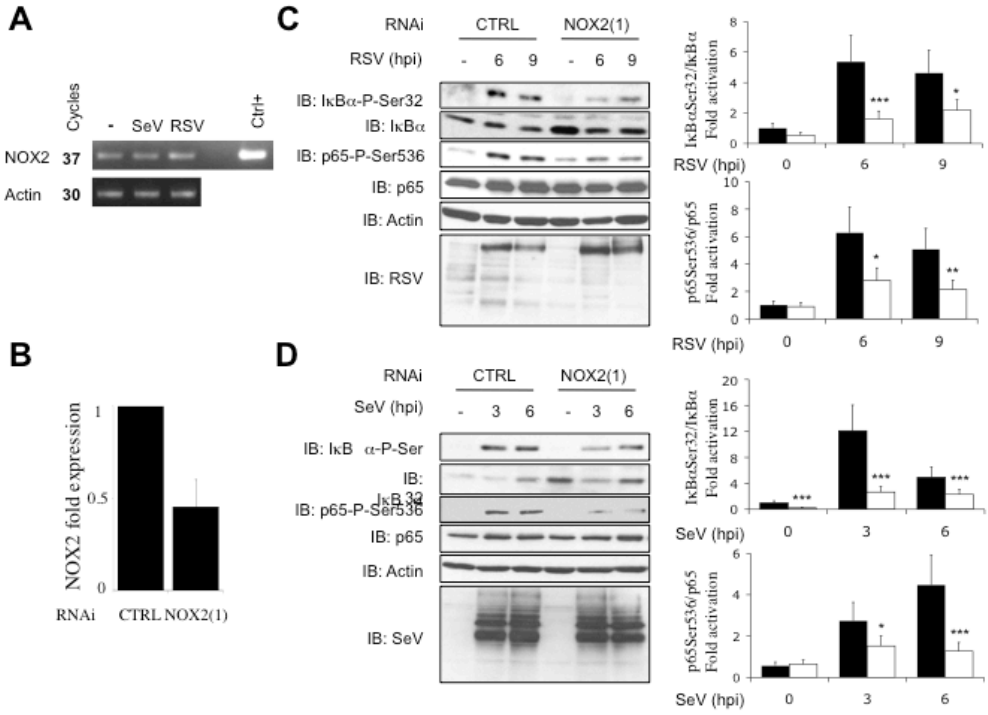
Fink *et al.*, Figure 7



Fink *et al.*, Figure 8



Fink *et al.*, Figure 9



2.2 IFNβ/TNFα synergism induces a non-canonical STAT2/IRF9-dependent pathway triggering a novel DUOX2 NADPH Oxidase-mediated airway antiviral response

2.2.1 Context

The detection of an invading virus by its target cell is the basis for the establishment of a major antiviral state. Hundreds of ISGs are specifically induced following viral infection, which serve to eliminate the virus from infected cells or diminish the spread of the virus to surrounding cells. During the course of our early studies of the antiviral response of AEC, an induction of gene expression of the DUOX2 NADPH oxidase family member was observed upon SeV infection. This result - virus-induced DUOX2 induction - added to previous data that demonstrated DUOX2 upregulation by RV infection or stimulation with a dsRNA mimetic polyI:C (Harper et al., 2005). At that point it was also known that DUOX2, a protein highly expressed in the airways, was associated with antibacterial function (Moskwa et al., 2007). From these results the hypothesis arose that DUOX2 might be a previously uncharacterized ISG family member serving in the antiviral defense. To study this hypothesis, two questions needed to be unraveled. Firstly, what were the regulatory mechanisms that drove DUOX2 expression during viral infection; and secondly, was DUOX2 implicated in an antiviral mechanism and what was the nature of its contribution to the antiviral state. Our results demonstrate that during AEC infection by SeV secreted IFNβ and TNFα synergize to engage a non-canonical signaling pathway that is IRF9- and STAT2-dependent, but entirely STAT1-independent. This pathway culminates in the upregulation of DUOX2 and its maturation factor DUOXA2, which belong to late-induced genes. Functional analysis revealed that DUOX2 is essential for AEC to mount a late antiviral state triggered by the synergism between IFNβ and TNFα, at least through regulation of type I and type III IFNs levels. Further, our results

reveal that the human pathogen Respiratory Syncytial Virus (RSV) is able to subvert DUOX2 induction. Thus DUOX2 a novel antiviral player in the respiratory mucosal linings.

2.2.2 Author contributions

The data presented in the following article results from my investigations started during my Ph.D. studies. Experiments were planned under the supervision of and guidance by my research supervisor Nathalie Grandvaux (NG). My research (KF) contributed significantly to the publication of this article. During the pre-submission phase I received assistance from Stefany Chartier (SC) in conducting experiments. Once in revision phase, Esperance Mukawera (EM) and Lydie Martin (LM) helped significantly to finalize necessary experiments. Esperance did all the RSV purifications throughout this study. I contributed, in collaboration with Nathalie Grandvaux, to the writing of the article. This article is accepted for publication in *Cell Research*.

Figure 1 : KF/SC (panel F and G)
Figure 2 : KF/SC (panel D)/ LM (panel E)
Figure 3 : KF/EM (panel D)/ SC (panel B)
Figure 4 : KF/LM (panel D)
Figure 5 : KF/EM
Figure 6 : KF
Figure 7 : KF
Figure 8 : KF/SC (panel C and F)
Purification RSV: EM

2.2.3 Article

IFNβ/TNFα synergism induces a non-canonical STAT2/IRF9-dependent pathway triggering a novel DUOX2 NADPH Oxidase-mediated airway antiviral response

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Abstract

Airway epithelial cells are initial key innate immune responders in the fight against respiratory viruses, primarily via the secretion of antiviral and proinflammatory cytokines that act in an autocrine/paracrine fashion to trigger the establishment of an antiviral state. The current paradigm is that the early antiviral state in airway epithelial cells primarily relies on IFNb secretion and subsequent activation of the ISGF3 transcription factor complex, composed of STAT1, STAT2 and IRF9, which regulates the expression of a panoply of Interferon Stimulated Genes encoding proteins with antiviral activities. However, in the setting of virus infections, the specific pathways engaged by the simultaneous action of different cytokines, as well as the thereof resulting distinct physiological outcome, are still ill-defined. Here, we unveil a novel delayed airway antiviral defense mechanism, which is initiated by the synergistic autocrine/paracrine action of IFNβ and TNFα and signals through a non-canonical STAT2- and IRF9-dependent, but entirely STAT1-independent, cascade. This pathway ultimately leads to the late induction of the DUOX2 NADPH oxidase expression. Importantly, our study uncovers that the development of the resulting antiviral state relies on DUOX2-dependent H_2O_2 production. Key antiviral pathways have proven to be targeted by evasion mechanisms evolved by various pathogenic viruses. In this regards, the importance of this novel DUOX2-dependent antiviral pathway is underlined by the observation that human Respiratory Syncytial Virus is able to subvert DUOX2 induction.

Introduction

The mucosal linings of the airways are constantly exposed to an array of microbial pathogens, including life-threatening respiratory viruses. Control of the host-microbe homeostasis at the mucosal epithelia is essential to prevent microorganisms-triggered inflammatory diseases. More than just a physicochemical barrier, airway epithelial cells (AEC) are also responsible for key immune responses in the fight against viruses. AEC rapidly recognize invading respiratory viruses to actively trigger the production of antiviral substances, including mucus, peptides and cytokines that limit invasion and spread of the pathogen. Additionally, AEC produce proinflammatory cytokines and chemokines that recruit and activate immune cells to the site of infection. Thus, the molecular pathways engaged upon viral infection of AEC, and the thereof resulting antiviral state, are crucial for the clearance of the pathogen and host recovery.

The current picture of the innate immune response proposes that in AEC, viral nucleic acids are sensed by pattern recognition receptors (PRR) of the Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) families. Downstream signaling cascades culminate into the activation of the NF-κB and IRF-3 transcription factors (TF), which regulate the expression of genes encoding proinflammatory cytokines, such as TNFα, and antiviral cytokines, primarily type I (α and b) and type III (λ1-3) interferons (IFNs) [1]. Secreted type I IFNs bind to their cognate receptors (IFNAR) resulting in the autocrine/paracrine activation of the JAK/STAT signaling pathway, which ultimately leads to the formation of the ISGF3 TF complex composed of STAT1, STAT2 and IRF9. ISGF3 activation is a prerequisite for the establishment of a robust antiviral state through the induction of numerous interferon-stimulated genes (ISGs), which encode antiviral proteins that modulate protein synthesis, cell growth arrest and apoptosis [2]. Understanding the molecular mechanisms underlying the establishment of the antiviral state in AEC is the focus of intensive research aimed at identifying novel antiviral genes and their regulatory pathway.

The NADPH oxidase enzymes Dual oxidase 1 and 2 (DUOX1 and DUOX2) were originally identified in the thyroid, but have since been shown to be expressed in epithelial tissues, including the microbe-exposed barrier epithelia, such as respiratory and intestinal tract, in mammals [3]. Increasing evidence support a role of DUOX1 and DUOX2 in the host defense against invading bacteria at the mucosal surfaces through generation of H_2O_2 [3, 4]. However, analysis of the inducibility profile of DUOX expression in AEC suggests that DUOX2 might also be part of the host defense against virus infection. While *DUOX1* is induced following stimulation with IL-4 and IL-13, typical T helper (Th) 2 cytokines, *DUOX2* is induced by the Th1 cytokine IFN-γ [5]. Additionally, *DUOX2* is upregulated following infection with rhinovirus (RV) or *Paramyxoviridae* viruses and in response to stimulation with poly (I:C), a synthetic double stranded RNA analog [6, 7].

In this study, we uncover that *DUOX2* is a late antiviral gene induced by an autocrine/paracrine pathway specifically triggered in AEC by the synergistic action of two major cytokines, IFNβ and TNFα, secreted upon Sendai virus (SeV) infection, a model of *Paramyxoviridae* viruses. Detailed analysis of *DUOX2* regulation allowed us to unveil that the combination of IFNβ and TNFα signals through a novel, non-canonical signaling pathway dependent on STAT2 and IRF9, but entirely independent on STAT1. Functional analysis revealed that DUOX2-derived H_2O_2 is essential for AEC to mount an antiviral state specifically triggered by the synergism between IFNβ and TNFα. Importantly, we also reveal that Respiratory Syncytial Virus (RSV), the most important etiological viral agent of pediatric respiratory tract diseases worldwide, has evolved mechanisms to counteract DUOX2 expression, allowing RSV to escape the DUOX2-mediated antiviral response. This observation highlights the importance of DUOX2 as a key molecule in the antiviral innate immune response.

Materials and Methods

Chemicals

The JAK inhibitor AG490 and the Tyk2-specific inhibitor Bayer-18 inhibitors were obtained from Enzo life Sciences and Symansis, respectively.

Cell culture

All media and supplements were purchased from Gibco, except for primary cell culture for which reagents were obtained from Clonetics. A549 cells (American Type Culture Collection, ATCC) were grown in F-12 nutrient mixture (Ham) medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and 1% L-glutamine. Calu-3 cells (ATCC) were grown under submerged conditions in MEM medium supplemented with 10% HI-FBS, 1% L-glutamine, 1% sodium pyruvate and 1% non-essential amino acids. For Air-Liquid Culture (ALI), Calu-3 cells were plated at a density of $0.2x10^6$ cells/cm^2 onto Greiner Transwell inserts coated with collagen VI (Sigma-Aldrich) for at least 16h. Calu-3 cells were kept under submerged conditions for 48h before medium in the apical compartment was removed. Cells were kept in ALI (ALI-Calu-3) culture for 10-14 days before conducting Transepithelial Electric Resistance (TEER) measurement with a Voltohmmeter (World Precision Instruments). Experiments were performed using ALI-Calu-3 having TEER measures equal to or higher than 800 $\Omega.cm^2$. NHBE were obtained from Clonetics, cultured in BEGM medium (Clonetics) and used until maximum passage 3. Vero cells (ATCC) were cultured in DMEM medium supplemented with 10% HI-FBS and 1% L-glutamine.

Virus infections

SeV Cantell strain was obtained from Charles River Laboratories. The initial stock of RSV A2 strain was obtained from Advanced Biotechnologies, *Inc*. The initial stock of recombinant RSV encoding GFP (RecRSV-GFP) was a generous gift from Dr. P. L. Collins (NIH, Bethesda). Amplification and purification of RSV and RecRSV-GFP was performed as previously described [8].

SeV infection was conducted at 40 hemagglutinin units (HAU) per 10^6 cells in serum

free medium (SFM) for 2h, after which the medium was supplemented with 10% HI-FBS. RSV or RecRSV-GFP infection was conducted at a MOI of 3 or 1, respectively, in medium containing 2% HI-FBS. Infection of ALI-Calu-3 was conducted the day after TEER measurement using viruses diluted in SFM (SeV) or 2% HI-FBS containing medium (RSV) and added apically onto Transwells. After 2h, the apical medium was taken off and the infection was pursued in ALI condition. SeV infection of NHBE was conducted with 40 HAU/10^6 cells in BEGM.

Preparation of supernatant from infected cells

For generation of supernatant from SeV- or RSV-infected cells, A549 cells were infected as described above. At 2 hours post-infection (hpi), the virus was taken off and the medium was replaced with Opti-MEM Reduced Serum media (Invitrogen). The infection was pursued for 22h. Thereafter, the supernatant was harvested and cell debris eliminated by centrifugation. Where indicated, the supernatant was treated with UV for 20min. To generate SN from A549 cells treated with Z-VAD-FMK (Calbiochem), A549 cells were pretreated with 0.1 mM Z-VAD-FMK or DMSO (vehicle) for 1h before infection. Z-VAD-FMK was present throughout the infection. For heat treatment, SN-SeV-UV was either left untreated or heated for 15 min at 80°C.

Stimulation with recombinant cytokines

Recombinant IFNβ and TNFα (Feldan) were used at a final concentration of 1000-5000 IU/mL and 10-50 ng/mL, respectively, in F12 nutrient mixture (Ham), supplemented with 2% HI-FBS. Where indicated, cells were pretreated with AG490 (100mM) or Bayer 18 (100mM) or the corresponding vehicle DMSO for 1 h before cytokine stimulation.

Virus titration by plaque forming unit assay

The supernatant of A549 cells infected with RecRSV-GFP was harvested 72hpi. Serial

dilutions of the supernatant were performed in DMEM (Gibco) containing 2% HI-FBS and used to infect confluent Vero cells for 2h. Following this period, the medium was replaced with 1% methylcellulose in DMEM containing 2% HI-FBS. Infection was pursued for 7 days and fluorescent lysis plaques were visualized using a Typhoon apparatus (Molecular Dynamics) and counted using the ImageQuantTL colony counting analysis tool.

siRNA Transfection

RNAi oligonucleotides (see **Table 1** for sequences) were purchased from Dharmacon, except for siDUOX2(1), which was from Invitrogen. Transfection was performed as previously described [9] using Oligofectamine reagent (Invitrogen) and pursued for 48h before viral infection or SN or cytokines stimulation.

Immunoblot analysis

Whole-cell extracts (WCE) were prepared on ice in Nonidet P-40 (Igepal; Sigma) lysis buffer [10], quantified by a Bradford protein assay (Bio-Rad), and resolved by SDS-PAGE, followed by immunoblot analysis. Proteins were immunodetected using anti-actin (Millipore), anti-ISG56 (IFIT1; Novus Biologicals), anti-IRF1 (Santa Cruz), anti-IRF9 (BD Transduction Laboratories), anti-parainfluenza (obtained from Dr. J. Hiscott, McGill University, Montreal, Canada), anti-PARP (Cell Signaling), anti-RSV (Chemicon International), anti-IκBα-P-Ser32, anti-IκBα, anti-STAT1-P-Tyr701, anti-STAT2-P-Tyr690, anti-STAT1, anti-STAT2 (all from Cell Signaling), and anti-a-tubulin (Santa Cruz) antibodies diluted in phosphate-buffered saline (PBS) containing 0.5% Tween (Sigma Aldrich) and either 5% nonfat dry milk or 5% BSA (Sigma Aldrich). For DUOX2 and TNFRI immunodetection, WCE were prepared at room temperature in 125mM Tris/HCl (pH 6.8), 10% glycerol, 2% SDS and 0.1 M DTT followed by sonication (2x20s) and heating to 70°C for 10 min. WCE were quantified using a RC/DC protein assay (Bio-Rad). 150µg were resolved by SDS-PAGE. DUOX2 was immunodetected using the anti-DUOX1/2 specific antibodies previously described

in [11]. TNFRI was immunodetected using the anti-TNFRI/TNFRSF1A antibodies (R&D Systems). The membranes were further incubated for 1h with horseradish peroxidase (HRP)-conjugated secondary antibodies (Kirkegaard & Perry Laboratories or Jackson Immunoresearch Laboratories). Immunoreactive bands were visualized by enhanced chemiluminescence using the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences) and detected using a LAS4000mini CCD camera apparatus (GE healthcare).

Quantitative RT-PCR (qRT-PCR) analyses

Total RNA was prepared using the RNAqueous-96 Isolation Kit (Ambion) following the manufacturer's instructions. Total RNA (1µg) was subjected to reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen). PCR amplifications were performed with the Fast start SYBR Green Kit (Roche). Sequences of oligonucleotides (Invitrogen) are presented in **Table 2**. Absence of genomic DNA contamination was analyzed using a reaction without reverse transcriptase. Detection was performed on a Rotor-Gene 3000 Real Time Thermal Cycler (Corbett Research). For *DUOX2*, *DUOXA2*, *IFNB, IL28, IL29, IFIT1, TNFα, β-actin* and *S9* genes qRT-PCR amplifications, standard curves of absolute quantification expressed as copy number and PCR efficiencies were obtained using serial dilutions of DUOX2-HA-pcDNA3.1 (a generous gift from Dr. Grasberger, University of Michigan, Ann Harbor, USA), DUOXA2-pCR4-TOPO, IL28-pCR4-TOPO, IL29-pCR4-TOPO, β-actin-pCR2.1-TOPO, ISG56-pCR2.1-TOPO, IFNβ-pCR2.1-TOPO, TNFα-pCR2.1-TOPO, and S9-pCR2.1-TOPO. Gene specific absolute mRNA copy numbers were normalized to β-actin or S9 mRNA absolute copy numbers. For *IFNAR1* real time amplification, serial dilutions of cDNA derived from IFNAR1 expressing A549 cells where used to determine primer efficiency and linearity of PCR reaction. IFNAR fold induction was calculated using the DD Cycle threshold (C_t) method [12].

