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# Host-pathogens interactions during RSV / S. pneumoniae infection immune response and p53 pathway

Daniela Bandeira Brancante Machado

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Soutenue publiquement le 12/12/2017, par :

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**Etude *in vitro* et *in vivo* des interactions  
hôte/pathogènes dans un modèle de co-infection  
VRS/*S. pneumoniae***

Host-pathogens interactions during RSV / *S. pneumoniae* infection:  
immune response and p53 pathway

---

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---

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# LIST OF ABBREVIATION

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|               |  |
|---------------|--|
| C. pneumoniae | <i>Chlamydomphila pneumoniae</i>                               |
| cRNA          | complementary RNA  |
| CRP           | C-reactive protein   |
| DBD           | DNA-binding domain   |
| F             | fusion protein   |
| G             | attachment glycoprotein  |
| H. influenzae | <i>Haemophilus influenzae</i>                                  |
| HA            | Hemagglutinin protein  |
| hCoV          | human Coronaviruses  |
| HD            | hinge domain   |
| hMPV          | human Metapneumoviruses  |
| hPIV          | human Parainfluenza viruses                                    |
| IAV           | Influenza A viruses  |
| IFN           | Interferon   |
| IL            | interleukin  |
| IP-10         | Interferon gamma-induced protein 10                            |
| IRF           | interferon regulatory factor                                   |
| ISGs          | Interferon-stimulated genes                                    |
| L             | subunit of the RSV-polymerase protein                          |
| M. pneumoniae | <i>Mycoplasma pneumoniae</i>                                   |
| MAPK          | mitogen-activated protein kinase                               |
| Mdm2          | mouse double minute 2  |
| mRNA          | messenger RNA  |
| MRSA          | methicillin-resistant <i>Staphylococcus aureus</i>             |
| N             | nucleoprotein  |
| NA            | Neuraminidase protein  |
| NF-kB         | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NHP           | Nonhuman Primates  |
| NLRs          | NOD-like receptors   |
| NP            | Nucleoprotein  |
| NS            | non-structural protein   |
| NTHi          | nonencapsulated strains of <i>H. influenzae</i>                |
| OD            | oligomerization domain   |
| P             | phosphoprotein   |
| PA            | acid polymerase protein  |
| PAMPs         | pathogen-associated molecular patterns                         |
| PB1           | basic polymerase 1 protein                                     |
| PB2           | basic polymerase 2 protein                                     |
| PCR           | polymerase chain reaction                                      |
| PCT           | procalcitonin  |
| PCV           | Pneumococcus conjugated vaccine                                |
| PKC           | protein kinase C   |

|                      |                                       |
|----------------------|---------------------------------------|
| PRD                  | proline-rich domain                   |
| PRRs                 | pattern recognition receptors         |
| RLRs                 | RIG-I-like receptors                  |
| RNPs                 | Ribonucleoprotein complexes           |
| RSV                  | human Respiratory syncytial viruses   |
| <i>S. aureus</i>     | <i>Staphylococcus aureus</i>          |
| <i>S. pneumoniae</i> | <i>Streptococcus pneumoniae</i>       |
| SH                   | small hydrophobic protein             |
| TA                   | Transactivation domain                |
| Th                   | helper T cells type                   |
| TLR                  | Toll-like receptor                    |
| TNF                  | Tumor necrosis factor                 |
| TRAIL                | TNF-related apoptosis-inducing ligand |
| vRNA                 | viral RNA                             |
| WHO                  | World Health Organization             |

# RESUME

---

Les infections aiguës des voies respiratoires inférieures constituent la troisième cause de décès dans la population mondiale, avec 3,2 millions de décès. Parmi cette mortalité, au tour de 1 million c'était des enfants de moins de 5 ans, représentant la première cause de mortalité dans ce groupe d'âge, selon l'OMS en 2015. Le virus respiratoire syncytial (VRS) est considéré comme un agent étiologique important dans le milieu pédiatrique et les estimations sont que ce virus cause 3 millions d'hospitalisations par an. Un aspect important du pronostic des infections virales est le rôle de co-infection bactérienne. La combinaison d'agents viraux et bactériens a été signalée entre le VRS et les bactéries *Streptococcus pneumoniae*. En raison de l'importance clinique de cette co-infection et le taux élevé de circulation du VRS, il est important de comprendre comment le système immunitaire est affecté à l'infection de ces deux agents pathogènes. Notre étude identifie la réponse immunitaire dans les macrophages, ainsi que les interactions entre le VRS et le facteur de transcription p53. Les résultats montrent un profil particulier à cette co-infection mixte dans les macrophages et des modifications dans la réponse immunitaire innée que nous a permis de mieux comprendre les mécanismes de pathogénèse du VRS dans les cellules épithéliales pulmonaires en regardant la régulation de p53. Dans la dernière partie, nous avons évalué l'impact direct de l'infection mixte chez les primates non-humain et ce modèle nous a montré les difficultés et complexités des établir une pneumonie sévère.

Mots-clés : VRS ; *S. pneumoniae* ; co- infection ; réponse immunitaire ; p53 pathway.

# ABSTRACT

---

Respiratory viruses play a leading role in the etiology of respiratory infections. Currently, respiratory syncytial virus (RSV) is generally considered to be the etiologic agent of respiratory disease in pediatric importance, as children can develop bronchiolitis and pneumonia when infected with the virus. The first RSV infection occurs in the first two years of life in about 95% of children, with the peak incidence occurring in the first few months of life. An important aspect of the prognosis of viral infections is the role of bacterial co-infection. The combination of viral and bacterial agents has been reported between RSV and *Streptococcus pneumoniae* bacteria. Because of the clinical importance of this co-infection and the high rate of RSV circulation, it is important to understand how the immune system is affected by the infection of both pathogens. Our study was designed to evaluate the immune response in macrophages, in addition to interactions between RSV and p53 transcription factor. The results show a particular profile of this mixed co-infection in macrophages and p53 regulation that implies several modifications in the innate immune response and that allowed us to better understand the mechanisms of pathogenesis of RSV in pulmonary epithelial cells. In the last part, we evaluated the direct impact of mixed co-infection in non-human primates and this model showed us the difficulties and complexities of establishing severe pneumonia.

Keywords: RSV; *S. pneumoniae*; mixed-infection; immune response; p53 pathway

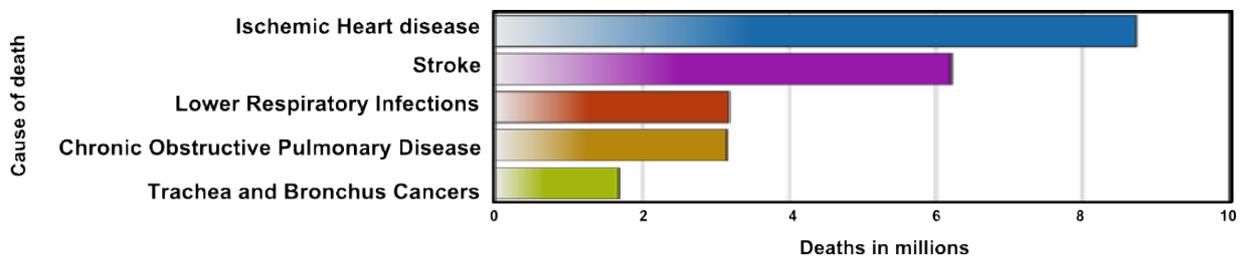
## *INTRODUCTION*

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Tous les résultats présentés ici ont été développés dans le cadre du projet de thèse visant à mieux comprendre les infections mixtes au cours de la pneumonie. Ce travail a eu la collaboration ferme et efficace entre les Laboratoire de Pathogènes Emergents et Virpath, permettant l'approfondissement et menant à des conclusions importantes dans la pathogenèse des virus respiratoires lors de la présence ou l'absence de bactéries dans des modèles in vitro et in vivo.

## **1.ACUTE LOWER RESPIRATORY TRACT INFECTIONS**

The respiratory tract constitutes a wide and critical frontier at the interface between the body and the environment. This complex organ system is divided into the upper airways and lower airways. The upper airways or upper respiratory tract includes the nose and nasal passages, paranasal sinuses, the pharynx, and the portion of the larynx above the vocal folds (cords). The lower airways or lower respiratory tract includes the portion of the larynx below the vocal folds, trachea, bronchi, and bronchioles. The lungs can be included in the lower respiratory tract or as a separate entity and include the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli [1]. Upper respiratory tract infections are less severe whereas lower infections are often associated with high mortality rates [2]. Acute lower respiratory tract infections constitute the third leading cause of human death worldwide with 3.2 million deaths in 2015 (**Figure 1**), and the first cause of mortality in children under five years of age, according to the World Health Organization (WHO) [3, 4].



**Figure 1 Causes of death worldwide.**

Lower respiratory infections constitute the third cause of death in the world population (red bar) being responsible for 3.2 million of deaths in 2015. Heart diseases (Ischemic heart disease – blue bar - and strokes – pink bar) were the most cause of deaths. Among the 5 main causes of global death described, lower respiratory infections are the only transmissible infectious disease [3].

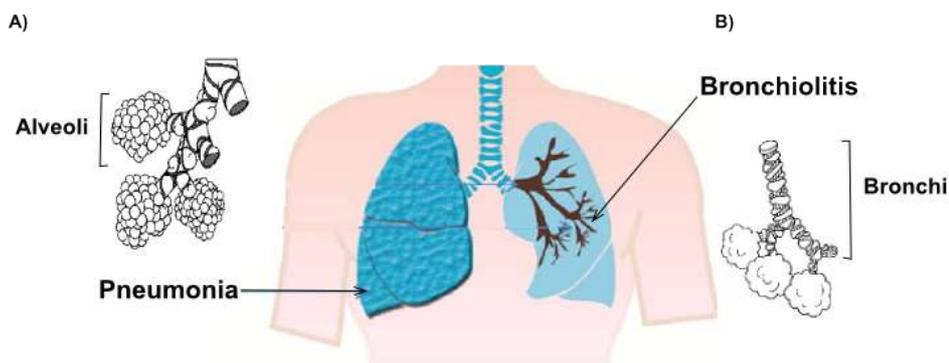
The diversity of pathological agents makes it difficult to prevent, diagnose and treat these diseases, contributing to high mortality rates [5-8]. These diseases can affect the general population, but severe cases and high mortality rates are found among children up to age 5, immunosuppressed adults and elderly [9-11].

Acute lower respiratory infections constitute a major global health burden due to the emergence of resistance to antimicrobial treatments, the presence of multiple pathogens and the recurrence of infections throughout life [12-14]. In this context, more knowledge about respiratory diseases and their etiological agents are very important to improve or propose novel prophylactic and therapeutic approaches.

## **1.1. Definition and clinical symptoms**

Respiratory tract infections are responsible for a variety of clinical features that range from milder manifestations, such as the common cold to acute lower respiratory tract infections, represented by bronchiolitis and pneumonia [15, 16]

Pneumonia is an inflammatory process that takes place in the alveolar spaces, whereas, in bronchiolitis, inflammation rather occurs in bronchi (**Figure 2**). The symptoms are relatively similar between pneumonia and bronchiolitis; they begin as a common cold (nasal congestion, high fever, and decreased appetite) and after 2 to 3 days it is possible to observe several complications of the disease. Pneumonia usually accompanies a dry cough, thoracic pain, and extreme tiredness while bronchiolitis accompanies a characteristic wheezing. At this stage of both diseases, cough is persistent and the difficulty of feeding is marked, besides the accelerated breathing. Apnea is a very common symptom in infants less than 2 months during bronchiolitis while confusion occurs in the elderly with pneumonia. Complications such as hypoxia (low oxygen level) and cyanosis (blue-tinged skin) are indicative of both severe diseases [16, 17].



**Figure 2 Differences between Pneumonia and Bronchiolitis.**

Lower respiratory tract diagram showing affected lung regions during respiratory tract infections. **A)** Pneumonia is characterized by infection in the alveoli airways and normally occurs in the terminal part of the lobular lung. **B)** Bronchiolitis is restricted in the bronchial region and remains near to principal bronchi. Adapted from Eugenia et al [18].

Bronchiolitis affects children younger than 2 years old with a high mortality rate among preterm infants, due to the characteristics of this age group in which the lung and immune system are functionally immature, forming ideal spots for viral infection [19]. Pneumonia affects all ages but is extremely severe in children up to 5 years of age, immunosuppressed adults and the elderly [20-22]. The most common pneumonia is community-acquired pneumonia, which is acquired in an extra-hospital environmental [6, 23, 24].

The pathogenesis of respiratory infections involves a complex interplay between virulence factors of a number of different pathogens – including bacteria, virus and/or fungi – and host response [25]. An overview of the etiological agents of pneumonia and bronchiolitis is presented in the following chapter and the host/pathogens interactions involved in these respiratory infections will be approached in the second part of this manuscript.

## 1.2. Etiology

The upper respiratory tract, mainly the nasopharynx, constitutes a rich and diverse niche in microbes. It is believed that most respiratory infections of the lower respiratory tract must have originated from this microbial niche. Thus, commensal microorganisms are also found in cases of severe disease, making it difficult to determine precisely etiological pathogens [2, 26].

## 1.2.1. Respiratory viral infections

The main viruses associated with lower respiratory tract infections are Influenza Viruses, human respiratory syncytial viruses (RSV), human parainfluenza viruses (hPIV), human metapneumoviruses (hMPV) and human Coronaviruses (CoV) [20, 27, 28].

### a) Influenza Viruses

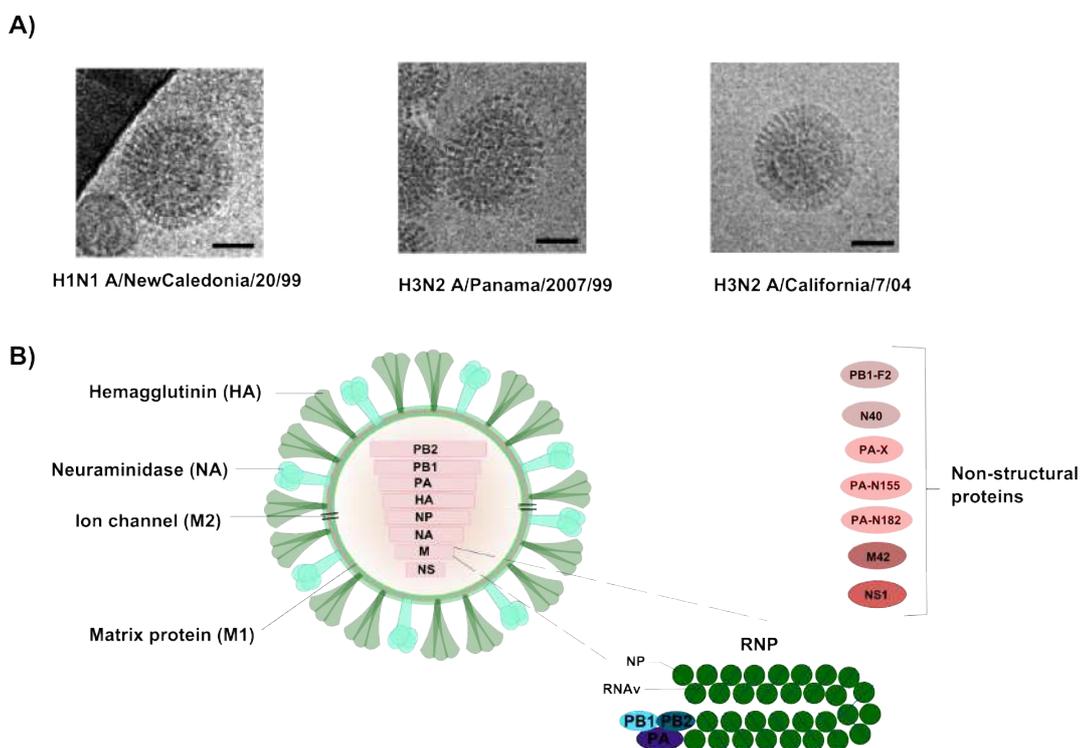
Influenza viruses cause an acute infection popularly known as Flu that has a high rate of recurrence and the ability to infect individuals in all age groups. Influenza viruses epidemics are estimated to result in about 3 to 5 million cases of severe illness, and about 250000 to 500000 deaths [29].

Influenza A, B and C viruses belong to the family *Orthomyxoviridae* [30]. Among these three types, Influenza A viruses (IAV) is responsible for the major pandemic and seasonal epidemics events being considered more virulent and genetically more variable [31]. IAV have the ability to achieve a large spectrum of animal reservoirs, among mammals and birds. Instead, Influenza B types are composed of two circulating lineages (Victoria and Yamagata) in the human population causing seasonal epidemics but without the capacity to promote pandemic events. Influenza C type is the less common and often only cause a mild infection in children [32-34].

IAV is divided into subtypes according to antigenic properties of the two viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Historically, three subtypes of HA (H1, H2, and H3) have acquired the ability to be transmitted efficiently among humans and seasonal subtypes H1 and H3 are most co-circulate detected [35]. However, other subtypes, such as H5, H6, H7, and H9 occasionally affect humans and are considered possible threats to a future pandemic [36]. IAV can cause pandemics when a strain which was not previously circulating among humans emerges and transmits among humans. As the majority of the population has no immunity against these viruses, the proportion of persons in a population getting infected may be quite large [37]. Also, zoonotic Influenza viruses can cause

sporadic severe infection in humans through direct contact with infected animals, such as swine or birds but rarely spread very far among humans [38].

IAV is spherical and elongated virions with a diameter varying between 95 to 120 nanometers (nm) and morphological virions are shown in **Figure 3A** [39]. In the viral envelope are inserted the surface glycoproteins HA and NA, and also the M2 channel, which is a protons channel. The viral envelope is constituted from host cell membrane and M1 proteins delimit the inner portion of the viral particle. The negative single strand RNA viral genome is composed of 8 segments and each single RNA strand is coated by nucleoprotein (NP) and associated to an RNA-polymerase-RNA-dependent complex composed by basic polymerase 1 (PB1), basic polymerase 2 (PB2) and acid polymerase (PA) [40]. IAV genome encodes up to 17 structural and nonstructural proteins that play different roles in the architecture and dynamics of viral replication [35, 40-43]. A schematic viral particle is represented in **Figure 3B**.

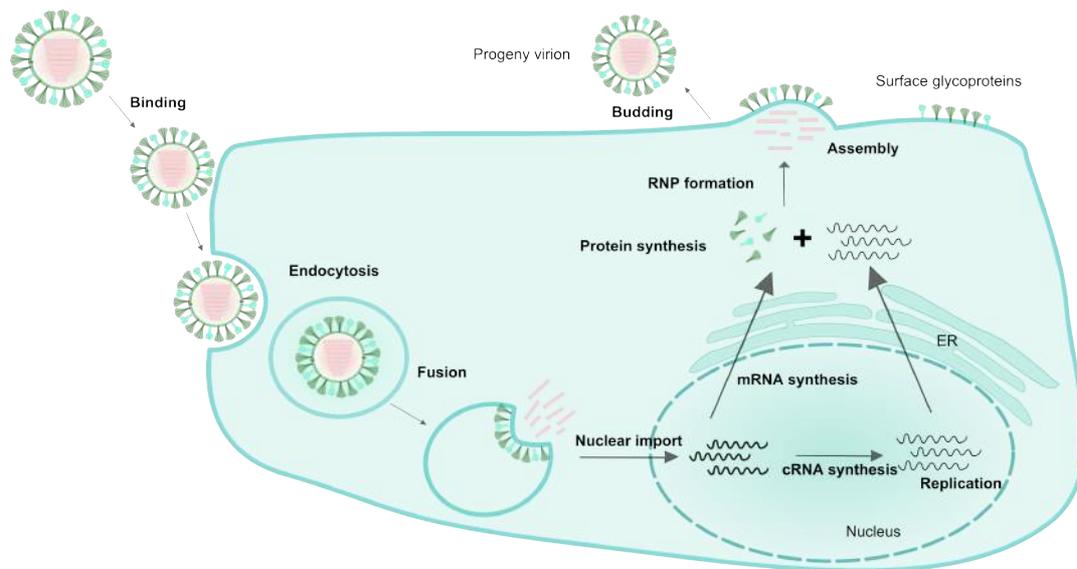


**Figure 3 Influenza virus particle.**

**A)** Cryo-electron micrographs (Cryo-EM) presenting different strains of Influenza A subtypes. **B)** Schematic structure of Influenza viral particle showing surface glycoproteins (HA, NA, and M2). Each segment of the viral genome is also represented and RNP complex is highlighted to show each protein that forms this complex. Also, nonstructural proteins are listed. Adapted from Moulès et al [39].

The HA glycoprotein possesses two primordial functions for the replicative cycle. First, the HA recognizes and binds to sialic acid receptors on the cell surface

and then allows the fusion of the viral envelope with the internal membrane of the endosome, allowing the release of vRNPs in the cytoplasm [44]. The other glycoprotein, NA is involved in the liberation of new virions from the surface of the host cell due to its sialidase activity, thus allowing viral dissemination [45]. A schematic representation of the different steps of the replicative cycle Influenza A viruses is presented in **Figure 4**.



**Figure 4 The Replicative cycle of IAV.**

IAV binds to the respiratory epithelial cells by residues of sialic acid on the cell surface. Then, the internalization occurs by endocytosis of the virus. The low pH in the endosome triggers the fusion of the viral and endosomal membranes, releasing the genome into the cytoplasm. The genome is imported into the nucleus where they serve as a template for translation and transcription of the viral genome. New proteins are synthesized from messenger RNA (mRNA) viral and the viral genome is replicated by means of a complementary RNA (cRNA) of positive sense. The newly produced vRNA assembles with the viral polymerase and the nucleocapsid protein to form the biologically active ribonucleoprotein complexes (RNPs). After packaging of the RNPs into new virions the virus is released from the cell surface by the action of the NA glycoprotein. Adapted from Dubois et al. [43]

Two important evolutionary mechanisms are involved in the replicative cycle of influenza viruses, the antigenic drift and shift, which allow it to evade the immune system and adapt to new hosts [31]. The antigenic drift mechanism results from point mutations inserted during the replicative process, caused by the low fidelity of the viral RNA-dependent RNA polymerase enzyme, facilitating rapid viral evolution [41]. This process allows viral antigenic evolution by the selection of new variants containing amino acid alterations in HA and NA proteins [41]. More drastic changes in the viral genome may occur, such as the rearrangement of gene segments

(antigenic shift), resulting in the emergence of a new variant with pandemic potential, as occurred with the A (H1N1) virus pdm09 [46]. The mechanism of antigenic shift occurs due to the rearrangement between the gene segments of viruses of distinct origins during the simultaneous infection of two viral particles in the same cell.

The seasonality of IAV is complex and influenced by a set of socio-economic (social, behavioral and cultural interactions), viral (the continuous process of generation and selection of new strains) and ecological/environmental factors [47]. Influenza viruses have their peak epidemic from May to September in the temperate regions of the Southern Hemisphere, between December and March in the temperate regions of the Northern Hemisphere, and throughout the year (with a higher incidence in the rainy season) in Tropical and subtropical regions [48].

Due to the evolutionary characteristics of influenza viruses, the annual impact on morbidity and mortality of their epidemics and their pandemic potential, the constant surveillance of these viruses as well as the rapid identification of new emergent strains are extremely important.

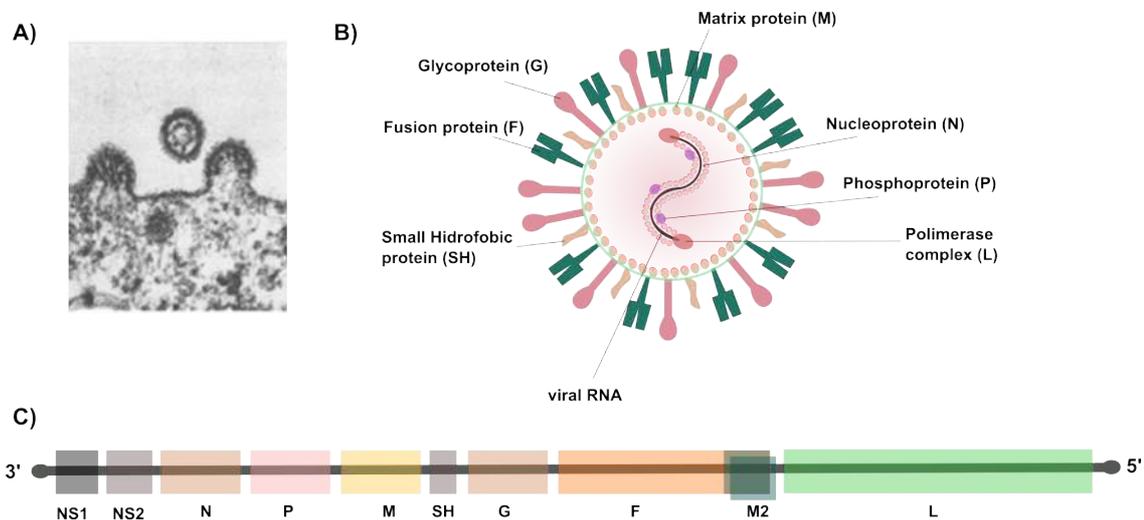
## **b) Respiratory Syncytial Virus**

The human Respiratory Syncytial Virus (RSV) is considered as an etiological agent of major pediatric importance in respiratory infectious disease such as pneumonia and bronchiolitis [49, 50]. The first RSV infection occurs in the first two years of age and the peak incidence usually occurs in the first months of life [51]. Also, RSV infection during the first year is associated with the development of recurrent wheezing, asthma, and others chronic lung diseases later in life [52, 53]. Cases of reinfection by RSV are common throughout life, but the clinical symptoms in children with older age and adults are milder nature [54]. Some studies associate RSV infection with a relevant morbidity and mortality in children with prematurity, bronchopulmonary dysplasia, and congenital heart disease, in the elderly [22] and in immunocompromised individuals [55, 56].

Human RSV is a member of the new family *Pneumoviridae* [57] with Bovine Respiratory Syncytial Virus and murine pneumonia virus. RSV particles have irregular spherical morphology, with a diameter around 100-350 nm (**Figure 5A**).

Long filamentous particles having a diameter from 60 to 200 nanometers and more than 10µm in length have also been described in the literature [54].

RSV possesses a negative, non-segmented single-stranded RNA genome [58]. Three surface glycoproteins are inserted at the surface of the viral envelope: the fusion protein (F), the attachment glycoprotein (G) and the small hydrophobic protein (SH). Two proteins compose the viral matrix and are known as M and M2-1. The nucleocapsid - a protein complex associated with the vRNA - is formed by the phosphoprotein (P), the nucleoprotein (N), the largest subunit of the polymerase (L) and the M2-2 transcription factor. RSV genome encodes 11 proteins, with two non-structural proteins, NS1 and NS2 which are expressed only during cell infection and are not packaged into the viral particle [59-65]. A schematic representation of RSV particle is represented in **Figure 5B** and RSV genome in **Figure 5C**.



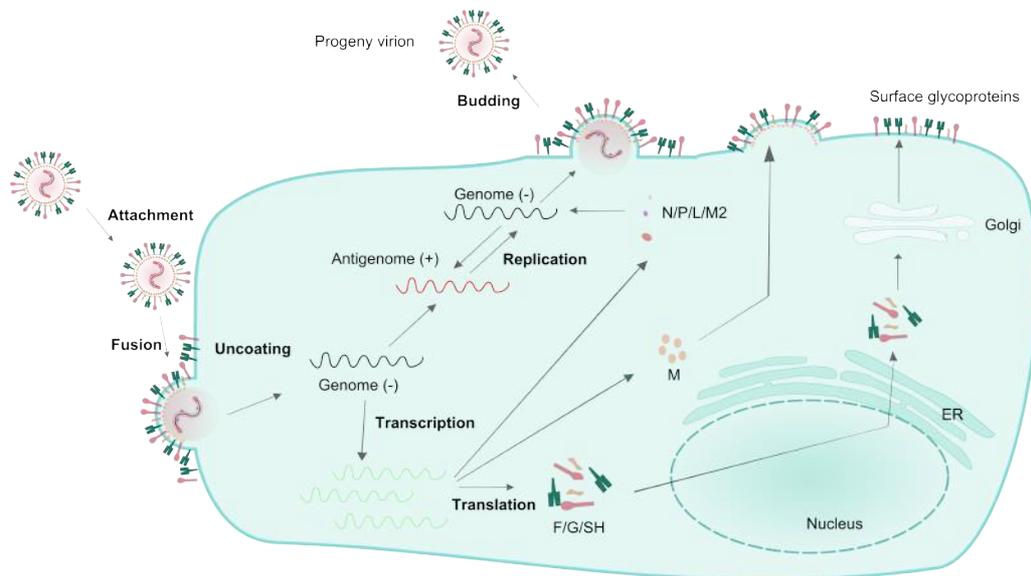
**Figure 5 Respiratory syncytial virus particle and genome.**

**A)** Electron transmission micrograph of different stages of the budding process of RSV particles in the cytoplasmic membrane. **B)** A schematic figure representing RSV viral particle with glycoproteins of the surface (F, G, and SH), matrix protein and viral RNA bound to N, P and L proteins. **C)** RSV genome showing representative proteins of each genome region. Adapted from Norrby et al. [66].

There are two major groups of the virus, RSV A, and B, based on differences in reaction with monoclonal antibodies against the major structural glycoproteins G and F, and also by genetic comparison analysis [54]. Each group was further subdivided into genotypes based on nucleotide sequence variability [54, 67].

Overall, the role of surface glycoproteins F and G in mediating receptor binding are not completely understood. Some studies have shown that heparin sulfate present at the cell surface is essential for RSV entry into continuous cell lines

and interactions with nucleolin, annexin II, ICAMs and Toll-like receptors may also be associated with the process of binding the RSV to cells *in vitro* [68-76]. The F protein also mediates the fusion of infected cells with adjacent cells that are not infected, contributing to the formation of large multinucleated cells called syncytia [77]. A schematic of RSV replication showing principle steps is represented in **Figure 6**.



**Figure 6 Replicative cycle of RSV.**

Replication cycle begins when viral surface proteins interact with a cell receptor. After viral attachment fusion between viral and cell membrane occurs in a pH-independent process. All RSV replication steps occur in the cell cytoplasm and viral maturation occurs with the assembly of the nucleocapsid by combining genomic vRNA with N protein, which is accompanied by the addition of P and L proteins for envelope assembly. Matrix proteins aggregate with the viral surface proteins in the cellular membrane and the complete viral particle is released by budding, taking a portion of the plasma membrane in a reverse process to penetration by fusion. In addition to transcription and translation of proteins, viral genome produces a positively stranded RNA intermediate, which serves as a template to generate copies of the viral genome. Adapted from Collins et al. [54]

Patterns of seasonality and duration of RSV outbreaks vary considerably between geographical regions. In temperate climates, epidemics have been described in the winter months [78-80] while in tropical regions, epidemics appear to occur in rainy seasons [81]. However, it is possible that the seasonality of the virus is not only related to climatic factors but also to socioeconomic factors increasing the risk of RSV contamination [82-84]. In most RSV epidemics reported, the co-circulation of different genotypes of groups A and B were detected [78, 79, 85].

### c) Others Human Viruses

Others respiratory viruses are capable to cause acute lower respiratory tract infections in humans such as:

Human Parainfluenza viruses (hPIVs) are common respiratory pathogens that induce acute respiratory tract diseases in infants and immunocompromised adults [86, 87]. Serological surveys have indicated that 80% of children are infected with hPIV-3 by 4 years of age and hPIV infections re-occur throughout life. The hPIV belong to a diverse group of enveloped single-stranded RNA viruses within the family *Paramyxoviridae* and based on genetic and antigenic analyses, hPIVs have been divided into four major subtypes (hPIV-1 to hPIV-4), with subtypes 1 and 3 being most frequently found in severe cases [88].

Human metapneumoviruses (hMPV) were first identified in 2001 and constitute a common cause of acute respiratory infection in individuals of all ages worldwide [89, 90]. hMPV is a member of the family *Pneumoviridae*, that also includes RSV, and two distinct hMPV genotypes, A and B, which can be divided into two subgroups: A1, A2, B1, and B2 circulate worldwide. hMPV and RSV share similar clinic features causing severe disease in the same range of age between children with an incidence around 15% of all respiratory tract infections [91-95].

Human Coronaviruses (HCoV) infections display a wide range of symptoms and their role in pediatric lower respiratory infections is still not clear [96, 97]. There are currently five coronaviruses (family *Coronaviridae*) known to infect humans and they are associated with both upper and lower respiratory tract infections in all age groups [96, 98-100]. Thus, the role of coronaviruses in pneumonia has not been completely clarified but HCoV 229E and OC43 have been recognized as causes of viral upper respiratory infection and were linked to pneumonia in children and immunocompromised adults [101, 102].

### 1.2.2. Respiratory bacterial infections

Etiological studies of acute lower respiratory tract infection identify a high prevalence of different types of bacteria, even more than viral detection. Among the most frequent bacterial causes of pneumonia are *Streptococcus pneumoniae*,

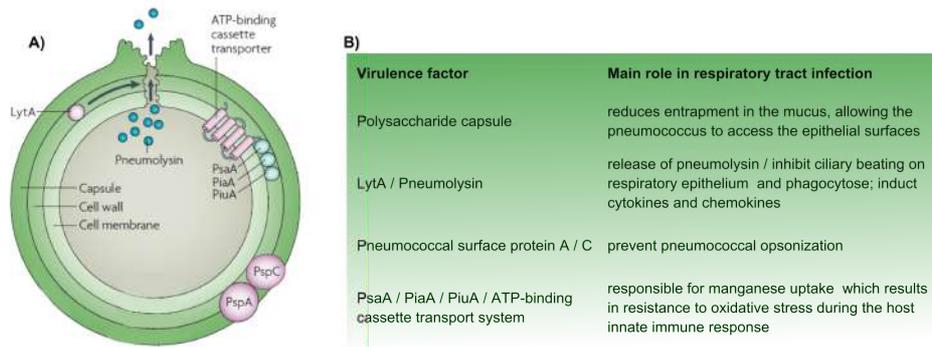
*Haemophilus influenzae*, and *Staphylococcus aureus*. All these pathogens are asymptomatic bacteria which carriage is well described in healthy individuals [103]. Also, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* are opportunistic bacteria considered as important pathogens causing pneumonia and bronchiolitis [104-110].

#### a) ***Streptococcus pneumoniae***

*Streptococcus pneumoniae* (*S. pneumoniae*) can colonize the nasopharynx asymptotically but is one of the leading causes of high mortality and morbidity in infants, the elderly and immunocompromised people [111-114]. Prior to the use of antibiotics, more than 75% of pneumonia cases were caused by *S. pneumoniae* [23]. However, nowadays, studies show that only 5 to 15% of pneumonia cases are caused by *S. pneumoniae* in developed countries and a higher proportion of cases described in low and income countries [23, 27, 115, 116]. *S. pneumoniae* induced-pneumonia is commonly named pneumococcal pneumonia.

*S. pneumoniae* is a gram-positive, encapsulated bacterium classified into 92 serotypes [117, 118] based on the composition of polysaccharide capsule. Despite this diversity, only a limited number of serotypes (around 20%) are responsible for almost 90% of all pneumococcal diseases and serotypes isolated in asymptomatic children generally reflect serotypes that cause disease [119]. The epidemiology of pneumococcal pneumonia exhibits a seasonal fluctuation with a peak incidence during the winter months [120].

*S. pneumoniae* is detected in the airways of healthy individuals [121] and carriage rates are around 60% to 80% in children under five years old [122]. Colonization state (the first step to infection) occurs when bacteria promotes adhesion on the mucosal surface of the nasopharynx. The surface of the bacterium consists of 3 structures with several virulence factors that could contribute to colonization and development of pneumococcal diseases as described in **Figure 7** [26, 123, 124].



**Figure 7 Pneumococcus surface structure and major virulence factors.**

**A)** Scheme of *S. pneumoniae* surface structure showing capsule, cell wall, and cell membrane. The principal's virulence factor PSpA, PspC, LytA, Pneumolysin and ABC complex are identified. **B)** The virulence factors showed in part A with each main role in respiratory tract infection. Adapted from Kadioglu et al. [123].

### b) *Haemophilus influenzae*

*Haemophilus influenzae* (*H. influenzae*) is a gram-negative bacterium also found in the upper and lower respiratory tract as commensal bacteria. *H. influenzae* can be divided due to differences in the capsular polysaccharide, with six different strains (a-f), and nonencapsulated strains (NTHi) [125]. Polysaccharide capsule is a major virulence factor in protecting the bacterium from phagocytosis and stimulating the inflammatory response [125]. *H. influenzae* serotype b is considered an important agent causing pneumonia in children under five years of age, elderly and immunosuppressed [126]. Also, viral-bacterial dynamics has been described suggesting that viral infection increases NTHi colonization [127-129].

### c) Others respiratory bacteria

Some studies have suggested that other bacteria, such as *Staphylococcus aureus*, *Mycoplasma pneumoniae*, and *Chlamidophyla pneumoniae*, may cause pneumonia with elevated mortality among HIV positive population [10, 130].

*Staphylococcus aureus* (*S. aureus*) is another commensal bacteria of the human nasopharynx that causes respiratory infections [131]. *S. aureus* is a gram-positive, facultatively anaerobic bacterium, usually without a capsule. This species of bacteria is widely distributed, being able to live in a wide variety of environments due to its tropism for several tissues. *S. aureus* is composed of several species and

subspecies [132], whereas methicillin-resistant *Staphylococcus aureus* (MRSA) is the principal responsible for hospital infections, among organisms resistant to antibiotics [133].

*Mycoplasma pneumoniae* (*M. pneumoniae*) are distributed globally and are the smallest prokaryotic microbes present in nature. Mycoplasma is divided into seven species that are pathogenic to humans, including *M. pneumoniae* [134] that accounts for approximately 20% of all pneumonia and higher rates correlated with the degree of immunosuppression accounts in the HIV-infected population [135].

*Chlamydia pneumoniae* (*C. pneumoniae*), with the two other species *C. psittaci* and *C. trachomatis*, are responsible for lung infections and *C. pneumoniae* remains a particular problem in the HIV-infected population [8, 130]. *C. pneumoniae* is an obligate intracellular pathogen that induces an inflammatory reaction which contributes to damages in epithelial respiratory tract [136, 137].

### **1.2.3. Mixed Respiratory infections**

The upper respiratory tract constitutes a dynamic and equilibrated microbiological niche, notably composed of commensal viruses and bacteria. Perturbation of this equilibrium, by the emergence of a pathogen and/or imbalance of the host immunity, can constitute the starting point of respiratory diseases [2, 26, 138]. Usually, the opportunistic bacteria, such as *S. pneumoniae*, *S. aureus*, and *H. influenza* are co-detected with respiratory viruses during lower respiratory tract infections [127, 139-142]. However, determining the contribution of viral/bacterial co-infection to disease severity is highly complex. There is an abundance of distinct viruses and bacterial species carried commensally in the nasopharynx and samples for laboratory diagnosis are generally contaminated with components of upper respiratory tract [143]. The use of the same pathways, cofactors, and the overlap in the inflammatory mediators produced by different pathogens create an opportunity for augmentation of the immune response during dual or sequential infection. The complexity of microbiome interactions in the airways possibly contributes to the susceptibility to exacerbations and the natural course of airway diseases [144, 145].

Thus, many aspects of the relationship between co-infection detection and disease severity remain unclear. However, in the literature, some studies have classified viruses/bacteria interactions in two distinct scenarios:

Bacterial superinfection is described when viral infection promotes favorable conditions to commensal bacteria causes an infection in the lower respiratory tract. The association of viruses and bacteria is described by epidemiological studies that show a high prevalence of bacteria in severe disease during seasonal epidemics of the respiratory virus [146]. During IAV pandemics, bacterial superinfection was observed in adults and children associated with increased morbidity and mortality [147-149]. Bacteria superinfection is also demonstrated by quantitative studies that show an increasing of commensal bacteria during a viral infection. For example, IAV and RSV infection increase colonization rates of *S. pneumoniae* and *H. influenzae* which can lead to secondary complications contributing to the disease severity [128, 142, 146, 150-155].

Mechanisms associated with viruses predisposing the respiratory tract to bacterial superinfection [156] are poorly understood with two potential explanations:

(I) Viral infection can increase bacterial adherence into epithelial cells, as described for IAV infection which is capable to exposes bacterial receptors on the surface of host cells by cleaving sialic acids residues in the upper respiratory tract [128, 157-161]. RSV, on the other hand, is thought to bind directly to *H. influenzae* and *S. pneumoniae* [129, 159, 162], increasing bacterial proximity to the epithelial monolayer and augmenting attachment to host cell receptors. Also, viral infection can induce disruption of epithelial cell tract enabling bacteria to access into deep epithelial cells [147].

(II) Viral infection can also predispose bacterial superinfection via the alteration of host's innate immune response. Viral replication may increase recruitment and activation of pro-inflammatory immune cells and may also directly affect the immune system [163-166]. Additionally, viral presence also affects the production and biological activity of cytokines [167] impairing bacterial clearance in its initial phase.

Bidirectional synergism or bacterial predisposition to viral disease is represented by an increased viral susceptibility to bacterial infection. It might be possible that microbial interactions may disturb the equilibrium of the microbiota, creating an opportunity for viral invasion and transmission in the lower respiratory tract.

Epidemiological studies show that pneumococcal conjugate vaccine not only reduced the incidence of pneumonia due to *S. pneumoniae* but also prevented approximately 33% of pneumonia associated with respiratory viruses [168]. Also, several studies have shown that the presence of a specific bacterial species may promote viral infection in the respiratory tract such as *S. pneumoniae* was shown to enhanced RSV infection *in vitro* and *in vivo* [144, 169, 170]. In addition, pre-exposure of epithelial cells to bacteria alters the response to subsequent viral infection, suggesting that bacterial presence could facilitate viral attachment to host cells [171].

### **1.3. Diagnostic of lower respiratory tract infections**

The diagnostic of pneumonia or bronchiolitis is performed through clinical examinations taking into account the history and the age of the patient. This clinical diagnosis should be performed according to WHO criteria [120]. In addition, to confirm the diagnosis of pneumonia, a chest X-ray can be performed, being able to show the extent of the disease and to identify the presence of complications that increase the severity of the disease. Usually, clinicians start the treatment without an etiologic detection due to the low sensitivity and/or lack of specificity of current diagnostic tools. In severe cases, laboratory tests capable to identify the pathogen are usually requested and a great effort has been made to improve etiological diagnosis methods [172, 173]. The quality of the collection, packaging, and transport of clinical samples are essential for an optimal diagnosis. In general, nasopharyngeal secretion samples are used for detection of viruses while blood samples are analyzed for bacterial detection [172, 173]. The most common laboratory detection methods are pathogen isolation, molecular detection, immunofluorescence and antibodies detection.

Pathogen isolation is a method where samples are incubated in different conditions and the most abundant pathogen is detected and identified. During a long time, this type of identification was considered the preferred method in diagnostics. For bacterial culture, the problems of this method are the false-negative detection due antibiotic pretreatment and nonculturable bacteria [174]. For viral isolation in cell culture, the support and conditions can vary a lot. Influenza virus can be isolated in embryonated chicken eggs or mammalian cell lineages (MDCK) while for RSV isolation, other mammalian cell lineages (HEp-2) are most common. The main disadvantage of these procedures is the relatively long period of time required between 7 to 10 days, depending on the pathogen [173].

Molecular detection is based on nucleic acid amplification and nowadays polymerase chain reaction (PCR) assay is considered a primordial technique for pathogen characterization. This method can be used directly on clinical samples and the rapid nature of the results can greatly facilitate investigation of outbreaks of respiratory illness. In addition, this method allows detecting multiples pathogens together being capable to identify different respiratory pathogens and its subtypes [173]. Also, it is possible to make quantitative analyses correlating pathogen load to the severity of disease [175, 176]. However, although PCR is highly specific, sensitivity has been shown to vary depending on the patient sample tested [177, 178].

Indirect immunofluorescence assay is the most common test in the diagnosis of various respiratory viruses. This technique is based on antibody staining of virus-infected cells in original clinical specimens and is a rapid and sensitive method for diagnosing viral infections [173].

Serological diagnoses are important approaches when clinical specimens are unobtainable or when a laboratory does not have the resources required for pathogen isolation. Serological methods such as the haemagglutination inhibition test are essential for many epidemiological and immunological studies and for evaluation of the antibody response following vaccination for Influenza virus, for example [173].

Biomarkers approaches can help to predict or to recognize potential cases of severity. Biomarkers are biological markers that function as indicators of a pathogen-related disease, or of disease severity [179]. The first biomarker proposed during an

infection were white cell count and erythrocyte sedimentary rate but nowadays they have been replaced by C-reactive protein (CRP) and procalcitonin (PCT) which have higher sensitivity and specificity for severity prognostic [180-199]. They seem to have suboptimal sensitivity and specificity for differentiated bacterial to viral pneumonia [187, 200, 201].

Several others biomarkers capable of identifying the etiology and predict complications, outcomes, and mortality of pneumonia have been studied. Tumor necrosis factor (TNF) receptor 2 and interleukin (IL)-10 characterization studies don't show success but tissue inhibitor of metalloproteinases has shown promise for the identification of bacterial pneumonia in children [195, 202-205]. Also, an association of different biomarkers like CRP, TRAIL, and IP-10 were described and might offer advantages in the differentiation of viral or bacterial pneumonia.

In addition, innovative technologies, including microarray-based whole genome expression arrays, proteomics, and metabolomics, can be a basis for biomarker discovery. For example, specific host responses induced show a microRNA bio-signatures that can be identified using microRNA analyses [199]. However, further studies are necessary before routine use of biomarker assays [199, 206].

## **1.4. Treatments for lower respiratory tract infections**

Treatments for lower respiratory tract infections depend on the nature of the etiological agent, resulting in antibiotic or antiviral treatment, in the case of bacterial or viral infections, respectively.

### **1.4.1. Antiviral treatments**

Viral replication is linked to metabolic processes of the host cells and safe antivirals offering benefits by reducing mortality as well as the duration of disease symptoms and complications are described. The available antivirals include classical

approaches focused on targeting the viral cycle and new antiviral strategies targeting the host instead of the virus [207].

### **a) Anti-influenza drugs**

According to their targets in the viral replicative site, anti-influenza drugs are classified into three groups: targeting M2 channel, neuraminidase inhibitors, or polymerase inhibitors.

Inhibition of viral envelope fusion to cell membranes by M2 proton channel blockers is represented by amantadine and rimantadine. These inhibitors were approved by health authorities but transmissible resistance variants rapidly emerged from patients after treatment and since 2009, they are not recommended for clinical use anymore [208].

Viral NA protein is an attractive target for drug action as it is essential for infectivity and has a highly conserved active site across influenza A and B viruses [31]. Inhibitors of NA, which block the sialidase activity of NA and prevent the release of new viral particles, are represented by zanamivir, oseltamivir, peramivir, and laninamivir [209]. This class of antiviral is approved for human treatments and oseltamivir is the most recommended worldwide. Viral resistance to oseltamivir was reported confirming the need for new antiviral therapies [210, 211].

Inhibitors of viral polymerases, which interrupt replication and transcription of the viral genome, include inhibitors of PB2 and NP. Some molecules such as nucleozin, naproxen, RK424 (NP inhibitors) and VX-787 (inhibitors of PB2) are in pre-clinical phases studies with promising results [212].

An alternative strategy less prone to antiviral resistance consists to target the host rather than the viral determinants. Fludase (DAS181) is inhibitory for a range of influenza A and B viruses, altering the ability of the virus to replicate efficiently. Potent antiviral properties during clinical trials with reduced inflammatory responses in mice and ferrets were described. Also, Fludase promotes protection against secondary pneumococcal infection of mice [213, 214]. Another example is the acetylsalicylic acid and its derivate demonstrate antiviral activity against influenza A viruses with some ongoing phase I/II clinical assays [215]. Also, the combination of

antiviral agents like oseltamivir with immune modulators like acetylsalicylic acids has been evaluated and shown to increase survival in animal models [212, 215, 216].

In addition, RNA-based screening studies or other similar high-throughput approaches are very helpful to identify new cellular targets. These studies provide a valuable library to select novel cellular drug targets [217, 218]. Targeting cellular rather than viral factors could be an important approach to prevent the problem of resistance to classic antivirals.

### **b) Anti-RSV drugs**

To date, no effective and accessible treatment for RSV is available. The only drug licensed is inhaled ribavirin. Ribavirin is an analog of purine nucleotides which inhibits viral replication. However, its use remains limited because of a lack of results proving its efficacy and suspicion of side effects. Ribavirin is sometimes used in some circumstances but is not recommended in most cases [219-222].

Some anti-RSV treatment targets viral replication cycle by intervention in membrane fusion and RNA synthesis. Membrane fusion inhibition, such as GS-5806, prevents virus-cell fusion and cell-cell syncytium formation and has shown promise results in early-phase clinical trials with efficacy and safety in hospitalized adults [223, 224]. Inhibitors of RNA synthesis during RSV replication are divided into two groups. Nucleoside analogs represented by ALS-8112 and ALS-8176 target the active site of the polymerization domain, and non-nucleoside inhibitors (BI-compoundD) bind to other regions of the polymerase. These molecules are in ongoing studies and show promising results but further evaluation is necessary to determine effectivity and safety [225-228].

Despite the importance of this viral pathogen, there are not adequate treatment options available. Thus, it is important to continue to identify and characterize possible targets for antiviral drugs.

## **1.4.2. Antibiotics**

Against bacterial infections, antibiotics are widely used worldwide. There are cytotoxic or cytostatic towards the microorganism and often act by inhibiting the

synthesis of a bacterial cell wall [229, 230]. For mild to moderate pneumonia suspected to be of bacterial origin, amoxicillin is recommended first-line therapy. Amoxicillin appropriately covers the most prominent invasive bacterial pathogen, *S. pneumoniae* [231]. During an atypical bacterial suspicion, a macrolide (azithromycin) is recommended [232]. In addition, a third-generation cephalosporin is recommended for a specific group of patients [5].

Due to the indiscriminate use of antibiotics, the emergence of antibiotic-resistant strains is considered a serious problem [230]. Thus, preventing lower respiratory tract infections through vaccination and prophylaxis is important.

## **1.5. Prevention of lower respiratory tract infection**

Prophylaxis is considered one of the best alternatives for combating respiratory tract infections. The prophylaxis method most common are vaccines, but passive immunization has also been overspread worldwide.

### **1.5.1. Viral prophylaxis**

The development of viral vaccines are based on attenuated, recombinant, inactivated and subunit composition strategies.

A traditional strategy that has worked for several pathogens involves the development of attenuated viral strains. Attenuation can be accomplished by serial passage or cold-adaptation and has the advantage of expressing most of the pathogen's antigens to improve immune response. Attenuated vaccines for Influenza viruses have been produced for more than 50 years, however, this vaccine has a restricted use in USA and Russia [233, 234]. Using this strategy, attenuated RSV strains have been developed [235, 236] but failed in some clinical trials [237, 238]. The disadvantage of this strategy is the that, in rare cases, the live attenuated vaccine strain can revert to its virulent wild-type, causing severe disease [239].

Alternatively, recombinant vaccines consisting viral protein expressed in other *in vivo* support have been increasingly used for vaccine development [240]. This

approach was described for influenza virus which HA protein was expressed in insect cells by baculovirus vectors [233]. For RSV, viral antigens expressed in other viruses, such as Sendai virus, vaccinia virus, adenovirus, and parainfluenza or in bacteria was studied. Despite promising results in murine studies, studies in adults showed relatively low capacity for inducing neutralizing activity [241-243], so they have not been advanced into clinical phases. Another approach of recombinant RSV vaccine carrying host cytokines capable to promote immune responses suggests that these formulations can modulate the immune response being effective alternatives for immunization against RSV [244-249].