Multiplex ELISA

SeV and RSV infections were conducted in Opti-MEM Reduced Serum media (Invitrogen). Where applicable, virus infection was performed 48h post-siRNA transfection. Fifty µL of SN were analyzed using the *VeriPlex*^{TM} Human Interferon Multiplex ELISA (PBL Interferon) according to the manufacturer's instructions. The ELISA plate was imaged with the Q-View Imager and data analysis was performed using the Q-View Software (Quansys Biosciences).

H_2O_2 measurement

Extracellular H_2O_2 production was measured using homovanillic acid (HVA)-based fluorimetric assay as previously described [13]. Briefly, following stimulation, cells were incubated in Krebs-Ringer-Hepes solution containing 0.44mM HVA and 0.1mg/mL horseradish peroxidase for 2h at 37 C. Where indicated, catalase was added at 400U/mL. At the end of the incubation period, fluorescence was quantified with an excitation wavelength of 315nm and an emission wavelength of 425nm on a HT Synergy (Biotek) plate reader. H_2O_2 concentration values were assigned using a H_2O_2 standard curve.

Statistical analyses

All quantification data are presented as the mean ± standard deviation (SD). Statistical significance for comparison was assessed using the Prism 5 software (GraphPad). Statistical significance was evaluated using the following p values: $p < 0.05$ (*), $p < 0.01$ (**) or $p< 0.001$ (***).

Results

SeV infection induces DUOX2 and DUOXA2 expression in AEC.

We previously reported using RT-PCR that SeV infection of the A549 alveolar epithelial cell line triggered induction of DUOX2 mRNA [7]. Here, a detailed characterization of DUOX2 mRNA and protein expression following SeV infection was performed in different cell line models of AEC and non-transformed primary normal human bronchial epithelial cells (NHBE). First, A549 cells were infected with SeV for various times. Quantitative RT-PCR (qRT-PCR) analysis revealed significant induction of DUOX2 mRNA levels starting at 24 hpi (**Figure 1A**). Interestingly, induction of the classic early antiviral *IFIT1* gene peaked between 6hpi and 9hpi (**Figure 1A**). Thus, *DUOX2* belongs to a category of late virus-induced genes. DUOX2 induction was confirmed at the protein level by immunoblot using anti-DUOX1/2 antibodies (**Figure 1B**). Although we and others previously established that DUOX1 is not expressed in non-infected and SeV-infected A549 cells [7, 14], the specific detection of DUOX2 was confirmed using small interfering RNA (siRNA)-mediated knockdown of DUOX2 (**Figure 3, 5 and 6**). Functional expression of DUOX2 at the plasma membrane is dependent on the concomitant expression of its maturation factor DUOXA2 [15]. *DUOX2* and *DUOXA2* genes are individually transcribed from the same bidirectional promoter [15], making *DUOXA2* expression pattern likely to resemble the one of *DUOX2*. In accordance with this observation, DUOXA2 mRNA was induced in SeV-infected A549 cells following a kinetic similar to that of DUOX2 (**Figure 1C**). Infection of A549 cells with UV-treated SeV, which is unable to replicate, failed to induce *DUOX2*, indicating that virus replication is essential for *DUOX2* expression (**Figure 1D**). To further demonstrate that *DUOX2/DUOXA2* genes are responsive to SeV in AEC, polarized Calu-3 cells cultured at an Air-Liquid Interface (ALI-Calu-3) forming a tight monolayer, were used. This serous gland cell line of sub-bronchial origin is widely used in the study of airway barrier function and ion secretion, and was more recently shown to be a suitable model for the study of virus infections [16]. Monolayers of Calu-3 exhibiting good integrity, as monitored by a transepithelial electric resistance (TEER) of $\geq$800 Ωcm^2, were used in the

experiments. Infection of ALI-Calu-3 with SeV on the apical side resulted in DUOX2 and DUOXA2 mRNA induction starting at 24hpi (**Figure 1E and G**) and detectable DUOX2 protein levels at 48hpi (**Figure 1F**)**.** Importantly, DUOX2 and DUOXA2 mRNA induction was confirmed in primary NHBE infected with SeV for 24h (**Figure 7A and B**). Altogether, these results demonstrate that *DUOX2/DUOXA2* are late-virus induced genes in human AEC.

SeV-mediated induction of *DUOX2/DUOXA2* results from an autocrine/paracrine mechanism.

The delayed induction of *DUOX2/DUOXA2* during SeV infection suggested that their expression might be dependent on a mechanism requiring *de novo* synthesis and/or secretion of regulatory factor(s). To test this hypothesis, the supernatant of SeV-infected A549 cells was harvested (SN-SeV) and submitted to UV treatment (SN-SeV-UV) to impair the capacity of newly secreted virions to replicate and thereby induce *DUOX2* as shown in **Figure 1D**. Stimulation of fresh A549 cells with SN-SeV-UV (**Figure 2A**) led to induction of DUOX2 and DUOXA2 mRNA to levels corresponding to 64 +/- 12% and 88 +/- 33% of those induced by the corresponding direct SeV infection, respectively (**Figure 2B and D**). Treatment with SN-SeV-UV also efficiently upregulated DUOX2 protein expression (**Figure 2C**). Heat inactivation of SN-SeV-UV abolished DUOX2 mRNA and protein induction (**Figure 2E**). These results pointed to a major role of factors of protein nature released into the SN of infected cells in the induction of *DUOX2/DUOXA2*. Next, the possibility that apoptosis of infected cells could contribute to the release of these factors was ruled out. The SN-SeV was generated in the presence of the pan-Caspase inhibitor Z-VAD-FMK to block apoptosis as demonstrated by the efficient inhibition of PARP cleavage (**Figure 2F**). Blockade of apoptosis failed to interfere with the capacity of SN-SeV-UV to induce DUOX2 mRNA levels (**Figure 2F**), thus demonstrating that *DUOX2* induction does not result from caspase-dependent apoptotic processes triggered by SeV infection. Altogether, these results highlight for the first time that *DUOX2/DUOXA2* induction in virus-infected AEC results from an autocrine/paracrine mechanism.

IFNβ and TNFα synergize to regulate *DUOX2* and *DUOXA2* expression.

Next, we sought to determine the identity of the soluble factor(s) responsible for SeV-mediated induction of *DUOX2/DUOXA2*. Type I and type III IFNs are the most abundant cytokines secreted following viral infection, but stimulation of A549 cells with recombinant IFNβ (**Figure 3A and B**) or IFNα2b, IL28 or IL29 (data not shown) failed to induce significant increase in DUOX2 or DUOXA2 mRNA levels. Interestingly, previous reports revealed that IFNβ can synergize with TNFα to induce a late antiviral state distinct from the early state induced by IFNβ alone [17, 18]. Thus, the possibility that *DUOX2/DUOXA2* induction could be driven by the combination of IFNβ and TNFα was addressed. Multiplex ELISA analysis confirmed the presence of both IFNβ and TNFα in the SN-SeV derived from A549 cells (**Figure 3C**). Interestingly, stimulation of A549 cells with a combination of recombinant IFNβ and TNFα led to a significant increase in DUOX2 and DUOXA2 mRNA and DUOX2 protein levels as compared to stimulation with either cytokine independently (**Figure 3A and B**). Similar results were observed in primary NHBE (**Figure 7B**). Several other combinations of IFNα, IFNβ or TNFα with IFNλ (IL28/IL29) were tested, but none of them resulted in DUOX2 induction (**Figure S1**). To demonstrate the importance of IFNβ and TNFα in the capacity of SN-SeV-UV to induce *DUOX2/DUOXA2*, knockdown of type I IFN receptor chain 1, IFNAR1, and TNFα receptor, TNFRSF1A, was achieved by siRNA. Downregulation of either of the receptors led to decreased DUOX2 mRNA induction following treatment with SN-SeV-UV as compared to control cells (**Figure 3D**). Importantly, the combination of IFNβ and TNFα, similarly to SeV infection, induced catalase-sensitive H_2O_2 production (**Figure 3E**). H_2O_2 induction was also dramatically reduced by silencing of DUOX2 using specific siRNA, thereby demonstrating that DUOX2 is a main source for IFNβ and TNFα-stimulated H_2O_2 generation (**Figure 3E**). Altogether, these results unveil the synergistic action of IFNβ and TNFα in the autocrine/paracrine regulation of *DUOX2/DUOXA2* during virus infection.

***DUOX2* induction is mediated by a non-canonical, STAT2/IRF9-dependent, but STAT1-independent pathway.**

To further identify the mechanisms involved in virus-mediated induction of *DUOX2* expression, a siRNA strategy was pursued to individually downregulate each subunit of the ISGF3 complex, STAT1, STAT2 and IRF9. Surprisingly, while knockdown of STAT2 and IRF9 strongly diminished SN-SeV-UV-induced DUOX2 mRNA and protein expression compared to control cells, STAT1 knockdown did not impair DUOX2 induction (**Figure 4A**). Similar results were obtained using a second STAT1-specific siRNA (**Figure 4B**). While STAT1(1) siRNA led to induction of DUOX2 mRNA expression, this increase was not reproduced with the second STAT1(2) siRNA and was therefore not considered significant. Contrarily, both STAT1-specific siRNA efficiently inhibited IFNg-induced IRF1 expression (**Figure 4C**). In the ISGF3 complex, activated STAT2 is phosphorylated on Tyr690. To evaluate whether STAT2-Tyr690 phosphorylation is also required in the non-canonical pathway leading to DUOX2 expression, the JAK kinases inhibitor, AG490, and the specific inhibitor of the JAK member Tyk2, Bayer-18, were used. Both inhibitors efficiently inhibited STAT2-Tyr690 phosphorylation in IFNβ and TNFα-stimulated cells and dramatically reduced DUOX2 induction (**Figure 4D**). Interestingly, kinetic analysis of STAT2 phosphorylation in response to a combination of IFNβ and TNFα or to either cytokine independently, revealed that TNFα alone is not sufficient to trigger STAT2 phosphorylation, but in its presence maximum IFNβ-induced STAT2 phosphorylation is achieved during an extended period of time. Additionally, TNFα alone is able to stimulate IRF9 expression (**Figure 4E**). The impact of TNFα on IRF9 expression and on STAT2 phosphorylation most likely contributes to the activation of the non-canonical STAT2/IRF9 pathway during costimulation by IFNβ and TNFα. TNFα is well known to trigger NF-κB-dependent gene expression. However, no differences were observed in IκBα phosphorylation and degradation in response to TNFα alone or in response to IFNβ and TNFα costimulation (**Figure 4E**). Additionally, inhibition of the NF-κB pathway through expression of the widely used super-repressor of NF-κB, IκBα2NΔ4 [19], did not prevent SN-SeV-UV-mediated induction of DUOX2 (**Figure**

S3) strongly suggesting that NF-κB is unlikely to be involved in IFNβ and TNFα-mediated induction of DUOX2. Hence, a non-canonical signaling pathway involving phosphorylated STAT2 and IRF9, but not STAT1, mediates the IFNβ and TNFα-dependent induction of DUOX2 in AEC during SeV infection.

DUOX2 is essential for AEC to mount an antiviral defense.

Whether *DUOX2* is part of the numerous virus-induced genes that allow the host to mount an antiviral response has not yet been assessed. To evaluate the antiviral role of DUOX2, SN-SeV-UV generated from A549 cells or a combination of IFNβ and TNFα were used to stimulate fresh A549 cells previously transfected with CTRL- or two different DUOX2-specific siRNA (siDUOX2(1) and siDUOX2(2)). In these conditions, target cells mount an antiviral response that differs by the presence or absence of DUOX2. The antiviral states were next monitored through their capacity to limit replication of recombinant RSV encoding GFP (RecRSV-GFP) (**Figure 5A**). As shown in **Figure 5B**, CTRL siRNA-transfected, SN-SeV-UV-treated cells efficiently restricted RecRSV-GFP replication as compared to CTRL siRNA-transfected cells. Importantly, in the absence of DUOX2, SN-SeV-UV-treated cells only partially impaired RecRSV-GFP replication compared to CTRL siRNA-transfected, SN-SeV-UV-treated cells. Thus, in the absence of DUOX2, cells mount a less efficient antiviral response following stimulation with SN-SeV-UV. Similar results were obtained in the context of IFNβ and TNFα costimulation (**Figure 5C**). Importantly, DUOX2 siRNA did not alter the antiviral state induced by IFNβ alone **(Figure 5C)**, which does not trigger DUOX2 expression (**Figure 3A**). This highlights the specific role of DUOX2 in the antiviral state mounted in response to IFNβ and TNFα costimulation. Additionally, the antiviral effect triggered by IFNβ and TNFα was abrogated by treatment with catalase, thereby demonstrating that the antiviral state relies on H_2O_2 production (**Figure 5D**). The role of DUOX2 in the establishment of the IFNβ and TNFα-dependent antiviral response was also confirmed in primary NHBE (**Figure 7B**). Altogether, these results are the first to unveil the contribution of DUOX2 to the development of the antiviral state.

Next, to start elucidating how DUOX2 enhances the antiviral state, Multiplex ELISA analysis of type I (α and β) and type III (λ1-3) IFNs levels, the main antiviral cytokines secreted during virus infection of AEC, in the SN of A549 cells transfected with CTRL-, DUOX2(1)-, or DUOX2(2)-specific siRNA and infected with SeV were performed. Interestingly, absence of DUOX2 significantly diminishes levels of IFNβ at 24hpi and 32hpi and those of IFNλ at 32hpi (**Figure 6A**). On the other hand, TNFα levels were not significantly changed (**Figure 6A**). Despite the observation that DUOX2(1)-mediated downregulation of DUOX2 expression tends to diminish TNFα levels, although not significantly, this was not confirmed when knockdown of DUOX2 was achieved using siDUOX2(2) (**Figure 6A**). Similar results were observed in primary NHBE (**Figure 7C**). Importantly, none of the cytokine levels were altered at 6hpi, thereby suggesting that DUOX2 controls sustained levels of IFNβ and IFNλ specifically at late stages of viral infection. Importantly, differences in cytokines levels could not be explained by a decrease at their respective mRNA levels (**Figure 6B**). Thus, our results demonstrate that DUOX2 is a key player in the establishment of an antiviral state triggered by the synergism between IFNβ and TNFα, at least through regulation of IFNβ and IFNλ protein levels at late time points of infection.

RSV interferes with the expression of DUOX2.

The aforementioned data clearly highlight a new antiviral pathway occurring in the airway epithelium mediated by the synergism between IFNβ and TNFα involving induction of DUOX2. Key antiviral pathways have proven to be targeted by evasion mechanisms evolved by pathogenic viruses, including RSV [20]. Thus, we next sought to determine whether RSV is capable of evading the DUOX2-dependent antiviral response. First, A549 cells were infected with RSV for various times. As shown in **Figure 8A**, RSV infection induced only weak levels of DUOX2 mRNA compared to SeV. These low levels of mRNA translated into undetectable levels of the DUOX2 protein, even when the multiplicity of infection (MOI) was increased from 3 to 10 (**Figure 8B**). Analysis of DUOXA2 mRNA expression levels again revealed a barely detectable induction during RSV infection, thus significantly lower than the one observed during SeV infection (**Figure 8C**). Similar results were observed in the ALI-

Calu-3 cell model (**Figure 8D-F**). Importantly, IFNβ and TNFα levels in the SN of RSV-infected A549 cells were close to those observed in the supernatant of SeV-infected A549 cells (**Figure 8G vs. Figure 3C**). Hence, the decreased *DUOX2* and *DUOXA2* induction during RSV infection could not be attributed to a deficiency in the production of IFNβ or TNFα. Interestingly, UV-treated supernatant derived from RSV-infected A549 cells (SN-RSV-UV), used as in **Figure 2A,** induced DUOX2 mRNA levels corresponding to 305 +/- 125% of those induced by direct RSV infection (**Figure 8H**) and detectable levels of DUOX2 protein (**Figure 8I**). Similarly to our observation in the context of SeV infection, knockdown of STAT2 and IRF9, but not of STAT1, prior to stimulation with SN-RSV-UV, impaired DUOX2 induction (**Figure 8J**). Altogether, these results demonstrate that cytokines secreted in response to RSV infection are capable of inducing *DUOX2*, but that the presence of virus in the cell interferes with *DUOX2* induction, allowing RSV to escape the DUOX2-mediated antiviral response.

Discussion

AEC are the first line of defense against respiratory virus infection. The efficiency of the innate antiviral state mounted by AEC critically influences the outcome of viral infection. In the current paradigm, the antiviral state in infected AEC is primarily dependent on type I IFN-mediated activation of the JAK/STAT signaling cascade ultimately leading to the transcriptional control of ISGs by the ISGF3 complex. Here, we highlight a novel antiviral pathway occurring in AEC, which is initiated by the synergistic autocrine/paracrine action of IFNβ and TNFα, and which signals through a non-canonical IRF9- and STAT2-dependent, but entirely STAT1-independent, cascade to establish a late antiviral state mainly controlled by the DUOX2 NADPH oxidase (**Figure 9**).

Virus infections trigger the secretion of multiple antiviral and proinflammatory cytokines, which bind to their cognate receptor to engage specific signaling pathways. Although it seems intuitive that secreted cytokines do not act independently, but rather simultaneously to foster the antiviral response, the specific outcome resulting from their cooperation has barely been described. The synergism between IFNβ and TNFα was first reported in 1988 [21]. However, it was only recently discovered, through microarray profiling, that IFNβ and TNFα synergize to drive the expression of a panel of delayed genes that define a distinct antiviral state. These genes are either not responsive to IFNβ or TNFα separately or are only responsive to either one of the cytokine when used separately [17, 18]. Here, we uncover that in AEC, *DUOX2/DUOXA2* belong to this category of delayed genes that are not significantly induced by IFNβ and TNFα alone, but are remarkably induced to high levels in response to the combination of IFNβ and TNFα. Previous attempts to identify the mechanisms underlying the specific regulation of genes dependent on IFNβ and TNFα synergism was performed through bioinformatic analysis of the promoters of this panel of genes in order to identify enrichment in specific TF binding sites. However, no specific TF was identified [17]. Here, through a targeted strategy using siRNA, we demonstrate for the first time that the synergistic action of IFNβ and TNFα engages a

specific STAT2 and IRF9-dependent, but STAT1-independent, signaling pathway. Interestingly, our results revealed that TNFα contributes to IRF9 induction and that TNFα synergism with IFNβ induces an extended maximum activation of JAK-mediated STAT2 phosphorylation. Thus, IRF9 induction and STAT2 phosphorylation likely contribute to activation of the non-canonical STAT2/IRF9 pathway during costimulation by IFNβ/TNFα. This is consistent with previous reports that identified TNFα-mediated activation of JAKs [22, 23]. Whether this STAT2- and IRF9-dependent pathway is responsible for the regulation of all other genes previously identified to be responsive to the combination of IFNβ and TNFα remains to be determined. Other studies proposed that TNFα synergizes with IFNβ through an autocrine/paracrine loop. Indeed, TNFα can induce the secretion of IFNβ in an IRF1-dependent manner, thereby controlling the expression of specific delayed genes through the classical JAK/STAT pathway [18, 24, 25]. However, this loop is unlikely to be involved in the induction of DUOX2 in our system, since TNFα alone is not sufficient to activate *DUOX2/DUOXA2* expression.

Previous reports described the capacity of STAT2 and IRF9 to activate gene transcription independently of STAT1 [26-30]. However, none of these studies have linked activation of the STAT2/IRF9 pathway to the specific synergistic action of IFNβ and TNFα. Interestingly, in the absence of STAT1, STAT2/IRF9 display only limited DNA-binding affinity for the typical ISRE sequence targeted by the IRF9 DNA-binding domain as part of the ISGF3 complex [26] and the specific DNA-binding consensus sequence for this non-canonical complex remains to be determined. The possibility that STAT2/IRF9 uses a yet uncharacterized consensus responsive element would explain the failure of the previous bioinformatics strategy aimed at identifying TFs acting downstream of IFNβ and TNFα, as it was based on databases of known DNA-binding consensus sites [17]. It is noteworthy to mention that a STAT2/IRF9-dependent induction of *RIG-G* gene expression in response to all-trans retinoic acid (ATRA) was reported [31]. Interestingly, ATRA was shown to be a potent inducer of *DUOX2* in AEC [32].

To date, regulation of *DUOX2/DUOXA2* expression was mainly investigated in thyrocytes, as well as in airway and gut epithelial cells following bacterial infection [6,

33-35]. Our observation that *DUOX2/DUOXA2* are induced following infection with SeV in two different AEC models, A549 and ALI-Calu-3, as well as in NHBE, adds to the picture of the previously reported RV-induced *DUOX2/DUOXA2* expression in primary AEC [6, 36-38]. Importantly, SeV is a member of the *Paramyxoviridae* family of negative sense single-stranded RNA (ssRNA) viruses, whereas RV belongs to the *Picornaviridae* family containing positive sense ssRNA. Furthermore, poly (I:C) used to engage Toll-like receptor (TLR) 3 also induces DUOX2 expression in primary AEC [6]. Thus, altogether these data strongly support the idea that the DUOX2-dependent antiviral pathway might be relevant to a broad number of respiratory viruses. Interestingly, infection by RSV barely triggered detectable induction of *DUOX2/DUOXA2*. In contrary, SN-RSV-UV was a potent inducer of *DUOX2/DUOXA2*, thus demonstrating that the presence of intracellular replicating RSV interferes with *DUOX2/DUOXA2* induction. This is of particular significance, as most human pathogenic viruses have evolved strategies to circumvent the key mechanisms of the innate antiviral response for their own replication needs [20]. Thus, the observation that *DUOX2/DUOXA2* expression is the target for viral evasion points to the importance of DUOX2 in the capacity of the host to mount an efficient antiviral response. In this line, we unveiled for the first time that DUOX2 is critical for the outcome of respiratory virus infections through its key function in the establishment of the antiviral state specifically induced by the synergistic action of IFNβ and TNFα secreted during SeV infection. The capacity of RSV to counteract this novel antiviral pathway adds to its previously recognized capacity to interfere with other key antiviral events, including RIG-I-dependent signaling or IKKε, TRAF3 or STAT2 stability [39, 40]. Altogether these evasion mechanisms contribute to the development of RSV-associated pathologies. Interestingly, DUOX2 expression is decreased in patients with Cystic Fibrosis (CF) [41]. Thus, our data sheds light into a potential mechanism for increased susceptibility of CF patients to respiratory virus infections [42].

Up to date, the demonstration of a role of DUOX2 in the antimicrobial defense was restricted to bacterial infections. This function of DUOX2 appears to be evolutionary conserved. In *Drosophila*, absence of the DUOX homologue at epithelial

surfaces profoundly alters the outcome of intestinal bacterial infection and fly survival [43]. The current model proposes that at airway surfaces, H_2O_2 in combination with the secreted lactoperoxidase (LPO) enzyme and thiocyanite forms the microbicidal compound hypothiocyanate. To date, studies based on the use of antioxidant enzymes support the necessity of H_2O_2 in the LPO-dependent antibacterial defense [44]. In this line, further studies suggest that DUOX2 is a source of H_2O_2 production in the airways [45, 46]. However, none of these studies clearly demonstrated a functional connection of DUOX2-generated H_2O_2 production in LPO-dependent airway antibacterial defenses. Whether the H_2O_2/LPO system contributes to an antiviral defense still remains to be clearly established. *In vitro* studies have shown that the products of LPO activity, hypothiocyanate and hypoiodous acid, possess virucidal properties [47-49]. However, no data are available showing this activity in an *in vivo* context. In our setting, the H_2O_2/LPO system is unlikely to play a role based on the following observations. First, LPO is not significantly secreted from *in vitro* cultured AEC [50] and all studies, which previously demonstrated an antimicrobial function via the LPO system, required addition of LPO in the experimental setup [44, 45, 49]. However, here we observe an antiviral effect of DUOX2 without the ectopic addition of LPO. Moreover, we did not observe an antiviral effect of the supernatant in a cell free system (**Figure S2**). In the same line, rather than a role in the synthesis of extracellular virucidal components, we identified a significant role of DUOX2 in the regulation of secreted levels of IFNβ and IFNλ, but not of TNFα, nor IFNα, IFNω, IFNγ, IL6, and IL1α (data not shown). Interestingly, DUOX2 specifically regulates IFNβ and IFNλ levels at late time points of infection (starting at 24hpi and 32hpi, respectively) but not at early times (6hpi). Hence, DUOX2 seems to play a role in sustaining the levels of IFNβ and IFNλ during viral infection. Further studies will be required to unveil how DUOX2 fosters sustained IFNβ and IFNλ levels in the SN of infected cells. An interesting working model would be that, through its capacity to diffuse across membranes, DUOX2-derived H_2O_2 could modulate intracellular signals regulating production and/or secretion of IFNβ and IFNλ. Such a mechanism was previously shown to be implicated in the DUOX2-dependent regulation of NF-κB activity, and

thereof resulting IL-8 levels and neutrophil recruitment [51]. The closely related homolog of DUOX2, DUOX1, which is also expressed in AEC was previously shown to regulate EGFR-dependent signaling [52, 53]. Additionally, DUOX1-derived H_2O_2 was shown to regulate intracellular protein phosphatase activities [54, 55]. Although intracellular signaling modulation is a likely mechanism, regulation of IFNs expression in our system was not due to altered transcriptional regulation, since IFNβ and IFNλ mRNA levels were not affected by the specific downregulation of DUOX2 by siRNA. Thus, it can be excluded that DUOX2 regulates the signaling cascades leading to NF-κB and IRF-3 TFs, which are key regulator of IFN-encoding genes. It is noteworthy to mention that a recent publication has placed DUOX2 as a potential regulator of proinflammatory responses via shedding of the soluble TNFR in human AEC following TLR3 stimulation by poly (I:C) [56]. Here, we did not observe an effect of DUOX2 downregulation on TNFR shedding during SeV infection (data not shown).

In conclusion, our study unveils a key function of DUOX2 in the establishment of a late antiviral state triggered by the synergistic autocrine/paracrine action of IFNβ and TNFα secreted during respiratory virus infection. Importantly, induction of the antiviral state by the combination of IFNβ and TNFα is specifically driven by a non-canonical STAT2/IRF9-dependent, STAT1-independent signaling pathway. Thus, our study reveals a novel antiviral signaling cascade that acts in a late fashion compared to the classical ISGF3-dependent antiviral pathway.

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Titles and legends to figures

Figure 1: DUOX2 and DUOXA2 are induced upon SeV infection of AEC.

In **A-C,** A549 cells were infected for the indicated times with SeV (40HAU/10^6 cells). In **D**, A549 cells were infected for the indicated times with SeV or UV-treated SeV (40HAU/10^6 cells). In **E-G,** polarized Calu-3 cells cultured for 10 days in ALI (ALI-Calu-3) and presenting an UAR $\geq$ 800 Ωcm^2 were infected with SeV (40HAU/10^6 cells) at the apical side for the indicated times. In **A, C, D, E and G** total RNA were extracted and DUOX2, IFIT1 or DUOXA2 mRNA absolute copy numbers were quantified by qRT-PCR. In **B and F,** DUOX2 protein expression was analyzed by immunoblot using anti-DUOX1/2-specific antibodies. In **D,** SeV N protein expression was detected using anti-parainfluenza antibodies. Equal loading was verified using anti-tubulin or anti-actin antibodies. All data are presented as mean ± SD. Statistical analyses were conducted using one-way ANOVA with Tukey post-test, except in D, where analysis was performed using a t-test. Statistical significances are presented compared to the non-infected control, except in D, where the SeV infected condition is compared to SeV-UV infected condition. *$p<0.05$, ***$p<0.001$. The dotted line in qRT-PCR graphs represents the threshold of detection. IB, immunoblot; NS, non specific; hpi, hours post-infection; UAR, Unit Area Resistance.

Figure 2: SeV mediates induction of DUOX2 and DUOXA2 through secreted proteins.

In **A,** a schematic representation of the timeline used to generate SN from infected cells is presented. See Experimental Procedures for details. In **B-D,** A549 cells were stimulated with untreated (SN-SeV) or UV-treated supernatant (SN-SeV-UV) from SeV-infected cells for 24h or the indicated time. SeV infection (40HAU/10^6 cells, 24h) was conducted for comparison. In **E**, cells were stimulated with SN-SeV-UV or SN-SeV-UV subjected to heat treatment. In **F**, A549 cells were treated for 24h with SN-SeV-UV generated from cells exposed to DMSO or 0.1 mM Z-VAD-FMK. In **B, D, E and F** DUOX2 or DUOXA2 mRNA expression were analyzed by qRT-PCR. In **B**,

STAT1-Tyr-701 phosphorylation, STAT1, and SeV protein levels were analyzed by immunoblot. In **C and E**, DUOX2 protein expression was analyzed. In **E**, IFIT1 expression was analyzed by immunoblot. In **F**, PARP cleavage was assessed in SeV-infected A549 cells treated with DMSO or Z-VAD-FMK, as well as in cells stimulated with SN-SeV-UV/DMSO or SN-SeV-UV/Z-VAD-FMK. qRT-PCR data are presented as mean ± SD. Statistical analysis was conducted using one-way ANOVA with Dunnett post-test, except in E and F, where a t-test was used. *p<0.05, **p<0.01, ***p<0.001. IB, immunoblot; NS, non specific; ns, non significant; SN, supernatant.

Figure 3: Costimulation by IFNβ and TNFα efficiently induces DUOX2 and DUOXA2 expression and DUOX2-dependent H_2O_2 production.

In **A and B,** A549 cells were stimulated with recombinant IFNβ or TNFα or a combination of both for 24h. SeV infection (40HAU/10^6 cells, 24h) was conducted for comparison. In **C**, A549 were infected with SeV (40HAU/10^6 cells) and IFNβ and TNFα levels in the supernatant were measured by Multiplex ELISA. In **D**, A549 cells were transfected with control siRNA (siCTRL) or siRNA targeting the IFN receptor chain 1 using a mixture of siIFNAR1(1) and siIFNAR1(2) and/or the TNFRSF1A receptor using siTNFRSF1A. Forty-eight hours post-transfection cells were stimulated with SN-SeV-UV for 24h. In **E**, A549 cells were stimulated as in A and where indicated were transfected with siCTRL or siRNA targeting DUOX2 (siDUOX2(1)) 48h prior to stimulation. In **A, B, and D**, DUOX2 or DUOXA2 mRNA absolute copy number were analyzed by qRT-PCR. In **A,** DUOX2 protein expression was analyzed by immunoblot. In **D**, IFNAR1 expression levels were analyzed by qRT-PCR and TNFRI levels were detected by immunoblot. In **E**, H_2O_2 production was analyzed using the HVA assay. Where indicated, catalase was added at 400 U/mL. All qRT-PCR and H_2O_2 measurement data are presented as mean ± SD. Fold induction were calculated over the corresponding non-stimulated condition. The pointed line in graphs represents the threshold of detection. Statistical analysis was conducted by one-way ANOVA using Tukey multiple comparison analysis. *p<0.05, **p<0.01, ***p<0.001. IB, immunoblot; NS, non specific; SN, supernatant.

Figure 4: DUOX2 induction is regulated in a STAT2/IRF9-dependent, STAT1-independent manner.

In **A-C,** A549 cells were transfected with siRNA specific for STAT1, STAT2 or IRF9. 48h post-transfection cells were stimulated with SN-SeV-UV for 24h in **A and B** or IFNg for the indicated time in **C**. In **D**, A549 cells were pretreated with AG490 (100µM) and Bayer-18 (100µM) or DMSO (vehicle) for 1h before stimulation with IFNβ and TNFα for 24h. In **A, B and D** DUOX2 mRNA absolute copy number was analyzed by qRT-PCR and DUOX2 levels were expressed as % of the siCTRL condition. In **A,** DUOX2 protein levels were analyzed by immunoblot. In **A-E,** STAT2-P-Tyr690, STAT2, STAT1-Tyr-701, STAT1, IκBα-P-Ser32, IκBα, or IRF9 protein levels were analyzed by immunoblot. All qRT-PCR data are presented as mean ± SD. Data were analyzed by one-way ANOVA with Dunnett post-test. **p<0.01, ***p<0.001. IB, immunoblot; NS, non specific; ns, non significant; SN, supernatant.

Figure 5: DUOX2 is necessary for the establishment of an H_2O_2-dependent antiviral state.

In **A,** a schematic outline of the experimental timeline used for experiments in **B-D** is presented. A549 cells were transfected with siCTRL, siDUOX2(1) or siDUOX2(2) before being stimulated with SN-SeV-UV from non-infected or SeV-infected A549 cells or with IFNβ and TNFα for 24h. DUOX2 expression was analyzed by immunoblot using DUOX1/2 antibody. Twenty-four hours post-stimulation with SN-SeV-UV or cytokines, cells were infected with recombinant RecRSV-GFP at a MOI of 1 for 72h and release of infectious virus particles was quantified by plaque forming unit assay. In **D**, catalase was added 6h prior to RecRSV-GFP infection. All data are presented as mean ± SD. In **B-D**, data were analyzed by one-way ANOVA with Dunnett post-test, siCTRL vs. siDUOX2(1) or siDUOX2(2) or in **D**, IFNβ+TNFα vs. IFNβ+TNFα+catalase, . *p<0.05, **p<0.01, ***p<0.001. SN, supernatant; ns, non-significant.

Figure 6: DUOX2 regulates late type I/III IFN secreted levels.

In **A,** A549 cells were transfected with siCTRL, siDUOX2(1) or siDUOX2(2) and 48h post-transfection, cells were infected with SeV (40HAU/10^6 cells) for the indicated times. DUOX2 expression was analyzed by immunoblot using DUOX1/2 specific antibodies. Release of IFNβ, IFNλ and TNFα was measured by multiplex ELISA. In **B,** A549 cells were transfected with siCTRL or siDUOX2(2) for 48h and infected as in A for the indicated times. IFNβ, IFNλ (IL-28A and IL-29) and TNFα mRNA absolute copy numbers were analyzed by qRT-PCR. Data were analyzed by two-way ANOVA with Bonferroni post-test. *p<0.05, **p<0.01, ***p<0.001. The dotted line in qRT-PCR graphs represents the threshold of detection. hpi, hours post-infection; ns, non significant.

Figure 7: DUOX2 induction controls a late IFNβ/TNFα-dependent antiviral state in NHBE cells.

In **A and C** NHBE cells were infected with SeV (40HAU/10^6 cells) for 24h. In **B and C**, NHBE cells were transfected with siCTRL or siDUOX2(1). In **B**, NHBE cells were stimulated with IFNβ and TNFα for 24h before infection with RecRSV-GFP and quantification of infectious virion release as described in Figure 5. Data were analyzed by one-way ANOVA with Dunnett post-test. In **C**, release of IFNβ, IFNλ and TNFα was measured by multiplex ELISA. Data were analyzed by two-way ANOVA with Bonferroni post-test. In A-C, DUOX2 or DUOXA2 expression levels were analyzed by qRT-PCR. *p<0.05, **p<0.01, ***p<0.001. The dotted line in qRT-PCR graphs represents the threshold of detection. hpi, hours post-infection; ns, non significant.

Figure 8: SN-RSV-UV triggers higher expression of DUOX2 than direct RSV infection.

In **A and C,** A549 cells were infected with RSV at an MOI of 3 for the indicated time points. SeV infection (40HAU/10^6 cells) was conducted for comparison. In **B**, A549 cells were infected with RSV at an MOI of 3 or 10 or with SeV at 40HAU/10^6 cells for 24h. In **D-F**, ALI-Calu-3 cells were infected with RSV as in A. In **G**, A549 cells were infected with RSV at an MOI of 3 for 24h and IFNβ/TNFα levels in the supernatant

were measured by Multiplex ELISA. In **H and I**, A549 cells were stimulated with SN-RSV or UV-treated supernatant, SN-RSV-UV, for 24h. RSV infection (MOI=3, 24h) was conducted for comparison. In **J**, A549 cells were treated as in Figure 4A and 48h post-transfection were stimulated with SN-RSV-UV for 24h. In **A**, **C, D, F**, **H, and J** DUOX2 or DUOXA2 mRNA absolute copy numbers were quantified by qRT-PCR. In **H and J**, these values are presented as % DUOX2 induction. In **B, E, I and J**, DUOX2 protein expression was analyzed by immunoblot. In **H**, STAT1-Tyr-701 phosphorylation, STAT1, and RSV protein levels were analyzed by immunoblot. In **J**, STAT2, STAT1 or IRF9 protein levels were analyzed by immunoblot. All qRT-PCR data are presented as mean ± SD. Data were analyzed by one-way ANOVA with Tukey post-test except in J, where a Dunnett post-test was used; *$p<0.05$, **$p<0.01$, ***$p<0.001$. The pointed line in qRT-PCR quantification data represents the threshold of detection. hpi, hours post infection; IB, immunoblot; MOI, multiplicity of infection; NS, non specific; SN, supernatant; UAR, Unit Area Resistance.