Inactivated vaccines are composed of purified virus chemically inactivated and are capable to generate humoral and cellular immunity. The annual Influenza vaccine around the world is an inactivated vaccine [31, 233, 234]. This vaccine, during decades, was composed of 2 IAV strains and one strain of Influenza B virus according to circulated subtypes detected by surveillance. In 2016, WHO decided to add another Influenza B virus subtype to try to increase vaccine coverage [34, 233]. Even if this Influenza vaccine has a great coverage and seroconversion, the constantly evolving of influenza viruses requires continuous global monitoring and annually reformulation of influenza vaccines [250, 251]. For RSV, during 60's, a clinical trial of a formalin-inactivated RSV vaccine not only failed to prevent RSV infection but caused an increase in severe disease [252]. This experience had a profound negative impact on subsequent RSV vaccine development and the immune mechanisms that led to enhanced disease in this clinical study are not yet fully elucidated, making difficult to advances into clinical evaluation of inactivated RSV vaccines [253].

For RSV vaccine, lability and heterogeneity in particle size represent obstacles for the formulation of a stable vaccine [254] and despite the many approaches developed and tested, there is still no vaccine defined for use in humans.

Thus, the prophylactic palivizumab is indicated in months prior to the seasonality of RSV for premature babies; children with congenital pathology or with chronic lung disease by WHO. Palivizumab is a humanized mouse IgG1 monoclonal antibody directed against a conserved epitope on the surface fusion protein of RSV. This passive prophylaxis shows a potent RSV neutralizing activity and has been

clearly demonstrated to protect against RSV. The administration of palivizumab in specific risk groups is limited by its expensive cost in many low and income countries [221]. Consequently, prevention of RSV infection is a public health priority, and global initiatives have advanced numerous efforts to expand the field [255]. Continued research into the pathogenesis of RSV disease and immune responses are important to contribute to the development of RSV vaccines.

### 1.5.2. Pneumococcal vaccine

The polysaccharide capsule from encapsulated bacteria is a major virulence factor and can be used as an antigen for vaccine development [256, 257]. However, that does not induce a complete response and cannot provide adequate protection against pneumococcal infection in children [24]. Thus, the polysaccharide was chemically conjugated to different bacterial protein [258] and the first conjugate vaccine used was composed of 7 different serotypes of pneumococcus (PVC 7). In 2009, two new conjugated vaccines were licensed for use with 10 and 13 different serotypes (PCV 10 and PVC 13). Serotypes coverage of each conjugated vaccine is shown in **Table 1**.

| <b>Pneumococcal vaccine</b> | <b>Serotypes</b>                                   |
|-----------------------------|--|
| <b>PCV 7</b>                | 4, 6B, 9V, 14, 18C, 19F, 23F                       |
| <b>PCV 10</b>               | 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F             |
| <b>PCV 13</b>               | 1, 3, 4, 5, 6B, 6C, 7F, 9V, 14, 18C, 19C, 19F, 23F |

**Table 1 Pneumococcal vaccine.**  
Serotypes included in each pneumococcal vaccines approved to use worldwide [259].

A reduction in pneumococcal disease among vaccinated children have been observed since the introduction of the first PCV vaccine in 2000 [151, 260-264]. In addition, vaccination in children has been shown to reduce pneumococcal disease among the elderly by preventing the transmission due the diminished of the carriage in general population [265-267].

The challenge of the pneumococcal vaccine is the existence of 92 serotypes since vaccinated individuals remain susceptible to serotypes not included in the

vaccine [268-270]. Also, changes of serotypes carried out in the nasopharynx of children [271] were detected and the potential risk of other serotypes infections make the pneumococcal disease an important problem of global health, especially in children and must be a priority.

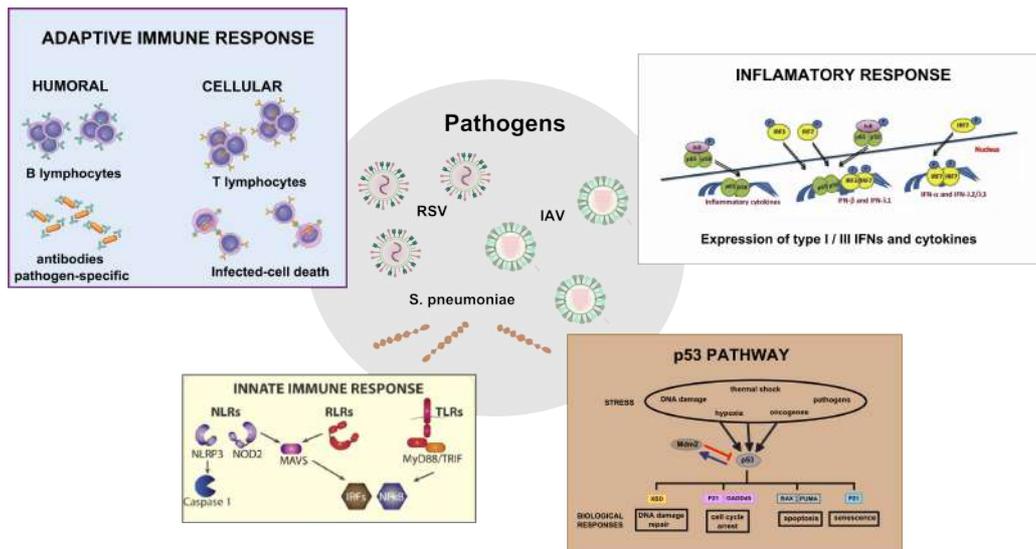
Therefore, new potential vaccines that effectively protect against pneumonia have been investigated and are undergoing clinical trials [24, 272-274].

## 2. Host-pathogen interactions

The human body is constantly exposed to microbes and prevention of opportunistic infections is made by physical or anatomical mechanisms (skin, mucosa), mechanical (cilia in respiratory tract cells, tight junctions) and biochemical (tears, saliva) barriers as well as cellular mechanisms including innate and immune responses [1].

The pathogenesis of respiratory infections involves the complex interplay between virulence factors, environmental conditions, the magnitude and temporal dynamics of the host response, and host susceptibility factors. The severity of disease is associated with an enhanced host immune response which is essential for pathogen control but can cause collateral damage to the tissues, leading to mortality in some cases. In addition, several pathogens evolved strategies to counteract or hijack host responses constitute by multiple elements including diverse cell types (epithelial cells, dendritic cells, macrophages, monocytes, and granulocytes), various pattern recognition receptors, a large array of cytokines and chemokines, cellular stress, and different pathways [25, 275].

Thus, in this chapter, immune response and others cellular mechanisms important during Influenza virus, RSV and *S. pneumoniae* infection was highlighted as resumed in **Figure 8**.



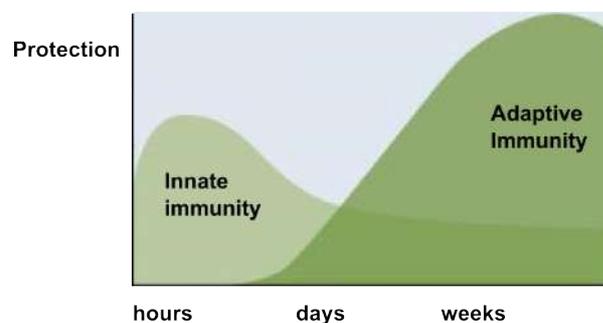
**Figure 8 Host-pathogen interactions.**

Important complex cell physiological processes in the control of pathogen infection showing an immune and inflammatory response, in addition to p53 pathway. all these cellular mechanisms are often targeted or modulated by pathogens during infection. Each pathway will be explained in the next part. Adapted from Sun et al. [275].

## 2.1. Immune response

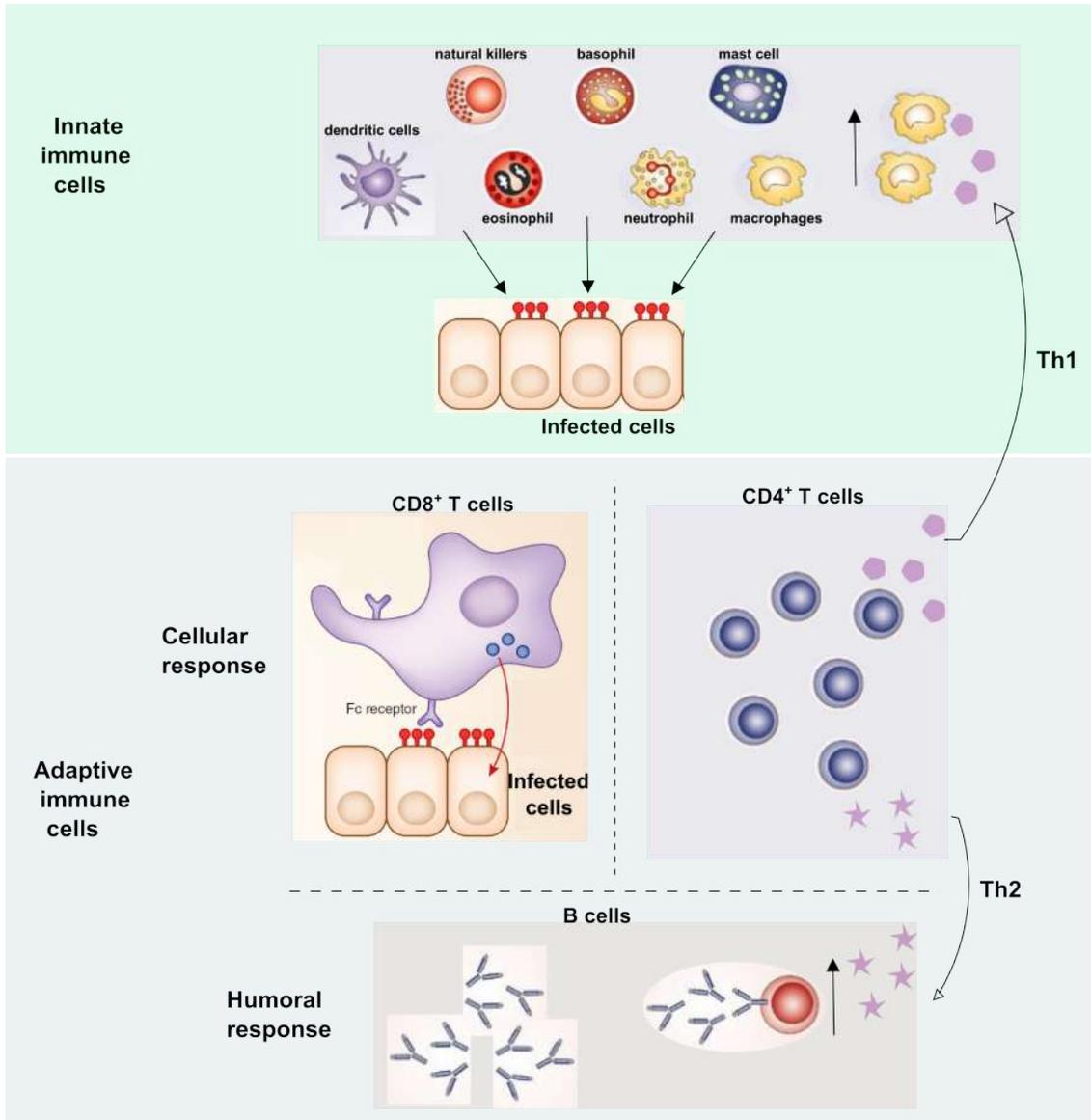
In the respiratory tract, epithelial cells are constantly in contact with potential pathogens having the important function to activate immune responses. The immune response is divided into innate and adaptive immunity. The innate response is the first line of defense induced by a pathogen and aims to control the infection locally and to activate an adaptive response. The adaptive response is specific to the pathogen, more systemic and durable [276]. A chronological course of innate and adaptive immunity is schematically showed in

**Figure 9.**



**Figure 9 Chronological course of innate and adaptive immunities.**

Innate mechanisms confer rapid protection, keeping the invading microorganisms under control until the development of adaptive immunity. It may take several days and even weeks for adaptive immunity to become effective.



**Figure 10 Innate and adaptive cells.**

Innate immune cells (macrophages, dendritic cells, natural killers, basophil, neutrophil and mast cells) exist before the invading microorganism, they are effector cells capable of recognizing several molecules of various pathogens. Adaptive cells are developed following exposure to a particular invading agent. It is able to react more quickly and more effectively to subsequent contacts, they specifically identify a molecule with high specificity. Adaptive immunity is divided into a cellular and humoral response. During the cellular response, CD8<sup>+</sup> T cells are capable to identify and kill infected cells while CD4<sup>+</sup> T cells increase macrophages quantity and/or increase antibodies expression by B cells (humoral response).

### 2.1.1. Innate immune response

The innate immune response begins as soon as the pathogen enters the target cell and is implicated in recognition and protection of infections. The innate system consists of different cells as shown in **Figure 10**.

The recognition of the pathogen by receptors is the first step in the host cell membrane during infection. This recognition is based on a limited repertoire of receptors called pattern recognition receptors (PRRs) that detect conserved microbial components known as pathogen-associated molecular patterns (PAMPs) [276]. This initial response triggered by infection is mediated by three major receptor families - PRRs: Toll-like receptors, RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) that will be detailed above.

Toll-Like Receptors (TLRs) are type 1 transmembrane proteins that are able to recognize PAMPs from bacteria, parasites, fungi, and viruses [277]. TLRs are one of the largest classes of PRRs with 10 receptors (TLRs 1-10). TLRs family is well conserved among organisms and homologous receptors are found in plants, insects and other vertebrates [276, 278]. Normally TLR 1, 2, 4, 5, 6 and 10 are expressed on the cell surface while TLR 3, 7, 8 and 9 are intra-vesicular [276, 279]. The expression profile of TLRs in different cell types may be tissue-specific and highlight the different role between cells of the immune system (macrophages, dendritic cells, neutrophils, B and T cells) and epithelial cells [277].

Viral attachment and fusion proteins, as well as the components of bacterial cell wall, are able to be recognized by these receptors. The summary of TLR receptors and pathogens ligands are described in **Table 2**.

| <b>TLR</b>                     | <b>Microbial ligands</b>   | <b>Reference</b>     |
|--------------------------------|--|----------------------|
| <b>TLR1</b>                    | Recognize lipoproteins; minority role in pneumococcus infection.   | [276]                |
| <b>TLR2</b>                    | Recognizes a large number of binders, such as bacterial peptidoglycans and lipoproteins, and surface viral proteins of RSV and IAV | [280, 281]           |
| <b>TLR3</b>                    | Viral recognition due double-stranded RNA viral of RSV and IAV.  | [280-288]            |
| <b>TLR4</b>                    | Viral recognition due to surface viral proteins of RSV and IAV; and bacterial lipopolysaccharide and pneumolysin.                  | [276, 280, 281, 289] |
| <b>TLR5</b>                    | Recognizes gram-negative bacteria and flagellin  | [290]                |
| <b>TLR6</b>                    | Recognize lipoproteins; minority role in pneumococcus infection  | [276]                |
| <b>TLR7</b>                    | Viral recognition due single-stranded RNA viral of RSV and IAV   | [280-283, 291, 292]  |
| <b>TLR8</b>                    | Recognizes single-stranded and double-stranded RNA; relevance during virus infection is unknown.                                   | [293]                |
| <b>TLR9</b>                    | Recognizes DNA; protector role against pneumococcus infection  | [276, 294]           |
| <b>TLR1/TLR2<br/>TLR2/TLR6</b> | Recognizes large number of bacterial binds, and a large range of viruses   | [295]                |

**Table 2 TLR and microbial ligands.**

TLR family represented by 10 receptors capable to recognize different microbial ligand.

Once the ligand is recognized, TLRs dimerize and initiate a signaling cascade leading to the activation of the proinflammatory response [296]. Signaling cascade is divided into two major families of transcription factors – Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and interferon regulatory factor (IRF) [25] which will be detailed in the next part of this manuscript.

TLR signaling is finely regulated to differentiate resident flora and to avoid excessive inflammation, which can cause damage to the tissue contributing to the severity of disease [288]. Thus, the activated cascade may be negatively regulated at different levels by different mechanisms such as protein phosphorylation and degradation [282, 297].

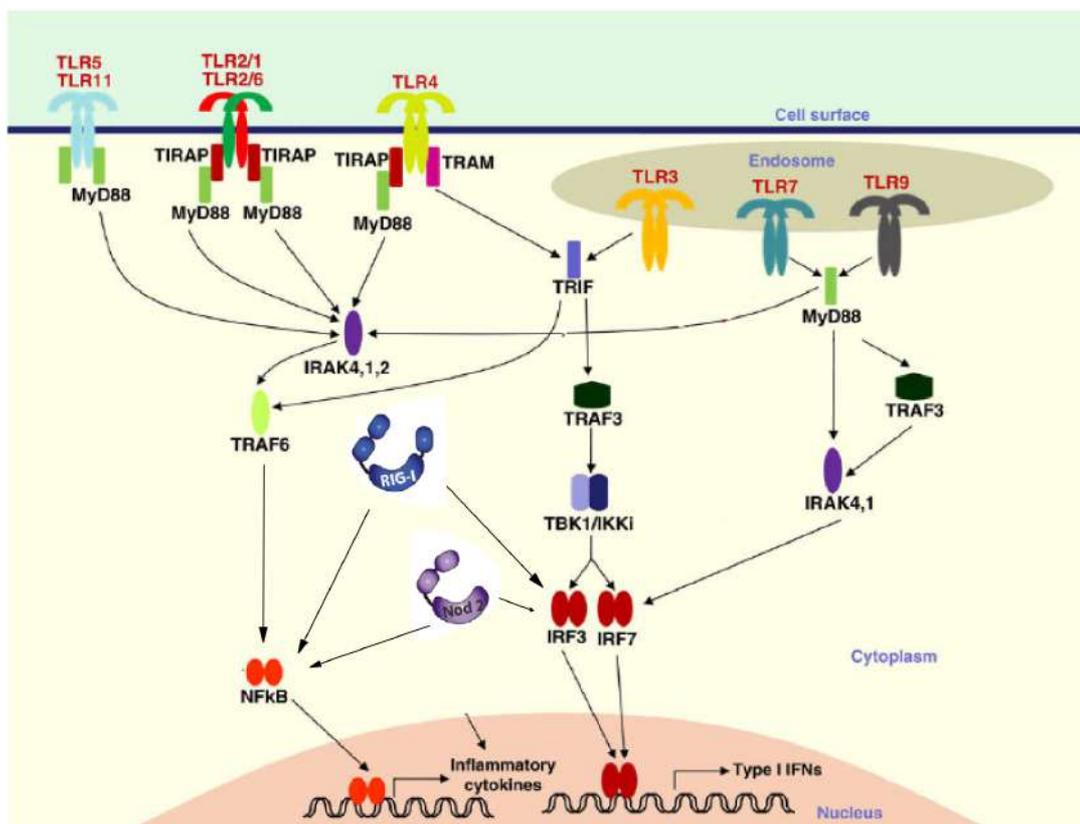
Another family of PRRs, RIG-I-Like Receptors (retinoic acid-inducible gene-I; RLRs) is intracellular helicases capable to detect [207] most respiratory viruses, including influenza viruses, RSV and hPIV, and others RNA viruses. Activated-RIG-I leads to the regulation of transcription factor IRF and NF- $\kappa$ B, inducing a very important antiviral response [280, 281, 298, 299].

NOD-Like Receptors (nucleotide oligomerization domain; NLRs) is another cytosolic PRR [207]. This family of more than 20 proteins is involved in various cellular processes of the immune response [300]. Some NLRs, including NLRP3, are

critical for the formation of inflammasome complexes which are involved in the proinflammatory responses [301, 302]. NLRP3 is expressed by monocytes, dendritic cells, neutrophils, macrophages, and human bronchial epithelial cell and is essential for the recruitment of innate immune cells during RSV and Influenza infection [303-307].

Another attendant of this family is the NOD2 receptor that should mediate the recognition of viral single-strand RNA. NLR NOD2 binds RSV genome and triggers the innate response, also described for Influenza virus and *S. pneumoniae*, leading to the production of type I IFN [276, 280, 281, 301].

All PRRs are represented in **Figure 11**.



**Figure 11 PRRs signaling pathway.**

Different classes of host PRRs (TLRs, RLRs, and NLRs) that trigger distinct signaling pathways culminating in the induction of IFNs and/or proinflammatory cytokines. Adapted from Kumar et al 2009 [277].

## 2.1.2. Adaptive immunity

The adaptive immunity is a durable response to pathogens and has been extensively studied for many years, contributing to vaccine development. Also, an exacerbated activation of this response contributes to the severity of the disease [308]. Overall, this response is highly specific being capable to recognize various antigens and to produces a specific immune response to each one. Once produced, memory cells have a long life and are able to recognize antigen for years. Thus, subsequent responses to the same antigen are usually faster, greater, and qualitatively different from the first. Adaptive immune cells are shown in **Figure 10** and are divided into two types: humoral and cellular response.

The adaptive humoral immune response is mediated by antibodies expression produced by B lymphocytes. Antibodies recognize bacterial antigens, neutralize the infection and eliminate these antigens by various effector mechanisms. During viral infection, the virus specific antibodies can block viral entry and subsequent establishment of infection. In Influenza virus infection, HA protein is neutralized by host antibodies while for RSV, F protein is probably the viral protein neutralized [275, 281].

The adaptive cellular immune is characterized by a T-lymphocyte-mediated response against intracellular microorganisms, such as viruses and some bacteria. T lymphocytes occur in two main classes when activated. The first, CD8<sup>+</sup> or cytotoxic, kill infected cells while the second class, the CD4<sup>+</sup> or helper, coordinate the immune response promoting the activation of B lymphocytes and macrophages.

CD8<sup>+</sup> cytotoxic T cells play an important role performing direct elimination of infected cells. They are recruited to the site of infection, where they recognize and eliminate the infected cell preventing the production of progeny. In the other hand, CD4<sup>+</sup> T cells produce various cytokines profile. The classic profile is helper T cells type 1 (Th1), which promote the activation of macrophages while Th2 cells, regulates antibodies production by B lymphocytes [309]. More recently, other profiles have been described, such as cells of Th17 and regulatory T cells, which control the inflammatory process [280, 281].

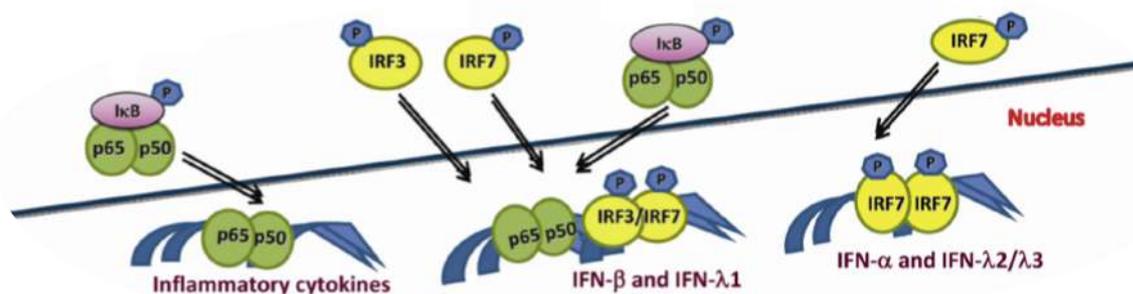
A particularity of the immune system in neonates is their reduced ability to produce Th1-responsive cytokines with normal or increased cytokine production to the response of type Th2 and Th17 [221]

During RSV infection, T and B cells are downregulated [310] leading to a poor induction of long-lasting immunity resulting in difficulties to the development of an effective vaccine [275, 311-314].

## 2.2. Inflammatory response

The severity of respiratory infections is closely linked to inflammation and tissue damage in the airways. Inflammation is induced by multiple signaling pathways such as NF- $\kappa$ B and interferon regulatory factor (IRF) - the two major transcription factors – and others more recently described like p53 pathway [285, 315, 316].

During mixed infection, both *S. pneumoniae* and RSV have been shown to stimulate inflammasome activation. The culmination of the multiple signaling pathways triggered by *S. pneumoniae* primarily results in an NF- $\kappa$ B-mediated inflammatory response, while RSV infection results in a strong interferon response. There is, however, the potential for considerable overlap between the signaling pathways triggered by each pathogen, which could lead to synergistic stimulation of inflammation during co-infection [304, 317, 318].



**Figure 12 Inflammatory responses.**

NF- $\kappa$ B pathway and Interferon pathway activation promote transcriptional activity of a number of genes responsible for inflammatory response. Adapted from Lester et al. [297]

### **2.2.1. NF-κB pathway**

The NF-κB family of transcription factors consists of five protein subunits that share an affinity for the κB DNA sequence motif and are divided into two classes: the NF-κB proteins (p105/p50 or NFκB1 and p100/p52 or NFκB2) and the Rel proteins (c-Rel, RelB, and RelA/p65) [319].

The NF-κB, composed of a p50 and RelA/p65 subunit, is considered a key transcription factor in antiviral cytokines production. Upon a variety of stimuli, such as viruses or bacteria, NF-κB migrates into the nucleus whereby it binds and activates target gene promoters of numerous cytokines [320] like ICAM, IL-1, IL-6, IL-8, and TNF-α, which are crucial for the response to inflammation [321].

There are a number of different mechanisms to regulate the transcriptional response downstream of NF-κB pathway activation and some viral proteins were described to interact with NF-κB pathway such as the NS2 protein of Influenza virus [318].

Antiinflammatory drugs such as aspirin, dexamethasone, and prednisone can indeed inhibit pathway activation, placing NF-κB as a prime target for therapies against inflammatory disease [319].

### **2.2.2. Interferon pathway**

Interferon (IFN) is a family of cytokine capable to produce an antiviral response, mediated by type I and type III, and to promotes macrophages activation by type II interferon [298].

Type I IFNs, including IFN-α and IFN-β, has an important role in restricts virus replication and spread. In addition, there is evidence that they are also produced in response to pneumococcal colonization resulting in a synergistic stimulation during mixed infection [298].

More recently, type III IFN (IFN- λ1, 2 and 3) have been proposed to have a crucial role in virus control in the respiratory tract, as they are expressed at higher levels than type I IFNs in human airway [322-325].

Due to IFN response importance in preventing viral infection, viruses have different strategies to evade to this response. For influenza viruses, the principal IFN

antagonist is NS1 and NS2 proteins that target the IFN system at multiple stages (Talon J, Horvath CM 2000)[283].

RSV also encodes two proteins, NS1 and NS2, which prevents the synthesis and signaling of type I and III IFNs. NS1 and NS2 work either individually or synergistically to block almost every essential step of the IFN pathways. These viral proteins promote the degradation of a number of elements of these pathways, including interferon regulatory factor and proteins coded by Interferon stimulate genes through proteasomal degradation [326-333].

#### **a) Interferon regulatory factors (IRFs)**

There are two major interferon regulatory factors: IRF 3 and IRF 7 in the antiviral response. IRF 3 is constitutively expressed while IRF 7 expression is enhanced significantly upon stimulation. Viral activation of the IRFs results in homo- or heterodimerization of IRF3 and/or IRF7 and subsequent nuclear translocation to bind to type I and III IFN promoters. In general, IFN- $\beta$  and IFN- $\lambda$ 1 are predominantly activated by IRF 3, while IRF 7 promotes the transcription of IFN- $\alpha$  and IFN- $\lambda$ 2 and 3 [334, 335]. During RSV infection, IRF3 degradation is promoted, reducing drastically IFN- $\beta$  expression [336].

#### **b) Interferon-stimulated genes (ISGs)**

The interferon signaling cascade induces the expression of many genes known as Interferon stimulated genes (ISGs). The transcription induction of nearly 1000 genes is regulated by IFNs and some are capable to encode direct antiviral effectors or molecules [337]. Some ISG already described to be modulated during virus infection are described in **Table 3**.

Further research on their expression and function is needed to better elucidate their contribution of ISG to the immune response during respiratory infection [283, 337, 338].

Some of the proteins encoded by ISGs have a well-defined role in the pathogenesis by a variety of mechanisms with virus, cell or tissue specific effects [283, 337]. For example, Mx proteins were among the first ISGs identified and MxA

protein prevents Influenza replication by the interaction with viral proteins such as PB2 and nucleocapsid [339, 340].

| ISG                                 | Virus  |
|-------------------------------------|--|
| <i>Eif2ak2 / Pkr, Oas1b, Rnase1</i> | Vesicular stomatitis virus, Vaccinia virus, flavivirus |
| <i>Isg15</i>                        | Chikungunya Virus, Influenza virus, Vaccinia virus     |
| <i>Ifit1</i>                        | West Nile virus, HCoV                                  |
| <i>Ifit2</i>                        | Vesicular stomatitis virus, West Nile virus            |
| <i>Ifitm3 / Ifitm</i>               | Influenza virus, RSV                                   |
| <i>Rsad2 / viperin</i>              | West Nile virus, Chikungunya virus                     |
| <i>Samhd1</i>                       | HIV  |
| <i>Bst2 / tetherin</i>              | Vesicular stomatitis virus                             |

**Table 3 ISG modulated during different virus infection.**

ISG already described in the literature to have an important role in viral infection. Adapted from Schoggins et al [337]

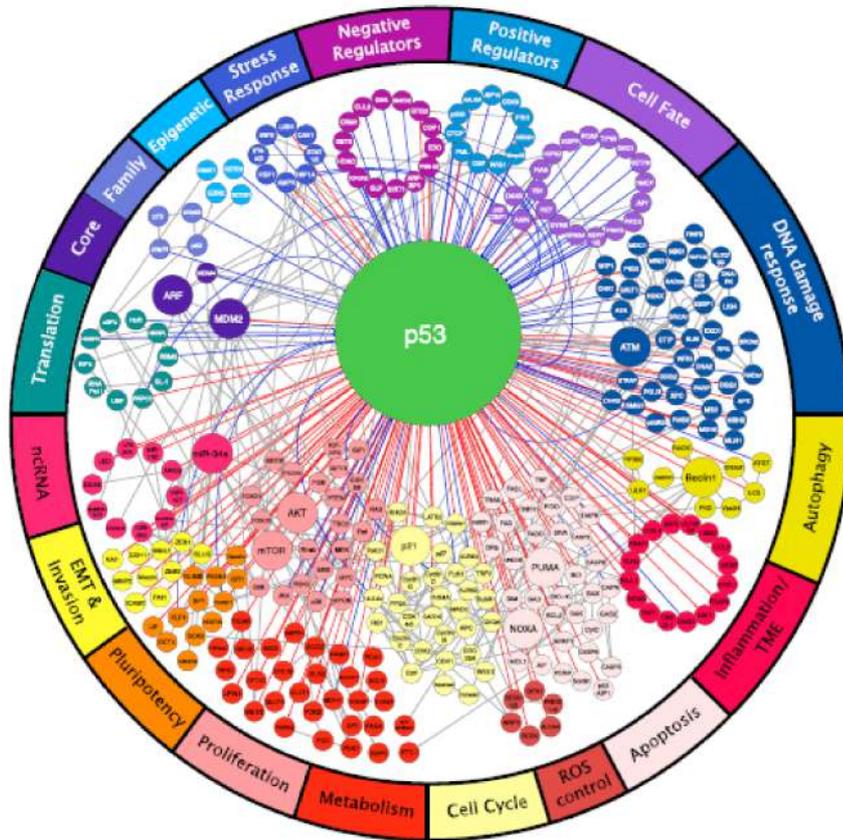
## 2.3. p53 pathway

The p53 protein, discovered in 1979, was described as "guardian of the genome" due to its major function as a tumor suppressor. The new classification "guardian of homeostasis" is maybe more appropriate, covering all known p53 biological activities [341-344]. The transcription factor p53 can activate several genes that regulate large quantities of cellular mechanisms in response to different types and intensity of stresses [345-347].

The p53 transcriptional activity binds to the promoter of the target to regulate gene expression [348-350]. However, even if this protein acts as a nuclear transcription factor, p53 can also play a role outside the nucleus through protein-protein interactions [351]. The p53 protein, formed by 393 amino acids, is expressed at low concentrations in various cell types and tissues and has a short half-life being continuously degraded [352-355].

Alterations in the structure and function of p53 are detected in a large number of cancers types and TP53 gene mutation is linked to the poor patient prognosis of cancer [356, 357]. Emerging role in various physiological processes and coordination of diverse cellular responses to different stress are shown in **Figure 13** [358]. In

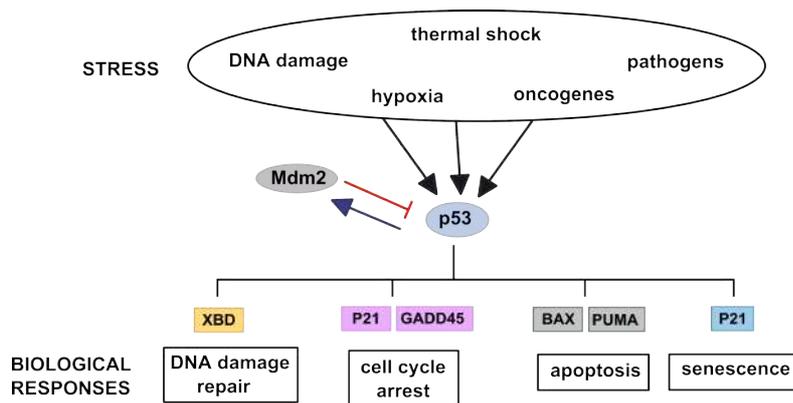
recent years, p53 regulation in several cell programs including cell metabolism, fertility, and immune regulation was described [345, 359, 360].



**Figure 13 The p53 network.**

Genes are represented by node and interactions by lines. P53 is activated by a large range of regulators, shows on blue line, which controls many distinct biological processes showed in the red line. Interactions are annotated as positive (arrow), negative (T-bar), or modifying (solid circle). Adapted from Kasthuber et al. [348].

In conclusion, there are different types of interactions with p53 for example, DNA damage promotes p53 activation driving cell-cycle arrest, senescence, or apoptosis [361, 362] while hypoxia, thermal shock, and oncogenes promote p53 stabilization [363, 364]. Also, pathogens can modulate p53 to optimize their infection as shown in **Figure 14**.



**Figure 14 Scheme of the p53 pathway.**

Different types and intensities of stresses activate p53 by mediators like Mdm2. Activated p53 initiates transcription of hundreds of target genes (transducers) that subsequently mediate cellular responses such as cell cycle arrest, apoptosis or senescence. Adapted from Vousden et al. [361].

### 2.3.1. Mechanisms of p53 regulation

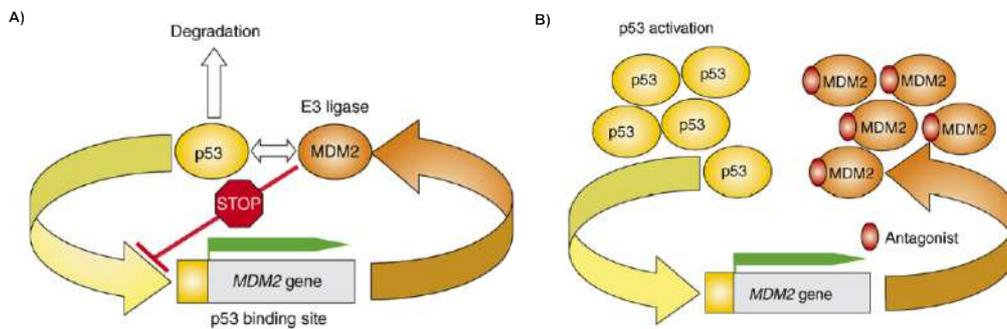
The regulation of p53 is complex and controlled by many factors including protein stabilization, activation, and posttranslational modifications. The most important posttranslational modifications of p53 include ubiquitinylation, phosphorylation, acetylation, and methylation [365].

#### a) Ubiquitinylation

Ubiquitinylation is a highly regulated, flexible and reversible process that mark proteins for degradation, changes in activity and re-localization [366]. Ubiquitinylation of p53 is mainly mediated by mouse double minute 2 (Mdm2) [366, 367] an E3-ubiquitin ligase protein which binds to p53, forming p53 / Mdm2 complex. This complex allows p53 degradation by the proteasome [368-370]. Controversially, the Mdm2 expression is transcriptionally regulated by p53 defining a negative-feedback loop, where p53 increases the expression of Mdm2, and this, promotes p53 degradation as showed in **Figure 15** [367, 371, 372]. Several others E3-ubiquitin ligases have been identified that could play a role of substitute/alternative of Mdm2 such as Cop1, Pirh-1, Pirh-2, Trim24, Arf-BP1, Topors, Chip, Carp1, Carp2, p300, and CBP. However, their role is not already understood [373-375].

Due to this major role of Mdm2 in p53 activation and the critical need for p53 stabilization during stress or cancer development, some studies look for Mdm2

inhibitors [376]. A small molecule Nutlin-3a, which can disrupt the p53 / MDM2 complex, has proven to be highly specific in inducing p53 stabilization in phase I clinical trials with promising results [377-379].



**Figure 15 p53/Mdm2 complex.**

**A)** P53 and MDM2 form a negative-feedback loop, where p53 induces transcription of MDM2, which represses the transcriptional activity and proteasomal degradation of p53, in unstressed cells. **B)** Stress or MDM2 antagonists lead to MDM2 degradation promoting p53 accumulation, resulting in cell cycle arrest and other p53 responses. If the stress can be resolved, the cells may return to the pre-stress state. If the p53 activating signal cannot be alleviated, p53 initiates specific responses, such as apoptosis or senescence [380].

## b) Phosphorylation

Phosphorylation of p53 or Mdm2 can occur on many different sites of these proteins. These modifications can be important in the activation of the p53 network [381]. The regulation and consequences of this phosphorylation are defined by different levels of stimuli for example, during a low DNA-damage, phosphorylation results in partial activation of p53 but in too severe damage, p53 is fully activated and leads to cell death [356, 366, 382-385].

## c) Other modifications

Several other post-translational modifications play an important role in the p53 regulation. Acetylation of p53 occurs on several lysine residues at the C-terminal domain and mainly results in increased DNA binding of p53, promoting the activation of its target gene [386]. Methylation in a specific region of p53 can suppress transcriptional activity of p53 while if it occurs in another region, it can activate p53. Also, some lysine residues of p53 that are modified by acetylation can also be methylated [386-390]. Other modifications of p53, such as prolyl isomerization and

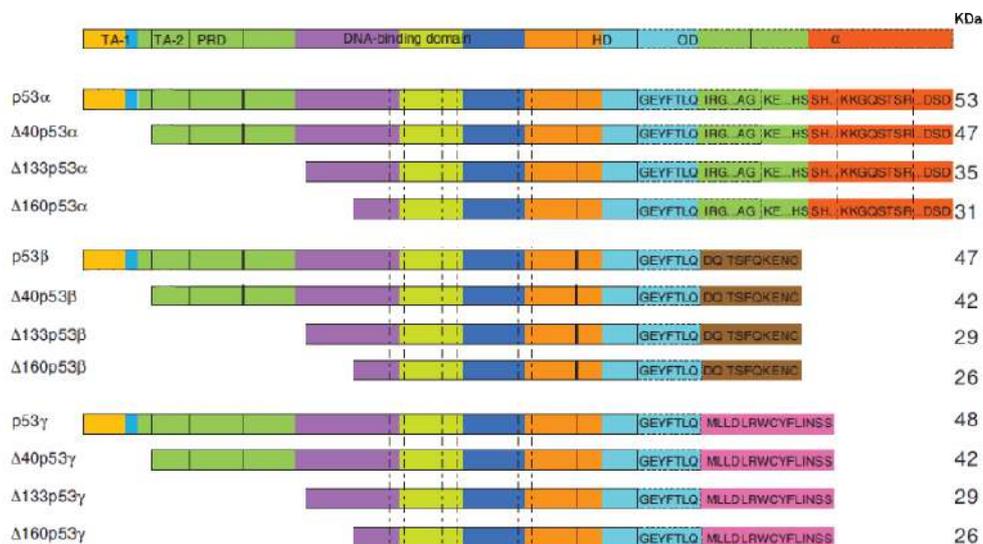
glycosylation, may also contribute to the regulation of p53 stability and activity [356, 366, 391].

## 2.3.2. p53 isoforms

TP53 gene encodes several isoforms of p53 due to the usage of multiple promoters, alternative translation initiation sites, and alternative splicing, as shown in **Figure 16** [392-396].

p53 $\alpha$  is the canonical full-length p53 protein and inclusion of alternative exons gives rise to p53 $\beta$  and p53 $\gamma$  protein isoforms. Some studies reported that these isoforms retain characteristics of a tumor suppressor and both expressions are lost in cancer tumors [392, 397]. Also, changes in the relative abundance of p53 isoforms have been implicated in senescence and aging as p53 $\beta$  was described to modulates p53 $\alpha$  transcriptional activity in response to stress and promotes apoptosis and senescence [392, 398-401].

These multiple p53 isoforms might contribute significantly to the generation of distinct p53 pathways [402]. Altogether, modulate p53 isoform expression and thus cellular response can be a therapeutic target to control p53 pathway.



**Figure 16 Schematic of the p53 isoforms.**

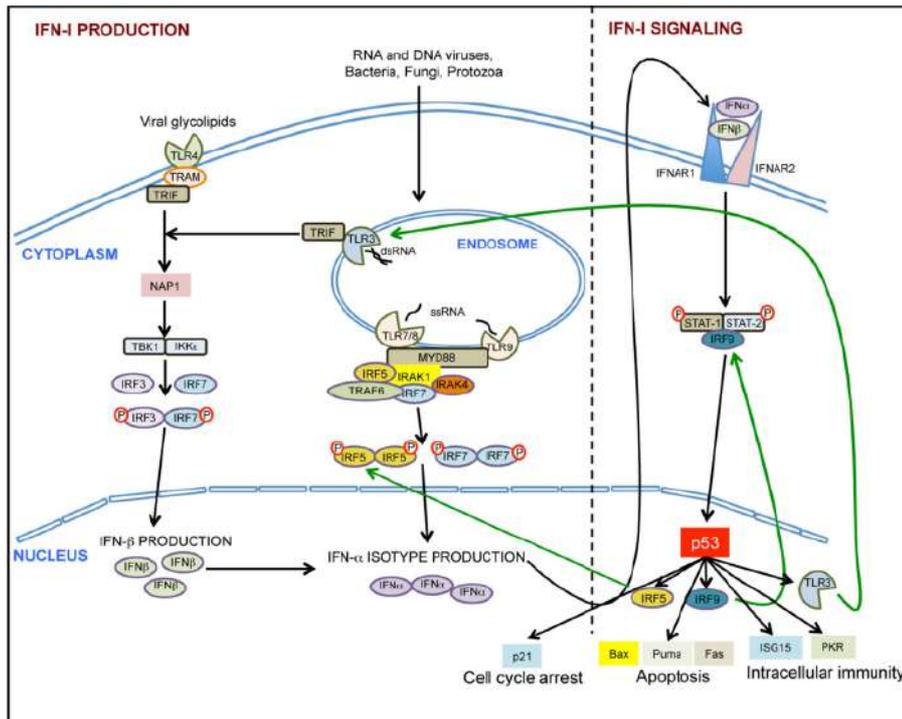
p53 $\alpha$  is composed of two transactivation domains (TA-1 and TA-2), a proline-rich domain (PRD), the DNA-binding domain (DBD), the hinge domain (HD), the oligomerization domain (OD), and a regulatory domain. The theoretical molecular weight of each protein isoform is indicated and the color of the protein domains represents different exon. Adapted from Joruz et. al. [395].

### **2.3.3. Biological responses induced by p53 target genes**

In the past two decades, a huge number of p53 target genes have been identified in various cell types. The classical role of proteins encoded by p53 target genes is apoptosis modulation [403-407] and cell cycle regulation [408]. Apoptosis is a type of programmed cell death activated by a variety of intracellular or extracellular death signals and is a common cellular response to infection. Multiple strategies to modulate, positively or negatively, the apoptotic responses of the host cell have been described for different pathogens [409, 410]. In addition to apoptosis, p53 promotes DNA repair by cell cycle and by modulation of the activity of various DNA repair systems [362].

Nowadays, the non-canonical p53 pathway show modulation in numerous aspects of cellular metabolism [411], inflammation pathway and immune response [348]. p53 changes the activity of multiple metabolic pathways, including glycolysis, mitochondrial oxidative phosphorylation and fatty acid synthesis via transcriptional and non-transcriptional regulation, promoting the preservation of cellular energy under conditions of nutrient restriction [412].

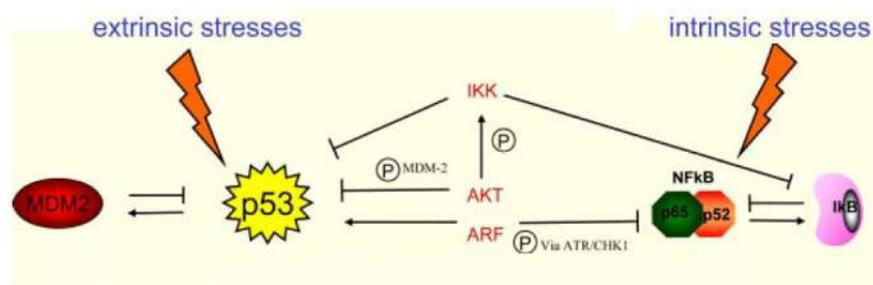
Another non-canonical role of p53 recently described is during inflammation and immune response [345, 413-415]. p53 inactivation in immune cells augments inflammation via multiple pathways, such as enhancing the production of inflammatory cytokines and chemokines in macrophages and promoting the differentiation and function of T cells. p53 also regulates many target genes involved in the immune response creating a loop of amplification of the immune response triggered by infection and mediated by p53. The antiviral role played by p53 limits virus replication, enhances antiviral activity modulated by IFN type I [416], and regulates TLR3 expression and function [417] as represented in **Figure 17**.



**Figure 17 p53 pathway role during the antiviral response.**

Viral activation of TLR 4 promotes IFN expression that induces p53 transcriptional activation. Target genes important for the antiviral response, such as TLR3, IRF5, ISG15, and IRF9, are up-regulated promoting important implications in the immune response. Adapted from Munoz-Fontela [345]

In addition, the most important transcription factor in immune response, NF-κB, is known to have opposite effects with p53. Some studies show that this balance NF-κB / p53 coregulates proinflammatory cytokine secretion [316]. Thus, NF-κB activity often shows a negative correlation with p53 while p53 can also suppress the NF-κB pathway.



**Figure 18 p53 and NF-κB modulation.**

During extrinsic or intrinsic stress, p53 and NF-κB pathway is activated and they can reciprocally regulate each other by different interactions. Adapted from Ak et al.[418]

### 2.3.4. Functional interplay between pathogens and p53

Modulation of p53 is an essential step for pathogens to extend the survival of the host cell, to access nutrients during intracellular replication and to modulate the immune response. However, targeting p53 may compromise the natural safety system of a cell promoting mutations and tumorigenesis, correlating to a number of pathogens that are considered oncogenic [419-421].

The p53 protein was discovered during a study with the oncogenic virus SV-40 which has a protein capable to physically interact with p53 and inhibit p53-mediated transcription [342]. Since then, a large number of other oncogenic and non-oncogenic pathogens were described to interact in different ways with p53.

The strategy of impairing the transcriptional activity of p53 is also used by others viruses, including hepatitis B virus, human T lymphotropic virus, hepatitis C virus and HIV, and some bacteria, like *Neisseria gonorrhoeae* [422, 423].

Another strategy of p53 inhibition was described by different pathogens, for example, human papillomavirus, adenovirus, Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus and some bacteria such as *Shigella flexneri*, *Helicobacter pylori*, and *Chlamydia trachomati* promoting proteasomal degradation [424-427]. In contrast to these pathogens, *Salmonella enterica* and *P.aeruginosa* induces acetylation of p53, which is a stabilizing modification that is associated with cell cycle arrest in infected cells [419, 428]. Some virus and their interactions with p53 pathway are shown in **Table 4**.

| Family                 | Virus               | Viral protein | Interplay with p53                                  | Functional effects                                     | Reference  |
|------------------------|---------------------|---------------|---|--|------------|
| <b>Bornaviridae</b>    | Borna disease virus | p24           | P53 inhibition                                      | Cellular response modification mediated by p53         | [429]      |
| <b>Bunyaviridae</b>    | Hantaan virus       | N             | P53 degradation                                     | Apoptosis prevention                                   | [430]      |
| <b>Coronaviridae</b>   | SARS-CoV            | -             | P53 down-regulation                                 | Inhibition of antiviral activity by p53                | [431]      |
| <b>Flaviviridae</b>    | Zika virus          | -             | P53 activation                                      | Apoptosis induction                                    | [432]      |
| <b>Paramyxoviridae</b> | hPIV-3              | -             | Induced down-regulation of p53                      | Apoptosis prevention                                   | [433]      |
| <b>Picornaviridae</b>  | Poliovirus          | -             | P53 degradation                                     | Inhibition of antiviral activity by p53                | [434]      |
| <b>Poxviridae</b>      | Vaccinia virus      | B1R           | P53 phosphorylation                                 | Cellular response modification mediated by p53         | [435]      |
| <b>Reoviridae</b>      | Avian reovirus      | p17           | P53 phosphorylation                                 | Apoptosis induction                                    | [436]      |
| <b>Retroviridae</b>    | HIV                 | Vif           | Inhibition of Mdm2-p53 interaction                  | Cell cycle arrest                                      | [437]      |
| <b>Rhabdoviridae</b>   | VSV                 | -             | P53 phosphorylation / inhibition of p53 acetylation | Apoptosis induction / Inhibition of antiviral response | [438, 439] |

**Table 4 Virus and their interplay with p53 pathway.**

Functional effects of virus interactions with p53 pathway showing different levels of interaction.

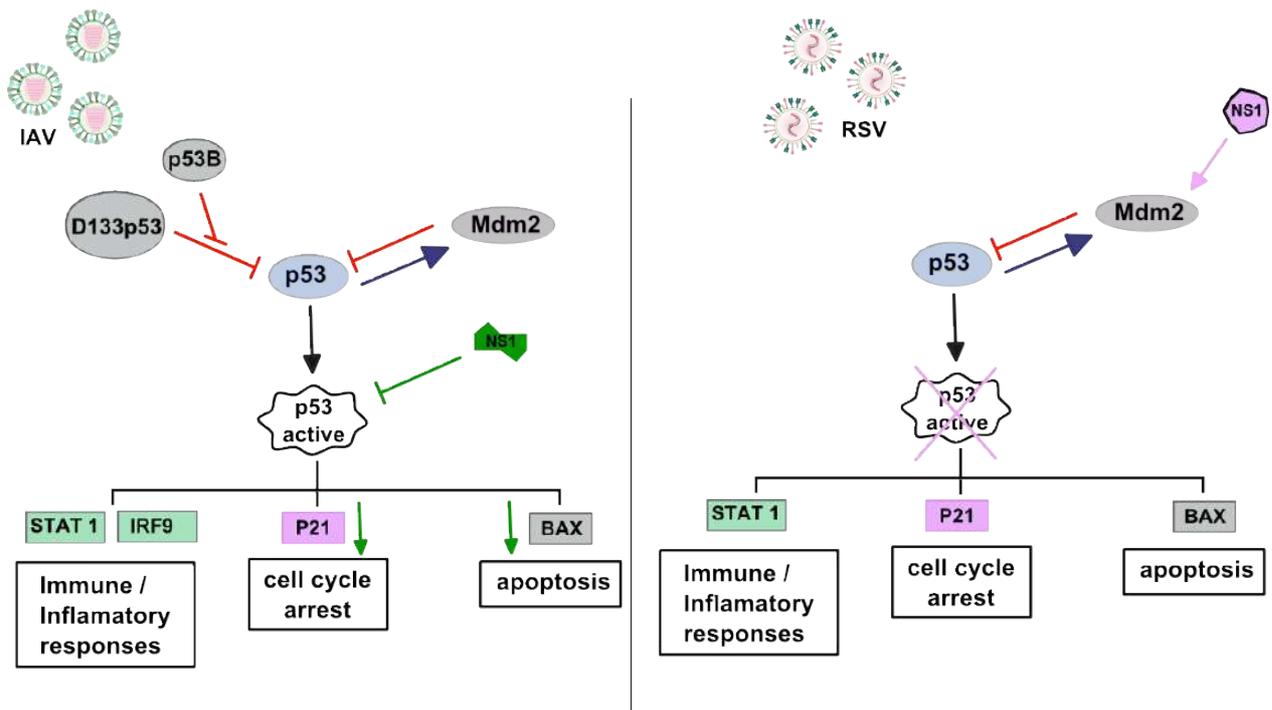
The functional interactions between influenza viruses or RSV and p53 have been reported in a limited number of studies, and infection of influenza viruses significantly alters the gene expression of several host factors belonging to the p53 pathway [438, 440-443].

Influenza viruses and RSV induce apoptosis in numerous cell types and the role of viral proteins which participates in the induction of cell death and cell cycle arrest was described in the literature [444-451].

Several levels of regulation of p53 transcriptional activity are affected during the time course of IAV infection. The global down-regulation of p53 target genes

during initial phases of influenza infection is in accordance with the inhibition of p53 pathway during this transient context [452]. After, IAV induces posttranslational modifications that stabilize the p53 protein throughout the course of infection [410, 452-454]. The enhance of p53 transcriptional activity limited viral replication and has an important role in the induction of the IFN response [410]. The viral protein NS1 participates in the stabilization of p53 [454, 455] while NP protein induces apoptosis and cell death [440]. p53 isoforms are involved in the regulation of these p53-dependent antiviral properties [452, 455]. Infection differentially modulates the expression of  $\Delta 133p53\alpha$  and p53 $\beta$  at both transcriptional and posttranscriptional levels. The modulation of  $\Delta 133p53\alpha$  and p53 $\beta$  isoforms play distinct roles in the viral cycle by acting as regulators of the p53-dependent antiviral activity [396]. Further investigations are needed to better understand p53 pathway during the time-course of infection.

Groskreutz and colleagues have shown that RSV induces the down-regulation of p53 during the time-course of infection, with a consecutive impact on apoptosis and survival of airway epithelial cells [441]. However, the underlying mechanisms of p53 modulation, possibly involving viral proteins, remain to be determined. The NS1 and NS2 RSV proteins play an essential role in suppressing apoptosis and facilitating virus growth through the inhibition of interferon responses [456], NS1 also interacted with components involved in cell cycle regulation and DNA damage repair, promoting a G1-phase arrest in the cell cycle [457]. The F protein triggers p53-dependent apoptosis in the late phase of acute infection [442] while P protein may inhibit apoptosis [458]. This discordance in the literature may reflect cell-specific responses to RSV infection or differences in the strain and infectivity of the virus [433]. The effect of viral modulation in p53 protein may be important targets for therapy in RSV infection. P53 modulation by IAV and RSV are represented in **Figure 19**.



**Figure 19 p53 pathway modulation by IAV and RSV.**

NS1 protein of IAV is capable to interact with p53 protein and inhibit transcriptional activity during infection. Also, an NS1 protein of RSV is capable to inhibit p53 by Mdm2 interaction.

## *OBJECTIVE*

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Ce travail de thèse se base principalement sur les interactions RSV / hôte dans le contexte d'infections simples ou de co-infections, en raison de l'importance clinique de l'infection mixte, du taux élevé de circulation de ces pathogènes et du manque d'arsenal thérapeutique existant.

En effet, il est important de comprendre comment le système immunitaire est modulé à différents niveaux, *in vivo* et *in vitro*, ce qui aide à mieux comprendre les mécanismes pathogéniques, identifier les biomarqueurs dans le contrôle de l'inflammation ou trouver des cibles potentielles pour de nouvelles stratégies antivirales.

This thesis work has mainly based on the interactions RSV / host in the context of simple infections or co-infections, due to the clinical importance of mixed infection, the high rate of circulation of these pathogens and lack of existing therapeutic arsenal.

Indeed, it is important to understand how the immune system is modulated at different levels, *in vivo* and *in vitro*, which helps to better understand the pathogenesis mechanisms, identify biomarkers in the control of inflammation or to find potential targets for new antiviral strategies.

## *RESULTS*

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Les résultats de la thèse sont divisés en trois chapitres pour faciliter la compréhension de chaque groupe de résultats. La connexion entre les chapitres est représentée dans la figure ci-dessous.

Les résultats du chapitre 1 obtenus au Laboratoire des pathogènes émergents (Fondation Mérieux) ont permis de consolider les résultats en cours du groupe et de renforcer le modèle de co-infection des macrophages en plus de favoriser l'identification des biomarqueurs.

Le chapitre 2 était le résultat d'un partenariat avec le laboratoire Virpath, et en profitant de l'expertise de ce groupe, nous visons à approfondir la connaissance de la co-infection dans des mécanismes importants pour la cellule, visant à corrélérer avec la réponse immunitaire trouvée dans le étude des macrophages (chapitre 1).

Le chapitre 3 a été développé en collaboration avec la société Cynbiose (incluant LPE et Virpath), pour examiner les différents niveaux d'interaction proposés et aussi pour appliquer les conclusions obtenues dans les études *in vitro* (chapitres 1 et 2).

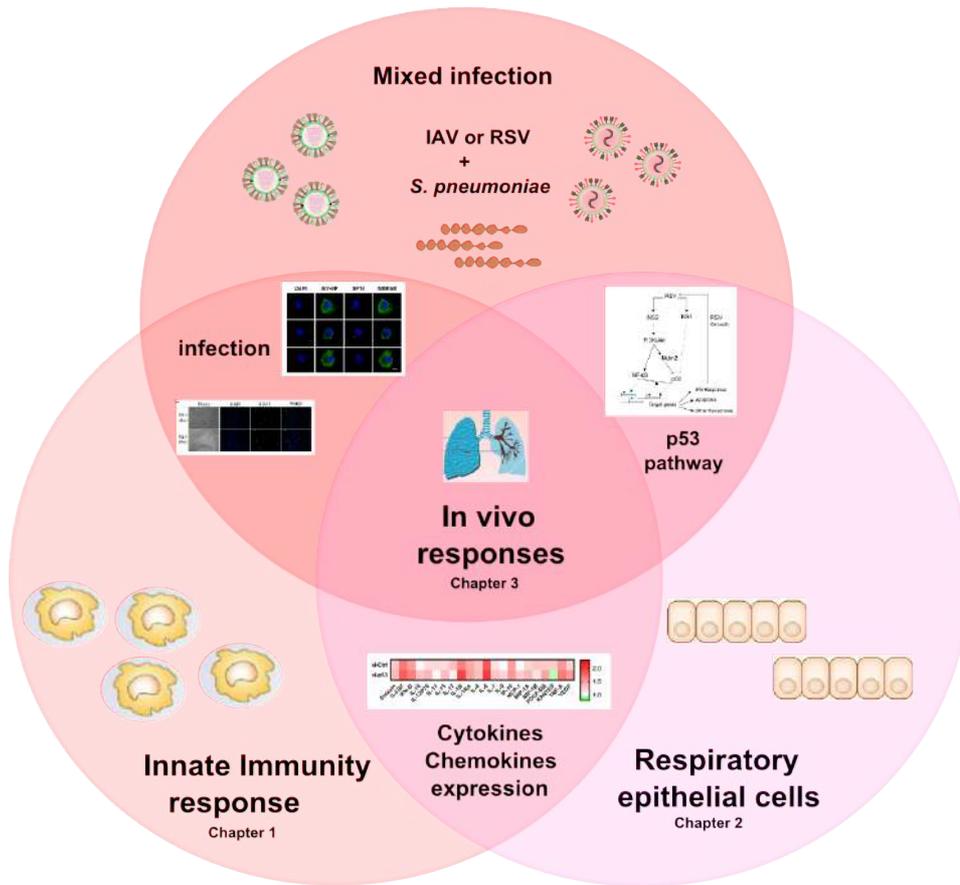
The results of the thesis are divided into three chapters to facilitate comprehension of each group of results. The connection between the chapters is represented in the figure below.

Chapter 1 results obtained at Laboratoire des Pathogenes Emergents (Fondation Mérieux) provided the consolidation of the results that were in progress by the group and strengthened the model of co-infection in macrophages in addition to promoting the identification of biomarkers.

Chapter 2 was the result of a partnership with the Virpath laboratory, and taking advantage of the expertise of this group, we aimed to deepen the knowledge of co-infection in important mechanisms for the cell, aiming to correlate with the immune response found in the macrophages study (chapter 1).

Chapter 3 was developed in collaboration with the company Cynbiose (including LPE and Virpath), to look the different levels of interaction proposed and also to apply the conclusions obtained in the *in vitro* studies (Chapter 1 and 2).

# Lower respiratory tract infection



# **Chapter 1 Host-viral interactions during single or mixed infections: role of innate immune responses**

In this chapter, two works will be presented, in which we observe an innate immune response during viral or mixed infection. The importance of each pathogen (IAV, RSV and *S. pneumoniae*) has already been highlighted by the introduction of this manuscript. These two works highlighted certain aspects of the *in vitro* host innate immune response during exposition by single or mixed infections.

The majority of respiratory viruses, including IAV and RSV, and bacteria are recognized in human airway respiratory cells by TLRs and RIG-I as reviewed in general introduction. This recognition leads to the initial production of antiviral inflammatory response and activation of immune cells like macrophages with an expression of cytokines and chemokines [459-461]. Individually, each pathogen interacts with immune cells and has different strategies to evade host immunity response. Influenza virus and RSV are capable to make modifications in cellular mechanisms during virus replication while *S. pneumoniae* possesses a number of virulence factors which may confound immune responses [462]. Together, viral–bacterial co-infection subverts many aspects of immunity, of which result in a failure to control the pathogens and contributes to severity disease [156, 309, 463-468].

Clinical evidence suggests that infection with particular combinations of pathogens results in the more severe clinical outcome [140, 168]. The most detailed mixed infection described in the literature is Influenza virus and *S. pneumoniae*. Bacterial superinfection after IAV pandemics was described and highlights the importance of Influenza infection in disease severity [147, 323, 469-472]. Thus, in our first study published in *Scientific Reports*, we set-up an optimization in IAV / *S. pneumoniae* macrophages infection and observed a potential biomarker and the mechanism involved in its expression after the double infection on these cells.

Another mixed infection that appears to be very important in disease severity in pediatrics community is RSV and *S. pneumoniae*. Some studies show bacterial superinfection as an important risk factor for hospitalization for RSV infection [128, 129, 473, 474]. Co-infections RSV / *S. pneumoniae* are frequently detected in respiratory diseases and pneumococcal bacterial load and carried serotypes can indicate clinical severity when co-infected with RSV [139-141, 473, 475-479]. Thus, in the second manuscript, we look mixed infection of RSV and pneumococcus in macrophage infection.

The main objective of these studies is to better understand how pathogens interactions and cell infection can lead to disease severity. For this purpose, we establish a macrophages model for each co-infection. Macrophages are strategically situated in the primary lung defense and play a central role in innate host defense. They are capable of initiating innate and adaptive immune responses. In addition, they are a primary source of inflammatory and immunomodulatory cytokines in lungs against invading pathogens. Some studies show that macrophages are able to limit RSV or Influenza virus replication participating in the clearance viral. *In vivo* studies which macrophages are depleted show an enhanced disease in both viral infections [459, 480, 481].

Several studies have also identified an important role for macrophages during the initial phase of host defense against *S. pneumoniae* even if this bacterium has a polysaccharide capsule which limits bacterial phagocytosis by macrophages [322, 323, 482].

The majority of mixed co-infection models described in the literature is realized using respiratory tract cells and focuses on interactions between the pathogens. It is well established that Influenza viruses enhance susceptibility to secondary bacterial infection promoting bacterial superinfection [309, 483]. Also, direct interaction of RSV and *S. pneumoniae* was shown to increase bacterial virulence [159]. In macrophages cells infected with Influenza virus decreased of binds and phagocytosis of *S. pneumoniae* was shown [309, 484-486]. In the literature, some mechanisms of RSV and other bacterial co-infection were already described, but the information is incomplete and scarce mainly when restricting information about RSV and *S. pneumoniae* co-infections is searched.

In addition to better thoughtful the role of macrophages during simple and mixed infection, we also tried to better understand cellular mechanisms and we described a set of potential biomarkers capable to indicate the severity of the disease or the etiology of infection. The relevance of new biomarkers to help diagnostics methods was highlighted in the diagnostic topic of introduction chapter of this manuscript. In our two articles, we confirmed the importance of biomarkers and we contribute to the literature with a description of new biomarkers. Also, to show the

close interactions between mixed infections and macrophages we described the cellular mechanisms and activated-pathway in the expression of these biomarkers.

In the last part of the first article, we performed a translational study to correlates *in vitro* experiments with clinical specimens of children with pneumonia. The correlations between severity of disease and pathogens were shown and confirmed the selected cytokine as a potential biomarker.

The results suggest potential biomarkers to generate an assay that could discriminate between single or mixed infection during pneumonia. Further research is needed to clarify how mixed infection modulates the cytokines/chemokines expression in macrophages. A better understanding of the host immune responses to mixed infections is important for designing vaccines and other preventative agents in the future. This group of results enriches the literature on respiratory infections and the role of macrophages in the response to mixed infection and promotes greater knowledge about the role of IP-10 as a potential biomarker.

**Article 1. Viral and bacterial co-infection in severe pneumonia**

# SCIENTIFIC REPORTS

OPEN

## Viral and bacterial co-infection in severe pneumonia triggers innate immune responses and specifically enhances IP-10: a translational study

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Mixed viral and bacterial infections are widely described in community-acquired pneumonia; however, the clinical implications of co-infection on the associated immunopathology remain poorly studied. In this study, microRNA, mRNA and cytokine/chemokine secretion profiling were investigated for human monocyte-derived macrophages infected *in-vitro* with Influenza virus A/H1N1 and/or *Streptococcus pneumoniae*. We observed that the *in-vitro* co-infection synergistically increased interferon- $\gamma$ -induced protein-10 (CXCL10, IP-10) expression compared to the singly-infected cells conditions. We demonstrated that endogenous miRNA-200a-3p, whose expression was synergistically induced following co-infection, indirectly regulates CXCL10 expression by targeting suppressor of cytokine signaling-6 (SOCS-6), a well-known regulator of the JAK-STAT signaling pathway. Additionally, in a subsequent clinical pilot study, immunomodulators levels were evaluated in samples from 74 children ( $\leq 5$  years-old) hospitalized with viral and/or bacterial community-acquired pneumonia. Clinically, among the 74 cases of pneumonia, patients with identified mixed-detection had significantly higher (3.6-fold) serum IP-10 levels than those with a single detection ( $P = 0.03$ ), and were significantly associated with severe pneumonia ( $P < 0.01$ ). This study demonstrates that viral and bacterial co-infection modulates the JAK-STAT signaling pathway and leads to exacerbated IP-10 expression, which could play a major role in the pathogenesis of pneumonia.

Community-acquired pneumonia (CAP) is a common inflammatory illness of the lungs that remains the major cause of pediatric mortality in low- and middle-income countries<sup>1</sup>. *Streptococcus pneumoniae* (SP) is the main causative agent of pneumonia; however, concomitant viral infection occurs in up to 66% of cases of CAP<sup>2,3</sup>. Clinical data suggest mixed infections are related to a higher severity of inflammatory disease<sup>4,5</sup> - especially in secondary pneumococcal infection following influenza virus (IAV) infection<sup>6-8</sup> - and mixed infection represents a relevant risk factor for pediatric intensive care hospitalization<sup>9</sup>. However, the mechanisms underlying the pathogenesis of mixed viral and bacterial infection remain poorly understood.

Assessment of induced blood immunomodulators during infection may assist clinical diagnosis and the management of severe CAP. Interferon (IFN)- $\gamma$ -induced protein 10 (IP-10/CXCL10) appears to contribute to the

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pathogenesis of several diseases and has been suggested as a potential biomarker of viral infection<sup>10,11</sup>, late-onset bacterial infection in premature infants<sup>12</sup>, and a promising biomarker of sepsis and septic shock<sup>13,14</sup>. Combined analysis of IP-10 and IFN- $\gamma$  has also been reported as a useful biomarker for diagnosis and monitoring therapeutic efficacy in patients with active tuberculosis<sup>15–17</sup>, and both remain detectable in the urine of patients with pulmonary diseases in the absence of renal dysfunction<sup>18</sup>.

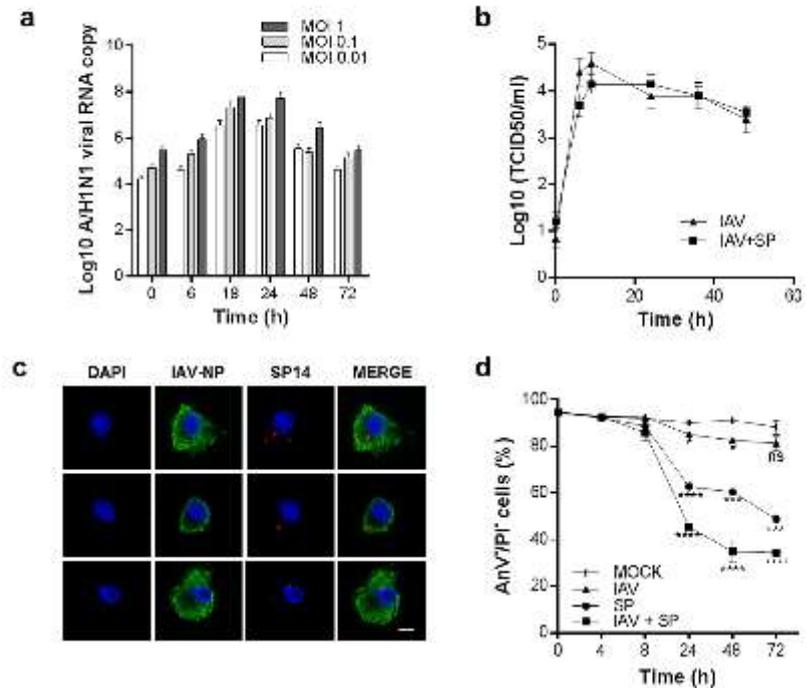
With airway epithelial cells<sup>19</sup>, resident alveolar macrophages (AMs) and blood monocytes-derived macrophages (recruited into tissues under inflammatory conditions<sup>20,21</sup>) represent a major line of defense against both pneumococcal (through their high phagocytic capacity<sup>22–24</sup>) and influenza infection<sup>25,26</sup>. So far, no studies have yet focused on the intracellular mechanisms that regulate IP-10 in human blood leukocytes during mixed IAV and SP infection. Several studies indicated that host non-coding small RNAs (including microRNAs) may function as immunomodulators by regulating several pivotal intracellular processes, such as the innate immune response<sup>27</sup> and antiviral activity<sup>28,29</sup>; both of these processes are closely related to toll-like receptor (TLR) signaling pathways.

In this study, we firstly investigated the *in vitro* intracellular mechanisms that mediate the innate immune response in IAV and/or SP infected human monocyte-derived macrophages (MDMs). Using this approach, we observed that mixed-infection of MDMs induces a synergistic production of IP-10 which can be related to a miRNA-200a/JAK-STAT/SOCS-6 regulatory pathway. Subsequently, in a retrospective analysis of clinical samples collected from children <5 years-old hospitalized with pneumonia, we confirmed that serum IP-10 level could be related to both viral and/or bacterial etiologies and disease severity.

## Results

**Characteristics of MDMs infected by IAV and/or SP.** Initially, we investigated *in vitro* the impact of single and mixed IAV and SP infection on MDMs. Firstly, active replication of IAV was assessed by qRT-PCR and quantification of new infectious viral particles in the cell supernatants (Fig. 1a,b). IAV titer increased over time after single infection with IAV and correlated with increased production of negative-strand IAV RNA. Maximum viral replication was observed at 18–24 hours post-infection, after which time both RNA replication and the quantity of infectious particles decreased. In this *in vitro* model, subsequent challenge of IAV-infected MDMs with SP had no significant impact on the production of new infectious viral particles (Fig. 1b). Together, these results indicate permissive and productive infection of MDMs by IAV. Secondly, we evaluated whether MDMs are permissive for both IAV and SP infection. The presence of pneumococci within IAV- and SP-infected primary MDMs was confirmed at 8 h post-infection (Fig. 1c), suggesting that MDMs are permissive for viral and bacterial co-infection in the early steps of infection. Importantly, confocal co-detection of mixed IAV and SP was only effective following 8 h post-infection due to the bactericidal impact of SP internalization within human macrophages (after 24 h, data not shown). Thirdly, we evaluated the impact of single and mixed infection with IAV and SP on MDM viability. Mixed infection significantly decreased cell viability ( $65.2 \pm 4.5\%$  total cell death at 48 hours post-infection;  $P < 0.0001$ ) compared to single SP and IAV infection ( $39.6 \pm 1.7\%$  and  $17.4 \pm 1.1\%$  total cell death, respectively; Fig. 1d). Taken together, these results confirmed human MDMs are permissive to mixed viral and bacterial infection.

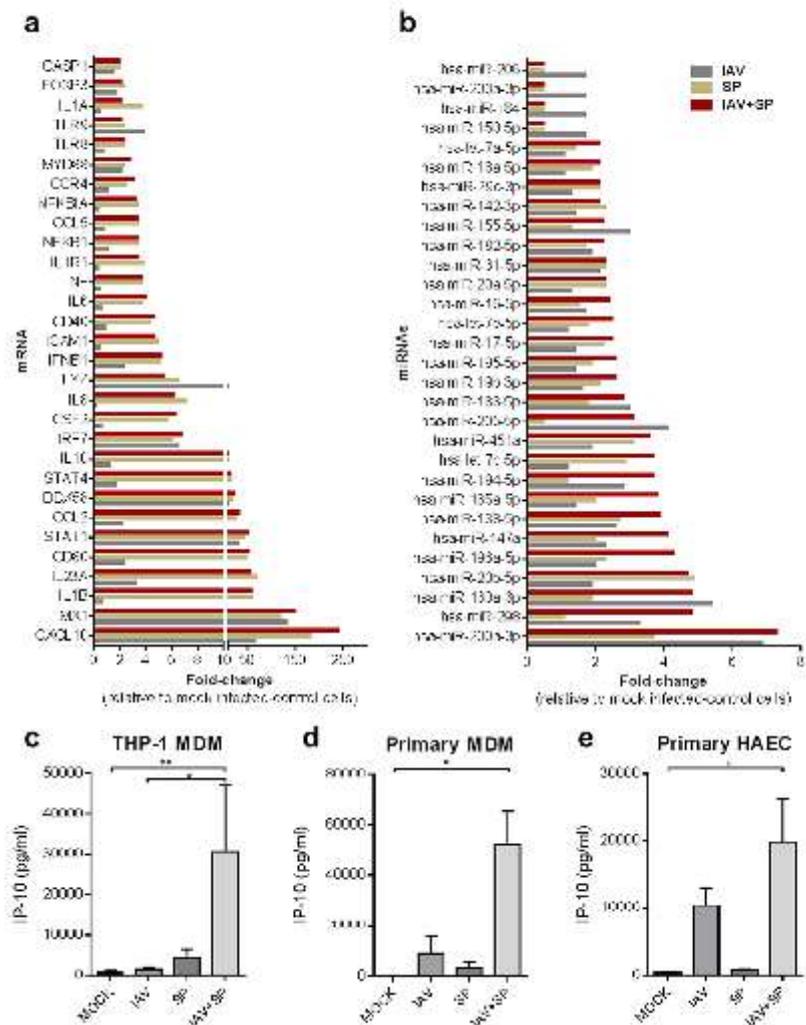
**mRNA, microRNA and protein expression profiling reveal an overall induction of the host innate immune response following IAV and/or SP infection of MDMs.** To investigate the innate immune response orchestrated by IAV- and SP-infected human MDMs, we firstly evaluated the expression of 84 genes involved in the innate and adaptive immune responses (Table S1); the major differentially-expressed genes are summarized in Fig. 2a. Expression profiling indicated an overall induction of genes related to the JAK-STAT, NF- $\kappa$ B and TLR signaling pathways. Indeed, all interferon-stimulated genes (ISGs) screened, including *CXCL10* (fold-change [FC] = 240.9), *CCL-2* (FC = 34.2) and *MX-1* (FC = 151.4) were upregulated following mixed infection compared to uninfected cells, most of which are closely related to *STAT-1* (FC = 52.3), *IRF-7* (FC = 6.8) and *IFNB1* (FC = 5.2) also found upregulated in mixed infected cells. Secondly, we investigated the endogenous microRNA expression profiles of IAV- and SP-infected MDMs. A selection of microRNAs that were found to be differentially-expressed under different infection conditions are shown in Fig. 2b and Table S2. miRNA-200a-3p was overexpressed after both single IAV (FC = 6.9), single SP (FC = 3.7) and mixed IAV/SP infection (FC = 7.3), indicating this miRNA may play a role in the innate immune response to viral and bacterial co-infection. Similar miRNA-200a-3p dysregulation profiles were obtained following IAV and/or SP infections of human macrophages-like (THP-1 monocytes-derived macrophages) or primary MDMs (data not shown). Thirdly, the secreted levels of various antiviral, pro-inflammatory and immunomodulatory cytokines/chemokines were assayed in IAV- and SP-infected- THP-1 and primary MDM cell supernatants. We observed a remarkable correlation between the mRNA and protein expression profiles of single or mixed infected MDMs especially regarding CXCL-10 and IP-10 expression. Indeed, the level of IP-10 was synergistically increased in the supernatant of IAV-infected THP-1 MDMs exposed to SP (mean:  $30,589 \pm 16,484$  pg ml<sup>-1</sup>) compared to single IAV infection ( $1,439 \pm 566.5$  pg ml<sup>-1</sup>) and single SP infection ( $4,472 \pm 2,001$  pg ml<sup>-1</sup>;  $P < 0.05$ ; Fig. 2c) at 24 hours after infection. In those cells, IP-10 expression reduced over time (48 to 72 hours), coinciding with a significant higher proportion of necrotic and apoptotic cells (Fig. 1d). The synergistic expression of IP-10 was similarly observed at 24 hours post-infection using primary MDMs (Fig. 2d). Significantly increased secretion of the other tested cytokines and chemokines was not observed post-infection, even in mixed infected MDMs (Fig. S1). Interestingly, a significant production of IP-10 was also observed in supernatants of primary human airway epithelial cells (HAEC) mixed-infected by IAV and SP compared to the single infections (Fig. 2e). Taken together, the mRNA and protein profiling results suggested that mixed viral and bacterial infection of MDMs induces a synergistic pro-inflammatory response related to the type-1 interferon and JAK-STAT signaling pathways, with IP-10 as signature of IAV/SP co-infection. Among all microRNAs screened, miR-200a-3p was the most



**Figure 1.** Single and mixed infection of human monocyte derived-macrophages (MDMs) by IAV and SP. (a) MDMs were infected with IAV at an MOI of 0.01, 0.1 or 1 and total RNA was extracted at 0, 6, 18, 24, 48 and 72 hours post-infection. The IAV A/H1N1 hemagglutinin (HA) gene was amplified by real-time qRT-PCR. (b) Influenza A/H1N1 viral load (TCID<sub>50</sub>) in the supernatant of single IAV-infected MDMs or IAV-infected MDMs following subsequent challenge with SP. (c) Confocal imaging of IAV and SP in mixed-infected MDMs at 8 hours post-IAV infection and 4 hours post-SP infection. DAPI, nuclear stain, blue. IAV, Influenza A virus nucleoprotein stain, green. SP, *S. pneumoniae* stain, red. Scale bar = 5  $\mu$ m. (d) Impact of single and mixed IAV and SP infection on MDM cell viability. Statistical analyses were performed using two-way ANOVA with Tukey's post-hoc test; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ .

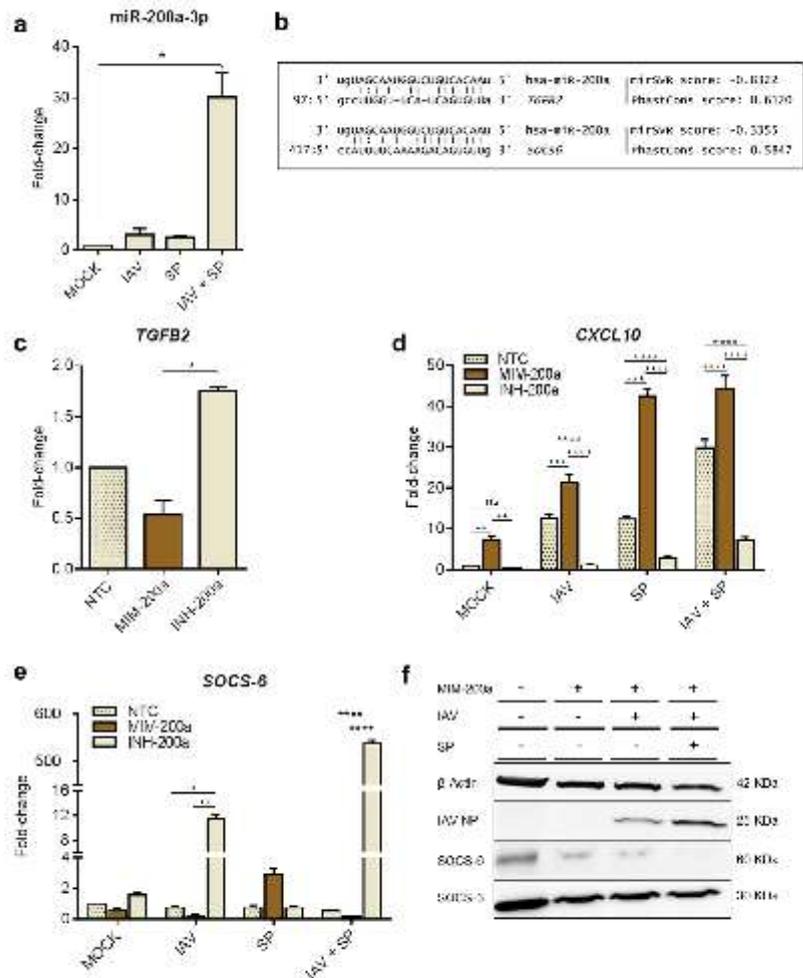
overexpressed in IAV/SP co-infection of human MDMs. In the remainder of this study, we decided to investigate the interconnection between miR-200a-3p expression and the innate immune response.

**Endogenous miRNA-200a-3p expression correlates with CXCL10 (IP-10) induction following mixed IAV and SP infection of human MDMs.** Using a specific Taqman probe assay targeting miR-200a-3p, we confirmed a significant upregulation of miR-200a-3p following mixed IAV and SP infection of human MDMs (Fig. 3a). In this experiment, a more marked up-regulation of miR-200a-3p was observed following IAV + SP compared to results obtained previously (Fig. 2b). This discrepancy has been attributed to the use of two different approaches to quantify miR-200a-3p expression. The use of a target-specific stem-loop reverse transcription primer in Fig. 3a allows a better sensitivity of miR-200a-3p detection compared to the non-specific fluorescent dye used in Fig. 2b. As the general trend was suggestive of a synergistic induction of miR-200a-3p in response to mixed infection (Fig. 3a), we hypothesized microRNA-200a-3p may play a role in the regulation of CXCL10 (IP-10), which was also synergistically upregulated in mixed-infected MDMs (Fig. 2c and d) and primary HAEC (Fig. 2e). To test this hypothesis, we investigated the effects of overexpressing (MIM-200a) or inhibiting (INH-200a) microRNA-200a in MDMs. As *TGF $\beta$ 2* is known to be targeted by miR-200a-3p (Fig. 3b, *in silico* alignment), we monitored its expression to assess the efficiency of mimicking/inhibiting miR-200a-3p in THP-1 MDMs. As expected, *TGF $\beta$ 2* mRNA was significantly downregulated in MIM-200a-transfected MDMs ( $PC = 0.57$ ) and upregulated in INH-200a-transfected MDMs ( $PC = 1.70$ ), compared to mock-transfected cells (Fig. 3c). Overexpressing miR-200a-3p (MIM-200a) in single and mixed IAV/SP-infected cells enhanced the levels of CXCL10 at 24 h post-infection, whereas partial inhibition (INH-200a) downregulated the expression of



**Figure 2.** Investigation of host mRNA, miRNA and protein expression levels during IAV and/or SP infection of MDMs or HAEC. (a,b,c) THP-1 MDMs were infected with IAV (MOI = 1) for 4h before SP (MOI = 1) was added. Total cellular mRNA (a) or miRNA (b) were extracted 24h post-infection, reverse-transcribed and amplified using specific RT<sup>2</sup> profiler PCR Arrays. Major differentially expressed mRNA or miRNAs are shown. (c-e) THP-1 MDMs, primary human MDMs or primary airway epithelial cells (HAEC) were infected with IAV (MOI = 1) for 4h before SP (MOI = 1) was added. The concentration of IP-10 in cell supernatant (24h post-infection) was determined by a multiplex immunoassay on a FlexMap3D Luminex platform. Values represent mean  $\pm$  SEM of four (c) biological replicates, four (d) independent experiments with different donors, three (e) biological replicates from one donor. Statistical analyses for each panel of experiments (c-e) were performed using a Kruskal-Wallis test (non-parametric, one-way ANOVA with Dunn's post-hoc test); \* $P < 0.05$ ; \*\* $P < 0.01$ .

*CXCL10* (Fig. 3d). These results suggested miR-200a-3p indirectly regulates *CXCL10* and led us to hypothesize that miR-200a-3p controls a potential repressor of the JAK-STAT signaling pathway.



**Figure 3. Functional analysis of the synergistic induction of miR-200a-3p in MDMs after mixed infection with IAV and SP.** (a) Preceding IAV infection elevates miR-200a-3p expression after mixed infection with SP. MDMs were infected with IAV for 4 hours, then infected with SP, incubated for 20 h and total endogenous miRNAs were purified. A specific PCR-probe assay targeting miR-200a-3p was used to assess the fold changes in miR-200a-3p expression in mock-transfected and infected cells. (b) *In silico* predictive target alignment showing that miR-200a-3p targets the 3' UTRs of both *TGFβ2* and *SOCS6*. (c) *TGFβ2*, (d) *CXCL10* and (e) *SOCS6* expression profiles in MDMs transfected with negative transfection control (NTC), miR-200a mimic (MIM-200a) or miR-200a inhibitor (INH-200a). At 18 h after transfection, the MDMs were singly or mixed infected as described previously. At 8 h post-IAV and/or SP infection, total mRNA was extracted and amplified by PCR using specific primers for the indicated genes. Values represent median ± IQR (a, c) or mean ± SEM (d, e) of three biological replicates. Statistical analyses were performed using a Kruskal-Wallis test (non-parametric, one-way ANOVA with Dunn's post-hoc test) for data presented in (a, c). An ordinary two-way ANOVA (with Tukey's post-hoc multiple comparison test) was used for data presented in (d, e). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . (f) Western blot analysis of SOCS-6, SOCS-3, IAV nucleoprotein (NP) and β-actin expression in MDMs transfected with negative transfection control (NTC), miR-200a mimic (MIM-200a) or miR-200a inhibitor (INH-200a), cultured for 18 h, and then infected as described previously. Cell lysates were harvested 24 hours post-infection.

**MiRNA-200a-3p indirectly regulates IP-10 expression by targeting SOCS6.** As shown in Fig. 2a, several JAK-STAT signaling pathway genes were deregulated in mixed IAV- and SP-infected human MDMs; therefore, we hypothesized that miR-200a-3p directly regulates a regulator of the JAK-STAT signaling pathway. Predictive target analysis indicated that the 3' UTR of suppressor of cytokine signaling-6 (SOCS6) may be targeted by miR-200a-3p (Fig. 3b). SOCS proteins constitute a class of negative regulators of JAK-STAT signaling pathways that are induced by both cytokines and TLR signaling. MiRNA-200a-3p was not predicted to target any of the other six members of the SOCS gene family. Transfection of human MDMs with MIM-200a downregulated SOCS6 (FC = 0.57) while inhibition of miR-200a-3p (INH-200a) upregulated SOCS6 (FC = 1.55), confirming that miR-200a-3p effectively regulates the expression of SOCS6 (Fig. 3c). Moreover, SOCS6 was synergistically downregulated in IAV- or IAV/SP-infected MDMs overexpressing miRNA-200a (Fig. 3e), suggesting that both infection and miR-200a-3p negatively regulate the expression of SOCS6. Finally, western blotting confirmed that expression of SOCS-6 sharply reduced following infection, especially after mixed IAV and SP infection (Fig. 3f). These results indicate miR-200a-3p is strongly induced in response to mixed viral and bacterial co-infection, which in turn leads to downregulation of the JAK-STAT regulator SOCS-6 at both the mRNA and protein levels and subsequent upregulation of IP-10.

**Clinical investigation of innate immune response related to pneumonia etiology.** The *in vitro* analyses demonstrated mixed IAV and SP infection of human MDMs and HAEC induced significant production of IP-10. As blood leukocytes and respiratory tract epithelial cells actively contribute to inflammation during pneumonia, we hypothesized the level of IP-10 in serum of patient with pneumonia may be both indicative of mixed respiratory infection and disease severity. As part of a prospective, hospital-based, multicenter case-control study on the etiology of pneumonia among children under 5-years-old, a total of 74 patients (44 male, 30 female) were included in this pilot evaluation. According to WHO guidelines, retrospective analysis indicated 44 (59.5%) children had clinical signs of non-severe pneumonia and 30 (40.5%) children had signs of severe pneumonia. The main patient characteristics at inclusion are shown in Table 1. Patients with severe pneumonia had significant more recorded episodes of dyspnea ( $P < 0.001$ ), cyanosis ( $P = 0.03$ ), lower chest indrawing ( $P < 0.001$ ), dullness to percussion ( $P < 0.001$ ) and lethargy ( $P < 0.001$ ) during chest examination than patient with non-severe pneumonia. Moreover, pleural effusions were significantly more observed among critically ill patients and the duration of hospitalization was significantly longer for the children with severe pneumonia than for those with non-severe pneumonia ( $P = 0.0015$ ). Two deaths occurred within the group of children retrospectively defined with severe pneumonia. Evaluation of the systemic inflammatory response of the 74 cases is shown in Table 2. Serum level of CRP, IP-10, PCT, G-CSF, IL-6, IL-8 and MIP-1 $\beta$  were significantly more elevated in serum samples from critically ill patients. Patients with severe pneumonia had significantly higher (4.2-fold) serum IP-10 levels than those with a non-severe pneumonia ( $P < 0.001$ ) suggesting IP-10 as a promising prognostic marker in pneumonia. Diagnostic accuracy measures for predicting pneumonia severity using blood-based biomarkers are summarized in Table S3. Briefly, in this study, the optimal IP-10 cut-off value for identifying patient with severe pneumonia was  $4,240 \text{ pg ml}^{-1}$ , with an area under the receiver operating characteristic curve of 0.69 (95% CI, 0.57 to 0.82,  $P < 0.001$ ). Defining as positive a serum IP-10 level above this cut-off resulted in a sensitivity of 63.3%, specificity of 63.6% and a positive likelihood ratio of 1.74. Prognostic values of IP-10 were close to procalcitonin (PCT; AUC = 0.70; 95% IC, 0.58 to 0.82,  $P < 0.001$ ) and IL-6 (AUC = 0.70; 95% IC, 0.58–0.83,  $P < 0.001$ ).

**IP-10 is significantly associated with viral and bacterial co-detection and pneumonia severity.** Multiplex PCR-based screening of respiratory and blood samples reveal a high variety of pathogen associations (Table 3). Respiratory viruses were detected in the nasal aspirates (NAs) of 63/74 patients (85.1%). Etiological bacteria of pneumonia (*S. pneumoniae*,  $n = 19$ ; *S. aureus*,  $n = 1$ ; or *H. influenzae* type B,  $n = 7$ ) were identified via real-time PCR in the blood samples of 27/74 (36.5%) of the patients. Multiplex PCR assays allowed the identification of respiratory bacteria in the blood of 19 patients with negative blood culture results. Among the 74 cases PCR-positive for respiratory pathogens, a single virus or bacteria were detected in the NAs of 7 (9.4%) and 3 (4.0%) patients, respectively; these 10/74 (13.5%) cases were defined as the single infection group. The mixed infection group included the 62/74 (83.8%) cases in which (1) multiple viruses and/or bacteria were identified in NAs (38/74; 51.3%) without any bacteria identified in blood samples or (2) one or more viruses and/or bacteria were identified in NAs and associated with a blood bacteremia (24/74; 32.4%). We evaluated whether IP-10 serum level could correlate with the viral and bacterial etiologies of pneumonia. Patients with mixed infection had significant higher (3.6-fold) IP-10 serum level than patient with single detection ( $P = 0.03$ ; Table 4). A stratified analysis reveals that the highest IP-10 serum level was observed among patients with both several respiratory pathogens identified (mixed-detection group) and severe pneumonia ( $14,427 \text{ pg ml}^{-1}$ , IQR (3,981–82,994)). In detail, a remarkable IP-10 serum level ( $142,531 \text{ pg ml}^{-1}$ ), representing 33-fold higher above cut-off value predicting pneumonia severity was observed in patient with hRV in NA co-detected with *S. pneumoniae* (serotype 14) in pleural effusion and blood. In concordance with our *in vitro* model of co-infection, a significant IP-10 level ( $90,338 \text{ pg ml}^{-1}$ ) was quantified in blood sample of patient with severe bacteremic pneumococcal (serotype 14) pneumonia with a positive co-detection of Influenza B virus in NA. Taken together, these results suggest that high serum IP-10 levels are significantly associated with mixed viral and bacterial detection and also related to pneumonia pathogenesis.

## Discussion

This study provides additional *in vitro* and clinical data to improve our understanding of the immunopathology of mixed viral and bacterial pneumonia (Fig. 4).

The *in vitro* model of influenza and pneumococcal superinfection of human MDMs demonstrated that mixed infection synergistically induced release of the pro-inflammatory chemokine IP-10, strongly suggesting human

| Patient characteristics                                   | NON-SEVERE PNEUMONIA<br>n = 44 |               | SEVERE PNEUMONIA<br>n = 30 |               | P           |
|---|--------------------------------|---------------|----------------------------|---------------|-------------|
| Age, months, median (IQR)                                 | 13.5                           | (6–26.7)      | 14                         | (8–28)        | 0.78        |
| Weight, kg, median (IQR)                                  | 9.4                            | (7.5–12.2)    | 10.5                       | (8.3–12.5)    | 0.41        |
| Gender, male  | 20                             | 45.5          | 24                         | 80.0          | <0.01       |
| <b>Clinical examination</b>                               |                                |               |                            |               |             |
| Axillary temperature, °C, median (IQR)                    | 37.6                           | (36.8–38.3)   | 37.9                       | (37–38)       | 0.56        |
| Breathing rate, cycles/min., median (IQR)                 | 49.5                           | (44.2–56.7)   | 56                         | (48.8–64.5)   | <b>0.02</b> |
| Cardiac rate, cycles/min., median (IQR)                   | 140.5                          | (127.8–152.8) | 152.5                      | (130.5–160.5) | <b>0.01</b> |
| Neutrophils, %, median (IQR)                              | 68                             | (56.5–78)     | 70                         | (54.5–78.5)   | 0.98        |
| Oxygen saturation, %, median (IQR)                        | 94                             | (92–97.7)     | 95                         | (90–96)       | 0.45        |
| <b>Chest examination</b>                                  |                                |               |                            |               |             |
| Cough, n (%)  | 44                             | 100           | 29                         | 96.7          | 0.41        |
| Dyspnea, n (%)  | 33                             | 75.0          | 30                         | 100.0         | <0.01       |
| Cyanosis, n (%)   | 2                              | 4.5           | 7                          | 23.3          | <b>0.03</b> |
| Lower chest indrawing, n (%)                              | 28                             | 63.6          | 29                         | 96.7          | <0.001      |
| Dullness to percussion, n (%)                             | 3                              | 6.8           | 12                         | 40.0          | <0.001      |
| Pulmonary crackles, n (%)                                 | 31                             | 70.5          | 27                         | 90.0          | 0.05        |
| Inability to drink, n (%)                                 | 3                              | 6.8           | 5                          | 16.7          | 0.26        |
| Vomiting, n (%)   | 6                              | 13.6          | 5                          | 16.7          | 0.75        |
| Prostration or lethargy, n (%)                            | 0                              | 0.0           | 18                         | 60.0          | <0.0001     |
| Convulsions, n (%)  | 0                              | 0.0           | 1                          | 3.3           | 0.41        |
| <b>Chest radiography interpretation</b>                   |                                |               |                            |               |             |
| Pleural effusion with parenchymatous infiltrate, n (%)    | 4                              | 9.1           | 15                         | 50.0          | <0.001      |
| Pleural effusion without parenchymatous infiltrate, n (%) | 1                              | 2.3           | 3                          | 10.0          | 0.30        |
| Generalized dense homogenous opacification, n (%)         | 1                              | 2.3           | 4                          | 13.3          | 0.15        |
| Localized dense homogenous opacification, n (%)           | 21                             | 47.7          | 11                         | 36.7          | 0.47        |
| Interstitial syndrome, n (%)                              | 19                             | 43.2          | 8                          | 26.7          | 0.22        |
| Alveolar infiltrate, n (%)                                | 6                              | 13.6          | 1                          | 3.3           | 0.23        |
| Pneumothorax, n (%)                                       | 1                              | 2.3           | 0                          | 0.0           | 1.00        |
| <b>Medical history</b>                                    |                                |               |                            |               |             |
| Heart condition, n (%)                                    | 6                              | 13.6          | 0                          | 0.0           | 0.08        |
| Lung disease, n (%)                                       | 12                             | 27.3          | 9                          | 30.0          | 0.80        |
| Tuberculosis, n (%)                                       | 1                              | 2.3           | 1                          | 3.3           | 1.00        |
| Asthma, n (%)   | 1                              | 2.3           | 1                          | 3.3           | 1.00        |
| Contracted ILP, n (%)                                     | 14                             | 31.8          | 15                         | 50            | 0.15        |
| Pneumococcal conjugate vaccine, n (%)                     | 11                             | 25.0          | 4                          | 13.3          | 0.25        |
| DPT–Hep. B–10b vaccine: 1 dose, n (%)                     | 28                             | 63.6          | 22                         | 73.3          | 0.45        |
| DPT–Hep. B–10b vaccine: 3 doses, n (%)                    | 20                             | 45.5          | 18                         | 60.0          | 0.24        |
| Influenza A/H1N1 vaccine                                  | 4                              | 9.1           | 8                          | 26.7          | 0.06        |
| <b>Hospitalization follow-up</b>                          |                                |               |                            |               |             |
| Pneumonia complication, n (%)                             | 5                              | 11.4          | 14                         | 46.7          | <0.001      |
| Length of hospitalization, days, median (IQR)             | 3                              | (2–5)         | 7                          | (3.8–12.3)    | <0.001      |
| Death, n (%)  | 0                              | 0.0           | 2                          | 6.7           | 0.16        |

**Table 1.** \*Within the two previous weeks before study inclusion. Significant *P* values (<0.05) are in bold.

blood leukocytes contribute to the immunopathology of pneumonia. Additionally, transcriptomics and omics analyses provided new data on the inflammatory pathways that are activated during mixed infection and related to synergistic induction of the pro-inflammatory chemokine IP-10 in mixed infected cells. Our observations are consistent with a recent study describing IP-10 induction as host-proteome signature of both viral and bacterial infections<sup>30</sup>. Of the differentially-expressed genes observed in mixed infected MDMs, the transcription factors *STAT-1* and *IRF-7* appear to play crucial roles in the regulation of interferon-stimulated genes including *CXCL10* (IP-10). By focusing on the intracellular mechanisms that regulate inflammatory pathways, we demonstrated a novel role for miRNA-200a-3p in the regulation of *CXCL10* (IP-10). These observations are consistent with previous reports showing that RNA virus infection upregulates miR-155 in macrophages and dendritic cells and also regulates suppressor of cytokine signaling 1 (*Socs1*), suggesting the existence of a miRNA/JAK-STAT/SOCS regulatory pathway during viral infection<sup>29</sup>. Our study suggests co-infection leads to overexpression of miR-200a-3p, which in turn targets and downregulates the JAK-STAT regulator *Socs-6* and consequently increases *CXCL10* (IP-10) expression. Interestingly, a complementary *in-silico* approach reveals that several microRNAs that were found dysregulated in our experiments of IAV and SP co-infection of MDMs or HAEC, might target several genes

| Blood-based biomarkers | NON-SEVERE PNEUMONIA n = 44 | SEVERE PNEUMONIA n = 30   | P               |
|------------------------|-----------------------------|---------------------------|-----------------|
| CRP*                   | 36<br>(12–96)               | 192<br>(24–192)           | <b>0.01</b>     |
| IP-10                  | 2,090<br>(1,033–8,040)      | 8818<br>(1,384–43,886)    | <b>&lt;0.01</b> |
| PCT                    | 0.3<br>(0.1–0.9)            | 1.46<br>(0.5–5.6)         | <b>&lt;0.01</b> |
| G-CSF                  | 62.6<br>(0–833)             | 684.2<br>(0–4,209)        | <b>0.03</b>     |
| IFN- $\gamma$          | 84.9<br>(0–391.8)           | 149.8<br>(0–308)          | <b>0.69</b>     |
| IL-10                  | 15.6<br>(0–72.4)            | 24.2<br>(0–87.1)          | 0.53            |
| IL-12p70               | 211.1<br>(84.8–411)         | 305<br>(93.2–407.9)       | 0.31            |
| IL-13                  | 10.9<br>(5.4–20.4)          | 13<br>(8.3–31.5)          | 0.17            |
| IL-1 $\beta$           | 1.9<br>(0.3–6.9)            | 4.4<br>(1.1–8.9)          | 0.18            |
| IL-1RA                 | 472<br>(116.2–1,015)        | 466.6<br>(165.9–1,289)    | 0.47            |
| IL-6                   | 116.4<br>(41.1–376.5)       | 559.8<br>(139.9–2,129)    | <b>&lt;0.01</b> |
| IL-7                   | 53<br>(31.1–84.1)           | 51.6<br>(17.3–76.0)       | 0.43            |
| IL-8                   | 49.1<br>(21.2–146.6)        | 117.2<br>(28.3–393.5)     | <b>0.03</b>     |
| IL-9                   | 51.4<br>(21.5–92.5)         | 68.8<br>(24.4–97.6)       | 0.37            |
| MCP-1                  | 26<br>(0–203.8)             | 108.1<br>(0–672.7)        | 0.19            |
| MIP-1 $\beta$          | 169.6<br>(127.6–251.1)      | 260<br>(150.5–381.3)      | <b>0.01</b>     |
| PDGF-BB                | 16,773<br>(9,624–28,393)    | 14,111<br>(7,055–26,165)  | 0.29            |
| RANTES                 | 34,286<br>(24,326–48,594)   | 36,218<br>(25,565–53,832) | 0.55            |
| VEGF                   | 1,741<br>(577.4–4,633)      | 3,100<br>(1,400–5,913)    | 0.11            |

**Table 2.** \*Only CRP data are expressed in mg L<sup>-1</sup> otherwise in pg ml<sup>-1</sup>. Values are expressed as median (IQR) in pg ml<sup>-1</sup>. Differences between groups were compared using unpaired Mann-Whitney tests; significant changes ( $P < 0.05$ ) are in bold.

of SOCS family and play similar role than miR-200a-3p. Indeed, miRNA-142-3p might target SOCS4, 5, 6 mRNA while miRNA-194-5p might target SOCS2, 3, 4, 5 and 7 mRNA. These observations underline that intra-cellular regulation of IP-10 is not limited to the contribution of a sole microRNA. A complex inter-relationship between numerous host microRNAs and inhibitors of the JAK-STAT signaling pathway occur to control host innate inflammatory response against viral and/or bacterial infections.