Figure 9: Model of the innate immune antiviral response triggered by IFNβ and TNFα in AEC. SeV infection of AEC triggers the secretion of IFNβ and TNFα. Binding of IFNβ to its cognate receptor activates the "classical" antiviral pathway mediated by the ISGF3 TF. Additionally, the synergism between IFNβ and TNFα induces late DUOX2 expression through a non-canonical antiviral signaling pathway. This pathway involves STAT2 and IRF9, but is entirely independent of STAT1. Late DUOX2 induction and H_2O_2 production is essential for the cells to mount an efficient antiviral state, at least in part through regulation of IFNβ and IFNλ levels at late time points of infection. The importance of this novel airway antiviral defense mechanism is underlined by the observation that pathogenic RSV is able to counteract DUOX2 induction suggesting that this virus has evolved a strategy to evade the DUOX2-dependent antiviral response.

Tables

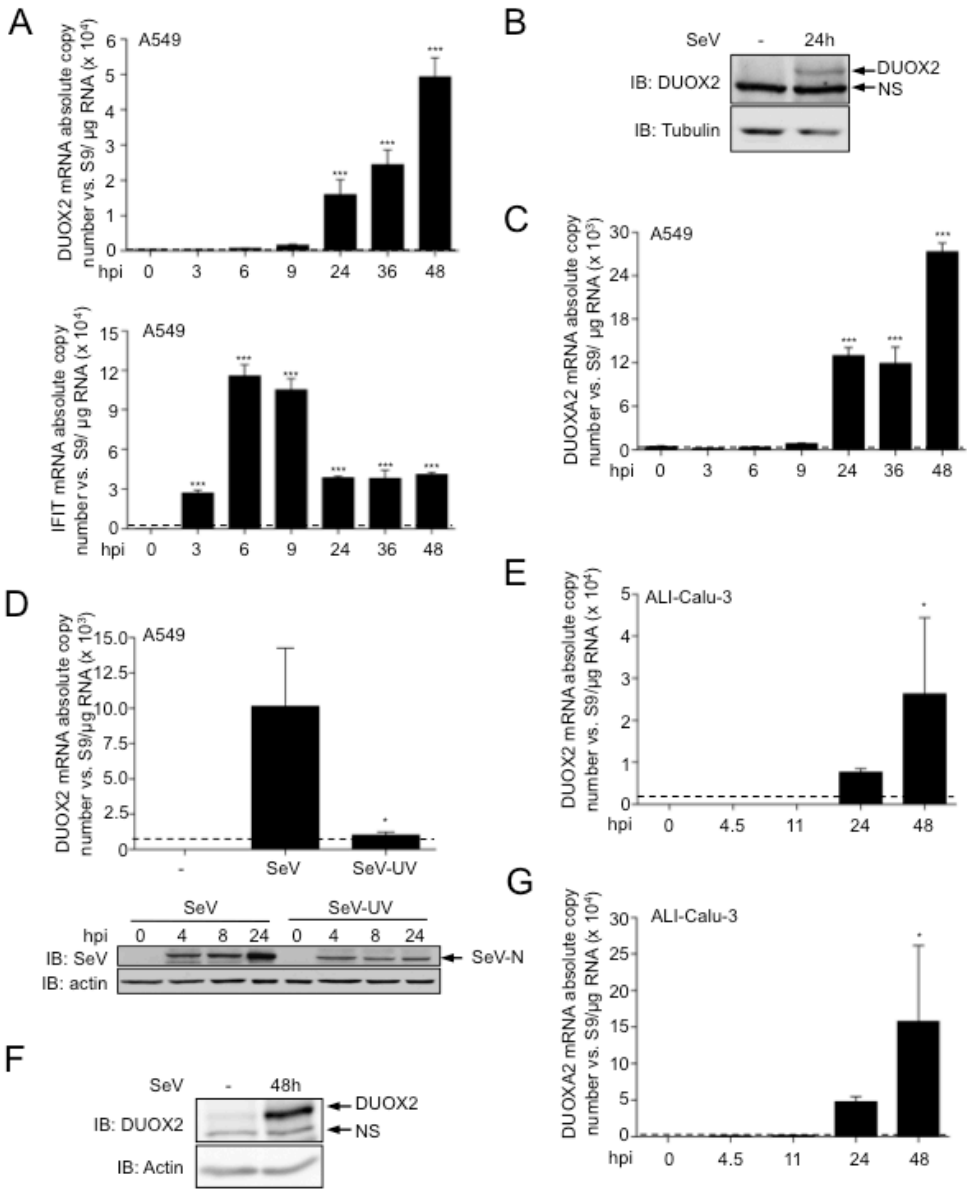
Table 1: siRNA oligonucleotide sequences used for siRNA knockdown

siRNA	Accession number	Antisense oligonucleotide sequence (5'-3')
CTRL		UGUGAUCAAGGACGCUAUGUU
STAT1(1)	NM_139266.2, NM_007315.3	GGCGUUAGGACCAAGAAGC
STAT1(2)	NM_139266.2, NM_007315.3	GAACCUGACUUCCAUGCGG
STAT2	NM_198332.1, NM_005419.3	UAACCAGGCAACUCAGUCCUU
IRF9	NM_006084.4	UACCUGACCAAGUCUCUGCUU
IFNAR1(1)	NM_000629.2	ACUAUAGACACCAAUUUUCUU
IFNAR1(2)	NM_000629.2	UCACUAUUGCCUUAUCUUCUU
TNFRSF1A	NM_001065.3	UAGUAGUUCCUUCAAGCUCUU
DUOX2(1)	NM_014080.4	UAAACAUUAGACGGGACUUAUCCUC
DUOX2(2)	NM_014080.4	UUAGGGUUGAGAUCACUCCUU

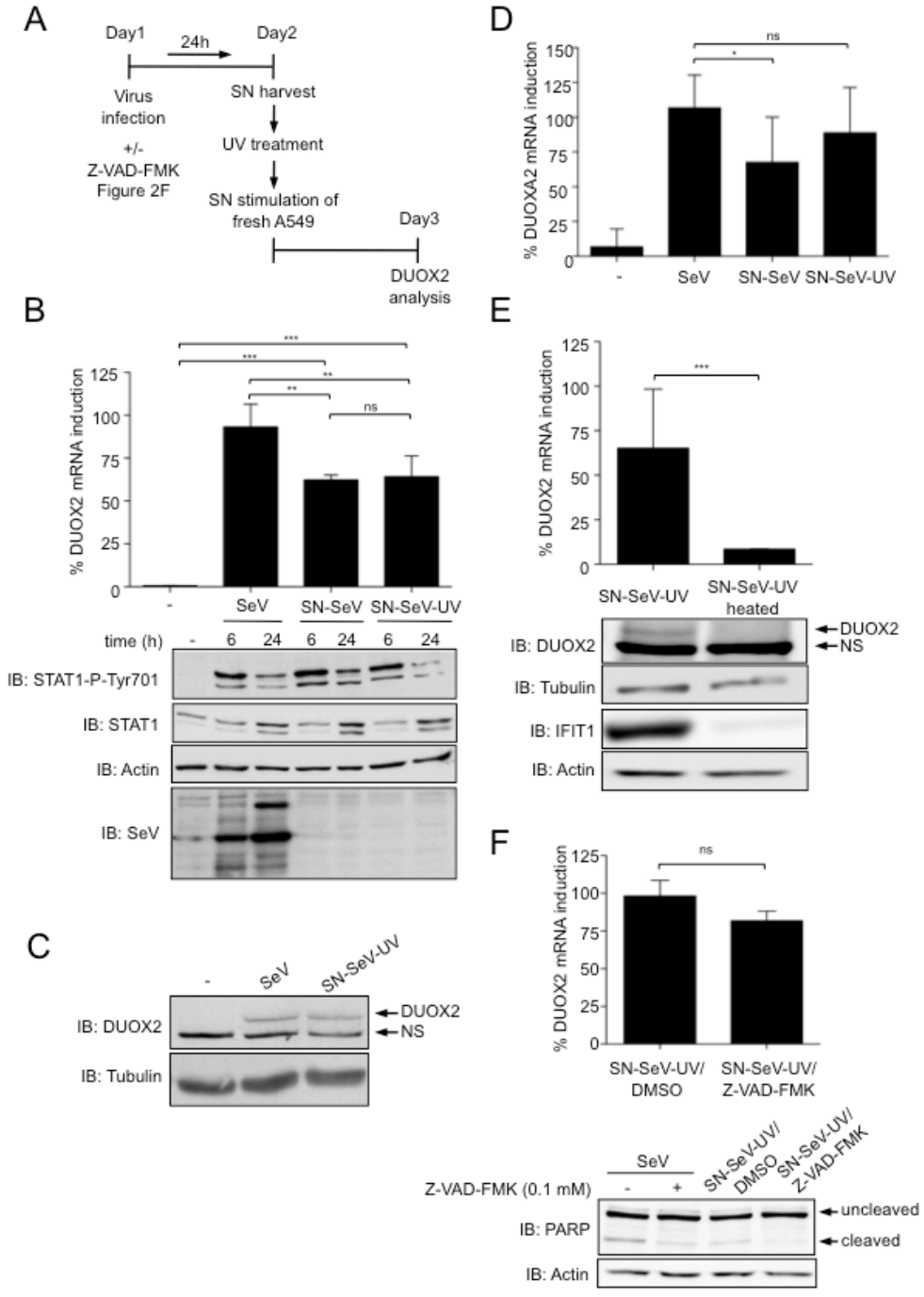
Table 2: Primer sequences used in qRT-PCR analysis

Gene	Accession number	Sense (S)/ Antisense (AS)	Sequence (5'-3')
DUOX2	NM_014080.4	S	ACCTAAGCAGCTCACAACT
		AS	CAGAGAGCAATGATGGTGAT
DUOX2	NM_014080.4	S	GATGGTGACCGCTACTGGT
		AS	CAGAGAGCAATGATGGTGAT
DUOXA2	NM_207581.3	S	TAACGGCTACAGACAGTGAG
		AS	CTGCTCTCAACGCTCTGG
IFNAR1	NM_000629.2	S	TGACCAGAAATGAACTGTGTCA
		AS	TTTAAATAGTTAAGAGCTTGCCCG
IFNB1	NM_002176.2	S	GAACTTTGACATCCCTGAGGAGATTAAGCAGC
		AS	GTTCCTTAGGATTTCCACTCTGACTATGGTCC
IFIT1	NM_001548.3	S	GCCCAGACTTACCTGGACAA
		AS	GGTTTTCAGGGTCCACTTCA
IL28A	NM_172138.1	S	CTCAGGTTGCATGACTGGTGG
		AS	GAGGCCTCTGTCACCTTCAAC
IL29	NM_172140.1	S	CTTCCAAGCCCACCACAACTG
		AS	CTTGAAGCTCGCTAGCTCCTG
TNFα	NM_000594.2	S	CAGAGGGCCTGTACCTCATC
		AS	GGAAGACCCCTCCCAGATAG
S9	NM_001013.3	S	CGTCTCGACCAAGAGCTGA
		AS	GGTCCTTCTCATCAAGCGTC
β-actin	NM_001101.3	S	GACTTCGAGCAAGAGATGG
		AS	AGCACTGTGTTGGCGTACAG

Fink *et al*., Figure 1



Fink *et al.*, Figure 2



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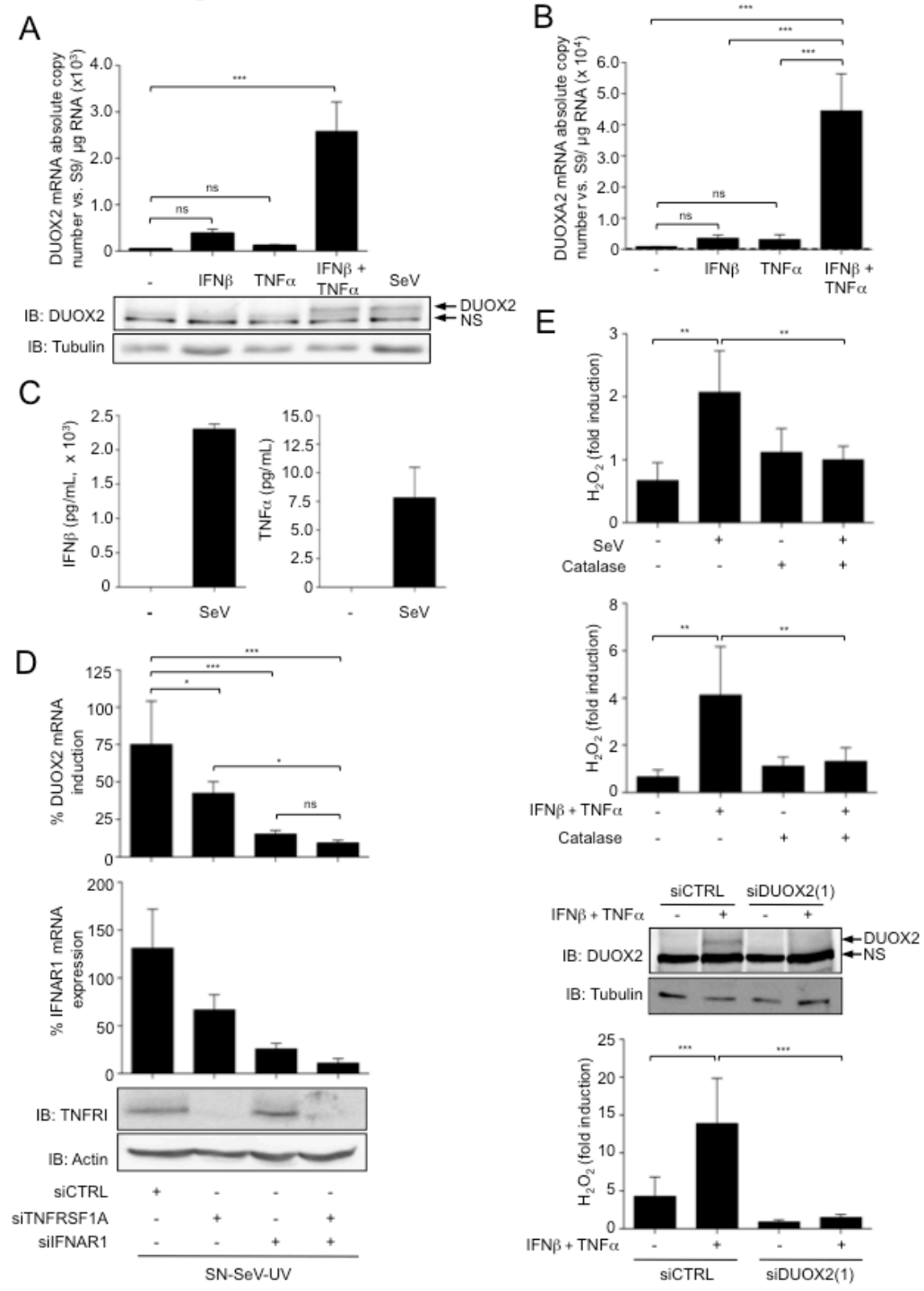

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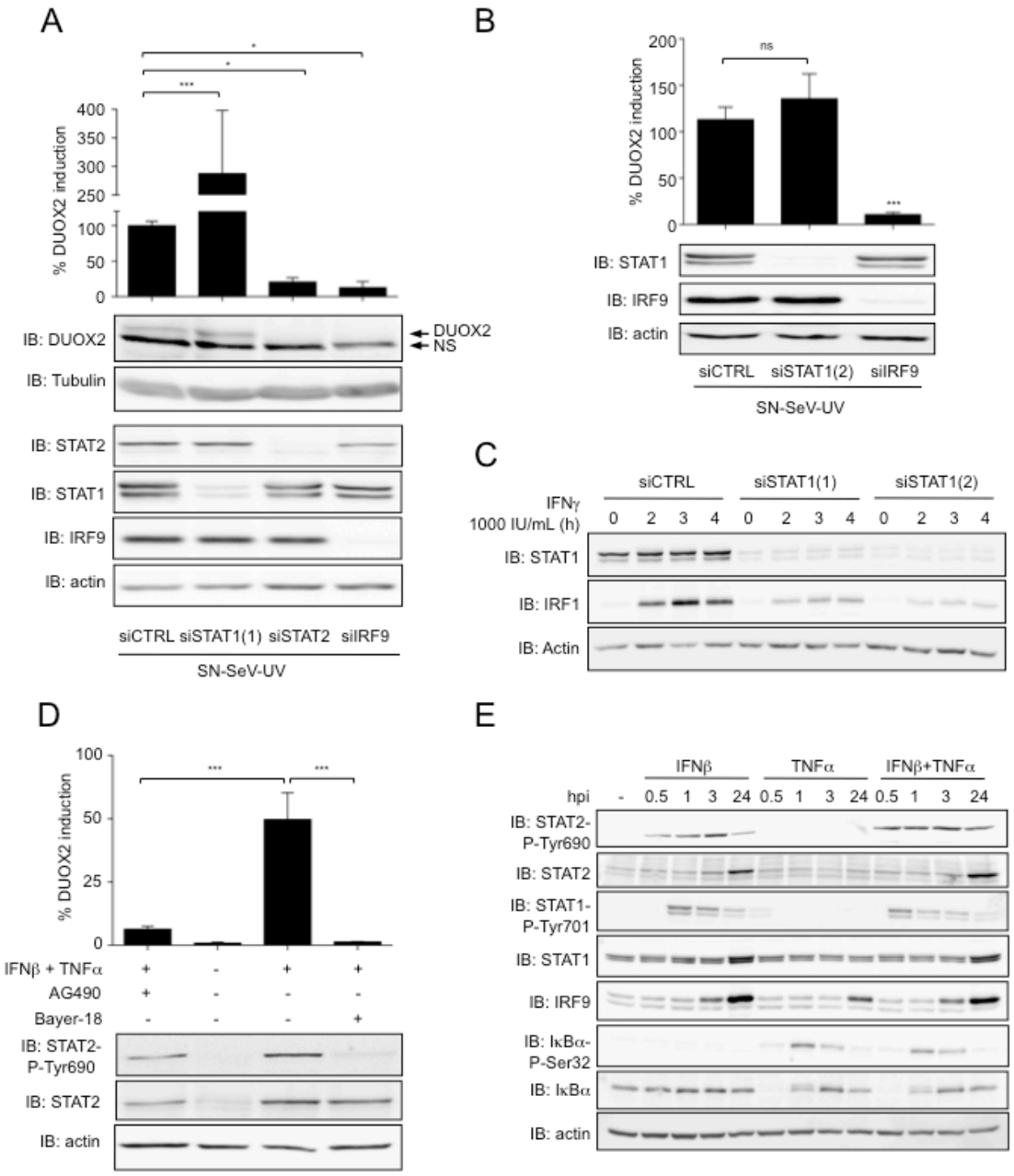