Clinically, the majority of pediatric CAP cases in this study were associated with both positive viral and/or bacterial detection. Respiratory microorganisms were detected in 97% of cases; 51.3% of which were viral-viral, viral-bacterial or bacterial-bacterial co-detected only in nasal aspirates, 32.4% of which co-detected in both nasal aspirates and blood samples. These data are consistent with previous etiological studies of pediatric CAP<sup>33–35</sup>. *S. pneumoniae* was the major bacteria identified in blood (19/74; 25.7%) and mainly co-detected with respiratory viruses in NAs (16/19; 84.2%). We observed a very high diversity of viral and bacterial associations in biological samples from children with pneumonia. In comparison with IAV and SP14 combination evaluated *in-vitro*, no pneumonia cases were singly influenza and pneumococcus infected, and no similar co-detection with those two pathogens has been clinically observed. Nevertheless, Influenza B (IVB) virus was identified in 5 patients and two of them had a positive SP co-detection in blood (one non-typable strain and one serotype 14 using our molecular typing test). IVB and SP14 combination seems to be the nearest pathogen co-detection to that *in-vitro* investigated. Clinically, this co-detection was associated with both a very high IP-10 expression and a very severe pneumonia case definition. Interestingly, our translational pilot evaluation reveals IP-10 expression can be induced by several different viral and/or bacterial combinations. As immune response to each pathogen is different, further *in-vitro* investigations using different pathogens associations are needed to better characterize the mechanisms involved in the immunopathology of pneumonia.

In this cohort, highest serum IP-10 levels were identified among patients with both several pathogen detected and severe pneumonia, suggesting a significant role of IP-10 on pneumonia pathogenesis. Indeed, high plasma levels of IP-10 have previously been reported in patients with sepsis<sup>12</sup>, and were associated with high mortality rate, especially among patients with CAP<sup>34</sup>. Additionally, the IP-10-CXCR3 axis has been related to acute immune lung injury and lymphocyte apoptosis during the development of severe acute respiratory syndrome (SARS)<sup>36,36</sup>. Moreover, an *in vivo* study that modeled influenza and pneumococcal superinfection in mice indicated that pro-inflammatory chemokines, including IP-10, play a crucial role in influenza-induced susceptibility to lung neutrophilia, severe immunopathology and mortality<sup>37</sup>. In this study, markedly elevated IP-10 (92,809 pg ml<sup>-1</sup>) combined with the highest PCT level (74.4 pg ml<sup>-1</sup>) were quantified in the serum sample of a child who died, in whom *S. pneumoniae* (serotype 9V) was identified in the blood (PCR and blood culture) and co-detected with *Haemophilus influenzae* type B in nasal aspirate. These observations suggest an interrelationship between co-detection, elevated serum IP-10 and the pathogenesis of pneumonia.

Several limitations of this pilot translational study need to be acknowledged before concluding mixed infection is related to elevated IP-10 and disease severity. Indeed, although viral shedding (e.g., of HRV and HBoV) is common in asymptomatic children, we were unable to evaluate the levels of immunomodulators in the serum samples of a control group. Moreover, although the samples were collected within the first 24 hours after admission, only a single blood sample was processed for each patient. Therefore, a larger, longitudinal study on the etiology and severity of pneumonia will be necessary to confirm these results. In conclusion, the present findings

| Group   | Viral and bacterial associations                               | n  | Description of viral and/or bacterial associations   |
|---|--|--|--|
| No etiology   | No pathogen detected   | 2  | -  |
| Single detection  | V <sub>nasal</sub>   | 7  | (AdV) <sub>nasal</sub> , n=1; (EV) <sub>nasal</sub> , n=1; (HRV) <sub>nasal</sub> , n=2; (RSV) <sub>nasal</sub> , n=1; (PIV) <sub>nasal</sub> , n=2  |
|   | B <sub>nasal</sub>   | 3  | (Sp_6AB) <sub>nasal</sub> , n=2; (Sp_22F) <sub>nasal</sub> , n=1   |
| Multiple detection of pathogen (in nasal aspirates)                   | xV <sub>nasal</sub>  | 1  | (HCoV_NL63 + HBoV) <sub>nasal</sub> , n=1  |
|   | xB <sub>nasal</sub>  | 3  | (Sp_3 + Sp_35F) <sub>nasal</sub> , n=1; (Sp_14 + Sp_19F) <sub>nasal</sub> , n=1; (Sp_3 + Sa) <sub>nasal</sub> , n=1  |
|   | V <sub>nasal</sub> + xB <sub>nasal</sub>                       | 10   | (HRV + Sp_6AB + Sp_5) <sub>nasal</sub> , n=1; (HMPV + Sp_9V + Sp_10A) <sub>nasal</sub> , n=1; (EV + Sp_6AB + Sp_9V) <sub>nasal</sub> , n=1; (HCoV_OC43 + Sp_7c + Sp_14) <sub>nasal</sub> , n=1; (HRV + Mp + Sp_NT) <sub>nasal</sub> , n=1; (RSV + Sa + Sp_19F) <sub>nasal</sub> , n=1; (AdV + Hib + Sp_14) <sub>nasal</sub> , n=1; (HRV + Sa + Sp_14) <sub>nasal</sub> , n=3   |
|   | xV <sub>nasal</sub> + B <sub>nasal</sub>                       | 8  | (HRV + AdV + Sa) <sub>nasal</sub> , n=1; (RSV + IVB + Sp_NT) <sub>nasal</sub> , n=1; (PIV-4 + HCoV_OC43 + Sp_NT) <sub>nasal</sub> , n=1; (HMPV + EV + Sp_16F) <sub>nasal</sub> , n=1; (AdV + HCoV_229E + Sp_14) <sub>nasal</sub> , n=1; (HMPV + HCoV_229E + AdV + Sp_16F) <sub>nasal</sub> , n=1; (IAV 2009 pdm + RSV + Sp_19F) <sub>nasal</sub> , n=1; (HBoV + HCoV_HKU + Sp_4) <sub>nasal</sub> , n=1  |
|   | xV <sub>nasal</sub> + xB <sub>nasal</sub>                      | 4  | (HBoV + IVB + PIV_1 + EV + AdV + Sp_6A + Sp_34) <sub>nasal</sub> , n=1; (PIV_3 + HMPV + Sp_16F + Sp_22F) <sub>nasal</sub> , n=1; (IVB + PIV_3 + Sp_Sg18 + Sp_20 + Sp_35B) <sub>nasal</sub> , n=1; (HPIV_4 + HRV + AdV + Hib + Sp_NT) <sub>nasal</sub> , n=1  |
|   | V <sub>nasal</sub> + B <sub>nasal</sub>                        | 12   | (HRV + Sp_NT) <sub>nasal</sub> , n=1; (HRV + Sa) <sub>nasal</sub> , n=1; (HRV + Sp_14) <sub>nasal</sub> , n=2; (RSV + Sp_6AB) <sub>nasal</sub> , n=3; (EV + Sp_19A) <sub>nasal</sub> , n=1; (RSV + Sp_23F) <sub>nasal</sub> , n=1; (AdV + Sp_14) <sub>nasal</sub> , n=2; (HCoV_OC43 + Sp_NT) <sub>nasal</sub> , n=1  |
| Multiple detection of pathogen (in nasal aspirates and blood samples) | B <sub>nasal</sub> + B <sub>blood</sub>                        | 2  | (Sp_14) <sub>nasal</sub> + (Sp_14) <sub>blood</sub> , n=1; (Sa) <sub>nasal</sub> + (Sp_NT) <sub>blood</sub> , n=1  |
|   | xB <sub>nasal</sub> + B <sub>blood</sub>                       | 1  | (Hb + Sp_9V) <sub>nasal</sub> , n=1 + (Sp_9V) <sub>blood</sub> , n=1   |
|   | V <sub>nasal</sub> + xB <sub>nasal</sub>                       | 2  | (PIV_3) <sub>nasal</sub> , n=1 + (Sp_NT + Hib) <sub>nasal</sub> , n=2  |
|   | V <sub>nasal</sub> + B <sub>nasal</sub>                        | 1  | (HRV) <sub>nasal</sub> + (Sp_20) <sub>nasal</sub> , n=1  |
|   | xV <sub>nasal</sub> + B <sub>nasal</sub>                       | 1  | (RSV + HRV) <sub>nasal</sub> + (Hb) <sub>nasal</sub> , n=1   |
|   | V <sub>nasal</sub> + B <sub>nasal</sub> + B <sub>blood</sub>   | 7  | (HRV) <sub>nasal</sub> + (Sp_NT) <sub>nasal</sub> + (Sp_NT) <sub>blood</sub> , n=1; (HBoV) <sub>nasal</sub> + (Hb) <sub>nasal</sub> + (Hb) <sub>blood</sub> , n=1; (HRV) <sub>nasal</sub> + (Sp_NT) <sub>nasal</sub> + (Sp_14) <sub>nasal</sub> , n=2; (PIV_3) <sub>nasal</sub> + (Sp_14) <sub>nasal</sub> + (Sp_14) <sub>blood</sub> , n=1; (EV) <sub>nasal</sub> + (Sp_NT) <sub>nasal</sub> + (Sa) <sub>nasal</sub> , n=1; (Hb) <sub>nasal</sub> + (Sa) <sub>nasal</sub> + (Sp) <sub>nasal</sub> , n=1 |
|   | xV <sub>nasal</sub> + B <sub>nasal</sub> + B <sub>blood</sub>  | 3  | (HBoV + RSV + IVB) <sub>nasal</sub> + (Sp_6AB) <sub>nasal</sub> + (Sp_NT) <sub>nasal</sub> , n=1; (IVB + PIV_4) <sub>nasal</sub> + (Sa + Sp_NT) <sub>nasal</sub> + (Sp_NT) <sub>blood</sub> , n=1; (HBoV + HCoV_HKU) <sub>nasal</sub> + (Sp_14) <sub>nasal</sub> + (Sp_NT) <sub>nasal</sub> , n=1  |
|   | xV <sub>nasal</sub> + xB <sub>nasal</sub> + B <sub>nasal</sub> | 2  | (IVB + HRV) <sub>nasal</sub> + (Sp_14 + Sp_6AB) <sub>nasal</sub> + (Sp_14) <sub>nasal</sub> , n=1; (HRV + AdV) <sub>nasal</sub> + (Sa + Sp_6AB + Sp_8) <sub>nasal</sub> + (Hb) <sub>nasal</sub> , n=1  |
|   | V <sub>nasal</sub> + B <sub>nasal</sub> + xB <sub>nasal</sub>  | 2  | (RSV) <sub>nasal</sub> + (Sp_17F) <sub>nasal</sub> + (Hb) <sub>nasal</sub> , n=1; (HRV) <sub>nasal</sub> + (Sp_7F) <sub>nasal</sub> + (Sp_NT) <sub>nasal</sub> , n=1   |
|   | V <sub>nasal</sub> + xB <sub>nasal</sub> + B <sub>nasal</sub>  | 1  | (HRV) <sub>nasal</sub> + (Sa + Sp_14) <sub>nasal</sub> + (Sp_14) <sub>nasal</sub> , n=1  |
|   | V <sub>nasal</sub> + B <sub>nasal</sub> + B <sub>blood</sub>   | 1  | (HBoV) <sub>nasal</sub> + (Sp_3) <sub>nasal</sub> + (Sp_3) <sub>blood</sub> , n=1  |
| V <sub>nasal</sub> + B <sub>nasal</sub> + B <sub>blood</sub>          | 1  | (HRV) <sub>nasal</sub> + (Sp_14) <sub>nasal</sub> + (Sp_14) <sub>blood</sub> , n=1 |  |
| TOTAL   |  | 74   |  |

**Table 3.** V: Virus; B: Bacteria; NA: Nasal aspirate; BI: Blood; PE: Pleural effusion; x: several. AdV: Adenovirus; EV: Enterovirus; HRV: Human rhinovirus; RSV: Respiratory syncytial virus; PIV: Parainfluenza virus; HCoV: Human coronavirus; HBoV: Human bocavirus; HMPV: Human metapneumovirus; IAV 2009 pdm: 2009 pandemic Influenza virus A/H1N1; IVB: Influenza virus B; Sp: *Streptococcus pneumoniae*, serotype 6 AB, 6A, 3, 4, 34, Sg18, 20, 23 F, 17 E, 35B, 35 E, 14, 9 V, 19 E, 10 A, 7c, 16 E, 19 A; NT: Non-typable; Sa: *Staphylococcus aureus*; Mp: *Mycoplasma pneumoniae*; Hib: *Haemophilus influenzae* type b.

suggest that mixed respiratory infections and IP-10 may play major, interconnected roles in the pathogenesis of pneumonia. Clinically, assessment and monitoring of induced IP-10 serum level may assist clinicians to improve diagnosis and patient management of severe community-acquired pneumonia.

**Materials and Methods**

**Viral and bacterial strains.** The seasonal influenza A/H1N1 virus (IAV; A/Solomon Islands/03/06) was obtained from the National Influenza Reference Center (Lyon, France). Encapsulated *Streptococcus pneumoniae* serotype 14 (SP) was obtained from the National Reference Center for Streptococci, Department of Medical Microbiology, University Hospital, Aachen, Germany.

**Cell culture conditions.** Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of four healthy donors (female/male ratio: 1:3; range of age: 21–65 years) using Ficoll-Paque plus (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) and monocytes were purified using magnetic CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated CD14+ cells was consistently > 90%, as determined by flow cytometry (Accuri C6; BD Biosciences, San Jose, California, USA). Primary MDMs were obtained after 6 days of differentiation in RPMI-1640 glutamax supplemented with 10% heat-inactivated FBS and

10 ng ml<sup>-1</sup> M-CSF (Miltenyi Biotec). THP-1 MDMs were obtained by culturing cells with 10 ng ml<sup>-1</sup> phorbol myristate acetate (PMA; Invivogen, Toulouse, France) for 72 hours. Human airway epithelial cells (HAEC, bronchial cell type) originated from a 54-years old woman with no pathology reported (batch number MD056501) were provided by Mucilair (Epithelix, Geneva, Switzerland). Sterility, tissue integrity (TEER), mucus production and cilia beating frequency have been certified by the company.

**Multiplex real-time qRT-PCR co-detection of IAV and SP.** Multiplex real-time qRT-PCR was performed to quantify IAV (*H1* hemagglutinin gene, provided by Pr AD Osterhaus from the Department of Virology, Erasmus Medical Center, 3015 GE Rotterdam, Netherlands) and SP (autolysin A gene, *LytA*, unpublished home-made primers and probe design) in MDMs. The primers and probes (*H1-F* 5'-GAATAGCCCCACTACAATTGGGTAA-3'; *H1-R* 5'-GTAATTCGCATTCTGGGTTTCT-3'; *H1-P* 5'-FAM-AAGATCCATCCGGCAACGCTGCA-BHQ1-3' and *LytA-F* 5'-GCGGAAAGACCCAGAAATTAG-3'; *LytA-R* 5'-GAATGGCTTCAATCAGTTCAAC-3'; *LytA-P* 5'-Cy5-TCTcAGcATcCCaACCGC-BHQ-2-3') were multiplexed using the AgPath-ID™ One-Step RT-PCR kit (Life Technologies; Carlsbad, California, USA) according to the manufacturer's instructions; the cycling program was adjusted to 50 °C for 15 minutes; 95 °C for 10 minutes; 40 cycles of 8 seconds at 95 °C (denaturation) and 34 seconds at 50 °C with fluorescence detection (annealing and extension). Absolute quantification of viral hemagglutinin and pneumococcal *LytA* copy number were calculated using the cycle threshold (Ct) values and in-house standard curves of A/H1N1 influenza virus and *S. pneumoniae* serotype 14.

**Co-detection of IAV and SP by immunofluorescence.** Single or mixed infected cells were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes at room temperature, permeabilized in PBS-0.1% Triton X-100, and incubated with PBS containing 1% BSA and 3% human serum to block non-specific binding. Mouse monoclonal anti-Influenza A Virus Nucleoprotein (#ab20921; Abcam, Cambridge, United Kingdom) and goat anti-mouse IgG H&L (AF-488, #A11029; Life Technologies) were used to detect IAV. Rabbit polyclonal anti-*Streptococcus pneumoniae* antibody (#ab20429; Abcam) and goat anti-rabbit IgG H&L (AF-647, #ab150083; Abcam) were used to detect SP. Cells were imaged using a Leica DMI 3000B microscope equipped for fluorescence imaging.

**Gene expression profiling.** Total cellular mRNA was purified using the RNeasy kit (Qiagen, Hilden, Germany). Reverse-transcription of total mRNA was performed using the RT<sup>2</sup> First Strand Kit (SABiosciences, Hilden, Germany). The expression of 84 genes involved in the human innate and adaptive immune responses was evaluated using the RT<sup>2</sup> profiler™ PCR Array (SABiosciences) according to the manufacturer's recommendations. The  $\Delta\Delta Ct$  method was applied to calculate the fold changes in gene expression for each gene relative to uninfected control cells using the web-based RT<sup>2</sup> profiler PCR Array Data Analysis software (SABiosciences).

**MicroRNA profiling array.** Total cellular microRNAs were purified using the miRNeasy Mini kit (Qiagen) and reverse-transcribed using the miScript Reverse Transcription kit (Qiagen). The profiling of 84 miRNAs was performed using the Human Immunopathology miScript miRNA PCR Array kit (Qiagen) according to the manufacturer's instructions. Data were analyzed using the miScript miRNA PCR array data analysis web portal.

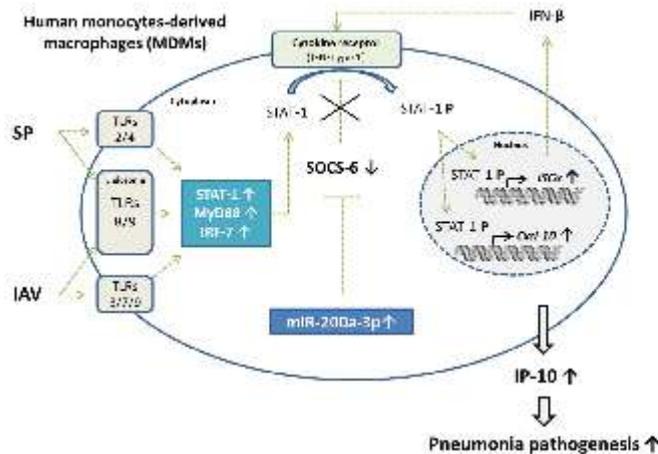
**In silico miRNA target prediction.** miRNA target genes were retrieved and compiled using TargetScan<sup>®</sup> and microRNA.org resource<sup>39</sup>. The interactions between miRNAs and intracellular pathways were predicted using DIANA-miRPath v2.0<sup>40</sup>.

**Monocyte-derived macrophage (MDM) co-infection model.** Human primary CD14<sup>+</sup> or THP-1 MDMs were seeded in 24-well plates (0.5 × 10<sup>6</sup> per well) in triplicate, exposed to Influenza A H1N1 (A/Solomon islands/3/2006) virus (IAV) under serum-free conditions for 1 hour and then cultured for 3 hours in fresh RPMI-1640 containing 2% FBS. *Streptococcus pneumoniae* (SP) serotype 14 was added at 4 hours after IAV infection. Gentamicin (10 µg ml<sup>-1</sup>) was added 2 hours after SP infection (i.e. 6 hours post-influenza infection) and maintained in the culture media throughout the experiment to kill extracellular bacteria and limit bacterial growth. Cell viability was determined by flow-cytometry using the FITC/Annexin V apoptosis detection kit (BD Biosciences), according to the manufacturer's instructions.

**Functional analysis of miR-200a-3p.** For miR-200a-3p inhibition/mimic assays, 1 × 10<sup>6</sup> THP-1 MDMs were transfected with either 30 nmol miRvana<sup>®</sup> hsa-miR-200a-3p inhibitor (#4464084), hsa-miR-200a-3p mimic (#4464066), miRNA Inhibitor Negative Control #1 (#4464077) or miRNA Mimic Negative Control #1 (#4464058; all Life Technologies) in OptiMEM (Life Technologies) using 3 µl Invitrogen RNAiMAX Lipofectamine/well in 12-well plates. After 18 hours, cells were washed and infected as previously described. The efficiency of the miR-200a-3p inhibitor/mimic was evaluated 24 hours after mixed infection by purifying total miRNAs (mirvana<sup>®</sup> miRNA Isolation Kit, Ambion, Life Technologies), reverse transcription and specific amplification of miR-200a-3p using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies, #4366596) combined with a specific miRNA probe assay (Life Technologies, #4427975). In this assay, fold changes have been defined by the  $\Delta\Delta Ct$  method using control RNU-44 and -48 as reference microRNAs. Total mRNA was purified from transfected and infected MDMs using the RNeasy kit (Qiagen) and specific primers were used to amplify transforming growth factor beta-2 (*TGFβ2*; F: 5'-CCATCCCGCCCACTTCTAC-3', R: 5'-AGCTCAATCCGTTGTTTCAGGC-3'), *SOCS6* (F: 5'-AAGAATTCATCCCTTGGATTAGTAAC-3', R: 5'-CAGACTGGAGGTCGTGGAA-3')<sup>41</sup>, *CXCL10* (IP-10; F: 5'-GTGGCATTCAAGGAGTACCTC-3', R: 5'-TGATGGCCTTCGATTCTGGATT-3') and β-Actin (F: 5'-CTCTTCCAGCCTTCTCTCT-3', R: 5'-AGCACTGTGTTGGCGTACAG-3'). Rabbit

|                                  | NON-SEVERE PNEUMONIA (n=44) |        |       |                  | SEVERE PNEUMONIA (n=30) |        |        |                  | TOTAL (n=74) |         |       |                  | P      |
|----------------------------------|-----------------------------|--------|-------|------------------|-------------------------|--------|--------|------------------|--------------|---------|-------|------------------|--------|
|                                  | n                           | (%)    | IP-10 | (IQR)            | n                       | (%)    | IP-10  | (IQR)            | n            | (%)     | IP-10 | (IQR)            |        |
| No respiratory pathogen detected | 2                           | (4.5)  | 652.1 | (637.6–666.6)    | 0                       | (0.0)  | 0      | 0                | 2            | (2.7)   | 652.1 | (637.6–666.6)    | —      |
| Single detection                 | 5                           | (11.4) | 2,932 | (903.8 – 10,388) | 5                       | (16.7) | 1,296  | (938.8 – 1,307)  | 10           | (13.5)  | 1,300 | (974.9 – 4,237)  | 0.53   |
| Multiple detection of pathogen   | 35                          | (79.5) | 2,167 | (1,173 – 7,953)  | 25                      | (83.3) | 14,427 | (3,981 – 82,994) | 62           | (83.8)  | 4,743 | (1,445 – 14,570) | < 0.01 |
| TOTAL                            | 44                          | (50.5) | 2,090 | (1,033 – 8,040)  | 30                      | (40.5) | 8,818  | (1,384 – 43,886) | 74           | (100.0) | 3,312 | (1,239 – 12,512) | < 0.01 |

**Table 4.** \*IP-10 values are expressed in  $\text{pg ml}^{-1}$ . IP-10 concentration differences between groups were compared using unpaired Mann-Whitney tests; significant changes ( $P < 0.05$ ) are in bold.



**Figure 4.** IAV and SP co-infection of MDMs exacerbates induction of IP-10: putative role for miR-200a-3p in regulating the JAK-STAT signaling pathway. Both the *in vitro* and clinical studies indicated IP-10 is associated with mixed viral/bacterial infection in pediatric community-acquired pneumonia. *In vitro*, miR-200a-3p was synergistically induced following mixed IAV/SP infection of human MDMs and found to indirectly regulate *CXCL10* (IP-10) expression by targeting the suppressor of cytokine signaling-6 gene (*SOCS6*), a well-known repressor of the JAK-STAT signaling pathway. *In vivo*, IP-10 was significantly elevated in the serum samples of pediatric patients with mixed viral/bacterial severe pneumonia compared to patients with single detection ( $P = 0.05$ ) and non-severe pneumonia ( $P < 0.01$ ).

polyclonal antibodies against  $\beta$ -actin (# ab8227), SOCS-6 (#ab13950), SOCS-3 (#ab16030) and goat polyclonal anti-influenza A/H1N1 (#ab119978, all Abcam) were used for Western-blotting.

**Multiplex immunoassay of inflammatory mediators.** Serum samples and cell culture supernatants were screened for the presence of 27 human cytokines and chemokines using the Bio-Plex Pro Human Cytokine Standard 27-Plex kit (Bio-Rad, Hercules, California, USA) on a FLEXMAP 3D<sup>®</sup> analyzer (Luminex, Austin, Texas, USA).

**Community-acquired pneumonia cohort.** As part of a prospective, hospital-based, multicenter case-control study on the etiology of pneumonia among children under 5-years-old (study protocol published in<sup>42</sup>), a total of 74 patients (44 male, 30 female) admitted to the Hospital Pediátrico Niños de Acosta Ñu, San Lorenzo, Paraguay, between 2010–2013 were included in our study. Briefly, pneumonia cases were defined by the presence of 1) cough and/or dyspnea, and 2) tachypnea, as defined by the World Health Organization (WHO) (age < 2 months:  $\geq 60$ /minute; age 2–11 months:  $\geq 50$ /minute; age 1–5 years:  $\geq 40$ /minute)<sup>43</sup>, and 3) absence of wheezing at auscultation, and, 4) first symptoms appearing within the last 14 days, and 5) radiological confirmation of pneumonia as per WHO guidelines<sup>44</sup>. Based on these primary criteria defining pneumonia cases, all 74 cases were retrospectively re-evaluated according to the WHO “Pocket book of hospital care for children”<sup>45</sup> criteria to evaluate pneumonia severity. Cases that died during the study, or who had at least one additional clinical signs including central cyanosis, dullness to percussion during chest examination, prostration/lethargy, pleural effusion observed on chest radiography were retrospectively included in the severe pneumonia group. Patients without any of these additional clinical signs were included in the non-severe pneumonia group.

**Clinical and molecular analysis.** Nasopharyngeal aspirates (NAs) and whole blood samples were collected from children within 24 hours of admission. Whole blood samples were used for complete blood counts, blood culture and multiplex real-time PCR to identify *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae* type B<sup>46</sup>. *S. pneumoniae* serotypes were defined using a 11 multiplex real-time PCR assay targeting the 40 most frequently represented serotypes or serogroups according to protocol developed by Messaoudi *et al.*<sup>47</sup>. Serum C-reactive protein (CRP; AssayPro, St. Charles, Missouri, United States) and Procalcitonin (PCT; VIDAS R.R.A.H.M.S; bioMérieux) were quantified from whole-blood samples. Multiplex real-time non quantitative PCR (Fast-Track Diagnostic, Sliema, Malta) was used to detect 19 viruses and five bacteria in the respiratory specimens (NAs and pleural effusions). Mixed detection was defined as 1) PCR-positive for multiple viruses in NAs, 2) positive blood culture or PCR-positive for multiple bacteria in blood or 3) PCR-positive for one or multiple viruses in NAs and one or multiple bacteria in blood (identified by PCR and blood culture).

**Ethical approval.** The study protocol, informed consent statement, clinical research form, any amendments and all other study documents were submitted to and approved by the Ethical Committee of the Instituto de Investigaciones en Ciencias de la Salud, the Universidad Nacional de Asunción (IICS-UNA) and the Hospital Pediátrico Niños de Acosta Nu. Informed consent was obtained from all subjects involved in this study. The clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

**Statistical analysis.** The Chi-square test and Fisher's exact test were used to compare categorical variables; continuous variables and non-normally distributed data were compared using the Mann-Whitney *U*-test; normally distributed data were compared using unpaired *t*-tests. Comparative analyses between experimental conditions (i.e., MOCK, IAV, SP or IAV + SP) were performed using one-way ANOVA with Tukey's post-hoc test or Kruskal-Wallis analysis with Dunn's post-hoc tests. Receiver operating curve (ROC) analysis was used to determine the optimal cut-off value for IP-10 to differentiate between non-severe and severe pneumonia cases.  $P < 0.05$  was considered statistically significant. All statistical analyses were performed using GraphPad Prism (La Jolla, California, United States).

## References

- Walker, C. L. E. *et al.* Global burden of childhood pneumonia and diarrhoea. *The Lancet* **381**, 1405–1416 (2013).
- Telles, J.-N. *et al.* Viral and bacterial pathogens identification in children hospitalised for severe pneumonia and parapneumonic empyema. *Pneumonia* **1**, 11–19 (2012).
- Honkinen, M., Lahti, E., Osterback, R., Ruuskanen, O. & Waris, M. Viruses and bacteria in sputum samples of children with community-acquired pneumonia. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **18**, 300–307 (2012).
- Jennings, L. C. *et al.* Incidence and characteristics of viral community-acquired pneumonia in adults. *Thorax* **63**, 42–48 (2008).
- Bello, S. *et al.* Inflammatory response in mixed viral bacterial community-acquired pneumonia. *BMC Pulm. Med.* **14**, 123 (2014).
- Morens, D. M., Taubenberger, J. K. & Fauci, A. S. Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness. *J. Infect. Dis.* **198**, 962–970 (2008).
- Madhi, S. A. & Klugman, K. P. A role for *Streptococcus pneumoniae* in virus-associated pneumonia. *Nat. Med.* **10**, 811–813 (2004).
- McCallers, J. A. Insights into the interaction between Influenza Virus and *Pneumococcus*. *Clin. Microbiol. Rev.* **19**, 571–582 (2006).
- Richard, N. *et al.* The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. *Pediatr. Infect. Dis. J.* **27**, 213–217 (2008).
- Sumino, K. C. *et al.* Detection of respiratory viruses and the associated chemokine responses in serious acute respiratory illness. *Thorax* **65**, 639–644 (2010).
- Quint, J. K. IP-10 as a biomarker for rhinoviral infections in asthma. *Thorax* **63**, 200–200 (2008).
- Ng, P. C. *et al.* IP-10 is an Early Diagnostic Marker for Identification of Late-Onset Bacterial Infection in Preterm Infants. *Pediatr. Res.* **61**, 93–98 (2007).
- Herzig, D. S. *et al.* The role of CXCL10 in the pathogenesis of experimental septic shock. *Crit. Care* **18**, R113 (2014).
- Punyadeera, C. *et al.* A biomarker panel to discriminate between systemic inflammatory response syndrome and sepsis and sepsis severity. *J. Emerg. Trauma Shock* **3**, 26–35 (2010).
- Ruhwald, M. *et al.* Evaluating the potential of IP-10 and MCP-2 as biomarkers for the diagnosis of tuberculosis. *Eur. Respir. J.* **32**, 1607–1615 (2008).
- Vantni, V. *et al.* IP-10 is an additional marker for tuberculous (TB) detection in HIV-infected persons in a low-TB endemic country. *J. Infect.* **65**, 49–59 (2012).
- Kabeer, B. S. A. *et al.* IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy. *BMC Infect. Dis.* **11**, 135 (2011).
- Canmas, A. *et al.* IP-10 detection in urine is associated with lung diseases. *BMC Infect. Dis.* **10**, 333 (2010).
- Vareille, M., Kjeninger, F., Edwards, M. R. & Regamey, N. The Airway Epithelium: Soldier in the Fight against Respiratory Viruses. *Clin. Microbiol. Rev.* **24**, 210–229 (2011).
- Jakubczak, C. *et al.* Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* **39**, 599–610 (2013).
- Varol, C. *et al.* Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity* **31**, 502–512 (2009).
- Frankie-Ullmann, G. *et al.* Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages *in vitro*. *J. Immunol.* **157**, 3097–3104 (1996).
- Marriott, H. M. & Dockrell, D. H. The Role of the Macrophage in Lung Disease Mediated by Bacteria. *Exp. Lung Res.* **33**, 493–505 (2007).
- Dockrell, D. H. *et al.* Alveolar Macrophage Apoptosis Contributes to Pneumococcal Clearance in a Resolving Model of Pulmonary Infection. *J. Immunol.* **171**, 5380–5388 (2003).
- Ghoneim, H. E., Thomas, P. G. & McCallers, J. A. Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *J. Immunol. Biol. Med.* **1950** 191, 1250–1259 (2013).
- Kim, H. M. *et al.* Alveolar Macrophages Are Indispensable for Controlling Influenza Viruses in Lungs of Pigs. *J. Virol.* **82**, 4265–4274 (2008).
- Rebane, A. & Akdis, C. A. MicroRNAs: Essential players in the regulation of inflammation. *J. Allergy Clin. Immunol.* **132**, 15–26 (2013).
- Terrier, O. *et al.* Host microRNA molecular signatures associated with human H1N1 and H3N2 influenza A viruses reveal an unanticipated antiviral activity for miR-146a. *J. Gen. Virol.* **94**, 985–995 (2013).

29. Wang, P. *et al.* Inducible microRNA-155 Feedback Promotes Type I IFN Signaling in Antiviral Innate Immunity by Targeting Suppressor of Cytokine Signaling 1. *J. Immunol.* **185**, 6226–6233 (2010).
30. Oved, K. *et al.* A Novel Host-Proteome Signature for Distinguishing between Acute Bacterial and Viral Infections. *PLoS One* **10**, e0120012 (2015).
31. Lahti, E. *et al.* Induced sputum in the diagnosis of childhood community-acquired pneumonia. *Thorax* **64**, 252–257 (2009).
32. Nascimento-Carvalho, C. M. *et al.* The role of respiratory viral infections among children hospitalized for community-acquired pneumonia in a developing country. *Pediatr. Infect. Dis. J.* **27**, 939–941 (2008).
33. Juvén, T. *et al.* Etiology of community-acquired pneumonia in 254 hospitalized children. *Pediatr. Infect. Dis. J.* **19**, 293–298 (2000).
34. Kellum, J. A. *et al.* Understanding the inflammatory cytokine response in pneumonia and sepsis: results of the Genetic and Inflammatory Markers of Sepsis (GenIMS) Study. *Arch. Intern. Med.* **167**, 1655–1663 (2007).
35. Jiang, Y. *et al.* Characterization of Cytokine/Chemokine Profiles of Severe Acute Respiratory Syndrome. *Am. J. Respir. Crit. Care Med.* **171**, 850–857 (2005).
36. Ichikawa, A. *et al.* CXCL10-CXCR3 Enhances the Development of Neutrophil-mediated Fulminant Lung Injury of Viral and Nonviral Origin. *Am. J. Respir. Crit. Care Med.* **187**, 65–77 (2013).
37. Damjanovic, D., Lai, R., Jayaraman, M., Hogaboam, C. M. & Xing, Z. Marked Improvement of Severe Lung Immunopathology by Influenza-Associated Pneumococcal Superinfection Requires the Control of Both Bacterial Replication and Host Immune Responses. *Am. J. Pathol.* **183**, 868–880 (2013).
38. Grimson, A. *et al.* MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell* **27**, 91–105 (2007).
39. Betel, D., Koppal, A., Agius, P., Sander, C. & Leslie, C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90 (2010).
40. Vlachos, I. S. *et al.* DIANA miRPath v2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res.* **40**, W498–504 (2012).
41. Lai, R.-H., Wang, M.-J., Yang, S.-H. & Chen, J.-Y. Genomic organization and functional characterization of the promoter for the human suppressor of cytokine signaling 6 gene. *Gene* **448**, 64–73 (2009).
42. Pico, V. S. *et al.* Multicenter case-control study protocol of pneumonia etiology in children: Global Approach to Biological Research, Infectious diseases and Epidemics in Low-income countries (GABRIEL network). *BMC Infect. Dis.* **14**, 635 (2014).
43. Aytoko, P. & English, M. Case Management of Childhood Pneumonia in Developing Countries. *Pediatr. Infect. Dis. J.* **26**, 432–440 (2007).
44. Chertan, T. *et al.* Standardized interpretation of paediatric chest radiographs for the diagnosis of pneumonia in epidemiological studies. *Bull. World Health Organ.* **83**, 353–359 (2005).
45. WHO | Pocket book of hospital care for children. WHO Available at: [http://www.who.int/maternal\\_child\\_adolescent/documents/9241546700/en/](http://www.who.int/maternal_child_adolescent/documents/9241546700/en/). (Accessed: 15th March 2015)
46. Albrich, W. C. *et al.* Pneumococcal colonisation density: a new marker for disease severity in HIV-infected adults with pneumonia. *BMJ Open* **4**, e005953 (2014).
47. Messaoudi, M. *et al.* The Relevance of a Novel Quantitative Assay to Detect up to 40 Major Streptococcus pneumoniae Serotypes Directly in Clinical Nasopharyngeal and Blood Specimens. *PLoS ONE* **11**, e0151428 (2016).

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#### Additional Information

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**Article 2. RSV infection in macrophages promotes IP-10 expression during bacterial co-infection**

1 Short communication

2 **RSV infection in human macrophages promotes**  
3 **CXCL10/IP-10 expression during bacterial**  
4 **co-infection**

5

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20 **Abstract:** Respiratory Syncytial Virus (RSV), a major etiologic agent of acute lower respiratory  
21 infection, constitutes the most important cause of death in young children worldwide.  
22 Viral/bacterial mixed infections are related to severity of respiratory inflammatory diseases, but the  
23 underlying mechanisms remain poorly understood. We have previously investigated the  
24 intracellular mechanisms that mediate the immune response in the context of influenza  
25 virus/*Streptococcus pneumoniae* (*Sp*) co-infection, using a model of human monocyte-derived  
26 macrophages (MDMs) (Hoffmann et al. 2016). Here, we set up and characterized a similar model of  
27 MDMs to investigate different scenarios of RSV infection and co-infection with *Sp*. Our results  
28 suggest that *Sp* contributes to a faster and possibly higher level of CXCL10/IP-10 expression  
29 induced by RSV infection in human MDMs.

30 **Keywords:** Respiratory Syncytial Virus (RSV); *Streptococcus pneumoniae* (*Sp*); Macrophages;  
31 co-infection; acute lower respiratory infection

32

33 **1. Introduction**

34 The common lower respiratory infections (LRIs), such as pneumonia and bronchiolitis,  
35 constitute the major cause of pediatric mortality worldwide but also contribute to high mortality  
36 rates among elderly [2] and immunocompromised patients [3, 4]. A wide variety of pathogens  
37 contribute to LRIs mainly including viruses and bacteria. Studies have underlined the relevance of  
38 mixed viral/bacterial infections as etiological risk factors in acute LRIs [5, 6] and their impact in  
39 severe LRIs. Viral and bacterial co-infections were extensively studied in the context of influenza  
40 viral infection, followed by pneumococcal infection [1, 7-10]. However, the mechanisms underlying  
41 pathogenesis in mixed infections remain poorly understood, notably in the context of other  
42 respiratory pathogens.

43 Respiratory syncytial virus (RSV) is the most important cause of death in very young children,  
44 and global RSV disease burden is estimated in approximately 200 000 deaths and over three million

45 hospitalizations per year. Currently, the most common causative agent of viral LRIs is RSV [11, 12].  
46 In contrast with Influenza viruses, prophylactic and therapeutics approaches for RSV remain very  
47 limited and new vaccine and antiviral strategies are urgently required. Among bacterial pathogens,  
48 *Streptococcus pneumoniae* (*Sp*) is the most common cause of severe pneumonia. It is a commensal of  
49 the nasopharynx and most carriage episodes do not result in disease [13] but pneumococcal  
50 pneumonia results in approximately half a million deaths in children younger than 5 years each year  
51 worldwide.

52 A large retrospective study performed recently provided evidence for an interaction between  
53 RSV and pneumococcal pneumonia [14]. In children with acute LRIs, RSV was associated with  
54 increased colonization by *Sp*, in a similar way to that was described in the context of influenza  
55 infection [15-19], and although we do not know the exact mechanisms of this interaction, it is  
56 assumed that it is due to the increase of attachment sites to the bacteria in the epithelial cells caused  
57 by viruses. The prevalence of RSV/*Sp* co-infection varies in approximately 40% of samples from  
58 pediatric RSV infections [20-22] and in vitro and in vivo studies show significant increase in disease  
59 severity in this context [15, 23-26].

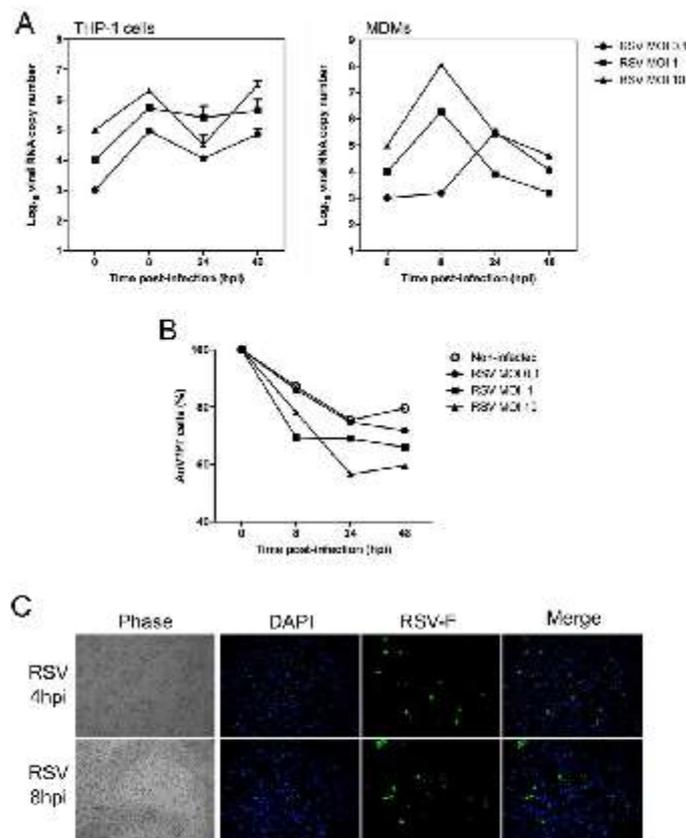
60 Although RSV infections involve several types of cells in the lung, the important role of  
61 macrophages in the host response has been demonstrated [27-29]. In the lungs, macrophages are  
62 strategically located and play a central role in the defense of the host against respiratory pathogens.  
63 They initiate innate and adaptive immune responses, besides being the primary source of cytokines  
64 and chemokines [30, 31]. The elimination of macrophages during a viral or bacterial infection is  
65 usually favorable to the pathogen and increases the severity of the disease, suggesting that  
66 macrophages are necessary for viral elimination and control of the disease [28, 31-33]. However,  
67 the underlying mechanisms of infection control by macrophages are not fully elucidated in the  
68 context of RSV/*Sp* mixed infections.

69 Recently, we have investigated the intracellular mechanisms that mediate the immune response  
70 in the context of influenza virus/*Sp* infection, using a model of human monocyte-derived  
71 macrophages (MDMs) [1]. This study revealed that mixed infections of MDMs induces a synergistic  
72 production of interferon- $\gamma$ -induced protein 10 (IP-10/CXCL10). This cytokine could constitute an  
73 interesting marker, as it was related to viral and/or bacterial etiologies and disease severity in a  
74 retrospective analysis of clinical samples [1]. McNamara and colleagues reported that IP-10/CXCL10  
75 is one the most abundant cytokines in bronchoalveolar lavages (BAL) from infants with RSV  
76 bronchiolitis [34]. In the present study, we wanted to set up and characterize a similar model of  
77 MDMs to investigate different scenarios of RSV infection and co-infection, focusing on IP10 as a  
78 reference indicator of the immune response.

## 79 2. Results and Discussion

80 First, monocytes were isolated from human peripheral blood mononuclear cells (PBMC) and  
81 differentiated into macrophages as previously described [1]. These human MDMs were then  
82 exposed to RSV (Long strain, ATCC VR-26) at different multiplicity of infection (MOI) to investigate  
83 the kinetics of infection by monitoring of genome copy number using a specific RT-qPCR on cell  
84 lysates harvested at different time-points (Figure 1A) As a comparison, immortalized monocyte-like  
85 THP-1 cells were infected in parallel with the same protocol. Our results confirmed that MDMs can  
86 be effectively infected by RSV, similarly to THPI-1, but that viral replication remains very limited in  
87 the case of MDMs. These observations were in good agreement with early works that showed that  
88 RSV replication in macrophages was possible but with a limited extent [35-36]. Indeed, we observed  
89 a similar kinetic profile whatever the MOI used, with a replication peak at 8hpi, followed by a  
90 stabilization or a decrease of genome copy number at 48hpi for THP-1 and MDMs, respectively  
91 (Figure 1A). In contrast to that is usually described in cell lines (e.g. Hep-2 or A549), we did not  
92 observe marked cytopathic effects in infected MDMs, but rather a slightly higher number of dying  
93 cells (data not shown). To complete these observations, we then monitored cell death during RSV

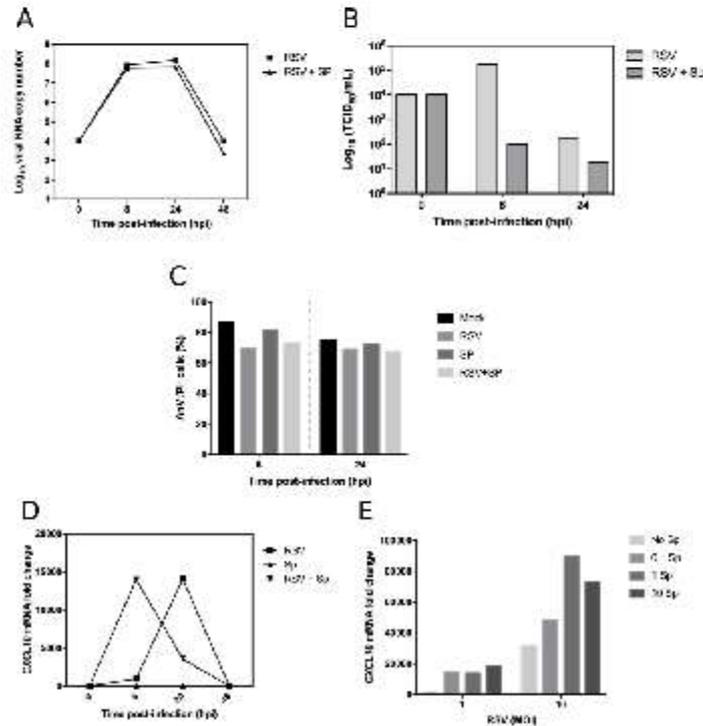
94 infection using flow cytometry (Annexin V/propidium iodide staining) (Figure 1B). Our results  
 95 confirmed that RSV infection increased cell death in MDMs, with a significant decrease of survival at  
 96 a MOI of 10 at 48hpi (Figure 1B). In parallel, we performed immunofluorescence microscopy of  
 97 RSV-infected MDM, using a specific antibody to detect RSV F protein (ab20745; Abcam, Cambridge,  
 98 UK) (Figure 1C). At a MOI of 1 at 8hpi, around 10-15% of cell monolayer was positive for RSV  
 99 antigen staining (Figure 1C), indicating an efficient infection, and possibly an initial limited viral  
 100 replication, as RSV F staining still progressively increase from early stages to 8hpi (data not shown).  
 101 Interestingly, the level of apoptosis in RSV infected cells (Figure 1B) is not completely correlated  
 102 with the number of infected cells (Figure 1C), suggesting that apoptosis is also induced in  
 103 non-infected cells. Altogether, these results indicate that human MDMs are susceptible to RSV  
 104 infection. Moreover, viral replication occurs, to a limited extent, in slight disagreement with  
 105 previous observations indicating abortive infection in lung macrophages [37-39].  
 106



107  
 108 **Figure 1. Characterization of RSV infection in human monocyte-derived macrophages (MDMs).**  
 109 Monocytes were isolated from human peripheral blood mononuclear cells (PBMC) and differentiated  
 110 into macrophages as previously described (1). RSV (Lon strain, ATCC VR-26) was propagated in HEp2  
 111 cells and isolated as described previously (43). A. THP-1 or MDMs cells were exposed to RSV Long at  
 112 different multiplicity of infection (MOI, 0.1, 1 and 10) to investigate the kinetics of infection over time.

113 Uninfected control replicates were exposed to cell culture medium. At different time-points, total cell  
114 extracts were harvested and analyzed by multiplex real-time RT-qPCR to quantify RSV (*F* fusion gene).  
115 The RT-PCR reaction was performed using the AgPath-ID™ One-Step RT-PCR kit (Life Technologies;  
116 Carlsbad, California, USA) according to the manufacturer's instructions. Samples with a cycle threshold  
117 (Ct) value of  $\geq 40$  were recorded as negative. A standard curve was prepared using serially diluted RNA  
118 extracts from a known quantity and used to calculate genomic copies/mL. **B.** In the same experimental  
119 condition, cell survival in infected MDMs was monitored using flow cytometry, using the FITC/Annexin  
120 V apoptosis detection kit (BD Biosciences), according to the manufacturer's instructions. Percent  
121 survival is expressed compared to viable cells measured at T=0hpi. **C.** Immunofluorescence microscopy  
122 of MDMs infected by RSV (MOI of 1) at 8hpi. Immunofluorescence protocol was performed as  
123 previously published (44). RSV F antigen (Green), Nuclei stained by DAPI (Blue). Each experiment was  
124 performed in duplicate, for two separate experiments.

125  
126 In a second step, we used this MDM model to study RSV/*Sp* mixed infection. We have  
127 previously demonstrated that human MDMs were permissive to *Sp* infection [1]. Using a similar  
128 experimental approach, MDMs were first infected by RSV at a MOI of 1 and then mock-infected or  
129 infected by *Sp* at 1 CFU/cell, 4h after viral infection. Viral kinetics were monitored by RT-qPCR for 48  
130 hpi (Figure 2A) and by measure of RSV viral load (TCID50/mL) in the supernatants (Figure 2B).  
131 Whereas the genome copy number remain unaffected by the subsequent challenge with *Sp*, we  
132 observed a significant decrease of viral production in the context of *Sp* co-infection, notably at 8hpi,  
133 with more than three log<sub>10</sub> difference of TCID50/mL in comparison with single RSV infection  
134 (Figure 2B).  
135



136 **Figure 2.** Characterization of RSV/*Sp* mixed infection in human monocyte-derived macrophages  
 137 (MDMs). Human MDMs were infected by RSV at a MOI of 1. After 4h of viral infection, cells were  
 138 mock-infected or infected by *Sp* at 1 CFU/cell. Encapsulated *Streptococcus pneumoniae* serotype 14  
 139 was obtained from the National Reference Center for *Streptococci* (Dept of Medical Microbiology, Aachen,  
 140 Germany). *Pneumococci* were opsonized in anti-pneumococcal immune serum and incubated for 30 min  
 141 at 37°C prior to infections for all experiments. MDM cells showed significant loss of viability following  
 142 overnight incubation with *S. pneumoniae*, and therefore gentamicin (10µg/ml) was added to all wells at  
 143 the 2-h time point to prevent bacterial overgrowth and loss of cell viability. A. At different time-points,  
 144 total cell extracts were harvested and analyzed by multiplex real-time RT-qPCR to quantify RSV. B. RSV  
 145 viral production in supernatants was performed by quantification of viral titers (TCID50/mL) with a  
 146 limit-dilution assay and using the Reed & Muench statistical method. Results are expressed as log<sub>10</sub>  
 147 TCID50/mL values. C. In the same experimental condition, cell survival in infected MDMs was  
 148 monitored using flow cytometry, with the FITC/Annexin V apoptosis detection kit (BD Biosciences),  
 149 according to the manufacturer's instructions. Percent survival is expressed compared to viable cells  
 150 measured at T= 0hpi. D. E. Co-infection alters the timing and extent of IP-10 expression in human  
 151 monocyte-derived macrophages (MDMs). Human MDMs were infected by RSV at a MOI of 1. After 4h  
 152 of viral infection, cells were mock-infected or infected by *Sp* at 1 CFU/cell. At different time-points, total  
 153 cell extracts were harvested and analyzed by multiplex real-time RT-qPCR to quantify CXCL10 mRNA  
 154 levels. Total mRNA was purified from transfected and infected MDMs using the RNeasy kit (Qiagen)  
 155 and specific primers were used to amplify CXCL10 (F: 5'-GTGGCAITCAAGGAGTACCTC-3', R:  
 156 5'-TGATGGCCTTCGATTCTGGATT-3') and β-Actin for normalization (F:  
 157 5'-CTCTCCAGCCTTCCTCTCT-3', R: 5'-AGCACTGTGTGGCGTACAG-3'). E. Similar strategy with

158 different combinations of RSV and *Sp*, at 8hpi. Each experiment was performed in duplicate, for two  
159 separate experiments.  
160

161 To complete these observations, we also monitored the impact of RSV and *Sp* single and mixed  
162 infections on MDM viability (Figure 2C). Interestingly, we did not observe a marked difference  
163 between single and mixed infections, with similar level of cell death around 20-30% (Figure 2C).  
164 Altogether, these results indicate that RSV particle release in supernatant is strongly reduced in  
165 presence of *Sp*, suggesting a possible activation of macrophage that could contribute to mitigation of  
166 viral production.

167 RSV infection is associated with increased expression of various cytokines [29]. After  
168 characterizing the simple and mixed infections in macrophages, innate immune response to single or  
169 mixed RSV infection in MDM cells was examined by measuring changes in the expression of the  
170 CXCL10 gene. The mRNA levels of CXCL10 were measured in the cells culture of mock, single or  
171 mixed infections at different time points after infection (Figure 2D). In our experimental conditions,  
172 *Sp* single infection did not induce the expression of IP-10, in contrast with our previously published  
173 study [1], most probably due to differences of protocols, which were adapted for RSV infection (e.g.  
174 lower cell confluency). In the context of single viral infection, we observed a dramatic increase of  
175 IP-10 expression at 14hpi, with a fold change of more than 14 000 compared to mock (Figure 2D).  
176 This high value could be explained by the relative low level of IP-10 expressed in mock (CT value =  
177 40) compared to infection (CT value = 27). Interestingly, this increase of CXCL10 expression was  
178 observed in the context of a very limited viral replication at the same time-point (Figure 2D and  
179 Figure 2B). A similar increase of CXCL10 expression was observed in the context of RSV/*Sp* mixed  
180 infection, but much earlier, at 8hpi (Figure 2D). This observation suggests co-infection induces an  
181 innate immune response more rapidly. To further investigate the possible synergy that could occur  
182 in term of activation of IP10 expression in response to mixed infections, we infected MDMs with 2  
183 different MOIs of RSV (MOI 1 and 10) together with 4 different CFU/cell of *Sp* (0, 0.1, 1 and 10  
184 CFU/cell) and monitored CXCL10 expression by RT-qPCR (Figure 2E). As expected, IP-10  
185 expression was stimulated with increasing quantity of viral inoculum. At a MOI of 1, CXCL10  
186 expression was strongly induced in presence of *Sp* (Figure 2E). Interestingly, at a MOI of 10, we  
187 observed a stronger increase of CXCL10 expression in the context of *Sp* co-infection (Figure 2E).  
188 Altogether, these results suggest that *Sp* could contribute to a faster and possibly higher level of  
189 CXCL10 expression induced by RSV infection in human MDMs.

### 190 3. Conclusion and Perspectives

191 In conclusion, we have shown that human MDMs constitute an interesting and biologically  
192 relevant experimental model to study RSV/*Sp* co-infections. Our data support that early stages of  
193 RSV infection strongly trigger the innate immune response in human macrophages cells, in good  
194 agreement with previously published studies [29, 40]. For example, Levitz and colleagues have  
195 recently described a strong induction of IL-6 and CCL5 in MDMs infected by different clinical  
196 isolates of RSV [40]. In a mice model, CXCL10 was demonstrated to have an important protective  
197 role to the host by reducing viral load and pathogenesis [41]. In the context of mixed infection, we  
198 have shown that expression of CXCL10 occurred earlier than in single RSV infection (16h delay,  
199 Figure 2E), suggesting a faster and possibly stronger response of macrophages, also with good  
200 agreement with other *in vitro* and *in vivo* studies [42].

201 We have shown that CXCL10 expression was strongly increased during infection, even if the  
202 level of viral replication appears very limited in MDMs. Based on this, we can speculate that  
203 strategies to control the immune response could constitute an interesting option in the treatment of  
204 patients with RSV infection. In this study, although RSV infection strongly mitigated by  
205 macrophages after 24hpi, we have shown that bacterial co-infection contributes to alter the timing  
206 and extent of innate immune response, as illustrated by CXCL10 expression. We previously obtained

207 quite similar results in a model of influenza/*Sp* co-infection infection in human MDMs, suggesting  
208 that common underlying mechanisms in different context of viral infection [1].

209 Future investigations may lead to the identification of key cell signaling pathways that mediate  
210 the host antiviral response to infection, which in turn could lead to the identification of novel drug  
211 targeting pro-inflammatory responses during infection by RSV. With increasing evidence of  
212 interactions between RSV and bacteria in the respiratory tract, it is important to better understand  
213 interconnections between pathogens and their combined effect on disease severity to develop future  
214 strategies for the prevention and treatment of RSV-induced severe acute LRIs.  
215

#### 216 4. Materials and Methods

217  
218 *Cell lines and culture conditions.* Hep-2 cells (ATCC CCL-23) were maintained in Eagle's minimal  
219 essential medium (EMEM) with 2 mM L-glutamine and 10% FBS and maintained at 37 °C, 5% CO<sub>2</sub>.  
220 THP-1 MDMs were grown in RPMI 1640 medium L-glutamine containing 10% FBS and maintained  
221 at 37 °C, 5% CO<sub>2</sub>. THP-1 cells (ATCC TIB-202) were induced to differentiate into macrophages by  
222 incubation with 10 ng/ml phorbol myristate acetate (PMA; Invivogen, Toulouse, France) for 72  
223 hours.

224  
225 *Monocyte isolation & differentiation.* Monocytes were isolated from human peripheral blood  
226 mononuclear cells (PBMC) and differentiated into macrophages as previously described [1].  
227

228 *Pathogen preparation.* RSV long strain (ATCC VR-26) was propagated in HEp2 cells.  
229 Encapsulated *Streptococcus pneumoniae* serotype 14 was obtained from the National Reference Center  
230 for *Streptococci* (Dept of Medical Microbiology, Aachen, Germany). *Streptococcus pneumoniae*  
231 was cultured in the brain and heart-infused medium (BHI) according to the manufacturer's instructions.  
232 Single colonies were expanded by resuspension in Todd-Hewitt and incubation at 37°C for 3–4 h to  
233 logarithmic phase. Bacteria were harvested by centrifugation at 1500 x g for 15 min at 4 °C. Bacteria  
234 were then resuspended in cell culture medium, at 1 CFU/cell.  
235

236 *Infection of Monocyte-Derived Macrophages (MDM) cells.* Cells were exposed to RSV Long at  
237 different multiplicity of infection (MOI) to investigate the kinetics of infection over time. Uninfected  
238 control replicates were exposed to cell culture medium. Fresh growth medium was applied, and  
239 cells were infected with bacterial strains at a different multiplicity of infection (MOI). Pneumococci  
240 were opsonized in anti-pneumococcal immune serum and incubated for 30 min at 37°C prior to  
241 infections for all experiments. MDM cells showed significant loss of viability following overnight  
242 incubation with *S. pneumoniae*, and therefore gentamicin (10µg/ml) was added to all wells at the 2-h  
243 time point to prevent bacterial overgrowth and loss of cell viability.  
244

245 *Multiplex real-time RT-qPCR.* Multiplex real-time RT-qPCR was performed to quantify RSV (F  
246 fusion gene) and *Sp* (autolysin A gene, *LytA*) in MDMs. The RT-qPCR reaction was performed using  
247 the AgPath-ID™ One-Step RT-PCR kit (Life Technologies; Carlsbad, California, USA) according to  
248 the manufacturer's instructions. Samples with a cycle threshold (Ct) value of ≥40 were recorded as  
249 negative. A standard curve was prepared using serially diluted RNA extracts from a known  
250 quantity and used to calculate genomic copies/ mL.  
251

252 *IP-10 mRNA expression.* For real-time quantitative PCR, total RNA was extracted using the  
253 RNeasy Mini Kit (Qiagen). Reverse-transcription was performed on 1 µg of total RNAs using the  
254 Superscript II enzyme (Invitrogen) at 42°C. Quantification of IP-10 mRNA levels was performed by  
255 real-time quantitative PCR using specific primers for IP-10; (F: 5'

256 -GTGGCATTCAAGGAGTACCTC-3', R: 5' TGATGGCCTTCGATTCTGGATT 3') and  $\beta$ -Actin (F: 5'  
257 -CTCTTCCAGCCTTCCTTCT-3', R: 5' -AGCACTGTGTTGGCGTACAG-3') for normalization.

258

259 **Apoptosis assay.** Cells were centrifuged and resuspended to be incubated with Annexin V-APC  
260 and propidium iodide (PI) (BD Biosciences) for 15 min. Cells were analyzed by flow cytometry  
261 (Accuri C6; BD Biosciences, San Jose, California, USA) and the data visualized using FlowJo 7.6.5  
262 software.

263

264 **Immunofluorescence Microscopy.** Infected cells were washed with PBS, fixed with 4%  
265 paraformaldehyde for 20 minutes at room temperature, permeabilized with PBS-0.1% Triton X-100,  
266 and incubated with PBS containing 1% BSA and 3% human serum to block non-specific binding.  
267 Reagents used for immunofluorescence in this study were goat anti-RSV (ab20745; Abcam,  
268 Cambridge, UK). Antibody incubations were performed for 2 hours, followed by three washes with  
269 PBS. Bound RSV primary antibodies were detected using FITC conjugated donkey anti-goat  
270 antibody (ab6881; Abcam, Cambridge, UK). Cells were imaged using a Leica DMI 3000B microscope  
271 equipped for fluorescence imaging. Images were rendered by ImageJ Software (NIH, Maryland,  
272 United States).

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274

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277

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279 M.M. performed the experiments; D.B., J. H., M.M., M.R-C, H.E., O.T. and G.P-B analyzed the data; M.R-C, H.E.,  
280 O.T. and G.P-B contributed reagents/materials/analysis tools; D.B., J. H., T.O. and G.P-B. wrote the paper. O.T.  
281 and G.P-B. are co-last authors.

282

283 **Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the  
284 design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in  
the decision to publish the results.

285

286 **Ethical statement:** Blood donation was obtained from healthy adult volunteers (Etablissement Français du  
287 sang, Lyon Gerland, France). Written informed consent from each subject were obtained (national procedure  
used for blood donations).

288

#### Abbreviations

|       |  |
|-------|--|
| RSV   | Respiratory syncytial virus              |
| Sp    | <i>Streptococcus pneumoniae</i>          |
| MDMs  | Human monocyte-derived macrophages       |
| LRI   | Lower respiratory infections             |
| IP-10 | interferon- $\gamma$ -induced protein 10 |

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#### References

- 290 1. Hoffmann J, Machado D, Terrier O, Pouzol S, Messaoudi M et al. Viral and bacterial co-infection in  
291 severe pneumonia triggers innate immune responses and specifically enhances IP-10: a translational  
292 study. *Sci Rep* 2016;6:38532.
- 293 2. Stupka JE, Mortensen EM, Anzueto A, Restrepo MI. Community-acquired pneumonia in elderly  
294 patients. *Aging Health* 2009;5(6):763-774.
- 295 3. Wong JL, Evans SE. Bacterial Pneumonia in Patients with Cancer: Novel Risk Factors and  
296 Management. *Clin Chest Med* 2017;38(2):263-277.

- 297 4. Vakil E, Evans SE. Viral Pneumonia in Patients with Hematologic Malignancy or Hematopoietic Stem  
298 Cell Transplantation. *Clin Chest Med* 2017;38(1):97-111.
- 299 5. Johansson N, Kalin M, Tiveljung-Lindell A, Giske CG, Hedlund J. Etiology of community-acquired  
300 pneumonia: increased microbiological yield with new diagnostic methods. *Clin Infect Dis*  
301 2010;50(2):202-209.
- 302 6. Honkinen M, Lahti E, Osterback R, Ruuskanen O, Waris M. Viruses and bacteria in sputum samples  
303 of children with community-acquired pneumonia. *Clin Microbiol Infect* 2012;18(3):300-307.
- 304 7. Morens David M, Taubenberger Jeffery K, Fauci Anthony S. Predominant Role of Bacterial  
305 Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza  
306 Preparedness. *The Journal of Infectious Diseases* 2008;196(7):962-970.
- 307 8. Huber VC, Peltola V, Iverson AR, McCullers JA. Contribution of Vaccine-Induced Immunity toward  
308 either the HA or the NA Component of Influenza Viruses Limits Secondary Bacterial Complications.  
309 *Journal of Virology* 2010;84(8):4105-4108.
- 310 9. O'Brien KL, Walters MI, Sellman J, Quinlisk P, Regnery H et al. Severe pneumococcal pneumonia in  
311 previously healthy children: the role of preceding influenza infection. *Clin Infect Dis*  
312 2000;30(5):784-789.
- 313 10. McCullers JA, Bartmess KC. Role of neuraminidase in lethal synergism between influenza virus and  
314 *Streptococcus pneumoniae*. *J Infect Dis* 2003;187(6):1000-1009.
- 315 11. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA et al. Global burden of acute lower respiratory  
316 infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis.  
317 *Lancet* 2010;375(9725):1545-1555.
- 318 12. Shi T, McAllister DA, O'Brien KL, Simoes EAF, Madhi SA et al. Global, regional, and national disease  
319 burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young  
320 children in 2015: a systematic review and modelling study. *The Lancet* 2017;390(10096):946-958.
- 321 13. Simell B, Auranen K, Käyhkö H, Goldblatt D, Dagan R et al. The fundamental link between  
322 pneumococcal carriage and disease. *Expert Review of Vaccines* 2014;11(7):841-855.
- 323 14. Weinberger DM, Klugman KP, Steiner CA, Simonsen L, Viboud C. Association between respiratory  
324 syncytial virus activity and pneumococcal disease in infants: a time series analysis of US  
325 hospitalization data. *PLoS Med*. 2015 Jan 6;12(1):e1001776.
- 326 15. McCullers Jonathan A, McAuley Julie L, Browall S, Iverson Amy R, Boyd Kelli L et al. Influenza  
327 Enhances Susceptibility to Natural Acquisition of and Disease due to *Streptococcus pneumoniae* in  
328 Ferrets. *The Journal of Infectious Diseases* 2010;202(8):1287-1295.
- 329 16. Nakamura S, Davis KM, Weiser JN. Synergistic stimulation of type I interferons during influenza  
330 virus coinfection promotes *Streptococcus pneumoniae* colonization in mice. *Journal of Clinical*  
331 *Investigation* 2011;121(9):3657-3665.
- 332 17. Short KR, Reading PC, Wang N, Diavatopoulos DA, Wijburg OL. Increased Nasopharyngeal Bacterial  
333 Titers and Local Inflammation Facilitate Transmission of *Streptococcus pneumoniae*. *mBio*  
334 2012;3(5):e00255-00212-e00255-00212.
- 335 18. Vu HTT, Yoshida LM, Suzuki M, Nguyen HAT, Nguyen CDL et al. Association Between  
336 Nasopharyngeal Load of *Streptococcus pneumoniae*, Viral Coinfection, and Radiologically  
337 Confirmed Pneumonia in Vietnamese Children. *The Pediatric Infectious Disease Journal*  
338 2011;30(1):11-18.
- 339 19. Janssen R, Bont L, Siezen Christine LE, Hodemaekers Hennie M, Ermers Marieke J et al. Genetic  
340 Susceptibility to Respiratory Syncytial Virus Bronchiolitis Is Predominantly Associated with Innate  
341 Immune Genes. *The Journal of Infectious Diseases* 2007;196(6):826-834.
- 342 20. Duttweiler L. Pulmonary and systemic bacterial co-infections in severe RSV bronchiolitis. *Archives of*  
343 *Disease in Childhood* 2004;89(12):1155-1157.
- 344 21. Thorburn K, Harigopal S, Reddy V, Taylor N, van Saene HK. High incidence of pulmonary bacterial  
345 co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis. *Thorax*  
346 2006;61(7):611-615.

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386  
387  
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389  
390  
391  
392  
393  
394  
395  
396  
397  
398
22. Hishiki H, Ishiwada N, Fukasawa C, Abe K, Hoshino T et al. Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients. *Journal of Infection and Chemotherapy: Official Journal of the Japan Society of Chemotherapy* 2011;17:87-90.
  23. Stark JM, Stark MA, Colasurdo GN, LeVine AM. Decreased bacterial clearance from the lungs of mice following primary respiratory syncytial virus infection. *Journal of Medical Virology* 2006;78(6):829-838.
  24. Stensballe Lone G, Hjulster T, Andersen A, Kalsoff M, Ravn H et al. Hospitalization for Respiratory Syncytial Virus Infection and Invasive Pneumococcal Disease in Danish Children Aged <2 Years: A Population-Based Cohort Study. *Clinical Infectious Diseases* 2008;46(8):1165-1171.
  25. Madhi SA, Klugman KP, The Vaccine Trialist G. A role for *Streptococcus pneumoniae* in virus-associated pneumonia. *Nature Medicine* 2004;10(8):811-813.
  26. Ampofo K, Bender J, Sheng X, Korgenski K, Daly J et al. Seasonal invasive pneumococcal disease in children: role of preceding respiratory viral infection. *Pediatrics* 2008;122(2):229-237.
  27. Haeberle HA, Takizawa R, Casola A, Brasier AR, Dieterich HJ et al. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. *J Infect Dis* 2002;186(9):1199-1206.
  28. Reed JL, Brewah YA, Delaney T, Welliver T, Burwell T et al. Macrophage impairment underlies airway occlusion in primary respiratory syncytial virus bronchiolitis. *J Infect Dis* 2008;198(12):1783-1793.
  29. Ravi LI, Li L, Sutejo R, Chen H, Wong PS et al. A systems-based approach to analyse the host response in murine lung macrophages challenged with respiratory syncytial virus. *BMC Genomics* 2013;14:190.
  30. Pribul FK, Harker J, Wang B, Wang H, Tregoning JS et al. Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. *J Virol* 2008;82(9):4441-4448.
  31. Benoit A, Huang Y, Proctor J, Rowden G, Anderson R. Effects of alveolar macrophage depletion on liposomal vaccine protection against respiratory syncytial virus (RSV). *Clin Exp Immunol* 2006;145(1):147-154.
  32. Franke-Ullmann G, Pfortner C, Walter P, Steinmuller C, Lohmann-Matthes ML et al. Alteration of pulmonary macrophage function by respiratory syncytial virus infection in vitro. *J Immunol* 1995;154(1):268-280.
  33. Koli D, Gupta MR, Sbrana E, Velayutham TS, Chao H et al. Alveolar macrophages contribute to the pathogenesis of human metapneumovirus infection while protecting against respiratory syncytial virus infection. *Am J Respir Cell Mol Biol* 2014;51(4):502-515.
  34. McNamara PS, Flanagan BF, Hart CA, Smyth RL. Production of chemokines in the lungs of infants with severe respiratory syncytial virus bronchiolitis. *J Infect Dis*. 2005 Apr 15;191(8):1225-32.
  35. Panuska JR, Cirino NM, Midulla F, Despot JE, McFadden ER Jr, et al. (1990) Productive infection of isolated human alveolar macrophages by respiratory syncytial virus. *J Clin Invest* 86: 113-9.
  36. Midulla F, Villani A, Panuska JR, Dab I, Kolls JK, et al. (1993) Respiratory syncytial virus lung infection in infants: immunoregulatory role of infected alveolar macrophages. *J Infect Dis* 168: 1515-9.
  37. Becker S, Quay J, Soukup J. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J Immunol* 1991;147(12):4307-4312.
  38. Becker S, Soukup J, Yankaskas JR. Respiratory syncytial virus infection of human primary nasal and bronchial epithelial cell cultures and bronchoalveolar macrophages. *Am J Respir Cell Mol Biol* 1992;6(4):369-374.
  39. Cirino NM, Panuska JR, Villani A, Taraf H, Rebert NA et al. Restricted replication of respiratory syncytial virus in human alveolar macrophages. *J Gen Virol* 1993;74 ( Pt 8):1527-1537.
  40. Levitz R, Gao Y, Dozmorov I, Song R, Wakeland EK, Kahn JS. Distinct patterns of innate immune activation by clinical isolates of respiratory syncytial virus. *PLoS One*. 2017 Sep 6;12(9):e0184318.
  41. Lindell DM, Lane TE, Lukacs NW. CXCL10/CXCR3-mediated responses promote immunity to respiratory syncytial virus infection by augmenting dendritic cell and CD8(+) T cell efficacy. *Eur J Immunol*. 2008 Aug;38(8):2168-79.

- 399 42. de Steenhuijsen Fijters WA, Heinonen S, Hasrat R, Bunsow E, Smith B et al. Nasopharyngeal  
400 Microbiota, Host Transcriptome, and Disease Severity in Children with Respiratory Syncytial Virus  
401 Infection. *Am J Respir Crit Care Med* 2016;194(9):1104-1115.  
402 43. Zlateva KT, Vijgen L, Dekeersmaeker N, Naranjo C, Van Ranst M. Subgroup prevalence and genotype  
403 circulation patterns of human respiratory syncytial virus in Belgium during ten successive epidemic  
404 seasons. *J Clin Microbiol* 2007;45(9):3022-3030.  
405 44. Terrier O, Carron C, De Chassez B, Dubois J, Traversier A et al. Nucleolin interacts with influenza A  
406 nucleoprotein and contributes to viral ribonucleoprotein complexes nuclear trafficking and efficient  
407 influenza viral replication. *Sci Rep* 2016;6:29006.  
408



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**Chapter 2 Host-viral interactions during single  
and mixed infections: role of the cell guardian  
p53**

Respiratory viruses have been shown to modulate the host response in a variety of mechanism while host cells have numerous strategies to resist infection, replication and virus spread using different cellular mechanisms to the elimination of pathogens. RSV infections are characterized by an extensive inflammatory response through the activation of cell networks and immunoregulatory genes. However, the mechanisms involved in the inducible expression, regulation of these immune modulators and the dynamics of the virus-host interface are not well-understood [25, 487].

Several different signaling pathways were described during RSV infection such as protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and NF- $\kappa$ B. MAPK are involved in RSV replication in human airway epithelial cells [488]. The precise trigger of NF- $\kappa$ B activation is uncertain and may involve oxidative stress, virus replication, viral nucleic acid, or expression of individual viral proteins. NF- $\kappa$ B is a family of inducible transcription factors that control or modulate the expression of several hundred genes, many of which are involved in a variety of activities including cell proliferation, differentiation, and death, as well as modulating the host immune response to viral invasion [489].

In addition to signaling pathways well studied in the literature, there are some others important pathway in the interactions between virus and host response responsible for regulating inflammatory cytokine and chemokine production,

To go forward into the knowledge between pathogen interactions and cellular mechanisms, we decided to look at the p53 pathway that, as was shown in the Introduction chapter of this thesis, participates in important cellular mechanisms and is a target of the pathogens. For this purpose, we performed a set of experiments on the respiratory tract of epithelial cells as they are known to express wild-type p53. These cells are the first site of virus infection and try to limit viral infection through cellular mechanisms. Thus, we can highlight the role of p53 during viral infection.

Studies on influenza virus and the interactions with p53 have been conducted since 2011 by the VirPath team and the results have already been published and are available in the literature. As the interactions are complex and involve several factors of transcription, a lot of work is still underway and in parallel of my thesis, I

contributed to the attached article in appendices which described Mdm2 interactions and influenza virus. This manuscript was submitted to *Scientific Reports* journal.

Due the epidemiological importance and urgency to develop an effective vaccine or RSV treatment, we investigated the role of p53 during this infection. To study the role of p53 in RSV infection we used several methodologies already described in the literature to identify and measure the intensity of p53 activity as well as using molecules and RNA silencing techniques to manipulate p53 activity over the course of infection. Also in this article, we used a cell lineage that does not have the TP53 gene, to better identify virus replication under these conditions.

In addition to the RSV results presented in the first article, we performed some experiments in parallel with parainfluenza 3 virus, because although it has recently been separated from the family and is no longer part of the RSV family, this virus has several structural similarities as well as a cycle replicative. Also, for this viruses, we started to look p53 isoforms expression.

After characterizing the interaction of RSV with the p53 pathway, we attempted to show the role of co-infection in the modulation of this pathway. Only preliminary results were presented in discussion due to difficulty in standardizing the co-infection. Adding the bacteria means more manipulation of the cells, with changes in the medium and more external changes, which causes stress in the cell and makes it difficult to identify the role of p53. As the Influenza virus is more easily manipulated *in vitro* and has mechanisms more accurate than compared to RSV, we also performed the co-infection of IAV and bacteria, to identify the role of the p53 protein.

**Article 1. Role of p53/NF-kB functional balance in RSV-induced inflammation and immune responses**

1 **Role of p53/NF-κB functional balance in RSV-induced inflammation and immune**  
2 **responses**

3

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23 **Keywords**

24 RSV ; p53 ; NF-κB ; virus/host interactions ; inflammation & immune response

25

26 **Short title**

27 **Role of p53/NF-κB balance in RSV infection**

28 ABSTRACT

29

30 The interplay between Respiratory Syncytial Virus (RSV) and the p53 pathway has only been reported  
31 in a limited number of studies, yet the underlying mechanisms of abrogation of p53 activity during the  
32 time course of infection, possibly involving viral proteins, remained unclear. Here, we demonstrate  
33 that RSV infection impairs global p53 transcriptional activity, notably via its proteasome-dependent  
34 degradation at late stages of infection. We also demonstrate that NS1 and NS2 contribute to the  
35 abrogation of p53 activity, and used different experimental strategies (e.g. siRNA, small molecules) to  
36 underline the antiviral contribution of p53 in the context of RSV infection. Notably, our study  
37 highlights a strong RSV-induced disequilibrium of the p53/NF-κB functional balance, which appears  
38 to contribute to the up-regulation of several proinflammatory cytokines and chemokines expression.

## 39 INTRODUCTION

40

41 Respiratory syncytial virus (RSV), discovered more than 60 years ago [1, 2], is one of the main causes  
42 of respiratory tract infection in infants and young children worldwide [3, 4], but also an important  
43 pathogen for the elderly and immunocompromised patients [5, 6]. In addition, RSV infection has been  
44 strongly associated with chronic respiratory diseases such as asthma, chronic obstructive pulmonary  
45 disease (COPD) or cystic fibrosis (CF)[7–9]. Despite numerous attempts and ongoing clinical trials,  
46 no efficacious RSV vaccine is yet available, and the specific therapeutic arsenal in the market is very  
47 limited and expensive (reviewed in [10, 11]). In this context, we urgently need to further increase our  
48 understanding of the multiple facets of RSV pathogenesis, including those involving complex levels of  
49 RSV/host interactions.

50 Our current knowledge of the cellular biology of RSV has been mainly obtained through studies  
51 focused on the immune and inflammatory responses. RSV infection was shown to upregulate the  
52 expression of host genes involved in the antiviral and cell-mediated immune responses, such as genes  
53 coding for interferons (IFN- $\alpha$ / $\beta$ ) or several cytokines/chemokines [12–14]. Interestingly, while  
54 the nuclear factor- $\kappa$ B (NF- $\kappa$ B)/REL family of transcription factors plays a central role in  
55 coordinating the expression of a wide variety of genes that control immune responses [15], several  
56 studies have highlighted the multiple levels of interplay between the NF- $\kappa$ B pathway and RSV during  
57 the time course of infection [16–22]. In that regard, viral non-structural proteins NS1 and NS2 have  
58 been shown to indirectly contribute to the RSV-induced activation of NF- $\kappa$ B [22–24].

59 Another key player in the regulation of inflammation and immune responses is the cellular gatekeeper  
60 p53 (reviewed in [25]). The interplay between RSV and this transcription factor has only been  
61 reported in a limited number of studies [26–28]. Groskreutz and colleagues have shown that RSV  
62 induces the down-regulation of p53 during the time-course of infection, with a consequent impact on  
63 apoptosis and survival of airway epithelial cells [26]. However, the underlying mechanisms of  
64 abrogation of p53 activity during the time course of infection, possibly involving viral proteins,  
65 remain to be elucidated.

66 In this study, we confirmed that RSV strongly impairs p53 transcriptional activity at late stages of the  
67 infection cycle, notably *via* a proteasome-dependent degradation. We also demonstrate that NS1 and  
68 NS2 contribute to the abrogation of p53 activity, and used different experimental strategies (*e.g.*  
69 siRNA, small molecules) to underline the antiviral contribution of p53 in the context of RSV infection.  
70 We finally propose a model by which RSV infection strongly induces a disequilibrium of the p53/NF-  
71  $\kappa$ B functional balance, which probably contributes to the up-regulation of several proinflammatory  
72 cytokines and chemokines.

73

## 74 RESULTS

75

76 **RSV impairs p53 transcriptional activity at late stages of infection.**

77 We firstly investigated the impact of RSV infection on endogenous p53, by performing mock or RSV  
78 (Long strain) infections in A549 human lung epithelial cells (Fig.1). At a multiplicity of infection  
79 (MOI) of 4, we measured an increase of p53 levels at 6 hours post-infection (hpi) (Fig. 1A, left panel),  
80 with more than 1.8-fold increase of p53 relative protein levels (RPL) compared to mock (Fig. 1B, left  
81 panel  $P < 0.0001$ ). In contrast, a slight but significant decrease on p53 RPL compared to mock was  
82 observed at 12 hpi (Fig. 1B, left panel). Moreover, a marked reduction of p53 RPL at late stages of  
83 infection (48 hpi) was also observed using different infection parameters (MOI of 1), with more than a  
84 60% decrease in RSV-infected cells compared to mock (Fig. 1A and 1B, right panel). To further  
85 investigate the impact of RSV on the endogenous p53 expression, we measured p53 mRNA levels by  
86 RT-qPCR in the same experimental conditions (Fig. 1C). Besides a moderate decrease at 6 hpi, p53  
87 mRNA levels in RSV-infected cells remained comparable to those measured in the mock control,  
88 regardless of the MOI and sampling time conditions (MOI 4 - 12 hpi, MOI 1, 24/48 hpi, Fig. 1C),  
89 hence indicating an absence of correlation between p53 mRNA and protein levels. We additionally  
90 measured global p53 transcriptional activity using a luciferase reporter plasmid (Fig. 1D). In all  
91 experimental conditions, our results indicated a statistically significant ( $P < 0.05$  to 0.01) decrease of  
92 luciferase activity in RSV-infected cells compared to mock. This decrease of p53 transcriptional  
93 activity in infected cells was notably marked at 48 hpi, with more than a 50% loss of luciferase  
94 activity compared to mock (Fig. 1D, right panel), in correlation with marked decrease of protein levels  
95 at the same time points (Fig. 1A and 1B, right panel). Altogether, our results indicate that p53  
96 transcriptional activity is impaired during the time-course of RSV infection, possibly by several  
97 mechanisms that could occur at the translational or post-translational levels during late stages of  
98 infection.

99

100 **RSV infection decreases p53 stability via a proteasome-dependent pathway.**

101 We then investigated the impact of RSV infection on p53 stability. We mock-infected or infected  
102 A549 cells at a MOI of 1 for 24 or 48 h, and then analysed p53 stability by monitoring protein levels  
103 over a 50-min period, after treatment with 50  $\mu$ M of cycloheximide (CHX) protein synthesis inhibitor  
104 (Fig. 2A). As expected, the estimated p53 half-life in mock was 20 min, in agreement with previous  
105 works using a similar cellular model and experimental conditions [29]. In contrast, we observed a  
106 faster decrease of p53 RPL in the context of RSV infection, with an estimated half-life below 10 min  
107 (Fig. 2A). Interestingly, after 10 min of CHX treatment at 48 hpi, p53 levels were undetectable by  
108 western blot (Fig. 2A). We then further investigated this strong destabilization of p53 at 48 hpi in  
109 presence of proteasome inhibitor MG132 and then observed that the decrease of p53 RPL was blocked

110 in this condition (Fig. 2B). Altogether, these results suggest that RSV infection decreases p53 stability  
 111 via a proteasome-dependent pathway.

112

113 **RSV non-structural proteins NS1 and NS2 both contribute to inhibit p53 transcriptional activity.**  
 114 As for many other respiratory viruses, RSV non-structural proteins (NS1 and NS2) are known to play  
 115 important roles in the modulation of cellular responses, like the IFN-response, and more largely in  
 116 RSV/host interactions [12–14, 30]. To investigate the potential impact of NS1 and NS2 on p53 activity,  
 117 we transfected A549 cells with 1  $\mu$ g of plasmids expressing RSV NS1 or NS2 fused to eGFP to  
 118 perform fluorescence confocal microscopy. After 24 h post-transfection, there was no clear impact of  
 119 NS1 or NS2 expression on p53 staining and subcellular localization (Fig. 3A). In parallel, A549 cells  
 120 were also transfected with either an empty plasmid or increasing amounts of plasmids expressing RSV  
 121 NS1 (Fig. 3B) or NS2 (Fig. 3C). After 48 h post-transfection, specific p53 global transcriptional  
 122 activity and mRNA expression were measured using a luciferase reporter and RT-qPCR, respectively.  
 123 Compared to mock-transfected cells, we observed a strong and significant (more than 90%) decrease  
 124 of luciferase activity in NS1-positive cells for both 0.2 and 2  $\mu$ g of NS1 transfected plasmid ( $P <$   
 125 0.0001, Fig. 3B). Interestingly, this decrease was not correlated with p53 mRNA expression, which  
 126 instead showed a slight up-regulation of expression in NS1-positive RSV-infected cells compared to  
 127 mock ( $P < 0.05$  and  $< 0.001$ , for 0.2 and 2  $\mu$ g, respectively Fig. 3B). In parallel, we also observed a  
 128 strong impact of NS2 expression on p53 transcriptional activity, as illustrated by more than 70 and 90%  
 129 reductions on luciferase activity ( $P < 0.0001$ ) compared to mock, for 0.2 and 2  $\mu$ g of transfected NS2,  
 130 respectively, and without strong impact on p53 mRNA expression (Fig. 3C). To further understand the  
 131 contribution of NS1/NS2 to the modulation of p53 activity, we transfected A549 cells with 2  $\mu$ g of  
 132 empty plasmid or NS1/NS2-expressing plasmids, and we assessed p53 stability as described before  
 133 (Fig. 3D). No differences between empty and NS1-transfected cells were observed, with an expected  
 134 p53 half-life of 20 min after the addition of CHX (Fig. 3D). In contrast, we observed a strong decrease  
 135 of p53 RPL in NS2-expressing cells compared to mock-transfected cells, with a resulting p53 half-life  
 136 below 10 min (Fig. 3D). Altogether, our results indicate that RSV non-structural proteins NS1 and  
 137 NS2 share the capacity to inhibit p53 transcriptional activity, possibly by distinct mechanisms, and  
 138 could constitute key contributors of the modulation of p53 in the context of RSV infection.  
 139 Interestingly, RSV NS2 downregulates p53 activity at the post-translational level, probably by  
 140 promoting its degradation, which occurs during infection (Fig 2).

141

142 **p53 expression-modulating approaches underline the antiviral contribution of p53 in the context**  
 143 **of RSV infection.**

144 To further investigate the role of p53 in RSV infection, we first evaluated the impact of endogenous  
 145 p53 silencing on viral production. A549 cells were transfected with either a non-specific siRNA (si-  
 146 Ctrl) or a pool of siRNAs targeting p53. Forty-eight hours post-transfection, cells were infected with

147 RSV at a MOI of 1 and viral supernatants and cell extracts were harvested at 48 hpi. As expected,  
148 western blots confirmed that p53 levels were reduced in cells transfected by si-p53 compared to si-Ctrl  
149 (Fig. 4A). We also observed that RSV genome copy numbers were significantly increased (2-fold  
150 increase,  $P < 0.001$ ) in supernatants of si-p53 transfected cells, compared to those of si-Ctrl  
151 transfected cells. This increase was confirmed by infectious titers (Fig. 4A). To complete our  
152 observations, we followed a transient expression approach in H1299 human lung cells (Fig. 4B),  
153 which constitutively lack the expression of full-length p53. H1299 cells were transfected with either  
154 an empty plasmid or a plasmid expressing p53. Forty-eight hours after transfection, cells were infected  
155 with RSV (MOI of 1) and the impact of transient expression on viral production was assessed at 48  
156 hpi. The adequate transient expression of p53 was confirmed by western blot. In these conditions, viral  
157 production was significantly lower in cells transfected with p53, with more than 2.5-fold decrease on  
158 genome copy numbers ( $P < 0.001$ ), and a 2 log<sub>10</sub> reduction on infectious viral titers (Fig. 4B). In  
159 parallel, we advantageously used Nutlin-3, a small molecule known to bind the Mdm2 E3-ubiquitin  
160 ligase, therefore inducing the stabilization and activation of endogenous p53 [31]. A549 cells were  
161 mock infected or infected with RSV (MOI of 1), in the presence of either Nutlin-3 (10  $\mu$ M) or control  
162 DMSO (Ctrl). Treatment with Nutlin-3 significantly decreased viral production compared to control,  
163 with almost 3-fold decrease on genome copy number ( $P < 0.001$ ) and a 3-log<sub>10</sub> reduction on viral  
164 titers (Fig. 4C). These results were consistent with our observations using siRNA and transient  
165 expression, suggesting an antiviral contribution of p53 in the context of RSV infection.

166

#### 167 RSV infection modulates the p53/NF- $\kappa$ B balance via a NS2- PI3K/Akt pathway.

168 The p53 and NF- $\kappa$ B pathways are known to be interconnected and responsible for reciprocal  
169 functional balance at the crossroads of several cellular processes [32]. Based on previously described  
170 data on the interplay between RSV and NF- $\kappa$ B [16–22], we mock infected or infected A549 cells in  
171 the conditions that lead to a marked decrease of p53 activity (MOI 1, 48 hpi, as described above), and  
172 then used a fluorescent reporter assay to quantify the phosphorylation of p65, as a surrogate of NF- $\kappa$ B  
173 activity (Fig. 5A). In these conditions, we observed a strong and significant increase on the relative  
174 fluorescence intensity (290-fold increase,  $P < 0.05$ ) (Fig. 5A), indicating that the RSV-induced  
175 decrease of p53 activity is inversely correlated with NF- $\kappa$ B activity. To complete this observation, we  
176 also performed similar experiment in the context of Nutlin-3 or DMSO (Ctrl) treatment. The  
177 stabilization and activation of p53 by Nutlin-3 in RSV-infected cells was associated with a significant  
178 reduction on fluorescence intensity compared to control (3.3-fold decrease,  $P < 0.001$ , Fig. 5A).

179 Additionally, we also monitored p65 phosphorylation in conditions that NS1 or NS2 transient  
180 expression (2  $\mu$ g of plasmid, 48 h post transfection) inhibits p53 transcriptional activity (Fig. 5B). We  
181 observed that both NS1 and NS2 induce a strong increase of p65 phosphorylation, reflecting an  
182 upregulation of NF- $\kappa$ B activity, in agreement with data published by Bitko and colleagues [23].

183 Altogether, these observations suggest that the NS1/NS2-induced modulation of p53 and NF- $\kappa$ B  
184 would be interconnected and inversely correlated.

185 To further understand the p53/NF- $\kappa$ B balance in the context of RSV infection, we focused our interest  
186 on Akt, an upstream factor of the PI3K pathway, whose phosphorylated form is known to  
187 simultaneously activate NF- $\kappa$ B signaling and inhibit p53 activity *via* Mdm2 [32]. We therefore  
188 infected A549 cells using the same viral parameters described above (MOI of 1), and cell lysates were  
189 harvested at 48 hpi and analyzed by western blot (Fig. 5C). In the context of RSV-infected cells, we  
190 observed an increase of phosphorylated form of Akt, with a Akt-Pho/Total Akt relative ratio of up to  
191 5-fold in comparison with mock-infected cells (Fig. 5C). These results suggest that the PI3K/Akt  
192 could be involved in the RSV-induced downregulation of p53 stability and consecutive transcriptional  
193 activity. In this way, we also observed an increase of Akt phosphorylation in NS1 and NS2-transfected  
194 cells, with Akt-Pho/Total Akt relative ratios of 2.7 and 1.9, respectively (Fig. 5D). These observations  
195 are correlated with the decrease of both p53 transcriptional activity (Fig. 3B and 3C) and protein level  
196 in western blots (Fig. 3D), and suggest that NS1 and NS2 could be involved in a PI3/Akt-dependent  
197 downregulation of p53.

198 Finally, NF- $\kappa$ B has been extensively documented for its regulatory role on a large panel of  
199 cytokine/chemokine expression. As our results indicate a likely modulation of the p53/NF- $\kappa$ B balance  
200 in the context of RSV infection, we investigated the contribution of p53 activity to the regulation of  
201 cytokines and chemokines expression during infection. To that end, A549 cells were pretreated with  
202 either a si-Ctrl or si-p53 for 48 h and then infected with RSV for 48 h (Fig. 5E). A panel of 22  
203 different cytokines and chemokines were quantified in supernatants by using antibody-based assay  
204 (Bio-Plex Pro, BioRad). Results are represented as a heatmap showing for each condition the fold  
205 change compared to the mock (si-Ctrl/si-p53 – Fig. 5E). While a large number of cytokines such as G-  
206 CSF, IL-1RA or IL6 appeared unaffected by the silencing of endogenous p53 expression (Fig. 5E),  
207 others were significantly deregulated, as IL-9, IL-10, VEGF and notably RANTES (Fig. 5E).  
208 Altogether, our results suggest that in the context of RSV infection, p53 could play a role as a  
209 regulator of cytokines and chemokines expression, that NS2 would inhibit in a PI3/Akt-dependent  
210 manner.

211

## 212 DISCUSSION

213

214 In response to stress, the transcription factor p53 rapidly accumulates in the nucleus where it regulates  
215 gene expression to maintain genomic and cellular integrity. The numerous genes regulated by p53 are  
216 involved in a large panel of biological processes, including cell cycle arrest, apoptosis or senescence  
217 [33]. In addition, a large number of studies clearly demonstrated that this “guardian of the genome”  
218 [34] or “cellular gatekeeper” [35] is also involved in the control of viral infections, acting at the  
219 crossroads of several signaling pathways and cellular responses, such as inflammation and immune  
220 response (reviewed in [25, 36]). On the other hand, viruses have developed a wide diversity of  
221 mechanisms to modulate/hijack p53 functions to achieve an optimal replication in their hosts [37]. For  
222 example, our group and others have shown that p53 stability and transcriptional activity were finely  
223 modulated by different multi-level mechanisms during the time course of influenza infection, notably  
224 *via* functional interactions between p53 and several viral proteins [29, 38–42]. However, our current  
225 understanding of the mechanisms of abrogation of p53 activity in the context of RSV infection  
226 remains quite limited.

227 Groskreutz and colleagues have demonstrated that RSV decreases p53 levels by enhancing p53  
228 degradation through the Akt-dependent activation of its negative regulator Mdm2 [26]. In the same  
229 pioneering study, the authors suggested that the decrease of p53 delays cellular apoptosis and prolongs  
230 cell survival, but without affecting viral replication [26]. Results obtained in the first part of our study  
231 are in good agreement with these initial observations, and then support that RSV infection decreases  
232 p53 stability *via* the Mdm2 – proteasomal dependent pathway (Fig. 2). Additionally, our results also  
233 indicate that this destabilization of p53 directly impacts its transcriptional activity at late stages of  
234 infection (Fig. 1) and is concomitant to a marked activation of the PI3K pathway upstream factor Akt  
235 (Fig. 5). On the other hand, the p53-targeting approaches used in our study (si-RNA, transient  
236 expression, small molecule Mdm2 antagonist), clearly underscore an antiviral contribution of p53 in  
237 the context of RSV infection (Fig. 4). In that regard, differences in inherent cell-specific responses to  
238 infection (HTBE *versus* A549 cells) or between the viral strains used (A2 *versus* Long RSV strain)  
239 may account for the divergence of results between our study and that of Groskreutz and al [26].

240 Importantly, our work also highlights the contribution of the two non-structural proteins NS1 and NS2  
241 to the inhibition of p53 transcriptional activity at post-translational level (Fig. 3). While the  
242 mechanism of NS1-induced inhibition remains to be deciphered, our results suggest that, in the case of  
243 NS2, this regulation occurs through the activation of Akt, hence promoting the degradation of p53 (Fig.  
244 3 and Fig. 5D, and proposed model Fig. 5F). We expect future work investigating the possible direct  
245 interaction between NS1 and p53 could better explain our observations. In the meantime, NS1 and  
246 NS2 are known to play important roles in the modulation of cellular responses like the IFN-response,  
247 and more largely in RSV/host interactions [12–14, 30]. Interestingly, two studies have also reported  
248 the possible contribution of other RSV proteins (Matrix and Fusion proteins) to the regulation of p53-

249 dependent cellular processes [27, 28]. Altogether, these observations clearly reinforce the idea that  
250 p53 constitutes a key factor in the host response to RSV infection, for which it would be a privileged  
251 target of RSV.

252 The second part of our study highlights that RSV infection deregulates the transcription factors p53  
253 and NF-κB in opposite ways, with a strong downregulation of p53 transcriptional activity (Fig. 1) and  
254 a strong activation of NF-κB activity (Fig. 5A) at the same time-point during infection. The interplay  
255 between RSV and the NF-κB pathway has been extensively studied, including the contribution of  
256 NS1/NS2 proteins [16–24]. Moreover, NFκB and p53 are known to reciprocally regulate each other,  
257 in a functional antagonism which involves a limited number of common functional interactors, like  
258 Akt and IKK (reviewed in [32]). In this way, our results indicate that RSV infection, notably through  
259 the NS1/NS2 activation of PI3K/Akt, induces a strong disequilibrium of the p53/NF-κB balance. In  
260 consequence, this alteration lead certainly to a large impact on the host transcriptional program  
261 mediated by these two key cellular transcription factors, and should consecutively affect apoptosis  
262 and/or innate immune responses to RSV infection, as we proposed as a model in Fig. 5F.

263 These new data about the interplay between RSV and p53 could be useful to complete our  
264 understanding of the role of NF-κB in the context of RSV infection. For example, it could contribute  
265 to explain the underlying antiviral activity of some molecules with NF-κB inhibitor properties in the  
266 context of RSV infection, such as curcumin or acetylsalicylic acid [43, 44]. The functional interactions  
267 between NS proteins and the p53/NF-κB balance will be further investigated using recombinant RSV  
268 constructs from which NS1/NS2 genes have been deleted. These future investigations may be of great  
269 interest to enrich our knowledge about the multiple functions of NS proteins, and to better design  
270 future NS-modified/deleted recombinant RSV vaccine candidates.

271 In conclusion, we have shown in this study that RSV infection strongly impairs p53 transcriptional  
272 activity, notably via the induction of its proteasome-dependent degradation by a NS2-PI3K/Akt  
273 upstream pathway, which in turn favors viral replication. This regulation loop is tightly interconnected  
274 with the NF-κB pathway, and future works dedicated to the regulation of cellular responses in the  
275 context of RSV infection, such as cytokine/chemokine responses, will need to simultaneously consider  
276 these two major players.

277

## 278 MATERIALS &amp; METHODS

279

280 *Cell lines and viral strain.* Human lung epithelial A549 cells (ATCC CCL-185) were maintained at  
 281 37 °C with 5% CO<sub>2</sub> in Dulbecco's modified Eagles's medium (DMEM), supplemented with 10%  
 282 heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL  
 283 streptomycin sulphate. Human respiratory syncytial virus Long strain (ATCC VR-26) was propagated  
 284 and titrated in HEp-2 cells (ATCC CCL-23).

285

286 *Stability, transactivation and RT-qPCR assays* - For the determination of p53 half-life, cells were  
 287 treated with cycloheximide (50  $\mu$ g/mL), and total protein lysates were harvested at different time  
 288 points and then analyzed by western blot. p53 relative protein levels (RPL) were determined by  
 289 densitometry analysis using the ImageJ software (<http://rsbweb.nih.gov/ij/>), as previously described  
 290 [38]. For transactivation assays, cells were transfected with 1  $\mu$ g of pG13-luc vector, harbouring the  
 291 firefly luciferase gene under the control of thirteen copies of the p53-binding consensus sequence (5'-  
 292 CCAGGCAAGTCCAGGCAGG-3') [45]. Transfection efficiency was normalized using a Renilla  
 293 Luciferase plasmid. Luciferase activity was measured in whole cell extracts using the Dual-Luciferase  
 294 Reporter Assay System (Promega), according to the manufacturer's instructions, and was expressed as  
 295 Relative Luciferase Units (RLU), compared to the mock-treated control. For real-time quantitative  
 296 PCR, total RNA was extracted using the RNeasy Mini Kit (Qiagen). Reverse-transcription was  
 297 performed on 1  $\mu$ g of total RNAs using the Superscript II enzyme (Invitrogen) at 42°C. Quantification  
 298 of p53 mRNA levels was performed by real-time quantitative PCR (RT-qPCR) as previously  
 299 described [38]. For the quantification of viral genome copy numbers, a fusion gene (F) specific RT-  
 300 qPCR reaction was performed using the AgPath-ID™ One-Step RT-PCR kit (Life Technologies;  
 301 Carlsbad, California, USA) according to the manufacturer's instructions. Samples with a cycle  
 302 threshold (Ct) value of  $\geq$  40 were recorded as negative. Genome copies/mL were calculated from a  
 303 standard curve, prepared using serially diluted RNA extracts of a known quantity.

304

305 *Antibodies and western blot* - Total proteins were extracted by scraping and syringing cells in 1x  
 306 NuPAGE LDS buffer (Invitrogen). Fifteen to thirty micrograms of total protein were then separated on  
 307 10% SDS-PAGE gels. The following antibodies were used: mouse monoclonal anti-Mdm2 (SMP14,  
 308 sc-965, Santa Cruz Biotechnology), anti-p53 (DO-1, Santa-Cruz Biotechnology) and anti-Akt (#9792,  
 309 Santa Cruz Biotechnology), rabbit polyclonal anti-Phospho Akt (ser473) (#4058, Santa Cruz  
 310 Biotechnology) and anti-Bax (#2774, Santa Cruz Biotechnology). In addition, an anti-Ku80 polyclonal  
 311 antibody was used as a loading control (#2753, Cell Signaling).

312

313 *Plasmid and siRNA transfection* - Plasmid transfections were performed using TransIT-LT1 reagent  
314 (Mirus), according to the manufacturer's instructions. NS1-EGFP and NS2-EGFP expression  
315 plasmids [46] were graciously obtained from Ralph Tripp (Department of Infectious Diseases,  
316 University of Georgia). pSV-p53 expression plasmid was a kind gift from Dr. Jean-Christophe  
317 Bourdon (Division of Cancer Research, University of Dundee, UK). Silencing of p53 was performed  
318 in A549 cells transfected with a siRNA specifically targeting p53 (si-p53)[39] and a non-specific  
319 siRNA (si-Ctrl, OR-0030-neg05, Eurogentec), as a control, by using Oligofectamine (Invitrogen).