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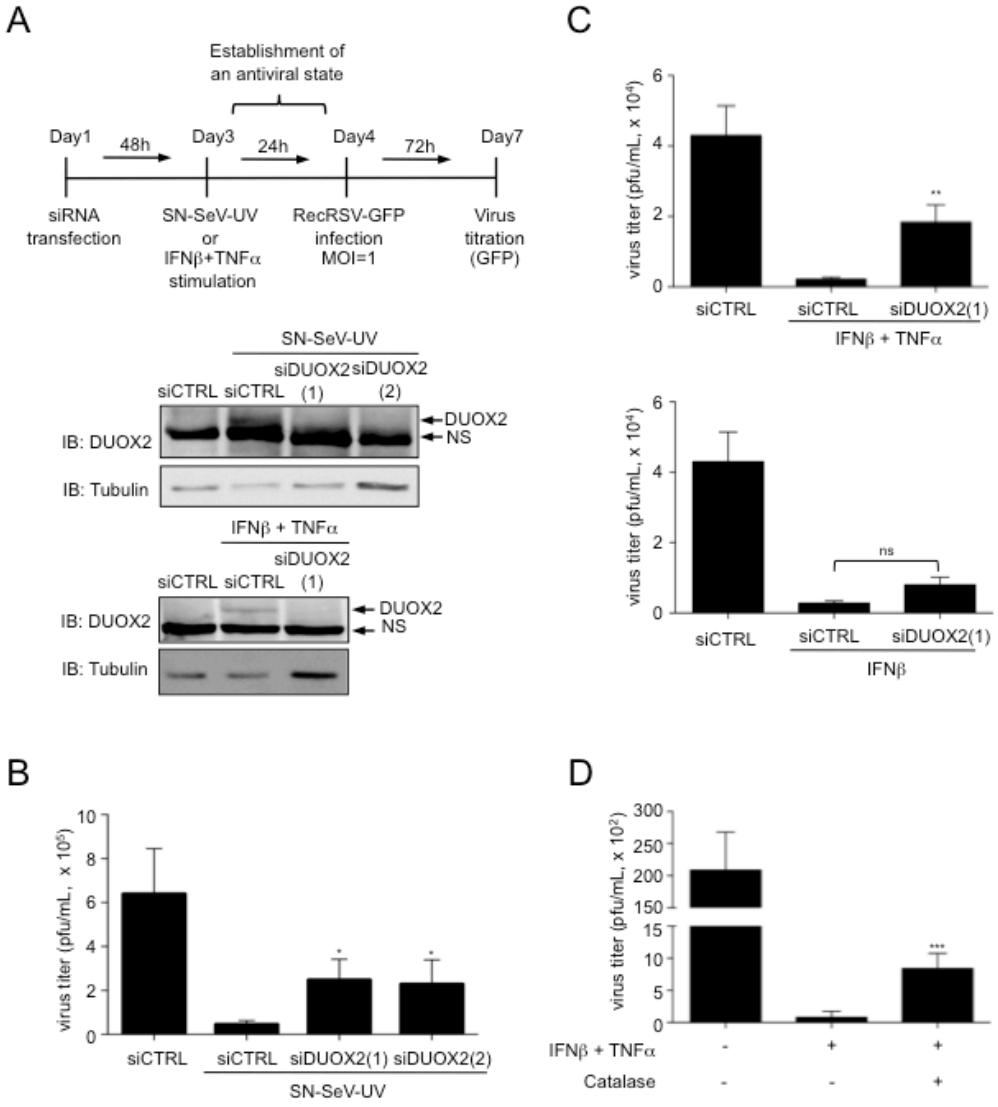

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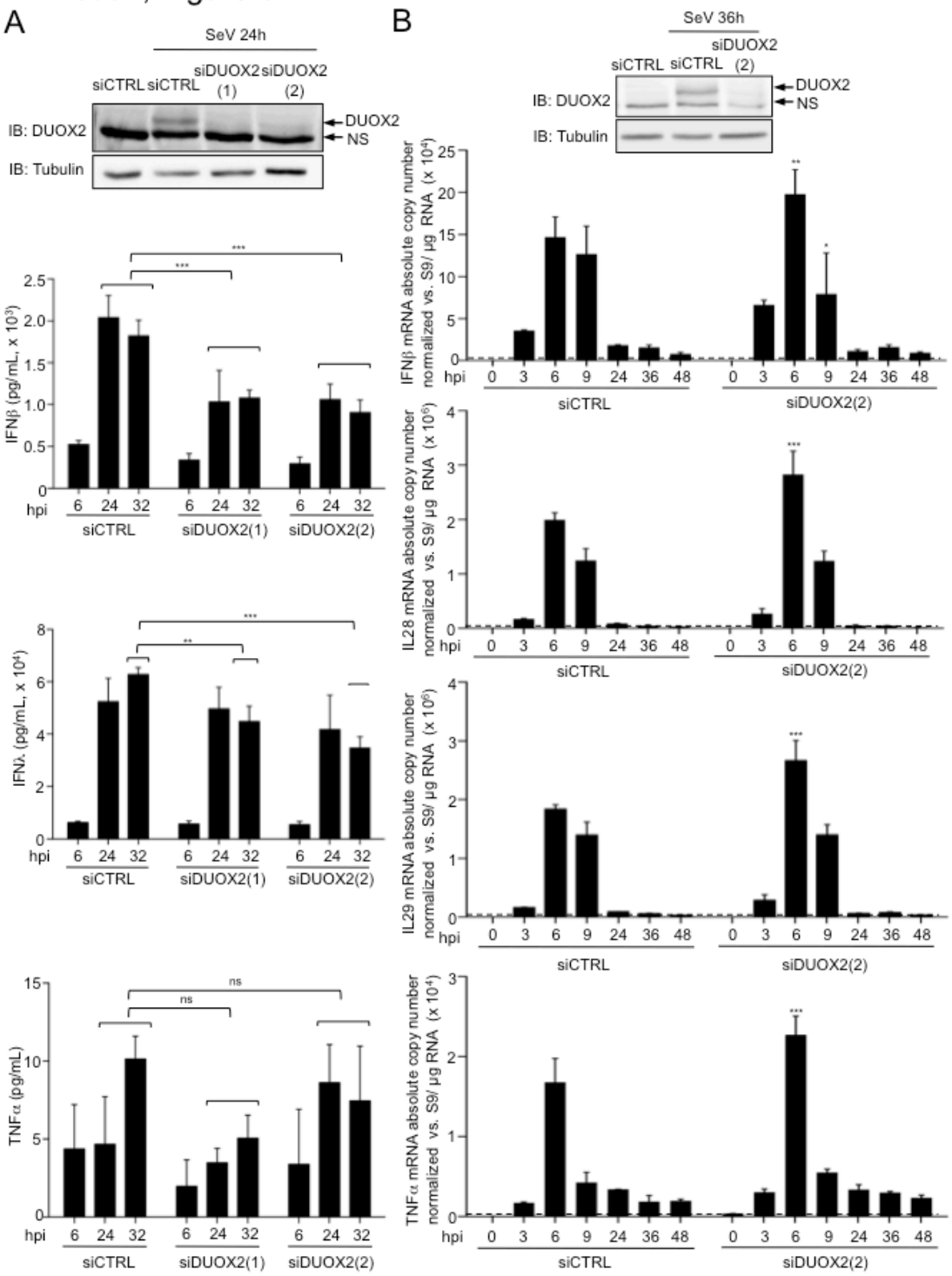

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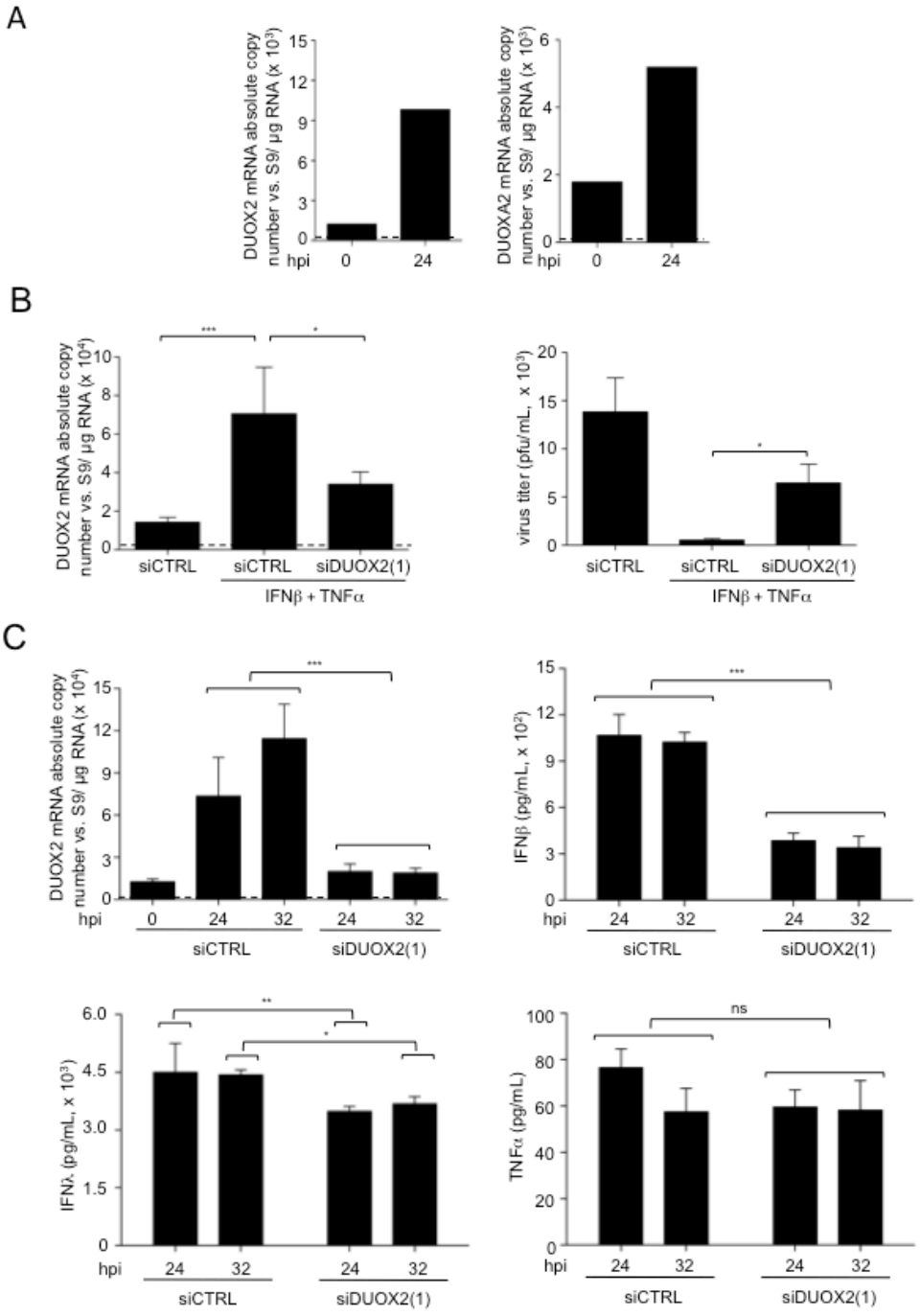

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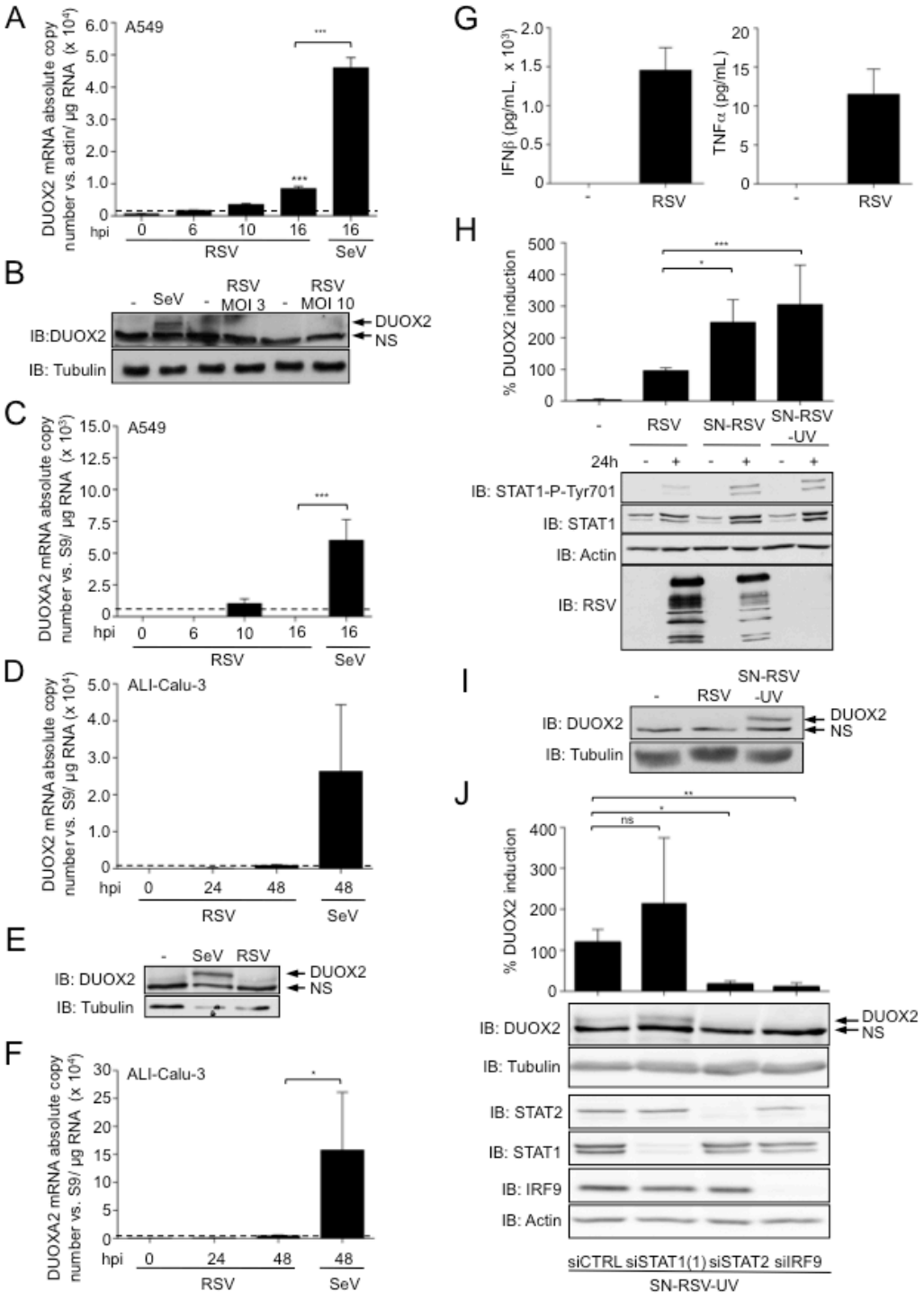

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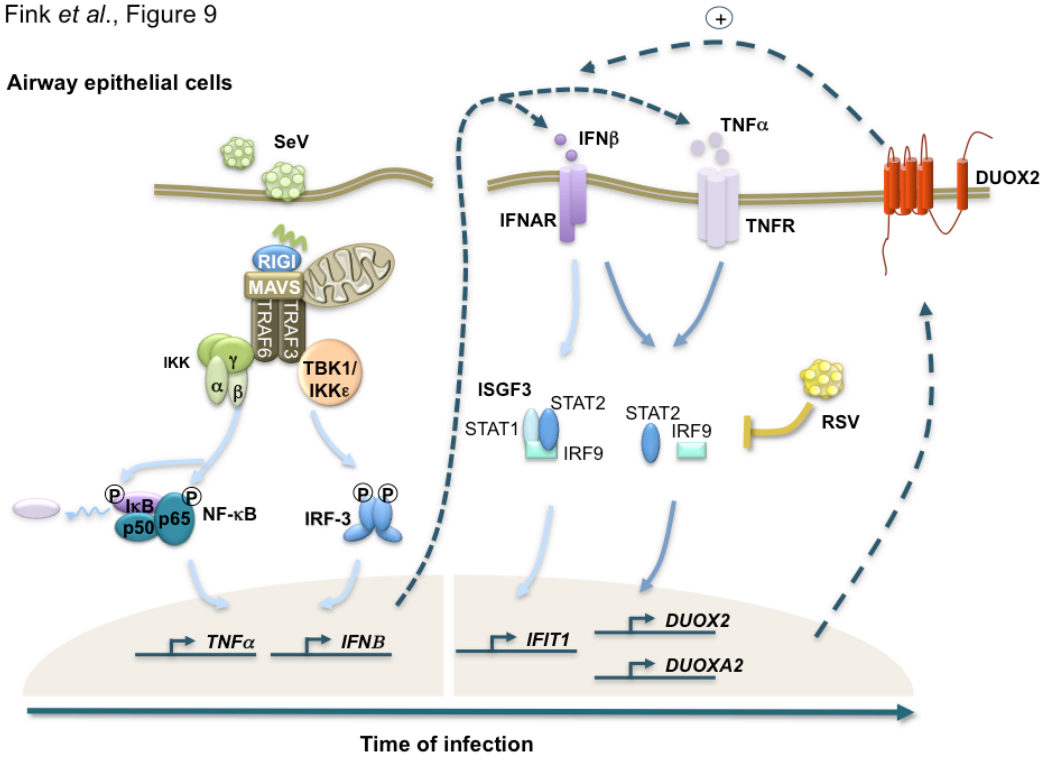
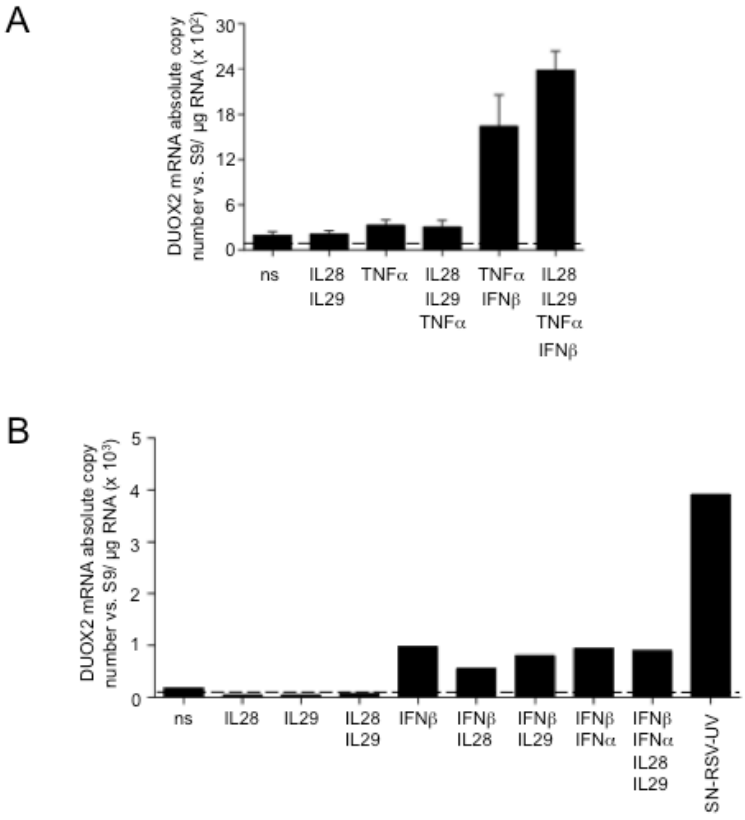


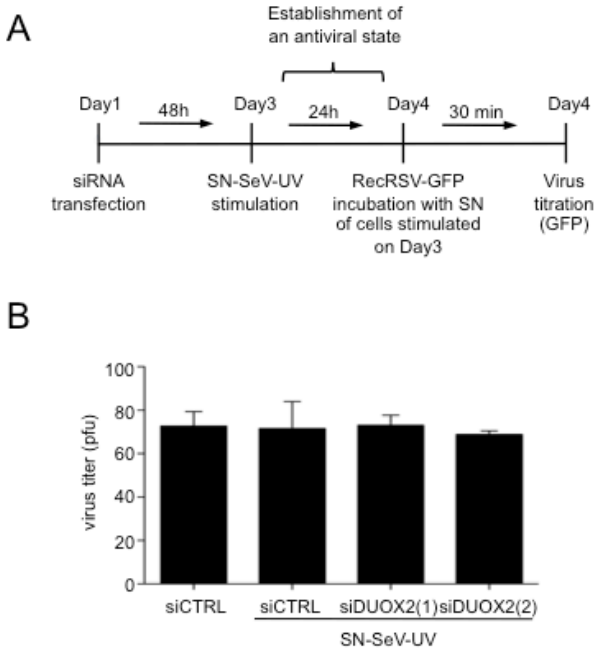
Fink *et al*., Supplemental Figure S1



Supplementary Information, Figure S1: IFNλ is not implicated in the induction of DUOX2 expression.