320

321 *Fluorescence immunostaining* - A549 cells grown on Lab-Tek II chamber slides (ThermoScientific)  
322 were fixed with 4% paraformaldehyde in PBS for 30 min. After washing with PBS, cells were  
323 permeabilized with 0.1% triton X-100 in PBS (PBS-T) for 15 min. Mouse monoclonal anti-p53 (DO-1,  
324 Santa-Cruz Biotechnology) was used as primary antibody. After 1h incubation, cells were washed in  
325 PBS-T and then incubated with goat anti-mouse coupled to AlexaFluor 633 (Molecular Probes,  
326 Invitrogen) for 30 min. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI,  
327 Invitrogen). After staining, coverslips were mounted using fluoromount G (Cliniscience) and observed  
328 with a confocal laser scanning microscope (Leica TCS-SP5X).

329

330 *NF- $\kappa$ B and Cytokines/chemokines immunoassays*

331 Cell culture supernatants were analyzed for the presence of 27 human cytokines and chemokines, as  
332 previously described [47], using the Bio-Plex Pro Human Cytokine Standard 27-Plex kit (Bio-Rad) on  
333 a FLEXMAP 3D® analyzer (Luminex, Austin, Texas, USA). The same technology was used for the  
334 monitoring of p65 phosphorylation, using the Bio-Plex Pro Phospho-NF- $\kappa$ B p65 kit (ser536) (BioRad).

335

336 *Reagents*. Small molecule Mdm2 antagonist Nutlin-3a (Calbiochem) was dissolved in DMSO,  
337 aliquoted, and stored at -20 °C. A549 cells were infected by RSV at a MOI of 1, in presence of  
338 DMSO (Ctrl) or a small-molecule Mdm2 antagonist (Nutlin-3, 10  $\mu$ M). Protein synthesis inhibitor  
339 cycloheximide (50  $\mu$ g/mL) and proteasome inhibitor MG132 (20  $\mu$ M) were purchased from Sigma-  
340 Aldrich (refs C7698 and M8699, respectively).

341

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**Author contributions**

MD, PA, HJ, TA, EH, LB, RCM, PBG and TO carried out the experiments and analysis of the results. RCM, PBG and TO participated in the conception and coordination of the study. MD, PA, RCM, PBG and TO designed the study and wrote the manuscript

**Conflict of interest**

The authors declare no conflict of interest.

## REFERENCES

1. Blount RE, Morris JA, Savage RE. Recovery of cytopathogenic agent from chimpanzees with coryza. *Proc Soc Exp Biol Med Soc Exp Biol Med NY N* 1956;92:544–549.
2. Chanock R, Roizman B, Myers R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. *Am J Hyg* 1957;66:281–290.
3. DeAntonio R, Yarzabal J-P, Cruz JP, Schmidt JE, Kleijnen J. Epidemiology of community-acquired pneumonia and implications for vaccination of children living in developing and newly industrialized countries: A systematic literature review. *Hum Vaccines Immunother* 2016;12:2422–2440.
4. Stein RT, Bont LJ, Zar H, Polack FP, Park C, *et al*. Respiratory syncytial virus hospitalization and mortality: Systematic review and meta-analysis. *Pediatr Pulmonol* 2017;52:556–569.
5. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med* 2005;352:1749–1759.
6. Pilie P, Werbel WA, Riddell J, Shu X, Schaubel D, *et al*. Adult patients with respiratory syncytial virus infection: impact of solid organ and hematopoietic stem cell transplantation on outcomes. *Transpl Infect Dis Off J Transplant Soc* 2015;17:551–557.
7. Pérez-Yarza EG, Moreno A, Lázaro P, Mejías A, Ramilo O. The association between respiratory syncytial virus infection and the development of childhood asthma: a systematic review of the literature. *Pediatr Infect Dis J* 2007;26:733–739.
8. Zwaans W a. R, Mallia P, van Winden MEC, Rohde GGU. The relevance of respiratory viral infections in the exacerbations of chronic obstructive pulmonary disease—a systematic review. *J Clin Virol Off Publ Pan Am Soc Clin Virol* 2014;61:181–188.
9. Somayaji R, Goss CH, Khan U, Neradilek M, Neuzil KM, *et al*. Cystic fibrosis pulmonary exacerbations attributable to respiratory syncytial virus and influenza: a population-based study. *Clin Infect Dis Off Publ Infect Dis Soc Am*. Epub ahead of print 9 March 2017. DOI: 10.1093/cid/cix203.
10. Griffiths C, Drews SJ, Marchant DJ. Respiratory Syncytial Virus: Infection, Detection, and New Options for Prevention and Treatment. *Clin Microbiol Rev* 2017;30:277–319.
11. Mazur NI, Martín-Torres F, Baraldi E, Fauroux B, Greenough A, *et al*. Lower respiratory tract infection caused by respiratory syncytial virus: current management and new therapeutics. *Lancet Respir Med* 2015;3:888–900.
12. Durbin JE, Johnson TR, Durbin RK, Mertz SE, Morotti RA, *et al*. The role of IFN in respiratory syncytial virus pathogenesis. *J Immunol Baltim Md* 1950 2002;168:2944–2952.
13. Ramaswamy M, Shi L, Monick MM, Hunninghake GW, Look DC. Specific inhibition of type I interferon signal transduction by respiratory syncytial virus. *Am J Respir Cell Mol Biol* 2004;30:893–900.
14. Tripp RA, Oshansky C, Alvarez R. Cytokines and Respiratory Syncytial Virus Infection. *Proc Am Thorac Soc* 2005;2:147–149.
15. Li Q, Verma IM. NF- $\kappa$ B regulation in the immune system. *Nat Rev Immunol* 2002;2:725–734.
16. Fiedler MA, Wernke-Dollries K, Stark JM. Inhibition of viral replication reverses respiratory syncytial virus-induced NF- $\kappa$ B activation and interleukin-8 gene expression in A549 cells. *J Virol* 1996;70:9079–9082.
17. Thomas KW, Monick MM, Staber JM, Yarovinsky T, Carter AB, *et al*. Respiratory syncytial virus inhibits apoptosis and induces NF- $\kappa$ B activity through a phosphatidylinositol 3-kinase-dependent pathway. *J Biol Chem* 2002;277:492–501.
18. Tian B, Zhang Y, Luxon BA, Garofalo RP, Casola A, *et al*. Identification of NF- $\kappa$ B-dependent gene networks in respiratory syncytial virus-infected cells. *J Virol* 2002;76:6800–6814.
19. Choudhary S, Boldogh S, Garofalo R, Jamaluddin M, Brasier AR. Respiratory syncytial virus influences NF- $\kappa$ B-dependent gene expression through a novel pathway involving MAP3K14/NIK expression and nuclear complex formation with NF- $\kappa$ B2. *J Virol* 2005;79:8948–8959.

20. Liu P, Li K, Garofalo RP, Brasier AR. Respiratory syncytial virus induces RelA release from cytoplasmic 100-kDa NF-kappa B2 complexes via a novel retinoic acid-inducible gene-I(middle dot)NF-kappa B-inducing kinase signaling pathway. *J Biol Chem* 2008;283:23169–23178.
21. Yoboua F, Martel A, Duval A, Mukawera E, Grandvaux N. Respiratory syncytial virus-mediated NF-kappa B p65 phosphorylation at serine 536 is dependent on RIG-I, TRAF6, and IKK beta. *J Virol* 2010;84:7267–7277.
22. Thornburg NJ, Hayward SL, Crowe JE. Respiratory syncytial virus regulates human microRNAs by using mechanisms involving beta interferon and NF- $\kappa$ B. *mBio*;3. Epub ahead of print 18 December 2012. DOI: 10.1128/mBio.00220-12.
23. Bitko V, Shulyayeva O, Mazumder B, Musiyenko A, Ramaswamy M, et al. Nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by an NF-kappaB-dependent, interferon-independent mechanism and facilitate virus growth. *J Virol* 2007;81:1786–1795.
24. Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. *J Virol* 2005;79:5353–5362.
25. Muñoz-Fontela C, Mandinova A, Aaronson SA, Lee SW. Emerging roles of p53 and other tumour-suppressor genes in immune regulation. *Nat Rev Immunol* 2016;16:741–750.
26. Groskreutz DJ, Monick MM, Yarovinsky TO, Powers LS, Quelle DE, et al. Respiratory syncytial virus decreases p53 protein to prolong survival of airway epithelial cells. *J Immunol Baltim Md 1950* 2007;179:2741–2747.
27. Eckardt-Michel J, Lorek M, Baxmann D, Grunwald T, Keil GM, et al. The fusion protein of respiratory syncytial virus triggers p53-dependent apoptosis. *J Virol* 2008;82:3236–3249.
28. Bian T, Gibbs JD, Örvell C, Imani F. Respiratory syncytial virus matrix protein induces lung epithelial cell cycle arrest through a p53 dependent pathway. *PLoS One* 2012;7:e38052.
29. Terrier O, Diederichs A, Dubois J, Cartet G, Lina B, et al. Influenza NS1 interacts with p53 and alters its binding to p53-responsive genes, in a promoter-dependent manner. *FEBS Lett* 2013;587:2965–2971.
30. Lay MK, Bueno SM, Gálvez N, Riedel CA, Kalergis AM. New insights on the viral and host factors contributing to the airway pathogenesis caused by the respiratory syncytial virus. *Crit Rev Microbiol* 2016;42:800–812.
31. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–848.
32. Ak P, Levine AJ. p53 and NF- $\kappa$ B: different strategies for responding to stress lead to a functional antagonism. *FASEB J Off Publ Fed Am Soc Exp Biol* 2010;24:3643–3652.
33. Lane D, Levine A. p53 Research: the past thirty years and the next thirty years. *Cold Spring Harb Perspect Biol* 2010;2:a000893.
34. Lane DP. Cancer. p53, guardian of the genome. *Nature* 1992;358:15–16.
35. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–331.
36. Sato Y, Tsurumi T. Genome guardian p53 and viral infections. *Rev Med Virol* 2013;23:213–220.
37. Lazo PA, Santos CR. Interference with p53 functions in human viral infections, a target for novel antiviral strategies? *Rev Med Virol* 2011;21:285–300.
38. Terrier O, Josset L, Textoris J, Marcel V, Cartet G, et al. Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway. *Virol J* 2011;8:285.
39. Terrier O, Marcel V, Cartet G, Lane DP, Lina B, et al. Influenza A viruses control expression of proviral human p53 isoforms p53 $\beta$  and Delta133p53 $\alpha$ . *J Virol* 2012;86:8452–8460.
40. Zhu Z, Yang Y, Wei J, Shao D, Shi Z, et al. Type I interferon-mediated immune response against influenza A virus is attenuated in the absence of p53. *Biochem Biophys Res Commun* 2014;454:189–195.
41. Yan W, Wei J, Deng X, Shi Z, Zhu Z, et al. Transcriptional analysis of immune-related gene expression in p53-deficient mice with increased susceptibility to influenza A virus infection. *BMC Med Genomics* 2015;8:52.
42. Nailwal H, Sharma S, Mayank AK, Lal SK. The nucleoprotein of influenza A virus induces

- p53 signaling and apoptosis via attenuation of host ubiquitin ligase RNF43. *Cell Death Dis* 2015;6:e1768.
43. Olivera A, Moore TW, Hu F, Brown AP, Sun A, *et al*. Inhibition of the NF- $\kappa$ B signaling pathway by the curcumin analog, 3,5-Bis(2-pyridinylmethylidene)-4-piperidone (EF31): anti-inflammatory and anti-cancer properties. *Int Immunopharmacol* 2012;12:368–377.
  44. Glatthaar-Saalmüller B, Mair KH, Saalmüller A. Antiviral activity of aspirin against RNA viruses of the respiratory tract—an in vitro study. *Influenza Other Respir Viruses* 2017;11:85–92.
  45. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, *et al*. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817–825.
  46. Bakre A, Wu W, Hiscox J, Spann K, Teng MN, *et al*. Respiratory Syncytial Virus (RSV) Non-Structural Protein-1 Modifies miR-24 Expression via TGF-beta. *J Gen Virol*. Epub ahead of print 7 August 2015. DOI: 10.1099/jgv.0.000261.
  47. Hoffmann J, Machado D, Terrier O, Pouzol S, Messaoudi M, *et al*. Viral and bacterial co-infection in severe pneumonia triggers innate immune responses and specifically enhances IP-10: a translational study. *Sci Rep* 2016;6:38532.

## FIGURES

**Figure 1. RSV strongly impairs p53 transcriptional activity at late stages of infection.** Human lung A549 cells were mock-infected or infected with RSV (Long strain) at a MOI of 4 or 1, and analyzed at 6-12 hpi (left panels) or 24-48 hpi (right panels), respectively. A. Cell lysates were analysed by western blot for the expression of p53 and Bax. Ku80 was used as loading control. B. p53 relative protein levels (p53 RPL) were measured by densitometry. C. p53 mRNA expression was measured by RT-qPCR. D. p53 transcriptional activity was monitored using a specific luciferase reporter assay. Relative Luciferase Units (RLU) reflect global transcriptional activity compared to the mock-infected control. All results were calculated from data from three independent experiments. \*, \*\*, \*\*\*, and \*\*\*\* for  $P$ -values < 0.05, 0.01, 0.001, and 0.0001, respectively.

**Figure 2. RSV infection decreases p53 stability via a proteasome-dependent pathway.** A. Human lung A549 cells were mock-infected or infected with RSV (Long strain) at a MOI of 1, and then analyzed at 24 and 48 hpi, respectively. After treatment with 50  $\mu$ M cycloheximide (CHX), p53 protein stability was assessed during a 50-min period, by monitoring p53 relative protein levels (RPL). Mean values  $\pm$  standard deviation from three independent experiments are presented. B. Human lung A549 cells were mock-infected or infected with RSV (Long strain) at a MOI of 1, and then analyzed at 48 hpi by western blot and densitometry in the presence/absence of proteasome inhibitor treatment (MG132 - 20  $\mu$ M), as indicated.

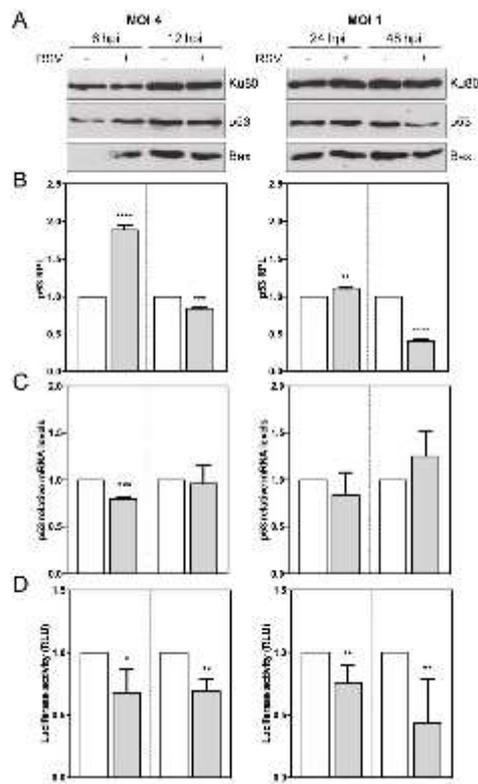
**Figure 3. RSV NS1 and NS2 both contribute to inhibit p53 transcriptional activity.** A. A549 cells were transfected with 1  $\mu$ g of plasmid expressing RSV NS1-eGFP or RSV NS2-eGFP. After 24 h post transfection, cells were fixed, immuno-stained and observed by immunofluorescence confocal microscopy. Expression of NS1 or NS2 (eGFP/green), p53 (red). Nuclei were counterstained with DAPI. Scale bar = 25  $\mu$ m. B. A549 cells were transfected with either an empty plasmid or two different amounts of plasmid expressing RSV NS1-eGFP (0.2 and 2  $\mu$ g). p53 global transcriptional activity and p53 mRNA expression were measured 48 h post-transfection, using a specific luciferase reporter assay (RLU) or RT-qPCR, respectively. C. In parallel, the same experimental strategy was followed with plasmid expressing RSV NS2-eGFP. All results in B and C were calculated from data from three independent experiments. \*, \*\*, \*\*\*, and \*\*\*\* for  $P$ -values < 0.05, 0.01, 0.001, and 0.0001, respectively. D. Stability assay in the presence of NS1 or NS2. A549 cells were transfected with either an empty plasmid (Empty) or plasmids expressing RSV NS1-GFP or RSV NS2-GFP (2  $\mu$ g). At 48 h post-transfection, p53 protein stability was assessed by monitoring relative protein levels (RPL) of p53 during a 50-min period, after treatment with 50  $\mu$ M cycloheximide (CHX). Mean values  $\pm$  standard deviation from three independent experiments are presented.

**Figure 4. p53-targeting approaches underline the antiviral contribution of p53 in the context of RSV infection.** A. Silencing of endogenous p53 mRNA expression in A549 cells increases RSV production. Forty-eight hours after si-RNA transfection using a control si-RNA (si-Ctrl) or a specific si-RNA (si-p53), A549 cells were infected by RSV (Long strain) at a MOI of 1 and viral production at 48 hpi was measured using two different methods: (i) determination of infectious titres in supernatants (log<sub>10</sub> TCID<sub>50</sub>/mL) by endpoint titration in Hep-2 cells (measured in quadruplicate in 2 independent experiments) and (ii) RT-qPCR (RSV genome copies/mL, measured in three independent experiments, \*\*\* for  $P$ -values < 0.001). B. Transient expression of p53 in H1299 cells (p53 null) decreased the level of viral production. Forty-eight hours after transfection, H1299 cells were infected by RSV at a MOI of 1 and viral production was measured at 48 hpi using the two complementary methods described in

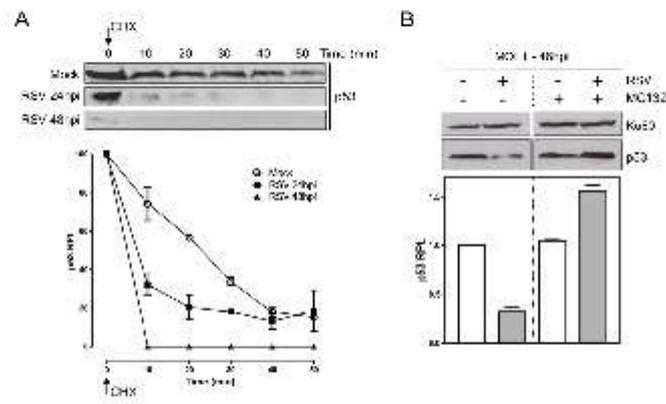
A. C. A549 cells were infected by RSV at a MOI of 1, in the presence of a small-molecule Mdm2 antagonist (Nutlin-3, 10  $\mu$ M) or DMSO (Ctrl). The level of viral production was measured at 48 hpi using the two complementary methods described in A.

**Figure 5. RSV infection modulates the p53/NF- $\kappa$ B balance via a NS2 – PI3K/Akt pathway.** A. Human lung A549 cells were mock-infected or infected with RSV (Long strain) at a MOI of 1 and analyzed at 48 hpi. p65 phosphorylation, considered as a reflect of NF- $\kappa$ B activity, was measured using a specific reporter assay and expressed as the relative value compared to mock. Measures were also performed in the context of treatment with DMSO (ctrl) or the small-molecule Mdm2 antagonist Nutlin-3 (10  $\mu$ M). \*, \*\*\*, and \*\*\*\* for *P*-values < 0.05, 0.001, and 0.0001, respectively. B. A549 cells were transfected with either an empty plasmid (Empty) or plasmids expressing RSV NS1-GFP or RSV-NS2-GFP. After 48 h post-transfection, the phosphorylation of p65 was measured using a specific reporter assay and expressed as the relative value compared to mock. \*, *P*-value < 0.05. C. Human lung A549 cells were mock-infected or infected with RSV (Long strain) at a MOI of 1 and analysed at 48 hpi. Cell lysates were analysed by western blot for the expression of both total Akt and phosphorylated Akt. Ku80 was used as loading control. The Akt-Pho/Total Akt ratio was determined using densitometry (3 separate experiments) \*\*\*\* for *P*-value < 0.0001). D. A549 cells were transfected with either an empty plasmid (Empty) or plasmids expressing RSV NS1-GFP or RSV-NS2-GFP. After 48 h post-transfection, cell lysates were analysed by western blot for the expression of both total Akt and phosphorylated Akt. Ku80 was used as a loading control. The Akt-Pho/Total Akt ratio was determined using densitometry (3 separate experiments, \* and \*\* for *P*-values < 0.05 and < 0.01, respectively). E. A549 cells, pre-treated by either si-ctrl or si-p53, were mock-infected or infected with RSV (Long strain) at a MOI of 1 and analysed at 48 hpi. A panel of human cytokines and chemokines was quantified using an antibody-based assay (Bio-Plex Pro – Human cytokines, BioRad). Results are represented as a heatmap showing for each condition the fold change compared to the mock (s-Ctrl/si-p53). F. Model proposed for the role of RSV infection on the p53/NF- $\kappa$ B balance. RSV infection modulates the p53/NF- $\kappa$ B balance *via* the activation of the PI3K/Akt – Mdm2 pathway, mainly through the NS1/NS2 proteins. This alteration of NF- $\kappa$ B balance in the context of infection further impacts (directly and/or indirectly) the regulation of a large panel of genes involved in different biological responses, including apoptosis as well as immune and inflammatory responses.

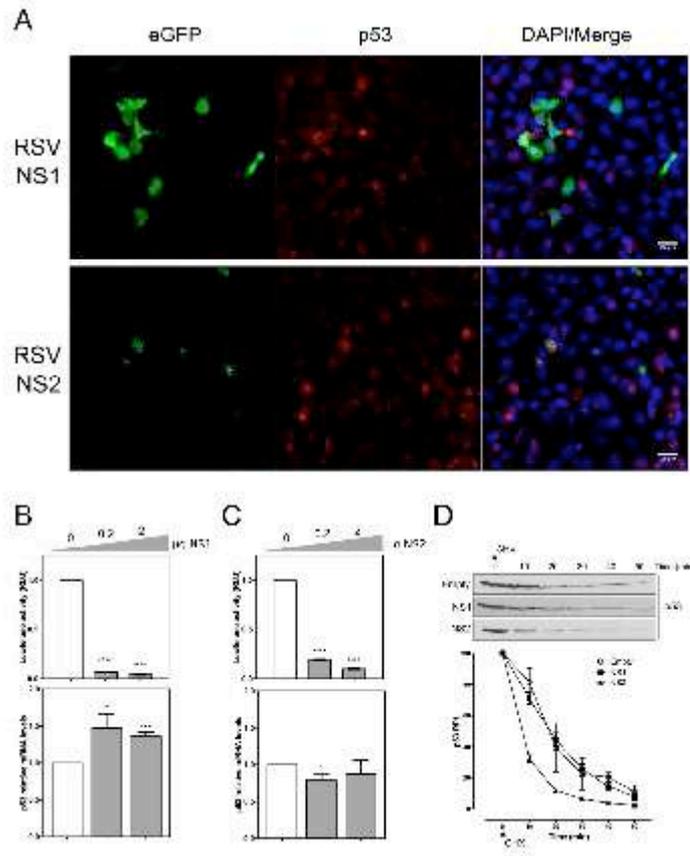
Figure 1



**Figure 2**



**Figure 3**



**Figure 4**

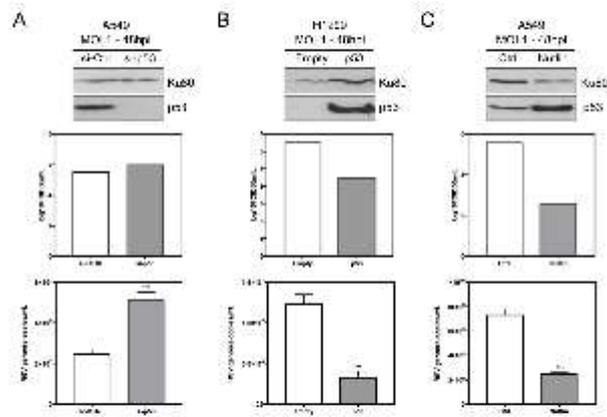
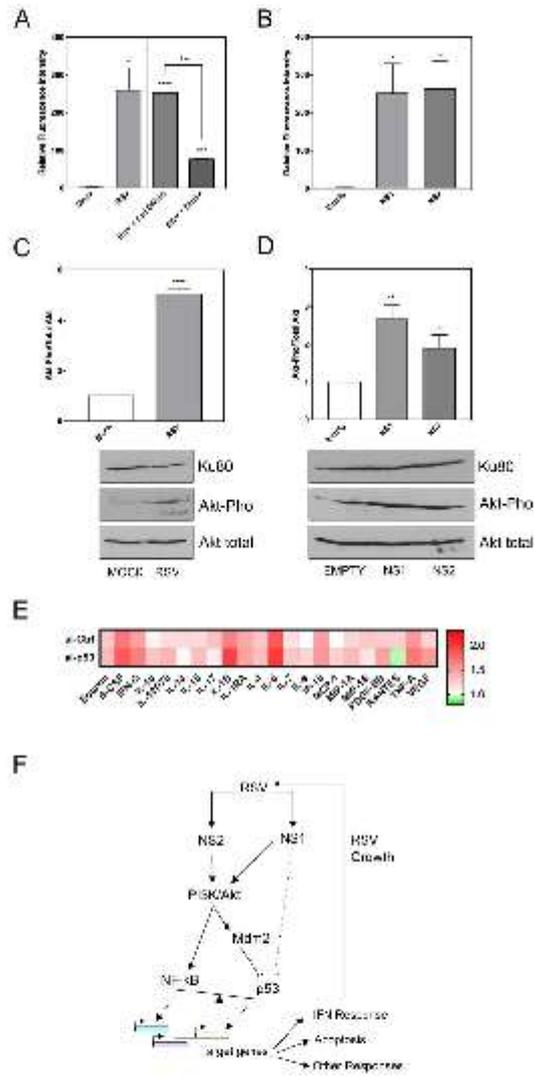


Figure 5



## **Chapter 3 Host-viral interactions during single and mixed infections: development of severe pneumonia model**

Pneumonia is a common inflammatory illness of the lungs which was described in the introduction part of this manuscript. The etiology of pneumonia can be either bacterial and/or viral, thus, mixed infection is a key element that promotes severe inflammatory disease and mortality.

Currently, the mechanisms involved in the lethal synergism between RSV and *S. pneumoniae* co-infection still remains misunderstood. We tried to develop a co-infection Nonhuman Primates (NHP) model of severe pneumonia susceptible to RSV and *S. pneumoniae* infection like the human. NHP lungs closely resemble the human lung anatomically and physiologically. NHP also exhibit immune responses to bacterial infections similar to the human and have some studies in vaccine development, including for *S. pneumoniae*.

RSV infection occurs an early age when maternal antibodies are present and in the failure of natural infection to prevent reinfection. A history of a formalin-inactivated RSV vaccine that enhanced disease in young children, and the lack of animal models that fully reproduce the pathogenesis of RSV infection in human difficult an efficacious vaccine development. RSV infection has already been observed in cynomolgus [490, 491]. Importantly, no study focus on pneumonia induced by mixed viral and bacterial infection in cynomolgus macaques. Very few new models are in development and no relevant models for respiratory diseases on primate are currently available due to the limited qualified centers in Europe.

In this study, we tried to develop a nonhuman primate model of severe pneumonia to evaluate pneumonia onset induced by mixed infection. Given the suboptimal performance of current diagnostic tests, we would like to characterize potential clinically relevant molecular profiles and to investigate the feasibility of identifying biomarker to severe pneumonia.

**Article 1. Establishing severe pneumonia in non-human primate model during mixed infection**

*Short communication*

**Establishing severe pneumonia in non-human primate during viral and bacterial infection: difficulties and challenges**

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### Abstract

1 Pneumonia is a serious respiratory disease spread throughout the world with high rates of  
2 mortality among very young children, elderly and immunocompromised. Clinical data suggest  
3 mixed respiratory infections are a relevant risk factor for pediatric intensive care hospitalization  
4 and death. To better understand the dynamics of host immune responses and pathogens the  
5 development of animal models with tissue interactions and immune system response homologous  
6 to those in humans is required. In this context, the aim of this study was to develop a model of  
7 severe pneumonia induced by RSV/*S. pneumoniae* co-infection that reflect the pneumonia  
8 disease in Human and allow the generation of relevant data. To expand the relevance of our  
9 findings and establish the utility of this model for the prevention and treatment of severe  
10 pneumonia, additional experiments are needed. In perspective, NHP model will help to describe  
11 several mechanisms involved in the pathogenesis of co-infection and identify prognostic  
12 biomarkers of severe pneumonia. Also, this model is important to validate findings in small  
13 animal models and may more closely predict vaccine and therapeutic efficacy for severe disease  
14 in humans.

**Keywords:** Pneumonia, Nonhuman Primates, mixed infections

15 Pneumonia is a serious respiratory disease spread throughout the world with high rates of  
16 mortality among very young children, elderly and immunocompromised [1, 2]. The etiology of  
17 pneumonia can be diverse ranging from viruses, bacteria or fungi, and often by the interaction of  
18 one or more pathogens [3, 4]. Respiratory syncytial virus (RSV) is responsible for the majority of  
19 cases of pneumonia in children [5]. Due to the importance of the disease caused by RSV, the lack  
20 of preventive measures and the high circulation of this virus in the world, the development of a  
21 vaccine is a priority. However, the lack of understanding of the correlation between the immune  
22 response and the severity of the disease has hampered the development of a safe vaccine.  
23 Pneumonia is also associated with the *Streptococcus pneumoniae* which represent a significant  
24 public health burden with an estimated 14.5 million cases of the serious pneumococcal disease  
25 worldwide [6]. Clinical data suggest mixed respiratory infections are a relevant risk factor for  
26 pediatric intensive care hospitalization and death [7], especially in secondary pneumococcal  
27 infection following RSV [8, 9]. Currently, the mechanisms involved in the interactions between  
28 RSV and *S. pneumoniae* co-infection remains misunderstood.

29 Commonly, an animal model of human respiratory infections includes small mammals like  
30 rodents because of their low cost, size, availability, the diversity of exploratory techniques and  
31 availability of reagents [10, 11]. To better understand the dynamics of host immune responses  
32 and pathogens the development of animal models with tissue interactions and immune system  
33 response homologous to those in humans is required.

34 Insights of the mechanism of immunity and interactions with RSV or *S. pneumoniae* have been  
35 described in cotton rats [12-16] and mice balb/c [17-29]. Ferrets are among one of the best  
36 models to study influenza viruses, but RSV is able to replicate only in the upper respiratory  
37 system, or in the lungs of immunodepressed ferrets [30]. However, RSV is not a true infectious  
38 pathogen of small animals (mice and ferrets) being semi- to not permissive to the infection and  
39 limiting virus replication in lung tissue. Additionally, immune response differences between  
40 these animals and humans are relevant.

41 Mixed infections were showed to correlates to the severity of pneumonia and in vivo study  
42 described that viral respiratory infections can enhance pneumococcal severity [31]. Also, *S.*  
43 *pneumoniae* has been shown to enhance RSV infection in differentiated human airway epithelial  
44 cells, in vitro, and in cotton rats [32]. Thus, correlations between RSV and *S. pneumoniae*

45 infection were already described in the literature but more information concerning disease  
46 development and severity is needed.

47 In the literature, studies with non-human primates (NHP) infected with RSV have been  
48 described. Chimpanzees are permissive to RSV and present a respiratory infection similar to  
49 humans [33-35]. Vaccines have already been tested on African green monkeys [36-41]. Several  
50 studies have tried to make a model with macaques and some studies have studied vaccine  
51 efficient in rhesus [42-44];

52 In other nonhuman primates like bonnet monkeys [45-47], owl monkeys [40, 48-50], Cebus  
53 monkeys [51] and Infant baboons [52] some groups have described RSV infection, but always  
54 requiring high doses of virus and few or no clinical signs of the disease [53]. The feasibility to  
55 mimic pneumococcal pneumonia in macaques has been already described elsewhere [54],  
56 showing that intranasal injection in infant rhesus macaques resulted in symptomatic  
57 pneumococcal pneumonia after 48 hours.

58 Thus, is possible to find RSV or Sp infected in vivo models but no one focused on pneumonia  
59 induced by mixed viral and bacterial infection in NHP. The NHP model constitutes a unique  
60 opportunity to 1) better understand the mechanisms involved in mixed viral and bacterial  
61 respiratory co-infections; 2) to identify prognosis biomarkers related to pneumonia severity and  
62 3) to develop novel therapeutic strategies. *Cynomolgus* macaques represent the major NHP  
63 resource for biomedical research and offer valuable information, relevant to humans, into disease  
64 mechanisms so as to develop new improved therapies, diagnostics, and vaccines.

65 In this context, the aim of this study was to develop a model of severe pneumonia induced by  
66 RSV/*S. pneumoniae* co-infection that reflect the pneumonia disease in Human and allow the  
67 generation of relevant data. Therefore, the NHP model was selected and more specifically the  
68 cynomolgus macaque (*Macaca fascicularis*), due to its genetic and anatomical similarity with  
69 Human that are relevant to describe several mechanisms involved in pneumonia pathogenesis,  
70 allow the identification and characterization of prognostic biomarkers associated with severe  
71 respiratory disease and also better understand host response to mixed infection. We described the  
72 difficulties and challenges of an adaptation of severe pneumonia in NHP model.

73

74 In order to develop the model of severe pneumonia in NHP, each macaque was infected as  
75 described in table 1 and monitored during 23 days to evaluate the symptomatic infection of SP

76 and RSV. To characterized pneumonia and disease severity, bronchoalveolar lavage (BAL)  
77 fluids, upper respiratory swabs, and blood samples were collected throughout the 23 days of  
78 infection and analyzed for bacterial culture and quantification.

79

80 BAL fluids, upper respiratory swabs and blood samples from control, single and co-infected  
81 macaques were collected and analyzed for viral and bacterial quantification. RSV replication was  
82 tracked in BAL and nasal swabs from all animals by real-time PCR but it was not possible to  
83 identify RSV in any of the samples collected. Sp quantities were assessed by a real-time PCR  
84 assay used by our group in the multicentric study on pneumonia among children. During the  
85 course of the study, Sp was detected by real-time PCR in nasal swabs in some samples but a  
86 continuous infection was not detected (Fig. 1A). In other studies, even with small animals, it is  
87 very hard to identify RSV in samples [55] and differences in virus detection methodology can  
88 explain the differences between our study and Grandin et al. [56], that used the same NHP model.  
89 For bacteria detection, Kraft et al. [54] were able to detect bacteria in samples with large  
90 quantities of bacteria.

91

92 Blood was cultured in the medium of bacterial culture, and the bacteria identified by Vitek  
93 technology, differing gram-positive or not bacterium. The only bacterium detected was  
94 sequenced and identified as a *Staphylococcus pettenoferi* in control 2 animal (justifying  
95 monocytosis on days 2 and 4). This bacterium causes gingivitis in monkeys and indicates that  
96 perhaps the acclimation time should be longer, or that only the manipulation of the monkeys for  
97 treatment, causes a decrease of the immune system and leaves the macaques susceptible to  
98 opportunistic diseases. Blood culture is an important diagnostic methodology utilized for  
99 bacterial detection during pneumonia in human but it is detected only in high bacterial  
100 concentrations studies [54] according to our results.

101

102 Blood was also analyzed in complete blood count to identify inflammation markers. During a  
103 bacterial single infection, we were able to detect monocytosis (Fig. 1A) and lymphopenia was  
104 found in RSV single and mixed infected. No differences in the white blood cell count were  
105 identified by Kraft et al. even in high doses of bacteria group like in our results. However, they  
106 described leukopenia after 48 hours which we don't detect but differences in quantities of

107 bacteria inoculated can explain these differences [54]. Another study was already showed that the  
108 proportion of neutrophils in BAL of monkeys infected with RSV increased only to 9%, [45],  
109 whereas neutrophils are the predominant cell in RSV infected children [57].

110

111 Weight, temperature, and behavior were also analyzed and there was no difference between the  
112 control and the infected ones. Clinical signs of disease are uncommon following RSV infection in  
113 NHP model, and they develop mild histopathological changes in the lung. Another important  
114 contribution to detect the development of pulmonary lesions and track the course of illness can be  
115 the use of thoracic imaging [58].

116

117 Respiratory tract biopsies (lungs, trachea, and bronchi) allowed us to observed macroscopic and  
118 microscopic histological evidence of injury and evaluate disease severity between single and co-  
119 infection. We observed that RSV and bacterial co-infection resulted in more severe lung  
120 histopathology. Single Sp infection was characterized by heterogeneity inflammation suggesting  
121 an acute infection but not classical pneumonia (Fig. 1B). Histopathological changes of RSV  
122 infection are characterized by bronchiolar epithelial cell necrosis and infiltration with a lot of  
123 neutrophils, eosinophils, and lymphocytes. Our results show a characterized inflammation  
124 developed near to bronchioles with large quantities of lymphocytes. All animals showed an  
125 important infection in left lung probably due to technical manipulation during inoculation steps  
126 and further research is needed to determine the optimal route of infection. In addition, direct  
127 introduction of pathogens into the lower respiratory tract maybe not represent natural exposures,  
128 but can help to maximize the severity of the disease.

129

130 Lung immune cell responses in nonhuman primates likely correlate better to those of humans  
131 than those of more genetically distant animals. A model of severe pneumonia following influenza  
132 and bacterial co-infection in nonhuman primates was already described [59, 60].

133

134 Mixed infection in our model did not progress to severe pneumonia. We believe that the health  
135 status of the animals protected them from more severe disease, similar to healthy humans infected  
136 with RSV and carriage of Sp. Despite this, mild pneumonia was found in our model. The severity  
137 of pneumonia is dependent upon the viral and bacterial strain used, pathogen dose, and route of  
138 infection and maybe high-dose of each pathogen is needed to induce more than mild illness in

6

139 primates. Another strategy for severe pneumonia model could be to develop immunodeficient  
140 NHP. To expand the relevance of our findings and establish the utility of this model for the  
141 prevention and treatment of severe pneumonia, additional experiments are needed. In perspective,  
142 NHP model will help to describe several mechanisms involved in the pathogenesis of co-  
143 infection and identify prognostic biomarkers of severe pneumonia. Also, this model is important  
144 to validate findings in small animal models and may more closely predict vaccine and therapeutic  
145 efficacy for severe disease in humans.  
146

147 **Figure legends**

148 **Table 1. Pathogens infection distribution by animal.** Cynomolgus macaques (*Macaca*  
149 *fascicularis*) were acclimatized to laboratory conditions for one month prior to first treatment.  
150 Then, RSV and/or Sp infected (n=4) and *pathogen-free* macaques (n=2) was inoculated with  
151  $1.10^6$  TCID<sub>50</sub>/mL of RSV-A and  $1.10^6$  CFU/mL of *S. pneumoniae* serotype 14 intranasal and  
152 intratracheal to maximize lower respiratory tract disease.

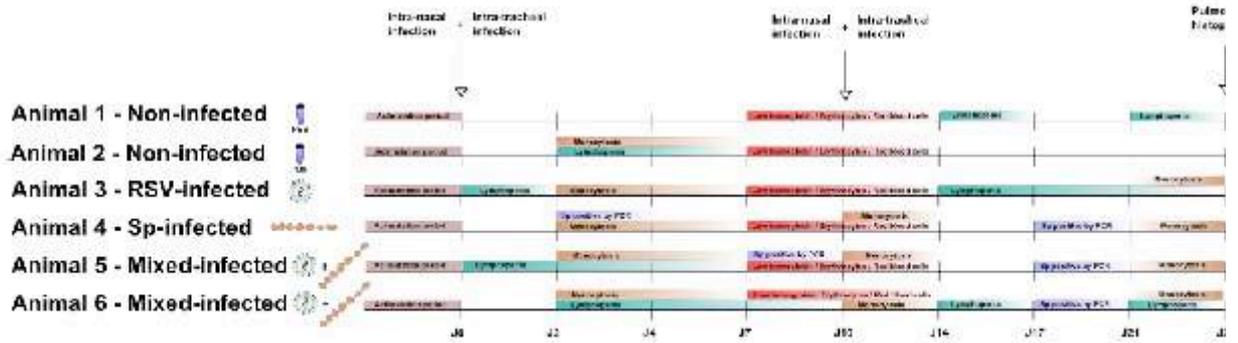
153

154 **Figure 1. RSV and/or Sp infections: study design and results.** A) Timeline showing infections  
155 time points, days of samples collection and lung histopathology. Colored bars resume analyzed  
156 results of all methodologies utilized. Blue bars represented lymphopenia; brown bars represented  
157 monocytosis and red bars low hemoglobin, erythrocytes and red blood cells. Samples positive for  
158 *S. pneumoniae* by PCR are shown in respective days of detection by purple bars. B)  
159 Histopathology of the lung of all infected animals showing *S. pneumoniae* infected an animal  
160 with slight signals of inflammation, RSV-infected characterized by lymphoid clusters and two  
161 co-infected animals showing areas of sub-pleural reinforcement (01) and congestive vessels,  
162 inflammatory cells, and hemorrhage (02).

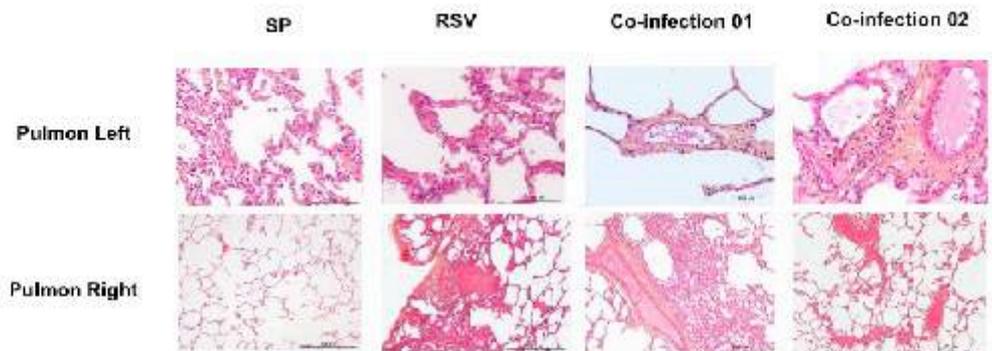
163

| Experimental group        | Delivered inoculum           |                   |
|---------------------------|------------------------------|-------------------|
|                           | RSV (TCID <sub>50</sub> /mL) | Sp14 (CFU/mL)     |
| Animal 1 – Non-infected   | X                            | X                 |
| Animal 2 – Non-infected   | X                            | X                 |
| Animal 3 – RSV-infected   | 1.10 <sup>6</sup>            | X                 |
| Animal 4 – Sp14-infected  | X                            | 1.10 <sup>6</sup> |
| Animal 5 – Mixed-infected | 1.10 <sup>6</sup>            | 1.10 <sup>6</sup> |
| Animal 6 – Mixed-infected | 1.10 <sup>6</sup>            | 1.10 <sup>6</sup> |

**A**



**B**



## Bibliography

1. WHO, W.H.O. *Pneumonia*. 2016; Available from: <http://www.who.int/mediacentre/factsheets/fs331/en/>.
2. Stupka, J.E., et al., *Community-acquired pneumonia in elderly patients*. *Aging Health*, 2009. 5(6): p. 763-774.
3. Johansson, N., et al., *Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods*. *Clin Infect Dis*, 2010. 50(2): p. 202-9.
4. Honkinen, M., et al., *Viruses and bacteria in sputum samples of children with community-acquired pneumonia*. *Clin Microbiol Infect*, 2012. 18(3): p. 300-7.
5. Miller, E.K., et al., *Viral etiologies of infant bronchiolitis, croup and upper respiratory illness during 4 consecutive years*. *Pediatr Infect Dis J*, 2013. 32(9): p. 950-5.
6. O'Brien, K.L., et al., *Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates*. *Lancet*, 2009. 374(9693): p. 893-902.
7. Richard, N., et al., *The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis*. *Pediatr Infect Dis J*, 2008. 27(3): p. 213-7.
8. Stensballe, L.G., et al., *Hospitalization for respiratory syncytial virus infection and invasive pneumococcal disease in Danish children aged <2 years: a population-based cohort study*. *Clin Infect Dis*, 2008. 46(8): p. 1165-71.
9. Ampofo, K., et al., *Seasonal invasive pneumococcal disease in children: role of preceding respiratory viral infection*. *Pediatrics*, 2008. 122(2): p. 229-37.
10. McCool, T.L. and J.N. Weiser, *Limited role of antibody in clearance of Streptococcus pneumoniae in a murine model of colonization*. *Infect Immun*, 2004. 72(10): p. 5807-13.
11. Bogaert, D., et al., *Impaired innate and adaptive immunity to Streptococcus pneumoniae and its effect on colonization in an infant mouse model*. *Infect Immun*, 2009. 77(4): p. 1613-22.
12. Boukhvalova, M.S. and J.C. Blanco, *The cotton rat Sigmodon hispidus model of respiratory syncytial virus infection*. *Curr Top Microbiol Immunol*, 2013. 372: p. 347-58.
13. Prince, G.A., et al., *The pathogenesis of respiratory syncytial virus infection in cotton rats*. *Am J Pathol*, 1978. 93(3): p. 771-91.
14. Shaw, C.A., et al., *The role of non-viral antigens in the cotton rat model of respiratory syncytial virus vaccine-enhanced disease*. *Vaccine*, 2013. 31(2): p. 306-12.
15. Boukhvalova, M., et al., *Treatment with novel RSV Ig RI-002 controls viral replication and reduces pulmonary damage in immunocompromised Sigmodon hispidus*. *Bone Marrow Transplant*, 2016. 51(1): p. 119-26.
16. Wong, D.T., et al., *Respiratory syncytial virus infection in immunosuppressed animals: implications in human infection*. *J Med Virol*, 1985. 17(4): p. 359-70.
17. van Diepen, A., et al., *Host proteome correlates of vaccine-mediated enhanced disease in a mouse model of respiratory syncytial virus infection*. *J Virol*, 2015. 89(9): p. 5022-31.
18. Shafique, M., et al., *Evaluation of an Intranasal Virosomal Vaccine against Respiratory Syncytial Virus in Mice: Effect of TLR2 and NOD2 Ligands on Induction of Systemic and Mucosal Immune Responses*. *PLoS ONE*, 2013. 8.
19. Stokes, K.L., et al., *Differential pathogenesis of respiratory syncytial virus clinical isolates in BALB/c mice*. *Journal of Virology*, 2011. 85: p. 5782-5793.
20. Aeffner, F., et al., *Double-stranded RNA induces similar pulmonary dysfunction to respiratory syncytial virus in BALB/c mice*. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 2011. 301: p. L99-L109.

21. Lindell, D.M., et al., *A Novel Inactivated Intranasal Respiratory Syncytial Virus Vaccine Promotes Viral Clearance without Th2 Associated Vaccine-Enhanced Disease*. PLoS ONE, 2011. 6: p. e21823.
22. Openshaw, P.J., *The mouse model of respiratory syncytial virus disease*. Curr Top Microbiol Immunol, 2013. 372: p. 359-69.
23. Byrd, L.G. and G.A. Prince, *Animal models of respiratory syncytial virus infection*. Clin Infect Dis, 1997. 25(6): p. 1363-8.
24. Rameix-Welti, M.A., et al., *Visualizing the replication of respiratory syncytial virus in cells and in living mice*. Nat Commun, 2014. 5: p. 5104.
25. Taylor, G., et al., *Respiratory syncytial virus infection in mice*. Infect Immun, 1984. 43(2): p. 649-55.
26. Graham, B.S., et al., *Primary respiratory syncytial virus infection in mice*. J Med Virol, 1988. 26(2): p. 153-62.
27. Bhoj, V.G., et al., *MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus*. Proc Natl Acad Sci U S A, 2008. 105(37): p. 14046-51.
28. Jafri, H.S., et al., *Respiratory syncytial virus induces pneumonia, cytokine response, airway obstruction, and chronic inflammatory infiltrates associated with long-term airway hyperresponsiveness in mice*. J Infect Dis, 2004. 189(10): p. 1856-65.
29. Mukherjee, S., et al., *IL-17-induced pulmonary pathogenesis during respiratory viral infection and exacerbation of allergic disease*. Am J Pathol, 2011. 179(1): p. 248-58.
30. Stittelaar, K.J., et al., *Ferrets as a Novel Animal Model for Studying Human Respiratory Syncytial Virus Infections in Immunocompetent and Immunocompromised Hosts*. Viruses, 2016. 8(6).
31. Thorburn, K., et al., *High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis*. Thorax, 2006. 61(7): p. 611-5.
32. Nguyen, D.T., et al., *Streptococcus pneumoniae Enhances Human Respiratory Syncytial Virus Infection In Vitro and In Vivo*. PLoS One, 2015. 10(5): p. e0127098.
33. Belshe, R.B., et al., *Experimental respiratory syncytial virus infection of four species of primates*. J Med Virol, 1977. 1(3): p. 157-62.
34. Crowe, J.E., Jr., et al., *A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV*. Vaccine, 1993. 11(14): p. 1395-404.
35. Collins, P.L., et al., *Evaluation in chimpanzees of vaccinia virus recombinants that express the surface glycoproteins of human respiratory syncytial virus*. Vaccine, 1990. 8(2): p. 164-8.
36. Bates, J.T., et al., *Immunogenicity and efficacy of alphavirus-derived replicon vaccines for respiratory syncytial virus and human metapneumovirus in nonhuman primates*. Vaccine, 2016. 34(7): p. 950-6.
37. Jin, H., et al., *Evaluation of recombinant respiratory syncytial virus gene deletion mutants in African green monkeys for their potential as live attenuated vaccine candidates*. Vaccine, 2003. 21(25-26): p. 3647-52.
38. Jones, B.G., et al., *Sendai virus-based RSV vaccine protects African green monkeys from RSV infection*. Vaccine, 2012. 30(5): p. 959-68.
39. Tang, R.S., et al., *Parainfluenza virus type 3 expressing the native or soluble fusion (F) Protein of Respiratory Syncytial Virus (RSV) confers protection from RSV infection in African green monkeys*. J Virol, 2004. 78(20): p. 11198-207.
40. Richardson, L.S., et al., *Evaluation of five temperature-sensitive mutants of respiratory syncytial virus in primates: I. Viral shedding, immunologic response, and associated illness*. J Med Virol, 1978. 3(2): p. 91-100.

## *DISCUSSION AND PERSPECTIVES*

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Au cours de mon travail de thèse, l'objectif général était de mieux comprendre les interactions multiples et complexes entre les pathogènes respiratoires et l'hôte, à différents niveaux, *in vitro* et *in vivo*. En effet, mon travail était centré sur différents pathogènes ou combinaisons d'entre eux (IAV, RSV, *S. pneumoniae*), sur différents modèles cellulaires *in vitro* (macrophages, cellules épithéliales pulmonaires humaines), et *in vivo* (primate non humain), et sur différents types des voies cellulaires (réponse immunitaire, voie p53 / NF-κB). Ces différents projets ont mis en évidence quelques points intéressants de discussion générale que je voulais aborder dans ce chapitre.

During my thesis work, the general objective was to better understand the multiple and complex interactions between respiratory pathogens and the host, at different levels, *in vitro*, and *in vivo*. Indeed, my work was focused on different pathogens or combination of them (IAV, RSV, *S. pneumoniae*), on different *in vitro* cellular models (macrophages, human lung epithelial cells), and *in vivo* (Nonhuman primate), and on different types of cellular pathways (immune response, p53/NF-κB pathway). These different projects have highlighted some interesting points of general discussion I wanted to address in this chapter.

### **Does viral-bacterial coinfection influence the severity of disease?**

A large number of clinical studies have described an enhanced severity of disease in the context of mixed infections, highlighting the importance of performing studies to better understand the role of each pathogen as well as host/pathogen interactions [156, 492-499]. In light of the major public health burden, pneumonia caused by *S. pneumoniae* following IAV infection has been extensively studied in children and adults with a correlation of disease severity [155, 500, 501]. In contrast, the clinical significance of bacterial co-infections during RSV remains relatively unclear. Children with RSV/bacterial co-infection have been found to be hospitalized for longer periods and required ventilator support for longer than sole RSV infections but more studies are needed to confirming this correlation [140, 141, 476].

In our translational study (article #1, chapter 1), the patient cohort studied was restricted (n=74) but the classification of non-severe and severe pneumonia was accurately defined, according to an exhaustive list of criteria. Thus, we managed to demonstrate an increase in disease severity in mixed infection cases. However, determining the precise contribution of each pathogen to disease severity by the clinical setting remained extremely difficult. Studies focused on the identification of etiological agents are difficult to compare due to a lack of standards for information on severity, In addition, variation in techniques, diagnostic cut-off values, and pathogens targeted between studies may confound the determination of the overall clinical significance of viral/bacterial co-infection.

In addition to clinical studies, several reports show the interactions between pathogens, suggesting a correlation with severity disease. For example, It was demonstrated that respiratory viral infection promotes an increasing of *S. pneumoniae* density in children with pneumonia [502]. Moreover, direct interactions between virus and bacteria are capable of increased virulence of the other pathogens, such as was described for RSV that is capable to interact with a protein of *S. pneumoniae* increasing bacterial virulence [159]. Bacterial-virus interactions were not studied in this thesis, however, the detection of the two pathogens in the same macrophage showed by immunofluorescence in article #1 (chapter 1) reveals that the model developed in Chapter 1 can be used in viral-bacterial interactions studies. Complementary studies, like immunoprecipitation, will be necessary to identify possible close viral-bacterial interactions.

A better understanding of pathogens involvement and their contribution to disease severity is required for the development of future strategies for the prevention and treatment of severe respiratory tract infection. *In vivo* studies constituted a good methodology for an attempt this goal confirming the necessity of *in vivo* models. Also, another important point to better understand the contribution of each pathogen for disease severity is to better understand interactions and strategies that the pathogen can utilize in cells during infection. For this attempt, I focused my interest on host/pathogen interactions at the level of p53, a key transcription factor at the crossroads between large numbers of cellular pathways – including innate immune responses.

## **Are macrophages activated cells during acute lower respiratory infections?**

Macrophages are a population of phagocytic cells composed of different phenotypes according to the local environment and differentiate stimuli [503, 504]. They are known to constitute long-lived reservoirs for some persistent viruses (e.g., HIV and Chikungunya virus)[505, 506] and several studies suggest that they contribute to allergic inflammatory responses and chronic respiratory disease, in addition to lower respiratory tract infections [507-510].

In the lungs, alveolar macrophages (AMs) are found in abundance and they are strategically situated as the first line of defense against respiratory pathogens playing a central role in innate host defense [480, 511, 512]. AMs constitute a subset of macrophages that develops from fetal liver monocytes and is phenotypically and functionally different from other tissue macrophages [513]. Alteration of the physiological function of AMs during infection, such as pattern of cytokines and chemokines profile, could lead to lung damage [514-517]

Numerous studies demonstrate that AMs play an important role in the response to infection [513, 517-520]. High expression of pro-inflammatory cytokines and up-regulation of many innate defense genes by AMs in response to IAV infection [513]. In addition, AMs are capable to control RSV infection with an important role in phagocytosis and cytokine production [513, 521, 522]. Also, they play important role in control bacterial infection [515, 523].

Despite the poorly characterization of human monocyte-derived macrophages phenotypes, some studies show comparable results between AMs and monocyte-derived macrophages [514, 524]. Results from article #1 (Chapter 1) described a reduction of phagocytosis of *S. pneumoniae* by viral infection confirming results already published in different macrophages subtypes [322, 480, 481, 484, 519, 525, 526]. An *in vivo* model could also contribute to this aspect since on obtaining bronchoalveolar aspirate the quantification and identification of active macrophages during co-infection could be performed.

To substantiate the important role of macrophages in co-infection, the expression of cytokines and chemokines by these cells was measured. In a model *in*

*vitro* developed in article 1 and 2 (Chapter 1), IAV and RSV infection altered biological functions in the monocytes-derived macrophages, inducing an antiviral state and bacterial co-infection show an exacerbated inflammatory response, mediated at least by IP-10 expression.

### **Importance of cytokines and chemokines expression during respiratory infections: a good strategy for the development of biomarkers of interest?**

The disease severity is not only determined by the causal agent, but also by interactions between the immune response and pathogen. The balance of this interaction is regulated through complex interactions between immune cells and pro- and anti-inflammatory cytokines [527]. Also, different pathogens may trigger different inflammatory responses depending on their intrinsic properties [528, 529]. It is well known that the cytokine storm is present in severe pneumonia, so, cytokines appear to be good biomarkers targeting the immune response. In chapter 1 (articles #1 and #2), mixed co-infection show an increase of cytokines expression when compared with RSV or IAV single infection, suggesting an increased inflammatory pathway. In addition, CXCL-10/IP-10 expression pattern found in bacterial and viral pneumonia highlight its importance as severity biomarker. IP-10 is expressed by macrophages in early response to infection and attracts activated T-cells to the site of inflammation IP-10 expression in macrophages infected with RSV was already described [513, 530, 531].

Also, Hayney et al described that an increased IP-10 concentration measured in serum of adult with pneumonia correlates with the severity, duration, and illness symptoms and concluded that IP-10 could serve as a useful marker or predictor of respiratory infections severity in adults [532]. In addition, Principi et al. described IP-10, in addition to CRP and TRAIL expression capable to distinguish bacterial from viral infections [481]. Thus, we propose that IP-10 is an important component during mixed infection and associated with others can be a good set of biomarkers.

Macrophages normally express cytokines in response to infection by pathogens. Often it is also activated by the cytokines expressed by the respiratory tract cells that are the primary site of infection. To continue studying the interactions

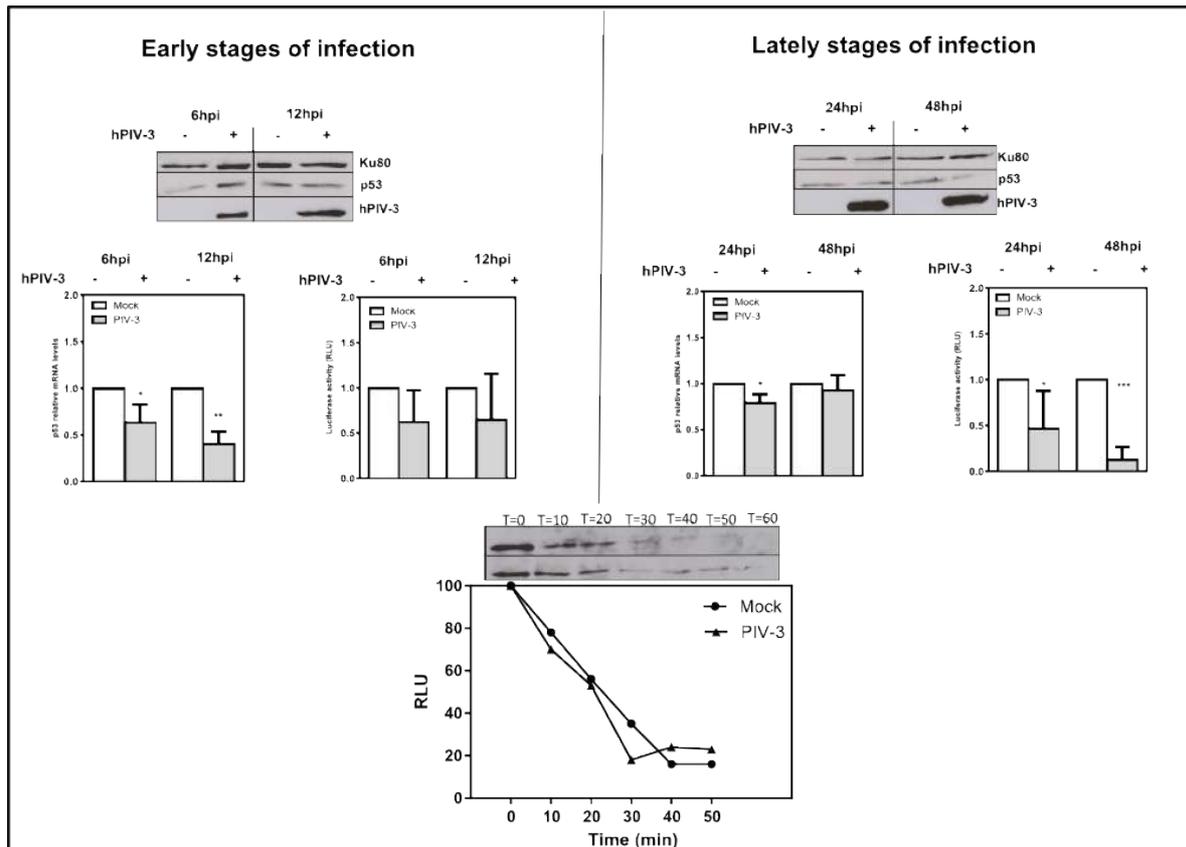
between RSV infection and the immune system, I have chosen to study an important cellular mechanism capable of modulating various pathways in the epithelial cells which play an important role in the immune response.

**Host-Pathogens interactions: Role of the master cell regulator p53 pathway during viral infection: similarities and differences between IAV, RSV, PIV-3, and mixed-infection.**

Respiratory tract epithelial cells are the first site of infection and promote the immune response activation. In the immune response, p53 not only promotes the activation of IFN-stimulated genes (ISGs) but also enhances IFN production in virus-infected cells [345].

The human parainfluenza viruses (hPIVs) belong to a diverse group of enveloped single-strand RNA viruses within the Paramyxoviridae family, a large group of viruses that was recently reformulated. The hPIV genome is quite the same of RSV genome, and viral replication is almost the same [533]. After RSV, hPIV type 3 is the leading cause of hospitalization for respiratory illness in young children from ages 0 to 2 years, with a marked increase among 1- to 2-year-olds [87]. Due to hPIV-3 importance and similarities with RSV, we decided to look for p53 interactions during hPIV-3 infection.

As part of complementary results, p53 protein and mRNA expression show that hPIV-3 promotes the downregulation of p53 by transcriptional level while the same group of results shows a posttranscriptional regulation of p53 during RSV infection in article 1 of Chapter 2. The decreased of p53 during these viral infections were already described by Ellis et al and Marques et al. [433, 534] with some contrarious results, probably due to differences in virus strain and time-course of infection. However, there are not many data in the literature that describe more details about these interactions.

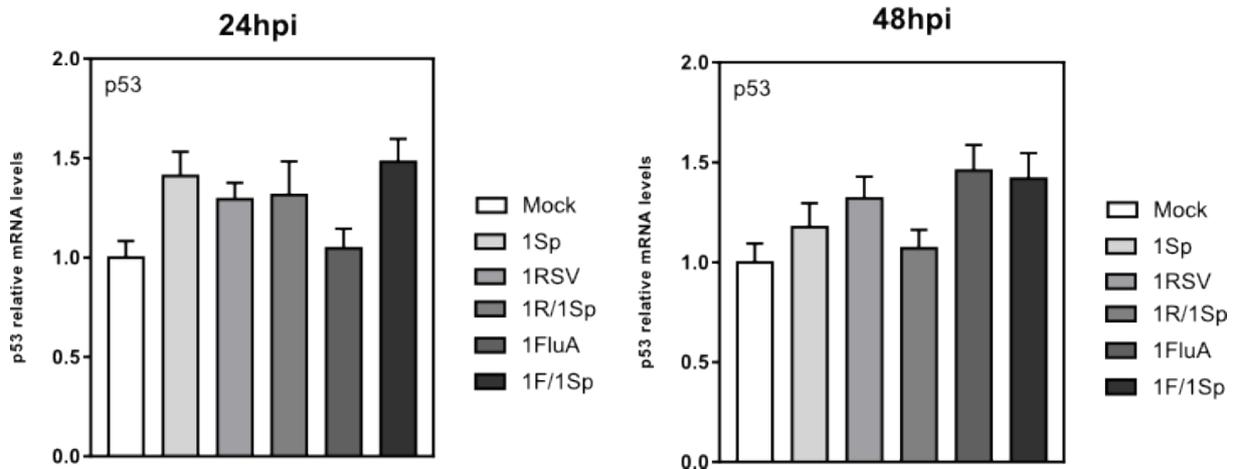


**Figure 20 Post-transcriptionally p53 modulation during hPIV-3 infection.**

The p53 pathway can be modulated during different stages of infection. A) Results from early stages of infection showing no differences in p53 protein expression by western blot, a decreased in mRNA expression without changes in the p53 transcriptional activity. B) During late stages of infection, western blot revealed a decreased of p53 proteins, while mRNA expression is stabilized but an important decreased of transcriptional activity of p53 is detected, suggesting a post-transcriptional impairment of p53 by hPIV-3. C) The half-life of p53 was measured with cycloheximide technique described by XX. hPIV-3 apparently, don't change half-life of p53, probably they don't promote p53 degradation.

The interactions between p53 and viral-bacterial infections are not available in the literature and even if preliminaries results show a tendency of increased mRNA p53 expression in IAV-*S. pneumoniae* infections, complementary results in future projects are needed.

## Co-infection



**Figure 21 A different pattern of p53 expression during viral and mixed infections.**

RT-PCR was used to quantify mRNA p53 expression in late stages of infection and the modifications apparently are up-regulation of p53 showing a synergism of expression during IAV and Sp infection.

The interplay between influenza viruses and p53 has been reported in different studies [438, 440, 452, 514] and influenza viruses modulate p53 transcriptional activity is modulated during time-course of infection, with a significant increase in early infection [455]. In contrast, this activity is significantly inhibited during the later stages of infection, correlating to elevated NS1 protein levels, which has been described to inhibit p53-mediated transcriptional activity [455]. Results in article #1 (Chapter 2) show that RSV infection modulates p53 transcriptional activity and an important role for non-structural protein NS1, contributing to inhibition of p53 activity by proteasomal degradation.

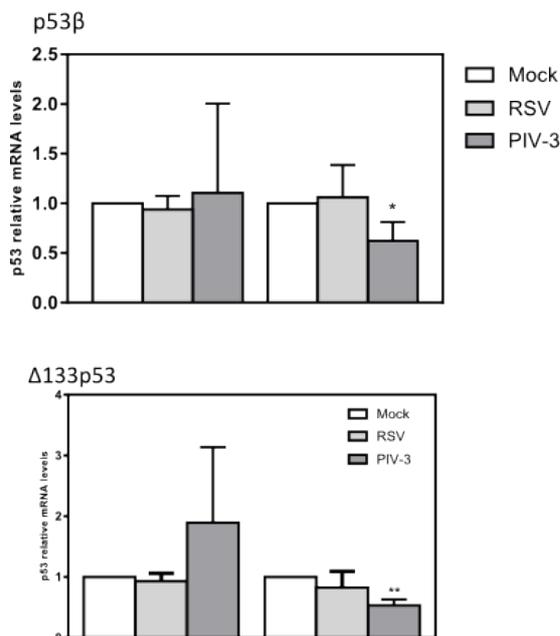
Further studies are required to understand the impact of RSV infection on p53 biological functions. To explore others possible p53 regulation by RSV and PIV-3 preliminary results shows the possible role of p53 isoforms in their self-regulation.

## Expression of p53 isoforms during viral infections: insights of p53 regulation

Since some interplays between p53 and respiratory viruses have been described, Terrier and colleagues investigated the role of  $\Delta 133p53\alpha$  and p53 $\beta$  in the context of an IAV infection [396, 402]. In the literature, it was reported that p53 $\beta$  can promote p53 transcriptional activity while  $\Delta 133p53\alpha$  inhibits in different cell lineages [397, 401, 402]. In addition, several studies reported a deregulation of p53 isoforms expression in human cancers [393, 395, 397-400].

For Influenza virus, distinct roles of the  $\Delta 133p53\alpha$  and p53 $\beta$  isoforms were described in the literature, showing a modulation of the expressions of  $\Delta 133p53\alpha$  and p53 $\beta$  at the transcriptional and posttranscriptional levels affecting viral production. Preliminary results can't show the impact of RSV in the expression of mRNA of these two isoforms, however, hPIV-3 show an impact on  $\Delta 133p53\alpha$  and p53 $\beta$  expression.

### Early stages of infections



In order to evaluate the role of p53 isoforms mRNA expression during RSV (light grey) or hPIV-3 (dark grey) infection, A549 cells were infected and results from 6 and 12 hours post-infection are shown. No significant differences in the isoforms expression were detected during RSV infection while hPIV-3 is probably capable to impaired p53 isoforms expression with a transcriptional strategy.

**Figure 22 Expression of p53 isoforms during RSV or hPIV-3 infection.**

During early stages of infections, no differences in p53 isoforms expression were detected during RSV infection. Controversially, hPIV-3 promotes a diminution of p53 isoform expression.

This preliminary results according to with the other preliminary result comparing hPIV-3 to RSV, showing that these viruses don't have the same strategies to regulate p53 activity. Further studies like inhibition of both p53 isoforms during infection to look the impact on viral replication and western blot analyses to conclude if these differences in mRNA level are representative of protein level are necessary to understand the impact of different p53 isoforms during viral infection and confirming the antiviral role of the p53 pathway.

### **Development of severe pneumonia model: application and difficulties**

A severe pneumonia model could be important to help to understand the development of severity and also to evaluate antiviral approaches and vaccine candidates due to proximity respiratory tract among non-human primates and humans.

Animal models of viral or bacterial infection that have been studied include the ferret and rodents, particularly cotton rats and mice [160, 273, 535-537]. Bacterial and host factors contributing to colonization have been defined in animal models and in a mouse acute pneumonia model [24, 123]. For RSV infection, they have shown viral replication and antibody response mimicking infection in humans but these models not develop the measurable clinical disease and cannot be utilized to model cellular immunity [14, 35, 38, 160, 535, 538]. Also, utilization of some non-human primates as a model of viral or bacterial respiratory infection was already described. For example, RSV-infected chimpanzees were used to evaluate live-attenuated RSV vaccine candidates, while African green monkeys and rhesus macaques have been used to model the FI-RSV vaccine-enhanced efficacy [538]. Therefore, currently available NHP models of RSV are not sufficiently permissive to use them as a gatekeeper for either efficacy or safety [539] and clinical symptoms are not well detailed and severe pneumonia was not described during RSV infection agreeing with results on article #1 (Chapter 3).

Differences in the route of inoculation were described to contribute to viral replication and disease severity [169, 243, 540]. In article 1 (chapter 3) results showed that even with an invasive inoculation of pathogens (intratracheal route) the

development of pneumonia was not severe. Thus, may be indicated that the quantities of the pathogen are the most important criteria for disease severity.

### **Improvement in the development of severe pneumonia in NHP model: particularities of RSV infection**

Different strains of RSV have already been described to infect primates, and reference strains and isolated clinical sample are usually adapted to the animal to be studied [540-542]The objective of this work was to develop a model of severe pneumonia caused by mixed infection, thus, a standard strain, well described and without passage in the animal was chosen. Its known that may be adapted strains can induce a more effective immune response and maybe help to explain why the severe disease was not detected.

Also, more studies of efficient entry of RSV are needed to help to identify fully permissive animal models. Initial attachment processes of RSV infection involve binding to glycosaminoglycans, or to C-type lectins which are abundantly expressed in many cell types across many species [54]. Thus, maybe are others necessary receptors that promote viral entry and viral tropism.

Severe pneumonia caused by RSV occurs mainly in newborns, so younger primates may potentiate the impact of infections, as described in humans. In summary, a possible strategy to optimize the severe pneumonia model may be, in addition to increasing the number of bacteria, use *in vivo* adapted virus and/or younger animals.

The literature describes the use of several species of monkeys as a model of respiratory infection, associated with good permissiveness and spontaneous production of the disease after a few days of infection.

## **Model of severe pneumonia, benefits, and challenges**

To conclude, a model of severe pneumonia can help the development of safe vaccines and treatment. NHP model can be used to evaluate therapeutics and pathogenesis for each pathogen and also in combination. Also, it can be used to study various aspects of pathogenesis, particularly the immune response patterns due to similarities in respiratory tract infection.

With the development of a model of severe pneumonia due to mixed infection, many innate immune response mechanisms can be the answer and drug targets can be explored. In view of all works presented in this thesis, *in vivo* model could be a strategy of research to clarify an innate immune response, cellular mechanisms, and complexities in disease severity development.

## *GENERAL CONCLUSION*

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Les infections des voies respiratoires inférieures sont causées par de nombreux pathogènes et l'impact des co-infections est un facteur important de la maladie grave. L'impact des co-infections sur la modulation de la réponse immunitaire innée a été étudié. A cette fin, un modèle pour l'étude in vitro de l'infection mixte dans les macrophages par RSV et *S. pneumoniae* a été développé et la modulation de la réponse inflammatoire par l'expression des cytokines a été étudiée. Les résultats démontrent que la co-infection des cellules immunitaires dérégule la réponse inflammatoire avec l'expression IP-10, identifiée dans notre étude, comme un pronostic biomarqueur potentiel de la pneumonie sévère.

En outre, le rôle de la voie p53 au cours de l'infection par RSV a été étudié pour mieux caractériser les pathogénies virales, les stratégies pour détourner les mécanismes cellulaires et la modulation de la réponse immunitaire. Un aspect important de cette modulation a été décrit soulignant la participation de deux protéines virales importantes.

En conclusion, l'étude pilote à l'établissement d'un modèle in vivo de pneumonie sévère a été développée montrant les difficultés et les défis de ce type de modèle. Les particularités des pathogènes pouvant contribuer au développement d'un modèle in vivo ont également été soulignées.

Lower respiratory tract infections are caused by many pathogens and the impact of co-infections is an important factor of the severe disease. The impact of co-infections on the modulation of the innate immune response was investigated. To this end, a model for the *in vitro* study of mixed infection in macrophages by RSV and *S. pneumoniae* was developed and the modulation of the inflammatory response by cytokines expression was studied. The results demonstrate that co-infection of immune cells deregulates the inflammatory response with IP-10 expression, identified in our study, as a potential biomarker prognostic of severe pneumonia.

In addition, the role of p53 pathway during RSV infection was investigated to better characterize viral pathogenesis, strategies to hijack cell mechanisms and modulation of immune response. An important aspect of this modulation was described highlighting the participation of two important viral proteins.

To conclude, the pilot study to the establishment of an *in vivo* model of severe pneumonia was developed showing difficulties and challenges of this type of model. Particularities of pathogens that can contribute to the development of an *in vivo* model were also highlighted.

## *APPENDICES*

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## **Article 1. Influenza A viruses alter the stability and antiviral contribution of host E3-ubiquitin ligase Mdm2 during the time-course of infection**

In parallel to the thesis, I contributed to another study that leads to a submission article. This work shows detailed interactions between Influenza virus and Mdm2 protein, highlighting the importance of p53 pathway during viral infection.

**Influenza A viruses alter the stability and antiviral contribution of host E3-ubiquitin ligase Mdm2 during the time-course of infection**

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**Abstract**

The interplay between influenza A viruses (IAV) and the p53 pathway has been reported in several studies, highlighting the antiviral contribution of p53. Here, we investigated the impact of IAV on the E3-ubiquitin ligase Mdm2, a major regulator of p53, and observed that IAV targets Mdm2, notably via its non-structural protein (NS1), therefore altering Mdm2 stability, p53/Mdm2 interaction and regulatory loop during the time-course of infection. This study also highlights a new antiviral facet of Mdm2 possibly increasing the list of its many p53-independent functions. Altogether, our work contributes to better understand the mechanisms underlining the complex interactions between IAV and the p53 pathway, for which both NS1 and Mdm2 arise as key players.

**Keywords**

Mdm2; E3-ubiquitin ligase; p53; apoptosis; influenza virus; virus-host interactions

## 1 Introduction

2

3 Influenza viruses are important pathogens responsible for recurrent seasonal epidemics and causing  
4 acute febrile respiratory illness<sup>1</sup>. Among the three types of influenza viruses (A, B & C), influenza A  
5 viruses (IAV) constitute a serious threat to human populations, with the potential to cause pandemics,  
6 as illustrated by the influenza A(H1N1) virus in 2009<sup>1,2</sup>. Influenza viruses rely on numerous host  
7 factors and pathways to achieve successful replication. However, despite important progress made  
8 throughout the last decade, notably with the contribution of high-throughput ‘omics’ approaches,  
9 many mechanisms underlying influenza-host interactions remain elusive<sup>3-6</sup>.

10 The transcription factor p53 is well-known for its role as a tumour suppressor, by rapidly  
11 accumulating in the cell nucleus in response to stress stimuli, hence modifying gene expression to  
12 regulate cell fate. Beyond this major function, p53 is also involved in the regulation of a large panel of  
13 biological processes<sup>7,8</sup>. Several studies have investigated the interplay between influenza viruses and  
14 different signalling pathways, such as the PI3K/Akt or the NF- $\kappa$ B<sup>9,10</sup>. Interestingly, the functional  
15 interactions between influenza viruses and p53 have only been reported in a limited number of studies,  
16 mainly focused on the antiviral facet of p53<sup>11-14</sup>. We have previously shown that several levels of  
17 regulation of p53 transcriptional activity are affected during the time course of IAV infection, possibly  
18 enabling a fine-tuning of p53-mediated biological responses in favour of viral replication<sup>14-16</sup>. We  
19 have particularly demonstrated that IAV modulate p53 transcriptional activity, notably through the  
20 contribution of viral non-structural protein NS1 to the stabilization of p53 in IAV-infected cells<sup>15</sup>.

21 Mdm2, considered as the main negative regulator of p53, is an RING-finger E3 ubiquitin ligase that  
22 binds to p53 to promote its ubiquitination and degradation<sup>17</sup>. Mdm2 and p53 participate in a negative  
23 feedback loop wherein p53 activates the transcription of Mdm2, which in turn inactivates p53 by  
24 directly associating with it and promoting its ubiquitination and proteasomal degradation<sup>17</sup>. Since p53  
25 is largely regulated at the post-translational level, the possible alteration of the Mdm2-p53 interaction,  
26 decrease of Mdm2 protein levels and/or subcellular delocalisation of Mdm2 have been suggested as  
27 the primary mechanisms for stabilization of p53<sup>17</sup>. Nevertheless, only a limited number of studies  
28 have investigated the role of Mdm2 in the context of infection by non-oncogenic viruses, such as

2

29 hantaviruses for example <sup>18</sup>. Although two different genome-wide RNAi studies, both performed in  
30 human lung epithelial cells, have highlighted Mdm2 as a crucial factor for IAV infection <sup>5,6</sup>, the role of  
31 Mdm2 in IAV infection remains unclear. In that regard, Wang and collaborators have recently shown  
32 that the stabilization of p53 in IAV infected cells was associated with a compromised Mdm2-mediated  
33 ubiquitination of p53 <sup>19</sup>.

34 The aim of the present study was to further characterise the mechanisms underlying the compromised  
35 activity of Mdm2 during IAV infection. Our results indicate that Mdm2 stability is modulated during  
36 infection, with a marked degradation at early stages of infection. Different experimental strategies also  
37 indicate an antiviral role played by Mdm2, possibly independently of both its E3-ubiquitin ligase  
38 activity and p53. Altogether, our results improve our comprehension of the role of Mdm2 during the  
39 time course of IAV infection.

40 **Results**

41

42 **Mdm2 expression is strongly impacted by IAV infection, mostly at the post-transcriptional level.**

43 We firstly investigated the impact of IAV infection on endogenous Mdm2 expression, by performing  
44 mock or influenza A (H3N2) infections in A549 human lung epithelial cells (Fig. 1). At a multiplicity  
45 of infection (MOI) of 0.1, we observed a dramatic decrease of up to 5-fold in Mdm2 endogenous  
46 protein levels at 2 h post-infection (hpi), compared to the mock-infected control. Although relative  
47 Mdm2 protein levels remained below 50% at least for the following 8 hours, they significantly  
48 increased (more than 1.5-fold increase of Mdm2 RPL compared to mock) at late stages of infection  
49 (24hpi, Fig. 1A). Similar observation at 24 hpi was confirmed using increasing MOIs in A549 cells  
50 (Fig. 1B). Interestingly, this increase on Mdm2 RPL was not correlated with a decrease of endogenous  
51 p53, suggesting a possible deregulation of p53/Mdm2 loop at late stages of IAV infection. Analogous  
52 experiments performed in H1299 cells, which possess a homozygous partial deletion of *TP53* gene,  
53 show a similar increase of Mdm2 protein levels at 24 hpi, hence advocating for a p53-independent  
54 nature of this phenomenon (Fig. 1B). These observations were confirmed with other influenza A  
55 strains and subtypes (data not shown). To further characterize the impact of infection on Mdm2  
56 expression, we mock-infected or infected A549 cells to reproduce different viral conditions of marked  
57 decrease (MOI 4, 4 and 8 hpi) or increase (MOI 0.1, 24 hpi) of Mdm2 RPL compared to mock and  
58 measured Mdm2 mRNA levels by RT-qPCR (Fig. 1C). Besides a slight decrease at early stages of  
59 infection (4 hpi), Mdm2 mRNA levels in H3N2 infected cells remained comparable to those observed  
60 in mock controls at 8 or 24 hpi (Fig. 1C). In a parallel experiment, we also measured the Mdm2  
61 promoter activity, using a luciferase reporter plasmid. Our results indicated a limited, yet statistically  
62 significant ( $p < 0.05$ ) increase of promoter activity in IAV-infected cells compared to mock at 8 and 24  
63 hpi. However, this increased activity did not appear to be correlated with Mdm2 mRNA and/or protein  
64 levels at the same time-points (Fig. 1C). Altogether, our results indicate that IAV strongly impacts  
65 endogenous Mdm2 expression, most probably at post-translational level.

66

67 **IAV not only alters Mdm2 protein levels but also modulate its nucleo-cytoplasmic localization**  
68 **during the time-course of infection.**

69 Since subcellular localization is known to play a major role in Mdm2 activity towards p53, and to  
70 further investigated the impact of IAV infection on Mdm2, we performed immuno-fluorescence  
71 confocal microscopy in A549 cells mock-infected or infected by influenza A (H3N2) virus at 8 or 24  
72 hpi (Fig. 2). In addition to specific Mdm2 immunostaining (red), we also used IAV non-structural  
73 protein NS1 (green) as an indicator of viral cycle advancement. In mock-infected cells, Mdm2 is  
74 mainly localized in nuclei (Fig. 2, panels a and e). In IAV-infected cells, Mdm2 immunostaining  
75 signal was slightly increased at 8 hpi, compared to mock-infected cells, with a visible nuclear  
76 accumulation (Fig. 2, panels i and m), and a co-localization with NS1 in several cells (Fig. 2, panels l  
77 and p). At late stages of infection (24 hpi), we observed a stronger increase of global Mdm2 staining,  
78 yet with partial localization in the cytoplasm (Fig. 2, panels q and u) as well as a relative exclusion  
79 between Mdm2 and NS1 respective stainings (Fig. 2, panels t and x). These observations confirmed  
80 that IAV strongly impacts endogenous Mdm2 protein levels, but also alters its nuclear-cytoplasmic  
81 localization.

82

83 **IAV infection modulates Mdm2 stability.**

84 We then investigated the impact of IAV infection on Mdm2 stability. To that end, we mock-infected  
85 or infected A549 cells in specific conditions, reproducing a scenario of marked decrease (MOI 4, 4 hpi)  
86 or increase (MOI 0.1, 24 hpi) of Mdm2 RPL and analysed Mdm2 stability by monitoring protein  
87 levels over a 1 h period after treatment with 50  $\mu$ M of cycloheximide (CHX) (Fig. 3A and Fig. 3B). At  
88 early stages of infection (4hpi), we observed a faster decrease of Mdm2 RPL on infected *versus* mock  
89 conditions, with an estimated half-life of 12 and 50 min, respectively (Fig. 3A). Conversely, Mdm2  
90 estimated half-life was almost 3 times higher in IAV-infected cells compared to mock (60 *versus* 20  
91 minutes, Fig. 3B) at late stages of infection (24hpi). We then further investigate the decrease of Mdm2  
92 at early stage of infection (4hpi). We observed less pronounced IAV-induced destabilization of Mdm2  
93 at 4 hpi in the presence of proteasome inhibitor MG132 (Fig. 3C). Moreover, ubiquitination assay in  
94 IAV-infected cells revealed an increase on Mdm2-ubiquitin conjugates between 0 and 6 h post-

5

95 infection (Fig. 3D). Altogether, these results indicate that IAV infection modulates Mdm2 stability,  
96 with a role of ubiquitin-dependent proteasomal degradation at early stages of infection.

97

98 **IAV NS1 expression contributes to IAV-induced Mdm2 destabilization, and consequently alters**  
99 **Mdm2/p53 interaction.**

100 We previously demonstrated that IAV non-structural protein NS1 contributes to p53 stabilization<sup>15</sup>.  
101 Although the underlying mechanism was not fully understood, Wang and collaborators proposed that  
102 IAV-induced p53 stabilization was associated with a compromised Mdm2-mediated ubiquitination of  
103 p53<sup>19</sup>. In an attempt to shed light on the potential role of NS1, we transfected A549 cells with either  
104 an empty plasmid or a plasmid expressing the influenza A (H3N2) NS1 protein and assessed Mdm2  
105 stability as described above but over a 4 h period (Fig. 4A). Compared to mock-transfected cells, we  
106 observed a significant decrease of Mdm2 RPL in NS1-expressing cells at 40 and 60 min time-points  
107 ( $p < 0.001$ , Fig. 4A). Indeed, the estimated Mdm2 half-life in the context of transient expression of  
108 NS1 was almost the half of that observed in mock conditions (35 versus 75 min, Fig. 4A). In parallel,  
109 we also observed that this NS1-induced destabilization was dependent of the quantity and kinetics of  
110 transient expression of NS1 (data not shown).

111 We then hypothesized that NS1 could constitute a major determinant involved in virus-induced  
112 destabilization of Mdm2. To further explore the potential contribution of NS1, we used reverse  
113 genetics<sup>20</sup> to produce 4 recombinant influenza viruses sharing the same A/PuertoRico/8/34 (H1N1)  
114 genomic background, but harbouring the NS1-coding segment (NS) issued from different IAV strains  
115 and subtypes: NS from A/PuertoRico/8/34 (H1N1, PR8), swine-origin A/Lyon/0969/09 (H1N, SO),  
116 A/Moscow/10/99 (H3N2, MO), A/Finch/England/20151/94 (H5N2, EN). A549 cells were mock-  
117 infected or infected with the different recombinant IAV at a MOI of 4 and cell lysates were harvested  
118 at 8 hpi for western blot analysis (Fig. 4B). The four recombinant IAV induced significant yet  
119 differential decrease on Mdm2 RPL ( $p < 0.0001$ ) at a similar stage of infection, as confirmed by NS1  
120 protein levels. Indeed, NS1 issued from H3N2 and H5N2 strains appeared to induce Mdm2  
121 destabilization more efficiently than their H1N1 counterparts (PR8, SO), suggesting a possible  
122 strain/subtype specificity in this process (Fig. 4B). In addition, we also evaluated the impact of NS1

123 expression on the interaction between p53 and Mdm2 by transfecting A549 cells with either an empty  
124 plasmid, or two different amounts of plasmids (1 and 4 µg) expressing NS1 issued from the H3N2  
125 strain (Fig. 4C). After 48 h post-transfection, cell lysates were analysed by co-immunoprecipitation  
126 assay using an anti-p53 polyclonal antibody. As expected, in the absence of NS1, Mdm2 was co-  
127 immunoprecipitated with the anti-p53 antibody. In contrast, the transient expression of NS1 sharply  
128 decreased Mdm2 co-immunoprecipitation, correlated with decreased Mdm2 protein levels in the input  
129 (Fig. 4C). In conclusion, our results underline NS1 as a key determinant in the IAV-induced  
130 destabilization of endogenous Mdm2, at early stage of infection.

131

132 **Silencing/transient expression experiments and small-molecule Mdm2 antagonists reveal an**  
133 **unexpected antiviral role of Mdm2.**

134 To further investigate the role of Mdm2 in IAV infection, we first evaluated the impact of Mdm2  
135 knockdown on IAV production. A549 cells were transfected with either a non-specific siRNA (si-Ctrl)  
136 or a pool of siRNAs targeting Mdm2 and/or p53. Forty-eight hours post-transfection, cells were  
137 infected with influenza A (H3N2) at a MOI of 0.01 and viral supernatants and cell extracts were  
138 harvested at 24 hpi. As expected, western blots confirmed that p53 and/or Mdm2 expression levels  
139 were reduced in cells transfected by specific siRNAs targeting p53 and/or Mdm2, respectively,  
140 compared to si-Ctrl treated cells (Fig. 5A). Moreover, viral production at 24 hpi was assessed by three  
141 different approaches: (i) quantification of influenza M genome copies by RT-qPCR, (ii) measure of  
142 infectious viral titres by endpoint titration in supernatants, and (iii) evaluation of NS1 protein levels by  
143 western blot. In agreement with previously published work validating the antiviral role of p53<sup>11,14,16</sup>,  
144 we observed that viral genome copy number was significantly increased (approximately 10-fold) in  
145 supernatants of si-p53 transfected cells, compared to that of si-Ctrl transfected cells (Fig. 5A). This  
146 result was confirmed by both infectious titres, with more than 10-fold increase on TCID50/mL under  
147 si-p53 treatment compared to si-Ctrl, and increased NS1 protein levels in western blot (Fig. 5A).  
148 Based on these initial observations, and considering Mdm2 is mainly recognised as a negative  
149 regulator of p53, we anticipated to observe the opposite effect with si-Mdm2. Surprisingly, we  
150 obtained very similar results between si-p53 and si-Mdm2 experimental conditions, independently of

151 the readout used (Fig. 5A). For example, we observed that viral genome copy number was  
152 significantly increased (more than 10-fold) in supernatants of si-Mdm2 transfected cells, compared to  
153 that of si-Ctrl transfected cells (Fig.5A). Interestingly, in the case of a combination of si-p53 and si-  
154 Mdm2 treatment, we observed a strong decrease of viral production compared to si-Ctrl, with more  
155 than 100-fold decrease in viral genome copies/mL and TCID50/mL, but without a marked difference  
156 of NS1 protein level in western blot (Fig. 5A). To complete these results, we performed a mirror  
157 experiment, using a transient expression approach in H1299 cells (Fig. 5B) that not only lack the  
158 expression of full-length p53 but also express Mdm2 at low levels. Cells were transfected with either  
159 an empty plasmid or a plasmid expressing p53 or Mdm2, or alternatively co-transfected with plasmids  
160 expressing p53 and Mdm2. Forty-eight hours post transfection, cells were further infected with  
161 A/Moscow/10/99 (H3N2) virus, and the impact of transient expression on viral production was  
162 assessed by measure of infectious titres, viral genome copy numbers and viral protein level (Fig. 5B).  
163 The transient expression of p53 and/or Mdm2 was confirmed by western blot. As expected, viral  
164 production was significantly lower in cells transfected with p53 ( $p<0.01$ ) compared to mock-  
165 transfected cells, in line with our previous observations<sup>16</sup>. Interestingly, a similar impact of Mdm2  
166 transient expression was observed, with almost 100-fold decrease on both genome copies/mL and  
167 TCID50/mL titres compared to mock-transfected cells, consistent with a marked decrease of NS1  
168 protein levels in western blot (Fig. 5B). The co-transfection of p53/Mdm2 was also associated with a  
169 decrease of viral production.

170 To further investigate the role of Mdm2 in IAV infection we used small Mdm2 antagonist molecules,  
171 such as Nutlin-3<sup>21</sup> or NSC66811<sup>22</sup>, which are known to bind Mdm2 in its p53-binding region,  
172 blocking Mdm2 regulatory activities towards p53, and hence inducing the stabilization and activation  
173 of p53<sup>23</sup>. We therefore evaluated the impact of these two Mdm2 antagonists on IAV production, by  
174 infecting A549 cells with influenza A/Moscow/10/99 (H3N2) virus at a MOI of 0.001, in the presence  
175 of either DMSO (control) or different concentrations of Mdm2 antagonists (Fig. 5C). Cells were pre-  
176 treated 14 h before infection to ensure good efficacy of Mdm2-antagonist treatment, and harvested at  
177 48 hpi. Treatment with Nutlin-3 significantly increased viral production compared to control, with up  
178 to 40-fold increase of log<sub>10</sub> RNA copies/mL for 10  $\mu$ M ( $p<0.05$ ) (Fig. 5C). A similar yet milder effect

179 was observed for the NSC66811 molecule, with 5-fold maximum increase on viral production for 2  
180  $\mu\text{M}$  ( $p < 0.001$ ). Of note, the same impact of small molecule antagonists on IAV production was  
181 observed in absence of p53, in HI299 cells, using lower doses of antagonists or shorter viral kinetics  
182 (data not shown). These results are consistent with our previous observations using si-RNA and  
183 transient expression approaches, suggesting a mostly p53-independent antiviral role of Mdm2.  
184 We then explored cellular processes regulated by p53/Mdm2 and therefore potentially impacted by  
185 Mdm2 antagonists. One striking observation is the impact of Nutlin-3 and NSC66811 on the early  
186 steps of IAV-induced apoptosis (Fig. 5C). We infected A549 cells by influenza A (H3N2) at different  
187 MOIs for 48h, in presence of control DMSO or Mdm2 antagonists at different concentrations, and  
188 using a specific luciferase assay to measure caspase 3/7 activity at 48 hpi. As expected, whereas  
189 higher MOIs were correlated with an increase of caspase 3/7 activity in DMSO-treated cells (control),  
190 the impact of IAV infection on apoptosis was strongly reduced, or almost completely attenuated in the  
191 context of Nutlin-3 or NSC66811 treatment (Fig. 5C). These results suggest a possible reduction of  
192 IAV-induced apoptosis by Mdm2 antagonists, creating a favourable context for IAV replication.  
193

194 **Discussion**

195 Mdm2 is a RING finger domain-containing protein with E3-ubiquitin ligase activity, mainly known  
196 for a central regulatory role through the binding to p53, which promotes its ubiquitination and  
197 degradation<sup>17</sup>. Whereas many studies have been dedicated to the interplay between p53 and viruses,  
198 including non-oncogenic viruses, only a limited number have really focused their interest on the  
199 Mdm2 partner, essentially explaining the stabilization of p53 observed in the context of infection  
200<sup>18,24,25</sup>.

201 In the context of IAV, several research groups, including ours, have described a stabilization of p53 in  
202 the context of viral infection<sup>11-13,15</sup>, with Wang and colleagues showing that this stabilization was  
203 associated with a compromised Mdm2-mediated ubiquitination of p53. A possible involvement of  
204 viral nucleoprotein (NP), that could impair p53/Mdm2 and consecutive Mdm2-mediated degradation  
205 was suggested<sup>19</sup>. In the context of a study demonstrating that viral NS1 inhibits p53 transcriptional  
206 activity during IAV infection, we have also shown that NS1 contributes to the stabilization of p53  
207 possibly via its direct interaction with p53, although to a limited extent<sup>15</sup>. We therefore hypothesized  
208 that both compromised ubiquitination of p53 and NS1 interaction may contribute towards IAV-  
209 induced stabilization of p53, but the underlying mechanisms remained unveiled.

210 Here, we showed that IAV alters Mdm2 expression mostly at the post-translational level, with an  
211 impact on its nucleocytoplasmic localization (Fig. 1 and Fig. 2). Although this observation is not in  
212 complete agreement with previous work by Wang *et al.*, indicating unchanged abundance or  
213 subcellular distribution of Mdm2 in IAV-infected cells compared with mock-infected cells<sup>19</sup>, this  
214 discrepancy may be explained not only by the differential origin of cellular models (simian/canine  
215 *versus* human in our work) but also by virus-related parameters (strains, MOI and kinetics) used. The  
216 initial degradation of Mdm2, followed by a stabilization at late stages of infection described in our  
217 study, partially correlates with the biphasic pattern of p53 observed by Shen and colleagues, notably  
218 with the transient p53 increase at the beginning of infection<sup>13</sup>.

219 Different experimental approaches in our study suggest that the p53/Mdm2 autoregulatory negative  
220 feedback loop is altered during the time course of IAV infection, notably at late stages (Fig. 1 and Fig  
221 5). This kind of modification has been already described in literature in different contexts of cellular

222 stressful stimuli, such as DNA damage, oncogenic or nucleolar/ribosomal stress <sup>26,17,27</sup>. Since we  
223 previously demonstrated that IAV infection induces a strong remodelling of the host nucleolus <sup>28,29</sup>, we  
224 cannot exclude a role of nucleolar/ribosomal stress in IAV-induced alteration of the Mdm2/p53 loop.  
225 Another non-exclusive hypothesis would be the involvement of viral proteins such as NP and NS1 in  
226 this mechanism, as we have recently demonstrated their functional interaction with host nucleolus and  
227 ribosome machinery at different levels <sup>28-30</sup>.

228 Our results show that NS1 contributes to Mdm2 degradation either directly or indirectly (Fig. 4),  
229 which is in line with our previous work on the induction of p53 stabilisation by transient expression of  
230 NS1 <sup>15</sup>. We then can hypothesize that NS1 might contribute to p53 stabilisation at different levels, *via*  
231 its interaction with p53 and/or its contribution to Mdm2 degradation. Although the underlying  
232 mechanisms remain to be investigated, several observations might be worth exploring. For example,  
233 our immuno-fluorescence confocal microscopy results (Fig. 2) indicate a progressive decrease of co-  
234 localization of Mdm2 and NS1 during the time course of infection, in correlation with the stabilization  
235 of Mdm2 observed at late stages of infection (Fig. 1 and Fig. 3), which suggests that NS1 contribution  
236 to Mdm2 degradation could occur in the nuclear compartment. Interestingly, NS1 has been shown to  
237 target other E3-ubiquitin ligases such as RING-domain containing TRIM proteins involved in innate  
238 immunity <sup>31</sup>, for which we cannot exclude a similar mechanism in the case of Mdm2. In addition, the  
239 differential effect of NS1 on endogenous Mdm2 in the context of infection by different recombinant  
240 IAV (Fig. 4B) suggests a virus strain/subtype specificity, which could be linked to specific NS1  
241 amino-acid differences that may impact the interaction, subcellular localization and/or functionalities  
242 of NS1. In addition, NS1 from different IAV strains/subtypes were shown to differentially activate the  
243 PI3K/Akt signalling pathway <sup>32,10</sup>, which is part of the upstream regulatory pathway of Mdm2 <sup>17</sup>. The  
244 possible role of NS1 functional interactions with upstream signalling pathway (MAPK, ERK,  
245 PI3K/Akt) and their consequence of Mdm2 underscores further validation.

246 The most striking result of our study is the unexpected inhibitory role apparently played by Mdm2, as  
247 suggested by silencing/transient expression experiments as well as small molecule Mdm2-antagonists  
248 (Fig. 5). This potential antiviral facet of Mdm2 is quite interesting, as Mdm2 is the main negative  
249 regulator of p53 <sup>17</sup> and p53 is known to contribute to the antiviral response <sup>33,34</sup>. However, several

250 results indicate that Mdm2 limits IAV production in a p53-independent manner (Fig. 5A). Indeed,  
251 Mdm2 has been reported to have p53-independent functions in a large panel of cellular processes such  
252 as cell cycle control, cell fate determination, or signalling pathways (i.e. NFκ-B)<sup>35,36</sup>, all of which  
253 could be involved in the property of Mdm2 to inhibit IAV infection.

254 Finally, we observed a significant increase of viral production in the context of treatment with Mdm2  
255 antagonists (Fig. 5B). In the light of the results obtained, we can hypothesize that these molecules,  
256 known to target Mdm2 in its p53-binding site, also block other Mdm2-related functions, including  
257 functions interfering with an optimal viral replication. After exploring several cellular processes  
258 potentially impacted by small Mdm2 antagonists, we found that apoptosis was possibly involved.  
259 Indeed, our results indicate a reduction of IAV-induced apoptosis by both Nutlin-3 and NSC66811 in  
260 comparison to mock-treated control (Fig. 5B). Whereas the induction of apoptosis was demonstrated  
261 to be essential for IAV propagation, notably at early stages of infection<sup>37</sup>, the limitation of IAV-  
262 induced apoptosis at late stages of infection could be also favourable to viral production, avoiding a  
263 premature death of the host cell.

264 In conclusion, we have shown that IAV targets the E3-ubiquitin ligase Mdm2, via its multifunctional  
265 protein NS1, altering Mdm2 stability and the p53/Mdm2 interaction and regulatory loop during the  
266 time-course of infection. This study also highlighted a new antiviral facet of Mdm2, which may be  
267 part of its p53-independent functions. Altogether, our work contributes to better understand the  
268 mechanism involved in the complex interactions between IAV and the p53 pathway, in which both  
269 NS1 and Mdm2 seem to constitute key players as well as cellular gatekeeper p53.

270

271 **Materials & Methods**

272

273 *Viruses and cells* – IAV strain A/Moscow/10/99 (H3N2) and recombinant viruses used in this study  
274 were cultivated and titered in MDCK cells and stored at – 80 °C, as previously described<sup>38,16</sup>. MDCK  
275 cells were purchased from Lonza (ATCC, CCL34) and were passaged twice weekly in serum-free  
276 Ultra-MDCK medium (Lonza) supplemented with 2 mM L-glutamine (Sigma Aldrich), penicillin (225  
277 units/ml) and streptomycin (225 µg/ml) (Lonza). Human lung epithelial A549 cells (ATCC CCL-185,  
278 wild type p53) and H1299 cells (ATCC CRL-5803, Homozygous partial deletion of TP53 gene) were  
279 maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Biowhittaker) supplemented  
280 with 100 units/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine and 10% fetal calf serum  
281 (Dutscher). All cells were maintained at 37 °C with 5% CO<sub>2</sub>. Sub-confluent A549 or H1299 cells  
282 were infected with influenza viruses at different multiplicity of infection (MOI, indicated in text and  
283 figure legend), After a 1h adsorption period in a minimal volume, DMEM supplemented with 10%  
284 heat-inactivated fetal bovine serum (Lonza), L-glutamine (2mM), penicillin (100U/ml), streptomycin  
285 (200 µg/ml) and 0.5 µg/ml trypsin was added, and cells were incubated at 37°C for different lengths of  
286 time. Mock-infected controls were realized with the same protocol, using DMEM instead of viral  
287 inoculum.

288

289 *Reverse genetics* - Reverse genetic system A/PR/8/34 (H1N1), and all NS reassortant viruses were  
290 generated by reverse genetic as previously described<sup>28,38</sup>. Four different recombinant IAV were  
291 generated using reverse genetics, using the same A/PuertoRico/8/34 (H1N1) genomic background, and  
292 harboring NS segment from different IAV strains; NS from A/PuertoRico/8/34 (H1N1, PR8), swine-  
293 origin A/Lyon/0969/09 (H1N, SO), A/Moscow/10/99 (H3N2, MO), A/Finch/England/20151/94  
294 (H5N2, EN). Recombinant viruses were generated by DNA transfection. Plasmids were mixed with  
295 Superfect reagent (Qiagen) in OptiMEM (GIBCO), according to the manufacturer's instructions and  
296 added to 293 T cells in six-well tissue culture plates. At 48 h post-transfection, viruses in the culture  
297 supernatant were harvested and used to infect MDCK cells to be amplified. After two passages, viral  
298 titers were measured using standard methods.