In **A**, A549 cells were stimulated with combinations of IL28 and IL29 (10ng/mL each), TNFα, and IFNβ (1000U/mL) for 24h. DUOX2 mRNA expression was analyzed by qRT-PCR. In **B**, A549 cells were stimulated with combinations of IL28, IL29, IFNβ and IFNα or SN-RSV-UV as in A and DUOX2 mRNA expression was analyzed by qRT-PCR.

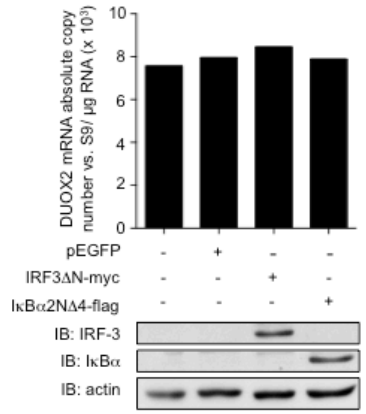
Fink *et al.*, Supplemental Figure S2



Supplementary Information, Figure S2: SN-SeV-UV does not have an antiviral effect on RecRSV-GFP.

In **A**, a schematic outline of the experiment used in B is presented. A549 were transfected with siCTRL, siDUOX2(1) or siDUOX2(2), and stimulated with SN-SeV-UV where indicated to establish an antiviral state. 24h post stimulation the supernatant was taken off and incubated, in a cell free system, with RecRSV-GFP for 30 min at 37°C. Then, confluent Vero cells were infected with the incubation mix and a plaque-forming assay was conducted. The results on RecRSV-GFP titer are presented in B.

Fink *et al*., Supplemental Figure S3



Supplementary Information, Figure S3: NF-κB and IRF-3 are not implicated in SN-SeV-UV mediated DUOX2 induction

A549 cells were transfected with dominant negative forms of IRF-3 (IRF3ΔN-myc) and IκBα (IκBα2NΔ4-flag) for 24h before being stimulated with SN-SeV-UV for an additional 24h. DUOX2 expression was analyzed by qRT-PCR. Expression of the dominant negative construts was evaluated using anti-myc and anti-flag specific antibodies.

3 Discussion

NOX2 - master regulator of early proinflammatory responses in virus-infected AEC

Following respiratory virus challenge AEC are the initial orchestrators of antiviral and proinflammatory defenses. A key event in triggering these defenses is the activation of TFs, such as NF-κB, that regulate the expression of antiviral and proinflammatory cytokines, which foster antiviral immune mechanisms. In order to properly activate these immune mechanisms, AEC signaling underlies a multitude of regulations. The recently recognized processes of redox-regulation are now emerging as control mechanisms in the early events following viral infection. In this doctoral thesis we present a role for the ROS-producing enzyme NADPH oxidase NOX2 in the activation of NF-κB and the regulation of downstream proinflammatory cytokines expression in *Paramyxoviridae*-infected AEC. NOX2 is thus a key molecule in the initiation of AEC-triggered inflammatory processes in the setting of respiratory virus infections.

Our studies on the redox-regulation of NF-κB revealed that the NADPH oxidase NOX2, but not NOX1, NOX5 nor DUOX2, which are expressed in the A549 AEC model, is a regulator of early proinflammatory signaling in *Paramyxoviridae*-infected AEC. On a molecular level, we demonstrated that NOX2 influences the virus-induced activation of the proinflammatory TF NF-κB. More specifically, in absence of NOX2, achieved by RNAi transfection, we observed a decreased phosphorylation of IκBα at Ser32. Further, NOX2 also had an impact on the phosphorylation of the p65 subunit of NF-κB at Ser536. We show that this phosphorylation is necessary to attribute full transactivation potential of NF-κB in *Paramyxoviridae*-infected AEC. Consequently, NOX2 influences NF-κB target gene expression. Indeed, using multiplex ELISA analysis, we demonstrate that knockdown of NOX2 affects levels of TNFα, as well as levels of IL-6, IFNβ, IFNλ, IL-6 and IP-10 (Annex - Figure A1). Of note, expression of IFNα, IFNω as well as IL-1α were not reproducibly affected. Thus, the NOX2 NADPH oxidase has a major impact on the AEC-induced proinflammatory response following respiratory virus infection via the regulation of NF-κB activity.

With regards to the mechanism by which NOX2 elicits its regulation on the NF-κB-activating events, our working hypothesis was that NOX2 might regulate the upstream kinase responsible for p65 Ser536 and IκBα Ser32 phosphorylation. Follow-up studies from our laboratory shed light on the exact mechanism by which NOX2 regulates NF-κB activation. Soucy-Faulkner *et al*. demonstrated that NOX2 was essential for the expression of mitochondrial adaptor MAVS, which lies upstream of both, p65 and IκBα phosphorylation events. Decreased MAVS expression leads to a limited signal transduction to IKKβ, the kinase responsible for p65 phosphorylation at Ser536 and IκBα phosphorylation at Ser32 in our context, consequently resulting into decreased NF-κB activation and target gene expression (Soucy-Faulkner et al., 2010; Yoboua et al., 2010). The study of Soucy-Faulkner *et al.* demonstrated that the effect of NOX2 on MAVS not only limits itself to the NF-κB activation, as NOX2 regulation of MAVS expression also influenced the activation of IRF-3 as well as IRF-3 target gene expression (Soucy-Faulkner et al., 2010). Thus, NOX2 cumulatively regulates the expression of a panoply of antiviral and proinflammatory mediators during the early phase of the antiviral response. NOX2 is thus a key element in sustaining early antiviral and proinflammatory action in *Paramyxoviridae*-infected AEC.

How NOX2 regulates MAVS mRNA expression levels is still unknown. A possible explanation would be that NOX2 has an activating effect on TFs regulating MAVS expression. Concerning the transcriptional mechanisms that control MAVS levels, it is well established that MAVS gene expression is not induced following virus infection. However, the field of MAVS transcriptional regulation has not yet been explored in much detail making the proposal of potential mechanisms for NOX2 action rather difficult. It is also possible that degrading processes and mRNA stability mechanisms of MAVS mRNA could be affected by NOX2. In this context it has been shown that MAVS mRNA stability changes upon ssRNA and dsRNA stimulation (Xing et al., 2012). Interestingly, NADPH oxidase activity has previously been linked to regulation of mRNA stability. NOX2 was responsible for decreasing IL-8, but not IL-6 and ICAM1 mRNA stability specifically following LPS, but not TNFα stimulation in endothelial cells (Al Ghouleh and Magder, 2012). Further, ROS derived from an unidentified NADPH oxidase positively regulate mRNA stability of the Kv 4.3

channel following Angiotensin II stimulation in cardiomyocytes as well as LPS-induced TLR4 mRNA half-life in vascular smooth muscle cells (Lin et al., 2006; Zhou et al., 2006). Although these data appear rather unrelated to the context of viral infection, they potentially suggest the implication of NADPH oxidases in the very specific events regulating MAVS mRNA stability.

The DUOX2 NADPH oxidase – guardian of the late antiviral state

DUOX2 induction is mediated by IFNβ and TNFα in late stages of viral infection

Whereas the NOX2 NADPH oxidase affects early antiviral and proinflammatory events of *Paramyxoviridae*-infected AEC, the role of the other NADPH oxidase family members expressed in our A549 model of AEC, notably NOX1, NOX5 and DUOX2, remained enigmatic. Our main focus of study was on DUOX2 function, as we and others had previously obversed an induction of DUOX2 expression in virus-infected AEC (Harper et al., 2005). To further characterize the importance of this virus-induced upregulation of DUOX2 expression, we conducted a detailed analysis of DUOX2 gene expression in SeV-infected AEC. This analysis revealed that DUOX2 expression augments gradually in a late-fashioned manner, contrasting the transient induction of a typically early ISG, *IFIT1*. Further, the gene expression of DUOX2 activator DUOXA2, necessary for DUOX2-dependent ROS production, increases in a similar fashion. This virus-triggered induction of DUOX2 and DUOXA2 expression has been confirmed by others in the setting of RV infection of primary airway epithelial cells (Chattoraj et al., 2011; Comstock et al., 2011; Harper et al., 2005; Schneider et al., 2010). Hence, DUOX2 and DUOXA2 are induced by respiratory virus infection with viruses from two quite distinct viral families, as RV is a positive-sense RNA virus from the *Picornaviridae* family. Adding to this, a viral dsRNA mimetic, polyI:C has also been shown to be a DUOX2 inducer (Harper et al., 2005; Yu et al., 2011). From these data we propose that DUOX2 and DUOXA2 induction and their function might

come into play during infection with other respiratory viruses and this will be a pertinent question to be addressed in future studies. Interestingly, with regards to RSV, infection does not lead to significant DUOX2 and DUOXA2 induction and we propose that RSV possesses a mechanism that interferes with efficient DUOX2 expression, an issue that will be discussed in more detail below.

The late-fashioned DUOX2 and DUOXA2 induction during SeV infection suggests that intermediary factors produced during the course of infection come into play to drive the induction of these two genes. Indeed, we found that heat-sensitive secreted factors are a major trigger of DUOX2 and DUOXA2 expression in virus infection since UV-treated supernatant from virus-infected cells was able to induce DUOX2 and DUOXA2 expression. More specifically, we provide evidence that IFNβ and TNFα, which are secreted from virus-infected cells, synergize to induce significant levels of DUOX2 and DUOXA2. To our knowledge, these are the first data linking the combination of IFNβ and TNFα stimulation to DUOX2 and DUOXA2 induction, as previously, among several cytokines, only IFNγ was described as a strong inducer of DUOX2 and DUOXA2 gene expression in primary bronchial epithelial cells as well as primary tracheobronchial epithelial cells in ALI culture (Gattas et al., 2009; Harper et al., 2005). Our data on IFNγ stimulation in the A549 AEC model does not support this result, which is potentially due to differences in the models used. Differential expression levels of the IFNγ receptor (IFNGR) in different AEC models could be one reason for these diverting results. TNFα is the only other cytokine associated with increases in DUOX2 expression. However, this was demonstrated in intestinal epithelial cells and a similar effect was not observed in primary AEC nor in our model of study (Harper et al., 2005; Lipinski et al., 2009).

As one considers the multitude of different cytokines secreted upon infection, it becomes evident that these, besides their independent action on their cognate signaling pathways, must also exert a complex combinatorial action fostering cross-talk between the individual cytokine-engaged signaling pathways. This is also the case for IFNβ and TNFα stimulation, whose combinatorial, synergistic action on ISG induction was first reported by Mestan *et al*. (Mestan et al., 1988). Importantly, a synergistic antiviral effect following IFNβ and TNFα co-stimulation was also observed in the context of

RSV infection (Merolla et al., 1995). As mentioned in the Introduction, IFNβ signals via a by IFNAR mediated JAK-STAT pathway, responsible for the induction of ISG expression. TNFα, via the TNFR signaling pathway, engages into NF-κB activation and consequent expression of various NF-κB target genes. Combined action of IFNβ and TNFα has previously been shown to foster the expression of quite a distinct set of delayed antiviral genes defining a novel antiviral state, different from the one induced by each cytokine separately (Bartee et al., 2009; Yarilina et al., 2008). The question remains on how IFNβ and TNFα signaling pathways intersect and how synergy is achieved. One possibility is that TNFα induces JAK-STAT signaling, leading to the induction of ISGs. Indeed, several data in the literature document that TNFα has the capacity to drive IRF1-dependent IFNβ gene expression, which thereafter leads to the activation of the IFN-dependent JAK-STAT pathway (Fujita et al., 1989; Tliba et al., 2003; Yarilina et al., 2008). However, this mechanism does not explain the synergistic action of IFNβ and TNFα on DUOX2 expression in our experimental context, since we do not observe TNFα-induced activation of the JAK-STAT pathway measured by STAT1 phosphorylation at Tyr701 and STAT2 phosphorylation at Tyr690, nor TNFα-induced DUOX2 expression. However, we do observe TNFα-induced expression of IRF1 and IRF9, suggesting that TNFα activates the expression of ISGs in a STAT phosphorylation-independent manner. Interestingly, IRF1 expression is increased significantly after costimulation of IFNβ and TNFα compared to individual stimulation suggesting that this protein could constitute a marker for cytokine synergy. Other studies report that TNFα is able to increase the expression of IFNAR2, the cognate IFN receptor, thereby potentializing the IFN-induced JAK-STAT pathway (Takayama et al., 2000). We do not exclude a regulatory mechanism of TNFR signaling on IFNAR expression, since our experiments demonstrated that cytokine-stimulated cells, which had decreased TNFRI receptor levels achieved by RNAi treatment also showed decreased mRNA expression levels of IFNAR1. Altogether, the interference of TNFR levels with IFNAR levels as well as the TNFα-induced IRF1 and IRF9 expression suggests that TNFα impacts on IFN signaling, which might explain IFNβ and TNFα synergy.

DUOX2 is regulated by a novel IFNβ- and TNFα-induced, STAT2/IRF9-dependent signaling cascade

The following issue we addressed in this work was the implication of specific TFs in the regulation of DUOX2 expression. Regarding this, our results show that DUOX2 expression is strongly dependent on the presence of STAT2 and IRF9, but does not depend on STAT1 in the regulation of its gene expression since treatment with STAT2- and IRF9-specific RNAi strongly abolished the expression of DUOX2 induced by the supernatant of virus infected cells as well as by IFNβ and TNFα costimulation (Annex, Figure A2). Previously, the STAT1-dependence of DUOX2 expression has been addressed in various cell types under different stimuli and it demonstrated a cell type-specific dependency pattern: whereas IFNγ-induced DUOX2 expression in pancreatic cancer cells was dependent on STAT1, it was STAT1-independent under the same stimulus in airway epithelial cells (Hill et al., 2010; Wu et al., 2011). Thus, our data, combined with the data of Hill *et al.* suggest that DUOX2 expression in the airway epithelium does not depend on STAT1 (Hill et al., 2010). The ISGF3 transcription factor that is activated during the course of IFN signaling consists of STAT1, STAT2, and IRF9. Since our data suggest the implication of STAT2 and IRF9, but exclude STAT1 implication in DUOX2 regulation we propose the importance of a non-canonical transcription factor complex that acts independently of STAT1, but is entirely dependent on STAT2 and IRF9. In support of this, the existence of such a STAT2/IRF9-dependent, STAT1-independent non-canonical pathway has been described previously (Hahm et al., 2005; Kraus et al., 2003; Perry et al., 2011; Sarkis et al., 2006).

The importance of TNFα in the regulation of DUOX2 induction suggested that NF-κB, which becomes activated downstream of TNFR activation, might be implicated in the regulation of DUOX2 induction. This transcription factor has recently been shown to regulate DUOX2 expression in the context of Lipopolysaccharide (LPS) stimulation in pancreatic cancer cells (Wu et al., 2013). However, our results do not suggest the implication of NF-κB in the regulation of DUOX2 expression upon viral

infection as we do not observe changes in DUOX2 levels upon inhibiting NF-κB activation in the context of stimulation with supernatant from SeV-infected cells.

From our study, the question regarding the composition of the TF(s) regulating DUOX2 arises. DUOX2 might likely be regulated by a STAT2/IRF9-containing complex consisting of a STAT2 homodimer and an IRF9 molecule. However, it is also possible that STAT2 heterodimerizes with a different STAT protein in order to regulate DUOX2 expression. From the seven mammalian STAT proteins, besides STAT1 and STAT2, STAT2 has only been shown to interact with STAT6 following the stimulation by IFNα (Gupta et al., 1999; Wan et al., 2008). Interestingly, STAT6 has recently been implicated in the regulation of DUOX2 in intestinal epithelial cells following IL-4 and IL-13 stimulation. Thus STAT6 could be a potential candidate for STAT2 heterodimer action (Raad et al., 2012). Further molecular studies will be needed to discern the exact composition of the DUOX2 regulating transcription factor(s) in our experimental setting.

Another unanswered issue from our study is if STAT2/IRF9 regulate DUOX2 gene expression directly, implying binding of STAT2/IRF9 complexes to the DUOX2 promoter. It is known that in the context of IFNγ stimulation of pancreatic cells, a STAT1-binding IFNγ-activating sequence (GAS) sequence plays an important role in DUOX2 regulation (Wu et al., 2011). The same group recently identified a NF-κB binding site that comes into play in pancreatic cells upon IFNγ and LPS stimulation (Wu et al., 2013). Further, a putative STAT6-binding GAS sequence has been identified in the DUOX2 promoter region, but its physiological importance remains to be demonstrated (Raad et al., 2012). Thus the DUOX2 promoter indeed contains target sequences for STAT binding. The *Drosophila* DUOX promoter also contains an ATF2-binding site, which underlies the regulation of a MAP kinase pathway following stimulation with bacterial extract. It is very likely that this site is conserved in the human DUOX2 promoter, since in intestinal epithelial cells DUOX2 expression is also increased following treatment with bacterial extract (Ha et al., 2009b). However, the importance of this pathway in the context of virus-induced DUOX2 expression remains to be explored. Regarding STAT2/IRF9-regulated DUOX2 expression, the

DUOX2/DUOXA2 bidirectional promoter region has not been studied in terms of the physiological existence of ISRE or IRF9 consensus sites that could be potential binding sites for these TFs. Furthermore, the nature of the consensus sequence of the STAT2/IRF9 containing complex is still obscure. Whereas a STAT2/IRF9-containing complex was described to bind to an ISRE in the context of ATRA-induced RIG-G expression, a different study demonstrated that STAT2/IRF9 containing complexes have only limited ISRE binding capacities (Bluyssen and Levy, 1997; Lou et al., 2009). During our preliminary DUOX2 promoter analyses using the MatInspector transcription factor binding site search tool from Genomatix (http://www.genomatix.de/matinspector.html), we did not localize any ISRE consensus sequence within the bidirectional promoter region of DUOX2, which only spans approximately 170 nucleotides (Cartharius et al., 2005; Christophe-Hobertus and Christophe, 2010; Xu et al., 2012) MatInspector is a software tool which uses a large library (> 600) of nucleotide position weight matrices to locate transcription factor binding sites in DNA sequences (Cartharius et al., 2005; Quandt et al., 1995). Further, the search engine did not detect any ISRE consensus sequence within -6000bp and +7000bp of the TSS of the DUOX2 gene (Annex – Figure A3). It is thus possible that the STAT2/IRF9 complex binds indeed a different sequence for DUOX2 regulation. On the other hand, it might be possible that the important regulatory region targeted by STAT2/IRF9 lies outside this quite extensive stretch of DUOX2 promoter. Future detailed studies of the DUOX2 promoter reactivity towards IFNβ and TNFα in combination with ChIP assays should give better insight on the nature of the transcription factor and its target sequence implicated in the regulation of DUOX2.

In this context, we also suggest that IRF1 could play a pertinent role in DUOX2 regulation. This TF has previously been linked to the STAT2/IRF9-dependent, STAT1-independent pathway of ATRA-induced RIG-G gene expression, which is reminiscent of the pathway regulating DUOX2 expression (Lou et al., 2009). Lou *et al.* placed IRF1 upstream of the STAT2/IRF9 complex, regulating IRF9 expression (Lou et al., 2009). Furthermore, other data suggest the importance of IRF1 following IFNβ and TNFα costimulation, as IRF1 is necessary for TNFα-induced IFNβ production (Yarilina et al., 2008). Additionally, as mentioned above, our results show a synergistic

induction of IRF1 expression following stimulation with IFNβ and TNFα, which is not or only weakly observed in the case of a simple stimulation with each cytokine. Altogether, these data suggest that IRF1 could indeed play a role in the regulation of DUOX2 by IFNβ and TNFα.

Our results, demonstrating that IFNβ and TNFα costimulation engages in a STAT2/IRF9-dependent, but STAT1-independent signal transduction pathway, suggest that IFNβ and TNFα costimulation might specifically engage into a novel non-canonical STAT2/IRF9-dependent signaling pathway. Importantly, a previous microarray study identified that the combination of IFNβ and TNFα is able to induce more than 850 novel host cell genes (Bartee et al., 2009). One hypothesis arising from our data is that these genes might be specifically regulated in a STAT2/IRF9-dependent, but STAT1-independent manner. Further studies are necessary to evaluate if this hypothesis holds true. Indeed, it would be very exciting to be able to attribute a specific gene expression signature to the combinatorial stimulation of IFNβ and TNFα. This being said, this also implies that during the costimulation of IFNβ and TNFα a certain switch exists that specifically turns on the STAT2/IRF9-dependent, STAT1-independent IFN-signaling axes. The nature of this exact mechanism remains to be characterized.

DUOX2 exerts an antiviral function in a ROS-dependent manner

The observed induction of DUOX2 during SeV infection as well as following IFNβ and TNFα treatment led us to believe that DUOX2 could be part of the IFN-regulated machinery necessary for antiviral defense. We addressed this question experimentally and showed that a decrease of DUOX2 achieved by RNAi treatment led to a decrease in the antiviral state as measured by an increase in viral load. This correlated with increased intracellular viral protein levels, therefore excluding a mechanism by which DUOX2 presumably inhibits viral budding from the cells (Annex – Figure A4). Together, these results indicate that DUOX2 is necessary for an efficient antiviral response. Using the antioxidant enzyme catalase, which targets H_2O_2 in the extracellular compartment, we demonstrated that the antiviral action of DUOX2

depends on the production of H_2O_2. Although the use of catalase, due to its non-specific nature regarding DUOX2 action, only hints that DUOX2-produced H_2O_2 is the antiviral mediator, these data, combined with the results obtained by the RNAi approach strongly suggest the importance of DUOX2-produced H_2O_2 in the establishment of the antiviral state.

The implication of DUOX2-produced ROS in the antiviral response has previously been addressed in several studies. As mentioned in the introduction, DUOX2, via its H_2O_2-producing activity, feeds an antibacterial H_2O_2/LPO/$OSCN^-$ system (Gattas et al., 2009; Moskwa et al., 2007). Based on the data of Mikola *et al.*, this system could also have antiviral action, as it has been shown that $OSCN^-$ specifically targets certain viruses *in vitro*. A more recent study by Fischer *et al.* addressed the importance of this system in the context or respiratory virus infection, notably RSV, and concluded that not $OSCN^-$, but rather hypoiodous acid, whose generation was achieved by supplementing the experimental setup with iodide, could be the potential antiviral agent generated by a DUOX2-driven system (Fischer et al., 2011). However, the implication of DUOX2 *per se* in this system was not proven, as catalase was the only agent used to demonstrate the implication of DUOX2-specific ROS. Regarding our experiments, we believe that DUOX2-derived H_2O_2 does not exert its antiviral function in an LPO-dependent manner. Firstly, LPO is not known to be secreted from *in vitro* AEC cultures (Conner et al., 2007). Secondly, using supernatants from DUOX2-expressing and non-expressing cells, achieved by application of DUOX2-specific and control RNAi, we do not see a differential antiviral effect of these upon incubation with RSV-GFP, thereby suggesting that the antiviral action in our system is not driven by a component in the supernatant fraction. Rather, we believe that DUOX2-generated H_2O_2 is able to cross the membrane and enter the cell to act on intracellular signaling pathways to indirectly foster antiviral action.

Such intracellular action of DUOX2-derived ROS has previously been demonstrated in different settings. First, DUOX2-derived ROS were implicated in TNFRI shedding from the plasma membrane following poly-I:C stimulation of AEC (Yu et al., 2011). However, we experimentally excluded this mechanism. In a similar manner, DUOX1-derived H_2O_2 is able to regulate intracellular signaling via

inactivation of protein tyrosine phosphatases. One report demonstrated increased STAT6 phosphorylation via inactivation of PTP1B (Hirakawa et al., 2011). A second study demonstrated increased T cell receptor (TCR) signaling via increased phosphorylation of ZAP-70 due to oxidative inactivation of SHP-2 (Kwon et al., 2010). These data demonstrate the possibility that DUOX2-derived extracellular ROS exhibit their biological effect intracellularly.

DUOX2 sustains the secretion of late type I IFNs

Regarding the mechanism by which DUOX2 enforces its antiviral function, our data demonstrated that DUOX2 is necessary for maintaining secreted IFNβ and IFNλ levels at late time-points from virus-infected AEC. Thus, DUOX2 fosters the antiviral state by sustaining the presence of late antiviral cytokines. Data showing that in AEC derived from chronic obstructive pulmonary disease (COPD) patients versus healthy donors, RV-induced DUOX2 levels correlate with an increase in expression levels of antiviral genes, such as IFNβ and IFNλ, support our results (Schneider et al., 2010). The diminution of IFNβ and IFNλ levels observed in DUOX2-RNAi treated AEC could explain the decreased antiviral state in these cells. To address the question of how DUOX2 could regulate extracellular protein levels, we analyzed these cytokines' mRNA expression. We did not observe a significant change in cytokine mRNA levels between control-RNAi and DUOX2-RNAi treated, SeV-infected cells. This suggests that DUOX2 influences the levels of these cytokines at a post-transcriptional level. Continuing this line of thought, further preliminary experiments show that DUOX2-RNAi treatment decreases intracellular IFNβ protein levels in SeV-infected A549, suggesting that DUOX2 might be implicated in the modulation of translational mechanisms that regulate the synthesis of these cytokines (Annex - Figure A5). The mammalian target of rapamycin (mTOR) pathway is a master regulator of protein translation. It is also implicated in the regulation of type I IFN and ISG expression (Cao et al., 2008; Colina et al., 2008; Costa-Mattioli and Sonenberg, 2008; Kaur et al., 2008). Interestingly, NADPH oxidase-derived ROS have been shown to modulate the mTOR driven pathways (Li et al., 2010). Hence, such data support the hypothesis that

IFNβ translation might indeed be regulated by a redox-sensitive mTOR pathway, which could be driven by DUOX2-derived ROS.

One remaining question from our studies concerns the effect that decreased IFNβ and IFNλ secretion might exert on the antiviral state. What are the consequences on ISG expression in the absence of DUOX2? Are the expression levels of all ISGs altered or does IFNβ and IFNλ diminution at late time points affect the expression of a specific subset of ISGs? First of all it needs to be mentioned that DUOX2 expression itself might be affected by the decrease in IFNβ and IFNλ levels as it could underlie the regulation of a feed-forward loop driven by IFNβ. Regarding the effect on ISG expression, taking into account current knowledge on ISG signatures and specificity of ISG expression in the context of different viral infections, we hypothesize that only a specific subset of ISGs will be affected by the fluctuations in IFNβ and IFNλ levels. In other words, specific antiviral mechanisms might be altered that are determinants in regulating the outcome of *Paramyxoviridae* infection.

The discussed antiviral function of DUOX2 could be supported by the presence of its activator DUOXA2, which is necessary for DUOX2-dependent ROS production. Our studies demonstrate that DUOX2 and DUOXA2 are both induced by autocrine/paracrine action of IFNβ and TNFα in virus infection. Thus, it would be very plausible that DUOXA2 knockdown by RNAi treatment results in a similar increase in viral replication as observed with DUOX2 RNAi.

When considering the more global events occurring in virus-infected airways, it needs to be mentioned that our experimental model, despite the specific insight it gives on AEC antiviral mechanisms, is at the same time limited and cannot be used to foresee the outcome of a viral infection in an *in vivo* setting. It is possible that the physiological consequence of DUOX2 action – the decrease in antiviral cytokine levels - also affects the hematopoietic cell compartment, targeting cells of the immune system that express the type I IFN receptor. Thus, DUOX2 might foster antiviral immunity not only within the AEC compartment. Additionally, it might contribute to the activation of innate immune players. Studies on animal knockout and chimera models will need to be conducted to cofirm this hypothesis. In this context, a recent investigation using DUOX2 knockout mice demonstrated that DUOX2 is driving TLR5-mediated

inflammation induced by flagellin stimulation. DUOX2 was necessary for recruitment of the neutrophil compartment into the lung. The use of such a model would certainly be pertinent in the context of a viral infection. However, data generated from it should also be analyzed with care as one has to consider that global DUOX2 deficiency in mice might lead to defects in the thyroid metabolism and thyroid hormone synthesis which could affect immunological responses in an unforeseeable manner.

RSV interferes with DUOX2 induction

Besides the use of SeV, we also analyzed the expression of DUOX2 and DUOXA2 following the infection with RSV, a pathogenic member of the *Paramyxoviridae* family. Our results demonstrate only weak DUOX2 and DUOXA2 induction in the setting of RSV infection. Interestingly however, when analyzing DUOX2 and DUOXA2 induction following treatment with UV-treated supernatant from RSV-infected cells, considerable levels were observed. This correlates with the fact that secreted IFNβ and TNFα concentrations from RSV-infected cells were in the similar range to those measured after SeV infection. We further observe that the magnitude of DUOX2 induction is inversely correlated with the quantity of replicating RSV, since supernatant from RSV-infected cells that had not been treated with UV and therefore still contained infectious replicating viral particles showed less potency in DUOX2 induction than supernatant from RSV-infected cells that had been treated with UV. These results suggest that the presence of replicating RSV inhibits DUOX2 expression. Since secreted IFNβ and TNFα levels in SeV infection, which efficiently induces DUOX2, and RSV infection are comparable, we speculate that RSV has in put place an inhibitory mechanism(s) that does not target the primary antiviral pathways leading to the initial secretion of these cytokines. Rather, in our experimental setting, RSV may target the STAT2/IRF9-dependent signaling pathway that is specifically induced by IFNβ and TNFα synergy. How exactly RSV achieves interference with this novel antiviral pathway will be subject to further studies. So far, from the literature it seems evident that several RSV viral proteins could be involved in such an inhibitory mechanism. NS1 and NS2 of RSV proteins have both been described to target STAT2

thereby shutting down IFN-induced antiviral JAK-STAT signaling (Elliott et al., 2007; Lo et al., 2005; Ramaswamy et al., 2006; Swedan et al., 2009). Besides NS1 and NS2, the RSV attachment protein G can also interfere with the JAK-STAT signaling pathway via modulation of SOCS-1 and SOCS-3 expression, which are negative regulators of the IFN-induced JAK-STAT pathway (Hashimoto et al., 2009; Moore et al., 2008). These members of the SOCS family could equally inhibit the STAT2/IRF9-dependent pathway described in our studies.

Several outcomes of RSV-mediated DUOX2 inhibition can be envisioned. The first scenario is that the inhibition of efficient DUOX2 induction by RSV might interfere with the generation of a complete antiviral state. Consequently, the antiviral response against RSV would not prove as effective, resulting in increased viral load and virus-related pathology. Interestingly, in CF patients, DUOX2 expression in the airways is decreased compared to healthy donors (Wright et al., 2006). The absence of DUOX2 could thus contribute to the increased susceptibility to respiratory virus infections observed in these patients (Zheng et al., 2003). Further, from several studies it is clear that DUOX2 is an effective player in antibacterial defenses, and, although so far only described in the gut, a regulator of homeostasis of commensal bacterial populations (Gattas et al., 2009; Ha et al., 2009b; Moskwa et al., 2007). Thus, inhibition of DUOX2 expression by RSV could not only have an effect on the infecting virus itself, but could also lead to an imbalance in airway resident bacteria and decreased antibacterial defenses against pathogenic bacteria. In this context, RSV infection often presents itself with complicating bacterial co-infections and one reason for this could be the interference of RSV with DUOX2 antibacterial defenses (Thorburn et al., 2006).

Study improvements

A major drawback of our study includes the issue of DUOX2-derived ROS and their function in the antiviral response. Our study was able to demonstrate an increase in H_2O_2 production following IFNβ and TNFα costimulation as well as SeV infection. This increase was shown to be DUOX2 dependent. Using the antioxidant catalase, we

demonstrate that H_2O_2 is indeed important in conveying an increased antiviral state. However, although the application of catalase can be taken as proof that extracellular H_2O_2 is implicated in the antiviral response, it does not however provide clear evidence for the implication of DUOX2-derived ROS in the antiviral defense. Indeed, these ROS could be generated by a different source. We propose that future studies using expression of inactive DUOX2 variants could deliver much clearer results on the implication of DUOX2-derived H_2O_2. In this context, patients suffering from congenic hypothyroidism (CH) are known for mutations in the DUOX2 genes. For instance, the Gly1518Ser mutation in the DUOX2 genes of such patients results in DUOX2 enzymatic inactivation (Hoste et al., 2010). Further, three other mutations have been described to cause inactivation of the DUOX2 enzyme: S965fsX994 deletion that causes a frameshift resulting in a termination signal within exon 22 of the DUOX2 gene; Tyr1150Cys as well as Ala728Thr mutations equally result in enzyme dead variants (De Marco et al., 2011). The application of DUOX2 expression plasmid constructs containing these mutations could give straightforward insight on the implication of DUOX2-derived ROS in the establishment of the late antiviral state.

NOX2 and DUOX2 – a united couple in the antiviral defense

As early and late antiviral responses are tightly intertwined, one cannot separate the action of NOX2 in the early phase of viral infection from DUOX2, which functions at late stages of viral infection. In other words, NOX2 and its influence on early antiviral signaling and antiviral proinflammatory genes expression will also determine if, during the late antiviral response, DUOX2 can be induced efficiently. This suggests a model by which initial NOX2-regulated NF-κB and IRF-3 activation drives an early antiviral response, characterized by IFN induction and ISG expression. DUOX2 is among these ISGs, and its expression might highly depend on this early action of NOX2. Once expressed, it sustains antiviral action via prolonged IFNβ and IFNλ stimulation in AECs (Figure 14). We have not evaluated DUOX2 expression in the context of decreased NOX2 expression and viral infection. However, based on the

effects we observed on IFNβ and TNFα cytokine levels in NOX2-RNAi conditions, it is very likely that DUOX2 expression would also be affected in these conditions.

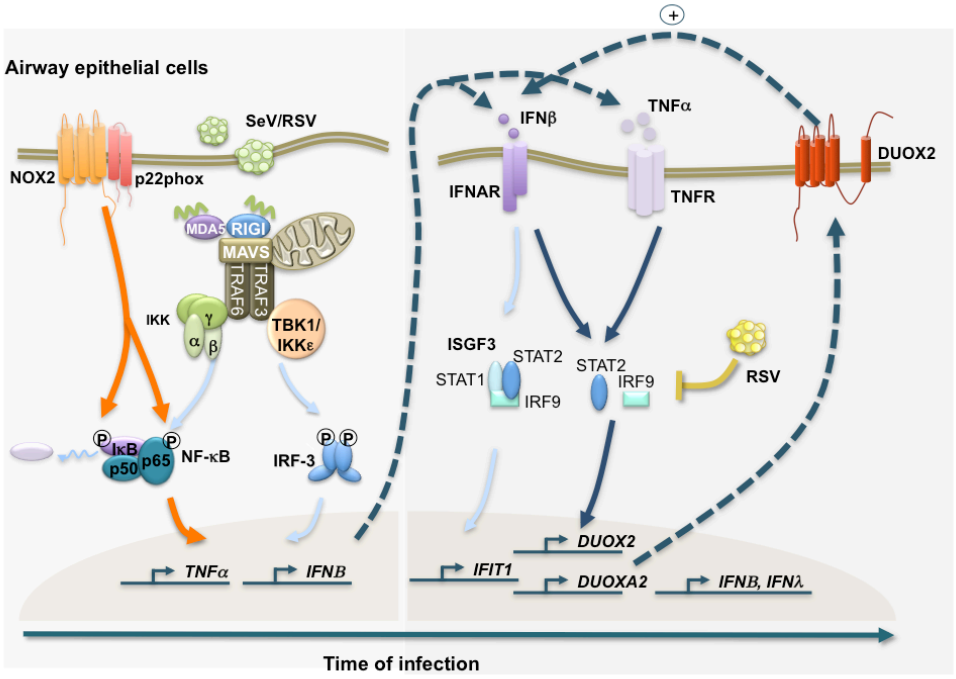


Figure 14: NOX2 and DUOX2 in the antiviral airway defense.

Schematic presentation resuming the findings of this doctoral thesis work. NOX2 regulates NF–κB activation and NF–κB regulated cytokines expression following *Paramyxoviridae* infection of AEC. DUOX2 is induced during the late phase of viral infection via synergistic action of secreted IFNβ and TNFα. Induction of DUOX2 expression relies on a non-canonical, STAT2/IRF9-dependent signaling pathway. DUOX2 is necessary for the establishment of an efficient antiviral state as it sustains the late expression of antiviral cytokines IFNβ and IFNλ. Pathogenic RSV evades the DUOX2-mediated antiviral mechanisms, as it has developed means of interfering with the DUOX2-inducing signaling cascade.

What is the beginning of it all?

The presented data raises the following fundamental question, which has not been resolved by the studies conducted during this thesis: how, upon AEC infection by viruses, are NOX2 and DUOX2 activated to be able to elicit their ROS-dependent functions? Is it possible that besides their putative basal ROS producing activity serving in housekeeping function, viral infection might trigger a signal that specifically turns on or increases NADPH oxidase activity? This scenario is very likely for the basally expressed NOX2 but might also be the case for DUOX2, as our observed elevated DUOX2 mRNA and protein levels in the context of AEC viral infection might additionally require an induction of DUOX2 activity in order to translate into a physiological, ROS-dependent change following viral infection.

In the case of NOX2, classical activation is driven by phosphorylation events of the regulatory subunits p47phox, p67phox, and p40phox. This culminates into the recruitment of these subunits to the cytochrome b_{558} membrane complex, thereby inducing molecular mechanisms that culminate into the ROS generating electron-transfer. Many kinases were shown to be involved in the phosphorylation events leading up to NOX2 activation in a diverse array of contexts. As mentioned, several PKC isoforms (PKCα, PKCδ, PKCβ, PKCγ, and PKCζ), PKA, p21 activated kinase, Erk1/2, AKT, PI3K, and possibly others come into play (Lam et al., 2010). Interestingly, most of these kinases also become activated during the course of viral infection, which could constitute the necessary signal to trigger NOX2 activation (Arnold and Konig, 2005; Marchant et al., 2010; Monick et al., 2001; Monick et al., 2004; Monick et al., 2005).

Further, it is very likely that close proximity of the NADPH oxidase with the viral entry site is necessary to trigger infection-dependent ROS production. This could be achieved via the existence of membrane microdomains where on the one hand virus entry is likely to occur and on the other hand NADPH oxidases are present. In this context it has been shown that IL-1R- and TNFR-mediated activation of NF-κB is driven by NOX2, which localized to plasma membrane lipid rafts and was necessary for TRAF6 and TRAF2 recruitment following receptor-mediated caveolin-dependent

endocytosis (Li et al., 2006; Li et al., 2009). Viral cell surface receptors, such as the RSV receptor nucleolin, have been shown to localize to such membrane microdomains. Thus, the RSV entry site could find itself in close proximity to NOX2, thereby laying the basis for an efficient signal transduction that links the event of viral entry with NADPH oxidase activation.

Additionally, the site where viral genome detection occurs might need to be in close distance to an NADPH oxidase. In this context, it has been known that certain PRRs find themselves in close proximity to NADPH oxidases. For instance, a complex formation of flagellin-sensing TLR5 and DUOX2 has been demonstrated previously (Joo et al., 2012). However, for *Paramyxoviridae* infection, it still remains to be demonstrated if NOX2 is indeed in close proximity to the virus-recognizing PRRs RIG-I and MDA-5 in AEC.

In the case of DUOX2, studies in the *Drosophila* model propose that DUOX2 basal enzymatic activity is necessary for the balance of symbiotic existence with commensal microorganisms in the gut, a pathway referred to as activity pathway. On the contrary, microbial infection leads to potentialisation of ROS production by DUOX by means of increasing DUOX expression via the expression pathway (Ha et al., 2009b). A similar mechanism can be pictured in the lung epithelium, where basal DUOX2 expression could keep a balance in airway resident microorganisms. Respiratory virus infection might lead to increased ROS production by induction of DUOX2 gene expression. Besides increases in DUOX2 expression levels, Ca^{2+} fluctuations might constitute a DUOX2 activating signal during virus infection. In this context, the *Drosophila* model has been helpful in delineating the dependency of DUOX activity on Phospholipase C β, which is necessary for generating inositol triphosphate (IP3) which in turn will lead to Ca^{2+} release from the endoplasmatic reticulum (Ha et al., 2009a). In this line of thought, it is noteworthy that DUOX2 activity is highly induced in thyroid cells following phorbol ester PMA stimulation in a manner dependent on PKC, which lies downstream of phospholipase C (Rigutto et al., 2009). It is known that certain viruses or viral proteins increase the cytosolic Ca^{2+} concentration (Brisac et al., 2010; Chami et al., 2006). This phenomenon has not yet been evaluated in the case of SeV or RSV infection. However, if changes in Ca^{2+} levels

should occur in *Paramyxoviridae* infection, these could plausibly constitute a triggering signal for DUOX2 activity.

4 Conclusion

" […] Much remains to be learned about these enigmatic proteins […] ". With this phrase, Pr. Vignais ended his last review on the phagocyte NADPH oxidase NOX2 (Vignais, 2002). And indeed, since the publication of these words, our scientific knowledge on NADPH oxidases has increased tremendously. Today, while I am writing this sentence, 11320 scientific publications can be found after my search for "NADPH oxidase", whereas at the beginning of 2002 there were only about 3000. The first non-phagocytotic oxidase, NOX1, was only discovered in 1999, approximately at the same time when the DUOXs were discovered in the thyroid (Dupuy et al., 1999; Suh et al., 1999). Today, NADPH oxidases are known to control processes such as cell transformation, angiogenesis, inflammation, immune activation and many others. This is where we stand. This is where this thesis adds yet another piece to the complex puzzle of NADPH oxidases.

We started our scientific journey with a hypothesis, namely that NADPH oxidases regulate the antiviral and proinflammatory mechanisms following *Paramyxoviridae* infection of AEC. Now, that we have ended our journey we can say that, yes, indeed, NADPH oxidases regulate antiviral and proinflammatory mechanisms in SeV- and RSV-infected AEC. We reveal that NOX2 is a major contributor to SeV- and RSV-induced inflammatory processes as it contributes to NF-κB and consequently virus-induced cytokine expression of AEC. Further, we demonstrate that AEC upregulate DUOX2 NADPH oxidase during the course of viral infection and that this upregulation contributes to a global antiviral resistance state. Our results further indicate that human pathogens, such as RSV, possess strategies to counteract this antiviral mechanism. To our knowledge, these are the first reports demonstrating the implication of NOX2 and DUOX2 in proinflammatory and antiviral processes in AEC.

We believe that these results lay an important foundation, not only for the understanding of the principles of AEC antiviral defense, but also for future studies. An issue that has not been addressed in this thesis is the exploitation of our results to improve lung immunity in respiratory virus infection. Could NOX2 constitute a potential target for calming the "cytokine storm" observed in pathogenic virus infections? Can airway antiviral immunity be increased by enforcing the DUOX2

system? If yes, how could this be achieved? Several sources propose that targeting NOX2 might indeed constitute a means to improve disease outcomes associated with respiratory virus infections (Vlahos et al., 2012). We argue that targeting NOX2 might not be such a straightforward issue. Although NOX2 clearly seems to be a regulator for post-infection inflammatory processes and our study adds to a broader understanding of the events taking place specifically in AEC, we still have unsufficient knowledge to whether NOX2 in AEC or in hematopoietic cells should be the target of intervention. In this context it should be stressed that inhibiting NOX2 might unlikely be specific to a certain compartment. Further, dampening NOX2 to downregulate the cytokine-environment might be a double-edged sword, considering that DUOX2 induction requires precence of IFNβ and TNFα. Further, other literature demonstrates that NOX2 supports RIG-I signaling by sustaining MAVS levels (Soucy-Faulkner et al., 2010). Thereby, NOX2 inhibition might have the negative side effect of hampering with AEC antiviral mechanisms.

Attempting to increase DUOX2-mediated antiviral responses might indeed be beneficial in RSV infection, since our results suggest that this virus interferes with the mechanisms that upregulate DUOX2 expression. The identification and targeting of the mechanism by which RSV interferes with this pathway should be a priority as it could significantly enhance airway antiviral immunity in RSV infection.

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6 Annex

Annex – Figure A1

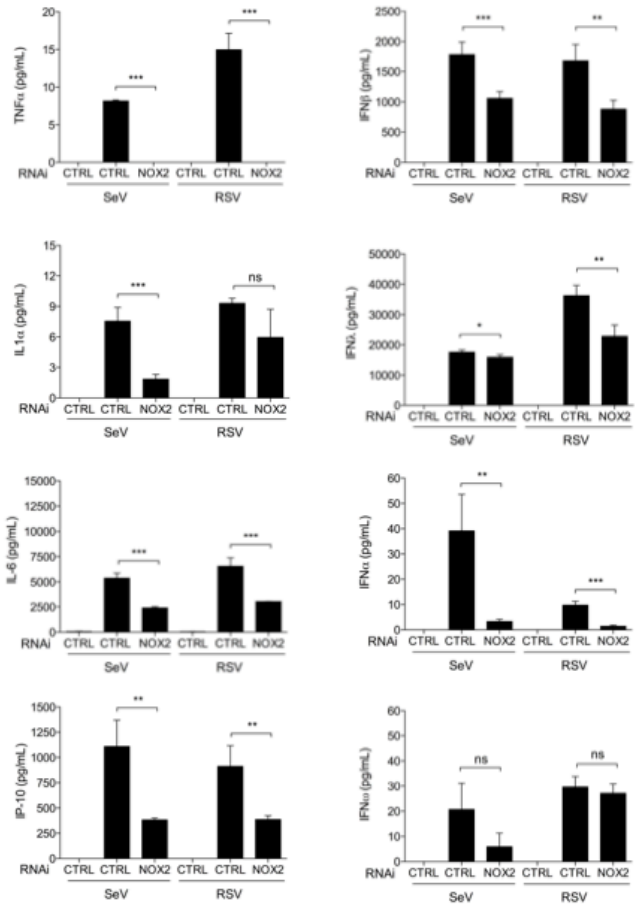


Figure A1: NOX2 regulates the secretion of antiviral and proinflammatory cytokines in SeV and RSV infection.
A549 cells were transfected with CTRL- or NOX2 specific RNAi. 48h post-transfection, cells were infected with SeV or RSV for 24h. Cytokine secretion was analyzed by multiplex ELISA. Data were analyzed by one-way ANOVA with Dunnett post-test, siCTRL-infected vs. siNOX2-infected. Ns, non-significant; *, p<0.05, **, p<0.01, ***, p<0.001.

Annex – Figure A2

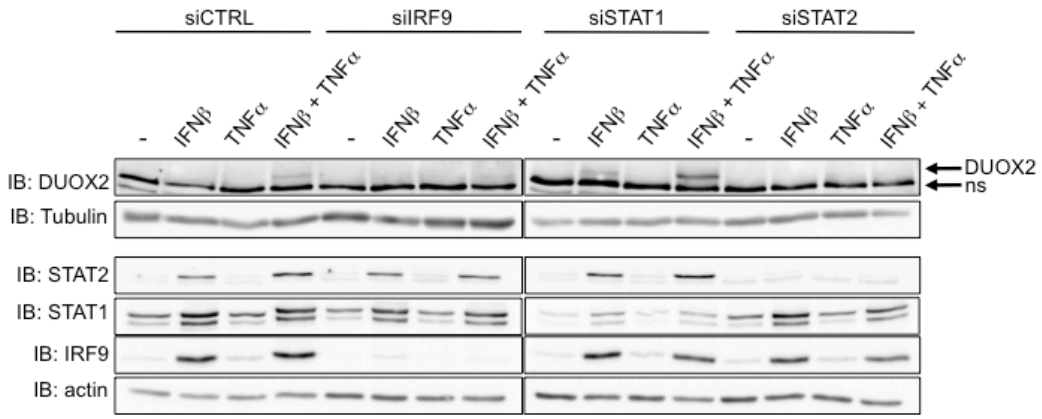
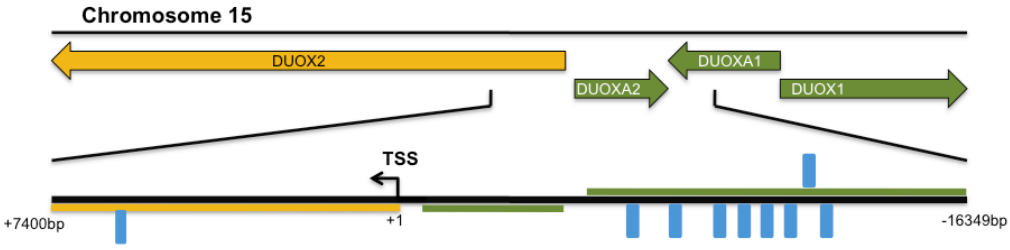


Figure A2: IFNβ and TNFα induced DUOX2 expression is regulated by STAT2 and IRF9 but not STAT1.
A549 cells were transfected with CTRL-, STAT1-, STAT2- or IRF9-specific RNAi. 48h post-transfection, cells were stimulated with IFNβ or TNFα or a combination of both for 24h. DUOX2, STAT1, STAT2 and IRF9 expression were analyzed by Western Blot.

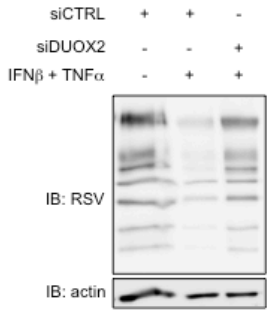
Annex – Figure A3



TF consensus site	Start position	End position	Strand
ISRE	6395	6415	-
ISRE	7706	7726	-
ISRE	9093	9113	-
ISRE	9949	9969	-
ISRE	10697	10717	-
ISRE	10980	11000	-
ISRE	11162	11182	-
ISRE	11233	11253	+
ISRE	12286	12306	-

Figure A3: ISRE consensus sites in the DUOX2/DUOXA2 promoter region.
Schematic representation of the genomic arrangement of DUOX2 and DUOXA2 genes, as well as the ISRE consensus sites present in the DUOX2/DUOXA2 promoter region. Analysis was conducted with the Genomatix transcription factor binding site research tool.

Annex – Figure A4



Annex – Figure A4: DUOX2 contributes to the antiviral state generated by IFNβ and TNFα.
A549 cells were transfected with siCTRL or siDUOX2 for 48h and stimulated with IFNβ and TNFα where indicated to establish an antiviral state. 24h post stimulation cells were infected with RecRSV-GFP for 72h. Viral protein content was analyzed by Western Blot with RSV-specific polyclonal antibodies.

Annex – Figure A5

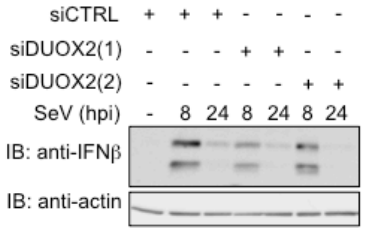


Figure A5: DUOX2 regulates intracellular IFNβ protein levels
A549 cells were transfected with CTRL-, DUOX2(1)-, or DUOX2(2)-specific RNAi. 48h post-transfection, cells were infected with SeV for the indicated times. Intracellular IFNβ levels were analyzed by Western Blot with IFNβ-specific antibodies.

Annex – Contribution to other scientific work

1. **Requirement of NOX2 and Reactive Oxygen Species for efficient RIG-I-Mediated Antiviral Response through Regulation of MAVS Expression**

   Anton Soucy-Faulkner, Espérance Mukawera, Karin Fink, Alexis Martel, Loubna Joan, Yves Nzengue, Daniel Lamarre, Christine Vande Velde, Nathalie Grandvaux.

   PLoS Pathogens, 2010, 6(6): e1000930.

   This article highlights a crucial role for NOX2 in the airway antiviral defense. It shows that NOX2 is essential in maintaining MAVS levels, thereby establishing the basis for efficient antiviral signaling. In the absence of NOX2, MAVS levels were decreased, which resulted in decreased IRF-3 activation and antiviral cytokines expression in the context of SeV infection in AEC. Previous data have indicated that IRF-3 activation following AEC infection might indeed be subject to NADPH oxidase-dependent redox regulation, however our work was able to identify the mechanism, by wich NOX2 sustains airway antiviral function in a redox-sensitive manner.

2. **Innate host defense: NOX and DUOX on phox's tail**

   Grandvaux Nathalie, Soucy-Faulkner Anton and Fink Karin.

   Biochimie, 2007, 89(9):1113-22.

   This review article published in 2007, discussed the at the time of publication still quite novel notion that NADPH oxidases not only functioned in phagocyte innate immunity, but were subtle regulators of innate immune processes in various cellular contexts.

3. **Inhibition of the beta interferon gene by the human herpesvirus 6 immediate-early 1 protein**

Joanna Jaworska, Annie Gravel, Karin Fink, Nathalie Grandvaux and Flamand Louis.

Journal of Virology, 2007, 81(11):5737-5748.

The study by Jaworska *et al*. studied the mechanism of Human Herpesvirus-6 (HHV-6) in the inhibition of the antiviral immune response. More specifically, HHV-6 immediate early protein-1 (IE1) was found to interfere with efficient IRF-3 activation. Thus work contributed significantly to the understanding of how HHV-6 evades antiviral immunity to establish successful infection. My work in this context consisted in the analysis of IRF-3 dimerization in the presence of IE1A and IE1B following transfection of TBK-1 to activate the antiviral signaling cascade.