299

300 *Immunofluorescence confocal microscopy* - A549 cells grown, and Mock or IAV-infected on Lab-Tek  
301 II chamber slides (ThermoScientific) were fixed with 4% paraformaldehyde in PBS for 30 min. After  
302 washing in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 15 min. Mouse  
303 monoclonal anti-Mdm2 antibody (SMP14, sc-965, Santa Cruz Biotechnology), and a rabbit polyclonal  
304 anti-NS1 (Kind gift of Dr Juan Ortin, CSIC, Spain) were used as primary antibodies in PBS-T. After  
305 incubation for 1 h, the cells were washed in PBS-T and then incubated with goat anti-mouse coupled  
306 to AlexaFluor 633 and/or goat anti-rabbit coupled to AlexaFluor 488 (Molecular Probes, Invitrogen)  
307 for 30 min, at concentrations recommended by the suppliers. Nuclei were counterstained with DNA-  
308 binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Invitrogen). After staining, coverslips  
309 were mounted with Fluoromount G (Cliniscience) and analyzed using a confocal laser scanning  
310 microscope (SP5 Leica).

311

312 *Antibodies and western blot* - Total proteins were extracted by scraping and syringing cells in 1x  
313 NuPAGE LDS buffer (Invitrogen). Fifteen to thirty micrograms of total proteins were then separated  
314 on 10% SDS-PAGE gels. The following antibodies were used: mouse monoclonal anti-Mdm2  
315 (SMP14, sc-965, Santa Cruz Biotechnology), anti-p53 (DO-1, Santa-Cruz Biotechnology) and anti-  
316 NS1 (sc-130568, Santa Cruz biotechnology) antibodies, and a sheep polyclonal anti-p53 antibody  
317 (SAPU, JC Bourdon, University of Dundee). In addition, an anti-Ku80 polyclonal antibody was used  
318 as a loading control (#2753, Cell Signaling).

319

320 *Stability, transactivation and RT-qPCR assays* - For the determination of Mdm2 half-life, cells were  
321 treated with cycloheximide (50 µg/mL), and total protein lysates were harvested at different time  
322 points and analyzed by western blot. Mdm2 relative protein levels (RPL) were determined by  
323 densitometry analysis using the ImageJ software (<http://rsbweb.nih.gov/ij/>). For transactivation assays,  
324 cells were transfected with 1µg of Mdm2-luc vector, possessing the firefly luciferase gene under the  
325 control of the partial promoter sequence of Mdm2 (Mdm2-luc)<sup>39</sup>. Transfection efficiency was  
326 normalized using a Renilla Luciferase plasmid. Luciferase activity was measured in whole cell

14

327 extracts using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's  
328 instructions, and was expressed as Relative Luciferase Units (RLU), compared to the control. For real-  
329 time quantitative PCR, total RNAs were extracted using the RNAeasy Mini Kit (Qiagen). Reverse-  
330 transcription was performed on 1µg of total RNAs using the Superscript II enzyme (Invitrogen) at  
331 42°C. Quantification of the levels of the different mRNAs of interest was performed by real-time  
332 quantitative PCR, as previously described (Terrier et al., 2011).

333

334 *Immunoprecipitation & Purification of His-tagged ubiquitin conjugates* – For immunoprecipitation,  
335 A549 cells were mock-infected or infected at different MOI. Cells were harvested 8 or 24 h post  
336 infection in a NP40 lysis buffer (NP40 (1%), NaCl (150mM), Tris-HCl (20mM)). Lysates were  
337 incubated with an anti-p53 polyclonal rabbit antibody (CM1, Novocastra Laboratories) on a rotating  
338 wheel overnight at 4°C. Protein G agarose beads (Life technologies) were then added to the lysate and  
339 incubated 1h at room temperature. The beads were pelleted and washed in NP40 lysis buffer.  
340 Antibody-antigen complexes bound to the beads were eluted in 1X NuPAGE LDS buffer (Invitrogen)  
341 and analyzed by western blot with an anti-Mdm2 antibody (SMP14, Santa Cruz biotechnology). For  
342 purification of His-tagged ubiquitin conjugates, cells grown in 150 mm dishes were transfected with a  
343 plasmid expressing his-tagged ubiquitin (pUb(His)6). The purification of His-tagged ubiquitin  
344 conjugates was performed as previously described<sup>40</sup>. Briefly, cells were harvested in a lysis buffer  
345 (Guanidinium-HCl (3M), NaH<sub>2</sub>PO<sub>4</sub> (0.1M), Tris-HCl pH8 (0.01 M), Tween 20 (0.05 %)). Lysates  
346 were mixed with Ni-NTA agarose beads (QIAGEN) and incubated on a rotating wheel 2 h at room  
347 temperature. Beads were then washed (Urea 8M, NaH<sub>2</sub>PO<sub>4</sub> (0.1M), Tris-HCl pH6.3 (0.01M), Tween  
348 20 (0.05%)) and His-tagged ubiquitin conjugates were eluted (Imidazole (200mM), Tris-HCl pH6.7  
349 (0.15M), glycerol (30%), β-mercaptoethanol (0.72M), SDS (5%)). The eluates were analyzed by  
350 western-blot with the appropriate antibodies.

351

352 *Plasmid and siRNA transfection* - Plasmid transfections were performed using TransIT-LT1 reagent  
353 (Mirus), according to the manufacturer's instructions. The His<sub>6</sub>-tagged ubiquitin expressing construct  
354 was a kind gift from Dr Mark Saville (Division of Cancer Research, University of Dundee, UK). The

355 NS1 (H3N2) plasmid (pCI-NS1 H3N2) was a kind gift from Dr Nadia Naffakh (Pasteur Institute,  
356 France). Silencing of MDM2 was performed in A549 cells transfected with the Smart Pool ON-  
357 TARGET plus Mdm2 (si-Mdm2) (L-003279-00-0005, Thermo Scientific), a siRNA specifically  
358 targeting p53 (si-p53)<sup>16</sup> and a non-specific siRNA (si-Ctrl, OR-0030-neg05, Eurogentec), using  
359 Oligofectamine (Invitrogen).

360

361 *Small molecule Mdm2 antagonists* - Two different small molecule Mdm2 antagonists were used in  
362 this study, Nutlin-3a (Calbiochem) or NSC66811 (Calbiochem)<sup>21,22</sup>. A549 cells were pre-treated for  
363 14h, and then infected by influenza virus A/Moscow/10/99 (H3N2) at a MOI of 0.001, in presence of  
364 DMSO or small molecules Mdm2 antagonists (Nutlin-3 or NSC 66811) at different concentrations.

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368

369 **Conflict of interest**

370 OT, JCB and MRC are co-inventors of a patent application deposited by University of Dundee, Centre  
371 National de la Recherche Scientifique, Université Claude Bernard Lyon 1 and Hospices Civils de  
372 Lyon ([FR20100059132 20101105](#) ; [WO2011FR52575 20111104](#)). The other authors declare  
373 that they have no competing interests.

## References

- 1 Paules C, Subbarao K. Influenza. *Lancet Lond Engl* 2017. doi:10.1016/S0140-6736(17)30129-0.
- 2 Neumann G, Kawaoka Y. The first influenza pandemic of the new millennium. *Influenza Other Respir Viruses* 2011; 5: 157–166.
- 3 Fukuyama S, Kawaoka Y. The pathogenesis of influenza virus infections: the contributions of virus and host factors. *Curr Opin Immunol* 2011; 23: 481–486.
- 4 Stertz S, Shaw ML. Uncovering the global host cell requirements for influenza virus replication via RNAi screening. *Microbes Infect Inst Pasteur* 2011; 13: 516–525.
- 5 König R, Stertz S, Zhou Y, Inoue A, Hoffmann H-H, Bhattacharyya S *et al.* Human host factors required for influenza virus replication. *Nature* 2010; 463: 813–817.
- 6 Karlas A, Machuy N, Shin Y, Pleissner K-P, Artarini A, Heuer D *et al.* Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature* 2010; 463: 818–822.
- 7 Lane D, Levine A. p53 Research: the past thirty years and the next thirty years. *Cold Spring Harb Perspect Biol* 2010; 2: a000893.
- 8 Bieganski KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. *Nat Rev Cancer* 2014; 14: 359–370.
- 9 Ludwig S, Planz O. Influenza viruses and the NF-kappaB signaling pathway - towards a novel concept of antiviral therapy. *Biol Chem* 2008; 389: 1307–1312.
- 10 Ehrhardt C, Ludwig S. A new player in a deadly game: influenza viruses and the PI3K/Akt signalling pathway. *Cell Microbiol* 2009; 11: 863–871.
- 11 Turpin E, Luke K, Jones J, Tumpey T, Konan K, Schultz-Cherry S. Influenza virus infection increases p53 activity: role of p53 in cell death and viral replication. *J Virol* 2005; 79: 8802–8811.
- 12 Zhirnov OP, Klenk H-D. Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. *Apoptosis Int J Program Cell Death* 2007; 12: 1419–1432.
- 13 Shen Y, Wang X, Guo L, Qiu Y, Li X, Yu H *et al.* Influenza A virus induces p53 accumulation in a biphasic pattern. *Biochem Biophys Res Commun* 2009; 382: 331–335.
- 14 Terrier O, Josset L, Textoris J, Marcel V, Cartet G, Ferraris O *et al.* Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway. *Virol J* 2011; 8: 285.
- 15 Terrier O, Diederichs A, Dubois J, Cartet G, Lina B, Bourdon J-C *et al.* Influenza NS1 interacts with p53 and alters its binding to p53-responsive genes, in a promoter-dependent manner. *FEBS Lett* 2013; 587: 2965–2971.
- 16 Terrier O, Marcel V, Cartet G, Lane DP, Lina B, Rosa-Calatrava M *et al.* Influenza A viruses control expression of proviral human p53 isoforms p53 $\beta$  and Delta133p53 $\alpha$ . *J Virol* 2012; 86: 8452–8460.

- 17 Ponnuswamy A, Hupp T, Fähræus R. Concepts in MDM2 Signaling. *Genes Cancer* 2012; 3: 291–297.
- 18 Park S-W, Han M-G, Park C, Ju YR, Ahn B-Y, Ryou J. Hantaan virus nucleocapsid protein stimulates MDM2-dependent p53 degradation. *J Gen Virol* 2013; 94: 2424–2428.
- 19 Wang X, Deng X, Yan W, Zhu Z, Shen Y, Qiu Y *et al.* Stabilization of p53 in influenza A virus-infected cells is associated with compromised MDM2-mediated ubiquitination of p53. *J Biol Chem* 2012; 287: 18366–18375.
- 20 Moulès V, Terrier O, Yver M, Riteau B, Moriscot C, Ferraris O *et al.* Importance of viral genomic composition in modulating glycoprotein content on the surface of influenza virus particles. *Virology* 2011; 414: 51–62.
- 21 Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004; 303: 844–848.
- 22 Lu Y, Nikolovska-Coleska Z, Fang X, Gao W, Shangary S, Qiu S *et al.* Discovery of a nanomolar inhibitor of the human murine double minute 2 (MDM2)-p53 interaction through an integrated, virtual database screening strategy. *J Med Chem* 2006; 49: 3759–3762.
- 23 Khoo KH, Hoe KK, Verma CS, Lane DP. Drugging the p53 pathway: understanding the route to clinical efficacy. *Nat Rev Drug Discov* 2014; 13: 217–236.
- 24 Henning W, Rohaly G, Kolzau T, Knippschild U, Maacke H, Deppert W. MDM2 is a target of simian virus 40 in cellular transformation and during lytic infection. *J Virol* 1997; 71: 7609–7618.
- 25 Santos CR, Vega FM, Blanco S, Barcia R, Lazo PA. The vaccinia virus B1R kinase induces p53 downregulation by an Mdm2-dependent mechanism. *Virology* 2004; 328: 254–265.
- 26 Haupt VJ, Schroeder M. Old friends in new guise: repositioning of known drugs with structural bioinformatics. *Brief Bioinform* 2011; 12: 312–326.
- 27 Zhou X, Liao J-M, Liao W-J, Lu H. Scission of the p53-MDM2 Loop by Ribosomal Proteins. *Genes Cancer* 2012; 3: 298–310.
- 28 Terrier O, Moulès V, Carron C, Cartet G, Frobert E, Yver M *et al.* The influenza fingerprints: NS1 and M1 proteins contribute to specific host cell ultrastructure signatures upon infection by different influenza A viruses. *Virology* 2012; 432: 204–218.
- 29 Terrier O, Carron C, De Chasse B, Dubois J, Traversier A, Julien T *et al.* Nucleolin interacts with influenza A nucleoprotein and contributes to viral ribonucleoprotein complexes nuclear trafficking and efficient influenza viral replication. *Sci Rep* 2016; 6: 29006.
- 30 Panthu B, Terrier O, Carron C, Traversier A, Corbin A, Balvay L *et al.* The NS1 protein from influenza virus stimulates translation initiation by enhancing ribosome recruitment to mRNAs. *J Mol Biol* 2017. doi:10.1016/j.jmb.2017.04.007.
- 31 Gack MU, Albrecht RA, Urano T, Inn K-S, Huang I-C, Carnero E *et al.* Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 2009; 5: 439–449.
- 32 Shin Y-K, Liu Q, Tikoo SK, Babiuk LA, Zhou Y. Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. *J Gen Virol* 2007; 88: 13–18.

- 33 Muñoz-Fontela C, Mandinova A, Aaronson SA, Lee SW. Emerging roles of p53 and other tumour-suppressor genes in immune regulation. *Nat Rev Immunol* 2016; **16**: 741–750.
- 34 Lazo PA, Santos CR. Interference with p53 functions in human viral infections, a target for novel antiviral strategies? *Rev Med Virol* 2011; **21**: 285–300.
- 35 Bohlman S, Manfredi JJ. p53-independent effects of Mdm2. *Subcell Biochem* 2014; **85**: 235–246.
- 36 Thomasova D, Mulay SR, Bruns H, Anders H-J. p53-Independent Roles of MDM2 in NF- $\kappa$ B Signaling: Implications for Cancer Therapy, Wound Healing, and Autoimmune Diseases. *Neoplasia NYN* 2012; **14**: 1097–1101.
- 37 Wurzer WJ, Planz O, Ehrhardt C, Giner M, Silberzahn T, Pleschka S *et al*. Caspase 3 activation is essential for efficient influenza virus propagation. *EMBO J* 2003; **22**: 2717–2728.
- 38 Moules V, Ferraris O, Terrier O, Giudice E, Yver M, Rolland JP *et al*. In vitro characterization of naturally occurring influenza H3NA- viruses lacking the NA gene segment: toward a new mechanism of viral resistance? *Virology* 2010; **404**: 215–224.
- 39 Juven T, Barak Y, Zauberman A, George DL, Oren M. Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. *Oncogene* 1993; **8**: 3411–3416.
- 40 Xirodimas D, Saville MK, Edling C, Lane DP, Lain S. Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 in vivo. *Oncogene* 2001; **20**: 4972–4983.

### Figure legends

**Figure 1. Mdm2 expression is strongly impacted by IAV infection, mostly at post-transcriptional levels.** A. Human lung A549 cells were mock-infected or infected by influenza A/Moscow/10/99 (H3N2) at a MOI of 0.1 and cell lysates were analyzed by western blot for the expression of Mdm2, p53 and IAV NS1 at different time-points post infection. Ku80 was used as a loading control. Mdm2 relative protein levels (Mdm2 RPL) were measured by densitometry and calculated from data from three independent experiments. \*\* and \*\*\* for  $P$ -value  $< 0.01$  and  $0.001$ , respectively. B. Alternatively, A549 or H1299 cells were mock-infected or infected by influenza A/Moscow/10/99 (H3N2) at different MOI and expression of Mdm2, p53 and IAV NS1 were monitored at 24 h post-infection by western blot, using the same approach. C. A549 cells were mock-infected or infected by influenza A/Moscow/10/99 (H3N2) at a MOI of 4 or 0.1 and cell supernatants and lysates were harvested at 4, 8 or 24hpi, respectively. Endogenous Mdm2 expression was measured at protein level by western blot, Mdm2 promoter activity was monitored using a luciferase reporter plasmid. In addition, Mdm2 mRNA expression was measured by RT-qPCR. \*, \*\*, \*\*\* for  $P$ -value  $< 0.05$ ,  $0.01$  and  $0.001$ , respectively.

**Figure 2. IAV alters Mdm2 protein level and its nucleo-cytoplasmic localization during the time-course of infection.** Immunofluorescence staining of Mdm2 (red) and influenza NS1 (green) in mock-infected (panels a to h) or infected with H3N2 virus at a MOI of 1 (panels i to x) was performed at different times, as indicated. Nuclei were counterstained with DAPI (blue, panels c, g, k, o, s, w). Merged fluorescent signals are presented in panels d, h, i, p, t and x. Cell details are enlarged (inset). White scale bar =  $10 \mu\text{m}$ .

**Figure 3. IAV infection modulates Mdm2 stability.** Stability assay in IAV-infected cells at 4hpi (A) and 24hpi (B). Human lung A549 cells were mock-infected or infected with influenza A/Moscow/10/99 (H3N2) with an MOI of 4 or 0.1 and analyzed at 4 and 24hpi, respectively. Stability was assessed by monitoring relative protein levels (RPL) of Mdm2 during a 1h period, after treatment

with 50  $\mu$ M cycloheximide (CHX). Mean values  $\pm$  standard deviation from three independent experiments are presented. \*\*\* for  $P$ -value  $< 0.001$ . C. Human lung A549 cells were mock-infected or infected with influenza A/Moscow/10/99 (H3N2) with an MOI of 4, in presence/absence of proteasome inhibitor MG132 (20  $\mu$ M), and harvested at 4 h post-infection, and analyzed by western blot. D. A549 cells were transfected with a plasmid expressing His-tagged Ubiquitin and then further infected 48 h post-transfection with influenza A/Moscow/10/99 (H3N2) with an MOI of 4, in presence of 20  $\mu$ M MG132. Cell lysates were harvested at different time-points after infection, ubiquitinated products were separated and analyzed by western blot using a specific antibody against Mdm2. In parallel, without MG132 treatment, Mdm2 protein levels were monitored at similar time-points. When necessary, Ku80 was used as a loading control for western blot.

**Figure 4. IAV NS1 expression contributes to IAV-induced Mdm2 destabilization, and consecutively alters Mdm2/p53 interaction.** A. Stability assay in presence of NS1. A549 cells were transfected with either an empty plasmid or a plasmid expressing NS1 from A/Moscow/10/00 (H3N2), and Mdm2 stability was evaluated at 48 h post-transfection. Stability was assessed by monitoring relative protein levels (RPL) of Mdm2 during a 1h period, after treatment with 50  $\mu$ M cycloheximide (CHX). Mean values  $\pm$  standard deviation from three independent experiments are presented. \*\*\* for  $P$ -value  $< 0.001$ . Ku80 was used as a loading control. B. Four different recombinant IAV were generated using reverse genetics, using the same A/PuertoRico/8/34 (H1N1) genomic background, and harboring NS segment from different IAV strains; NS from A/PuertoRico/8/34 (H1N1, PR8), swine-origin A/Lyon/0969/09 (H1N, SO), A/Moscow/10/99 (H3N2, MO), A/Finch/England/20151/94 (H5N2, EN). Human lung A549 cells were mock-infected or infected with these different IAV with an MOI of 4 and cell lysates were harvested at 8 h post-infection for western blot analysis. Mdm2 relative protein levels (Mdm2 RPL) were measured by densitometry and calculated from data from three independent experiments. \*\*\*\* for  $P$ -value  $< 0.0001$ . Ku80 was used as a loading control. C. Impact of NS1 transient expression on the interaction between p53 and Mdm2. A549 cells were transfected with either an empty plasmid, or two quantities of plasmids expressing NS1 (1 and 4  $\mu$ g NS1 H3N2). After

48 h post-transfection, cells lysates were analyzed using a co-immunoprecipitation assay using an anti-p53 polyclonal antibody.

**Figure 5. An unexpected antiviral contribution of Mdm2 revealed by silencing/transient expression experiments and small-molecule Mdm2 antagonists.** **A.** Knock-down of p53 and/or Mdm2 mRNA expression in A549 cells differentially modulates levels of IAV production (Left panel). Forty-eight hours after si-RNA transfection using a control si-RNAs (si-Ctrl) or specific siRNAs (si-p53/si-Mdm2), A549 cells were infected by A/Moscow/10/99 (H3N2) at a MOI of 0.01 and the level of viral production at 24 h post-infection was assessed using three different experiments: (i) determination of infectious titers of supernatants ( $\log_{10}$  TCID<sub>50</sub>/mL) by endpoint titration in MDCK cells (measured in quadruplicate in 2 independent experiments; \*\*\* for  $P$ -value < 0.001), (ii) RT-qPCR ( $\log_{10}$  RNA copies/mL, measured in three independent experiments), and (iii) western blot. Ku80 was used as a loading control; **B.** Transient expression/co-expression of p53 and/or Mdm2 in H1299 cells decreases the level of viral production (Right panel). Forty-eight hours after transfection, A549 cells were infected by A/Moscow/10/99 (H3N2) at a MOI of 0.01 and the level of viral production at 24 h post-infection was assessed using similar methods. **C.** Impact of small molecule Mdm2 antagonists on viral production and IAV-induced apoptosis. Human lung epithelial A549 cells were infected by influenza virus A/Moscow/10/99 (H3N2) at a MOI of 0.001, in presence of DMSO or small molecules Mdm2 antagonists (Nutlin-3 or NSC 66811) at different concentrations. The level of viral production at 48 h post-infection was evaluated by RT-qPCR ( $\log_{10}$  RNA copies/mL). The impact of small molecule antagonists on IAV-induced caspase3/7 activity at 48 h post-infection was monitored using a luciferase reporter assay, using three different MOI (0.1, 0.01 and 0.001).

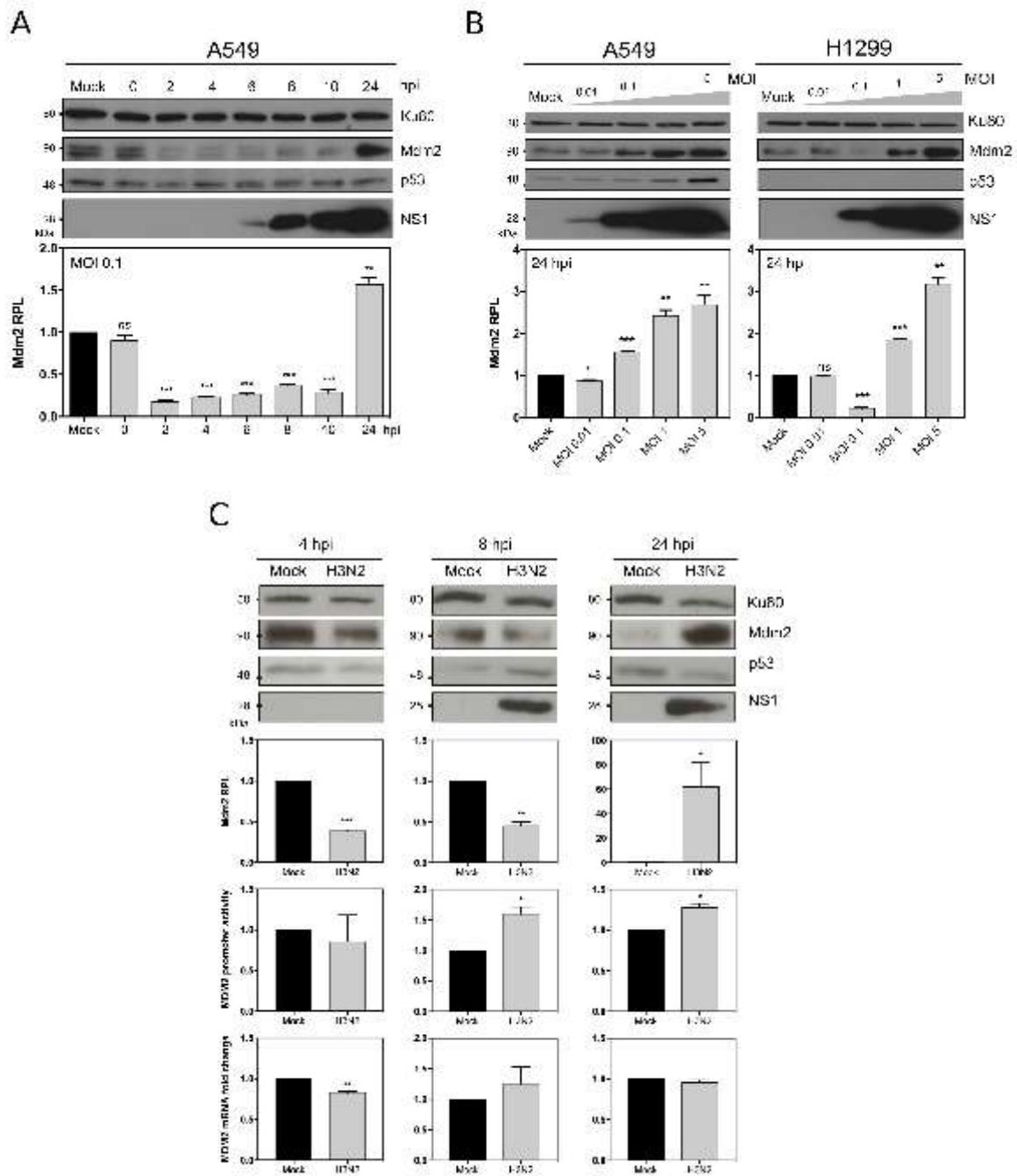


Figure 1

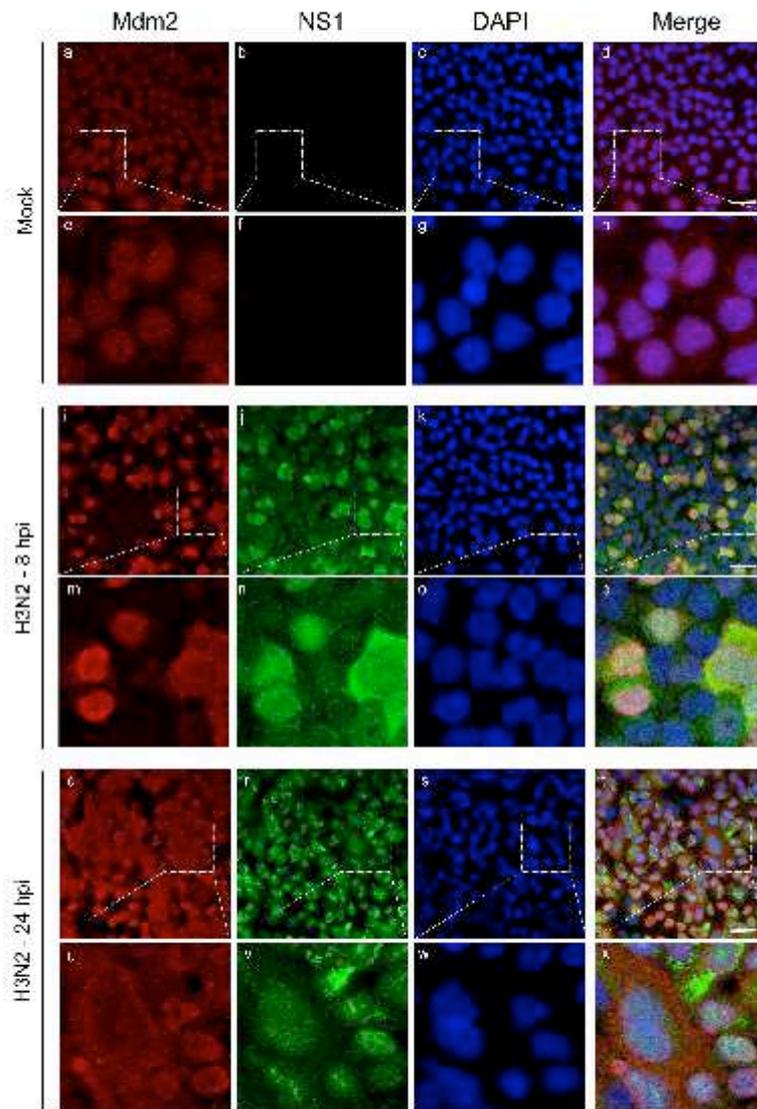


Figure 2

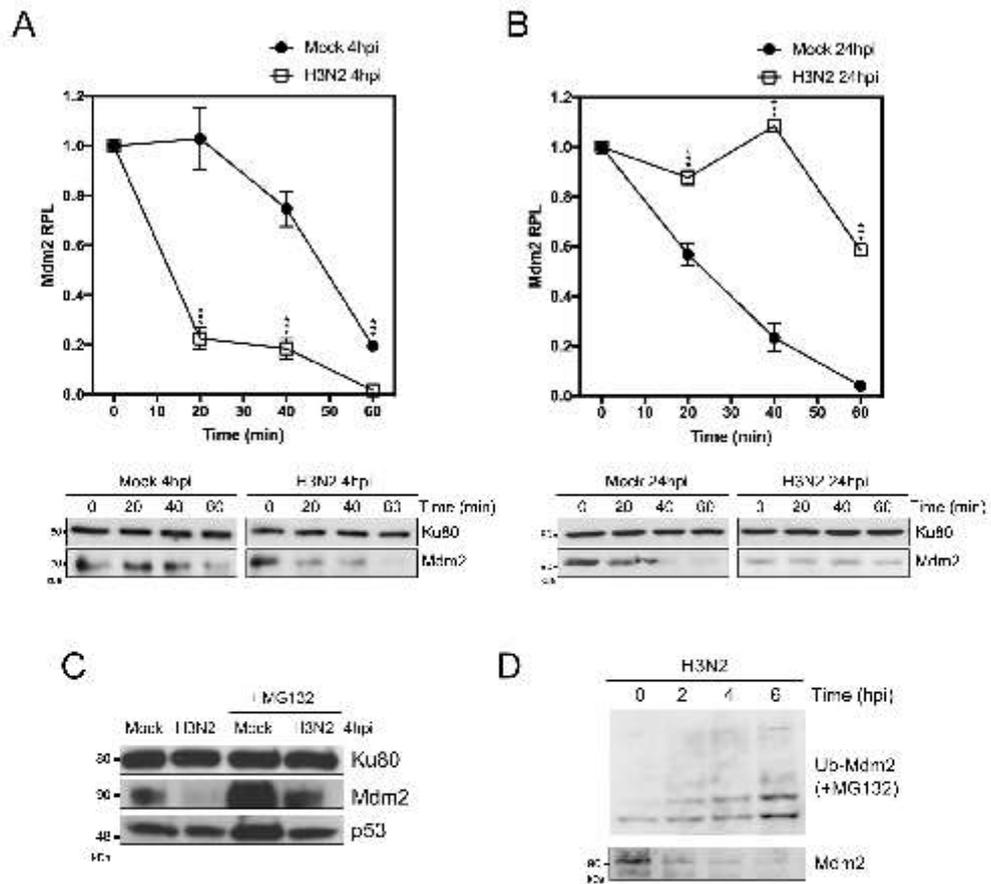


Figure 3

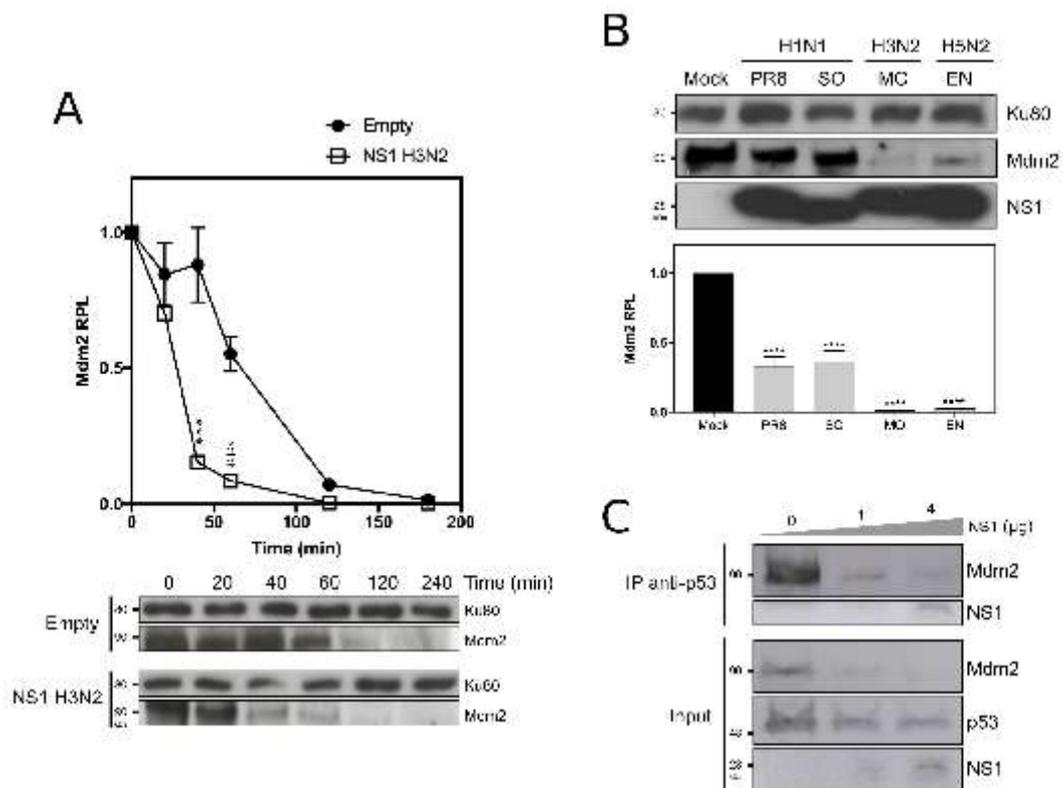


Figure 4

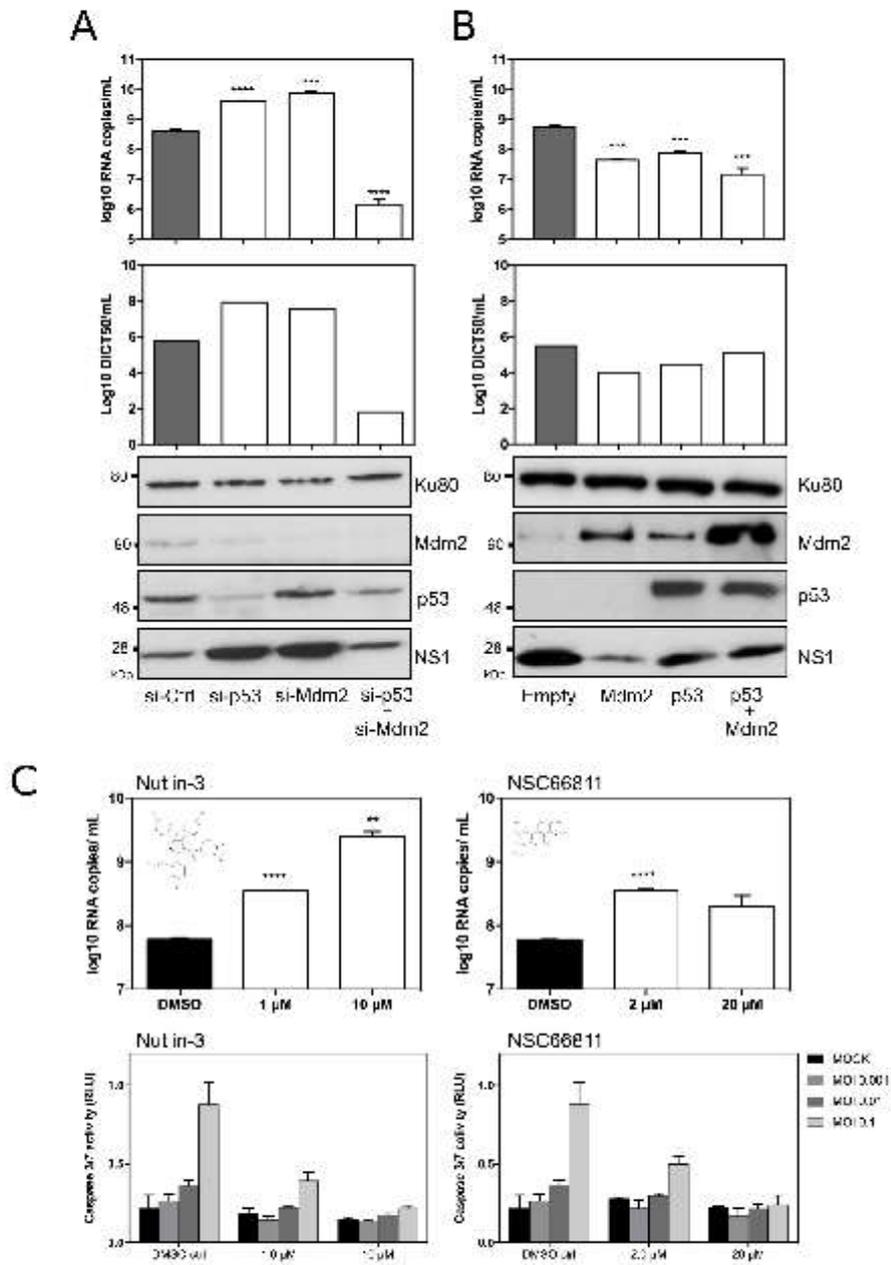
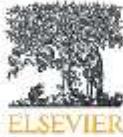


Figure 5

## **Article 2. Phylogenetic analyses of influenza A (H1N1)pdm09 hemagglutinin gene during and after the pandemic event in Brazil**

Also, in parallel to my thesis, I participated in some evolutionary analyses of Influenza virus. The Brazilian national reference center of Influenza virus work on the surveillance and evolutionary analysis of influenza virus detected in Brazil. The collaboration with this laboratory, where I did my master degree, is still established and I contributed to this article published by the Brazilian team.



## Phylogenetic analyses of influenza A (H1N1)pdm09 hemagglutinin gene during and after the pandemic event in Brazil



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### ABSTRACT

Pandemic influenza A H1N1 [A(H1N1)pdm09] was first detected in Brazil in May 2009, and spread extensively throughout the country causing a peak of infection during June to August 2009. Since then, it has continued to circulate with a seasonal pattern, causing high rates of morbidity and mortality. Over this period, the virus has continually evolved with the accumulation of new mutations. In this study we analyze the phylogenetic relationship in a collection of 220 A(H1N1)pdm09 hemagglutinin (HA) gene sequences collected during and after the pandemic period (2009 to 2014) in Brazil. In addition, we have looked for evidence of viral polymorphisms associated with severe disease and compared the range of viral variants with the vaccine strain (A/California/7/2009) used throughout this period.

The phylogenetic analyses in this study revealed the circulation of at least eight genetic groups in Brazil. Two (G6-pdm and G7-pdm) co-circulated during the pandemic period, showing an early pattern of viral diversification with a low genetic distance from vaccine strain. Other phylogenetic groups, C5, C6 (including 6B, 6C and 6D subgroups), and G7 were found in the subsequent epidemic seasons from 2011 to 2014. These viruses exhibited more amino acid differences from the vaccine strain with several substitutions at the antigenic sites. This is associated with a theoretical decrease in the vaccine efficacy. Furthermore, we observed that the presence of any polymorphism at residue 222 of the HA gene was significantly associated with severe, fatal cases, reinforcing previous reports that described this residue as a potential virulence marker.

This study provides new information about the circulation of some viral variants in Brazil, follows up potential genetic markers associated with virulence and allows infer if the efficacy of the current vaccine against more recent A(H1N1)pdm09 strains may be reduced.

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### 1. Introduction

Influenza viruses are important human pathogens, causing seasonal epidemics and occasional pandemics with high rates of morbidity and mortality annually (World Health Organization, 2014a). During April 2009 a new influenza A subtype H1N1 virus emerged simultaneously in Mexico and the United States, which rapidly spread worldwide, causing the first pandemic of the 21st century (Dawood et al., 2009; Smith et al., 2009). This virus was the product of multiple reassortment events involving viruses from swine, birds and humans and was responsible for more than 18,000 deaths worldwide (Smith et al., 2009; World Health Organization, 2010a). In Brazil, pandemic influenza A (H1N1) virus [A(H1N1)pdm09] was first detected in May 2009 and it spread rapidly across the country. Despite public health containment measures being

adopted to control viral transmission, a mortality rate of 4.6% among severe acute respiratory infections (SARI) cases were reported (Secretary of Health Surveillance – Brazilian MoH, 2010). After the pandemic period, A(H1N1)pdm09 began to circulate as a seasonal virus together with influenza A/H3N2 and influenza B virus lineages, causing annual epidemic seasons during winter and considerable morbidity and mortality (Secretary of Health Surveillance – Brazilian MoH, 2012; Secretary of Health Surveillance – Brazilian MoH, 2013a, 2013b, 2014).

Influenza viruses have a segmented single stranded RNA genome and exhibit an evolutionary plasticity. During replication process, gradual genetic changes (antigenic drift) occur due to the lack of proof reading of the RNA polymerase complex (Carrat and Flahault, 2007; Medina and Garcia-Sastre, 2011). It contributes to the ability of the virus to evade recognition by the immune system and ensure its consistent circulation within human populations (Carrat and Flahault, 2007; Medina and Garcia-Sastre, 2011). Surveillance of emerging viral variants is necessary to inform decisions on the vaccine composition. Currently, the vaccine used in Brazil contains two subtypes of influenza

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A, H1N1 and H3N2 and one influenza B lineage. Since the emergence of A(H1N1)pdm09, the H1N1 vaccine component has remained the same in the Northern and Southern Hemisphere (World Health Organization, 2014b) and the anti-influenza vaccine coverage in Brazil has always been over 80% in the target population (Ministry of Health, 2015). Influenza vaccine effectiveness may be influenced by a variety of factors associated with recipient or with the viral strain chosen to compose the anti-influenza vaccine (Houser and Subbarao, 2015; Van Loveren et al., 2001). The intrinsic capacity of the recipient to respond to the vaccine may be modulated by many characteristics such as age, sex, nutritional and immunological conditions, genetic factors, and presence of chronic disease (Van Loveren et al., 2001). Influenza vaccine effectiveness is mostly affected by the high mutation rate of influenza virus. Effectiveness figures of 30–60% against influenza have been reported, depending on the antigenic match between vaccine and circulation strain (Gupta et al., 2006; Houser and Subbarao, 2015). The rate of amino acid change of influenza means that the antigens included in the annual vaccination have to be changed on a regular basis to provide protection against current strains. The selection of strains is based on the detection of new variants and assessment of antigenic change using ferret antisera and sera from vaccinees. Studies have attempted to evaluate the use of sequence based amino acid changes to predict these antigenic changes (Glinsky, 2010; Lee et al., 2010; Mesquita et al., 2014; Resende et al., 2014; Souza et al., 2013). Viral mutations may also cause an increase in viral fitness leading to a greater transmissibility, pathogenicity and to the emergence of viral variants resistant to antiviral drugs (Glinsky, 2010; Lee et al., 2010; Mesquita et al., 2014; Resende et al., 2014; Souza et al., 2013). The HA gene is the major viral antigen and is responsible for entry into the host cells. Analyses of this gene provide important data on virulence and temporal and geographical distribution of strains (Smith et al., 2004). Some mutations in the HA of A(H1N1)pdm09 virus have been suggested as potential virulence markers, as: K-2E and Q293H (Glinsky, 2010; Potdar et al., 2010); D222G (World Health Organization, 2010b). However, the substitution of aspartic acid (D) to glycine (G) or mixed viral populations at residue 222 of the HA gene presented a strong association with severe and fatal cases (Chen et al., 2010; Kuroda et al., 2010; World Health Organization, 2010b; Resende et al., 2014). Regarding the phylogenetic dynamic of the A(H1N1)pdm09 virus, previous studies showed a n early diversification of this virus into seven phylogenetic groups during the pandemic period, based on specific mutations in the viral genome (Nelson et al., 2009; Potdar et al., 2010). After the pandemic, a major diversification of these strains occurred and new phylogenetic groups were identified based on mutations in the HA gene (European Centre for Disease Prevention and Control, 2011, 2013).

In this study, we described the long-term molecular epidemiology of influenza A H1N1pdm09 virus, which circulated in the different Brazilian geographical regions from 2009 to 2014. Based on HA gene we looked for mutations that could be associated with severe clinical outcomes and with anti-influenza vaccine failure.

## 2. Material and methods

### 2.1. Samples

Using the Brazilian Influenza Surveillance Network, nasopharyngeal aspirates (NPAs) were collected from patients with influenza-like

illness (ILI) and hospitalized patients suffering from SARI. These samples were sent to the Laboratory of Respiratory Viruses at FIOCRUZ in Rio de Janeiro, a National Influenza Center (NIC) for World and Health Organization (WHO) and for the Ministry of Health (MoH), where this study was conducted.

A total of 220 representative Brazilian A(H1N1)pdm09 strains were chosen for this study. These selected samples were representative of the viruses circulating in the three distinct Brazilian geographic/climatic regions, namely: Northeastern ( $n = 38$ ), Southeastern ( $n = 37$ ) and Southern ( $n = 145$ ), covering the pandemic period ( $n = 65$ ) and the following epidemic seasons 2011 ( $n = 9$ ), 2012 ( $n = 49$ ), 2013 ( $n = 74$ ) and 2014 ( $n = 23$ ). These samples were collected from patients with a median age of 26 years, being 63% of individuals ranging from 16 to 50 years-old, and 54.1% were females. Among these patients only 138 (62.7%) provided detailed information about clinical outcomes, 44 cases were mild, 67 severe and 27 were from fatal cases, and only 14 patients reported chronic disease.

Samples were manipulated anonymously. Patient identifiers including personal information (name and address) and hospitalization number were removed to protect patient confidentiality. This study was conducted within the scope of the National Influenza Epidemiological Surveillance Program/MoH, as part of a global and Federal public health policy for Influenza control and prevention in Brazil. For the reasons, a formal approval from our Ethical Committee was not required.

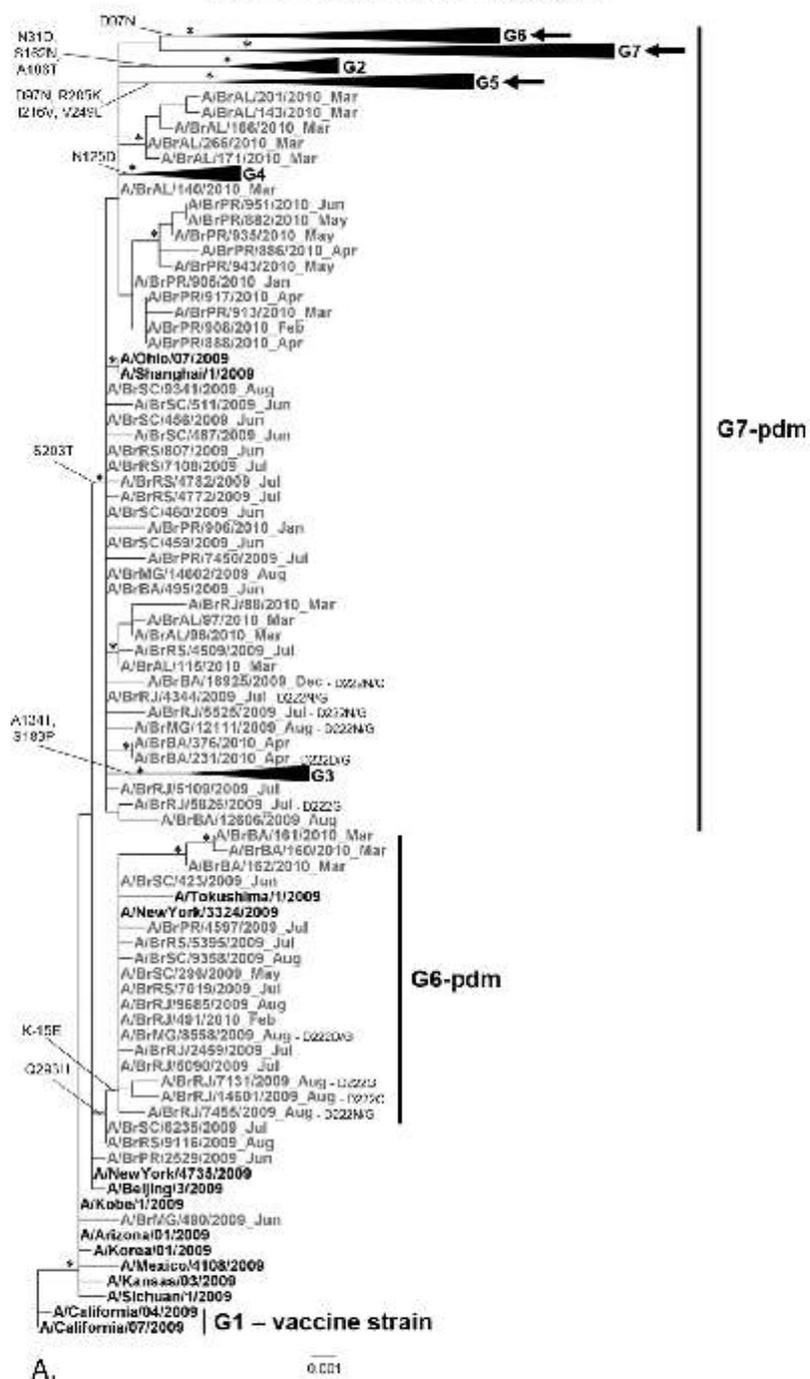
### 2.2. RNA extraction, amplification and sequencing of the HA gene

Viral RNA was extracted from NPAs using a QIAmp viral RNA mini kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. cDNA was synthesized using Super Script III enzyme (Life technologies, Gaithersburg, California, USA). Thereafter whole HA sequence was obtained by amplification of four PCR products using Taq Platinum DNA polymerase enzyme (Life technologies, Gaithersburg, California, USA) and primer sets described previously by CDC Influenza division (World Health Organization, 2009). DNA products were purified with a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced using an automated ABI 3130XL Genetic Analyzer (Applied Biosystems, California, USA). Sequences were assembled and the comparisons with the reference sequence (A/California/7/2009) were performed with Sequencher software 5.1 (Gene Codes, Ann Arbor, MI). Mixed nucleotide polymorphisms at residue 222 found with the Sanger sequencing method were checked using a customized pyrosequencing (PSQ) protocol described previously (Levine et al., 2011). Brazilian sequences were submitted to the Global Initiative on Sharing Avian Influenza Data (GISAID) database and are available with the accession numbers: EPI178726 to EPI178945.

### 2.3. Data set construction, genetic and phylogenetic analyses

A data set with 266 HA sequences was constructed, including Brazilian sequences ( $n = 220$ ) and other representative sequences ( $n = 46$ ) obtained from GISAID database or from influenza virus sequence database of National Center for Biotechnology Information (NCBI) (Supplementary table 1). These representative sequences have specific amino acid signatures in the HA gene that defined the phylogenetic groups, from pandemic (G1, G2-pdm to G7-pdm) and post-

**Fig. 1.** Maximum-Likelihood (ML) phylogenetic tree of the HA whole gene (1701 nucleotide) of 220 Brazilian influenza A (H1N1)pdm09 viruses (gray) detected from 2009, 2010, 2011, 2012, 2013 and 2014 seasons and 46 representative sequences (black) of the groups G1, G2-pdm to G7-pdm and G2 to G7. The geographical location of each Brazilian sequence is shown after the first bar at the sequence name. Br indicates Brazil and the next acronym indicates the state where each sample was collected (AL, Alagoas; BA, Bahia; SE, Sergipe; MG, Minas Gerais; RJ, Rio de Janeiro; ES, Espírito Santo; PR, Paraná; RS, Rio Grande do Sul; and SC, Santa Catarina). Amino acid changes associated with these specific groups were indicated in the nodes using H1 numbering without signal peptide. The best-fitting nucleotide substitution model used was Hasegawa–Kishino–Yano (HKY) + gamma distribution among the sites. ML reliability of branches was evaluated using approximate likelihood-ratio test (aLRT) and an inferior branch cut-off value was 0.9 (asterisk on the branches). 1A shows groups from pandemic period and branches, which represent groups from post-pandemic period were collapsed. The arrows in 1A indicate where the Brazilian sequences were found. 1B, 1C and 1D, show the G5, G7 and G6 and its subgroups, respectively.



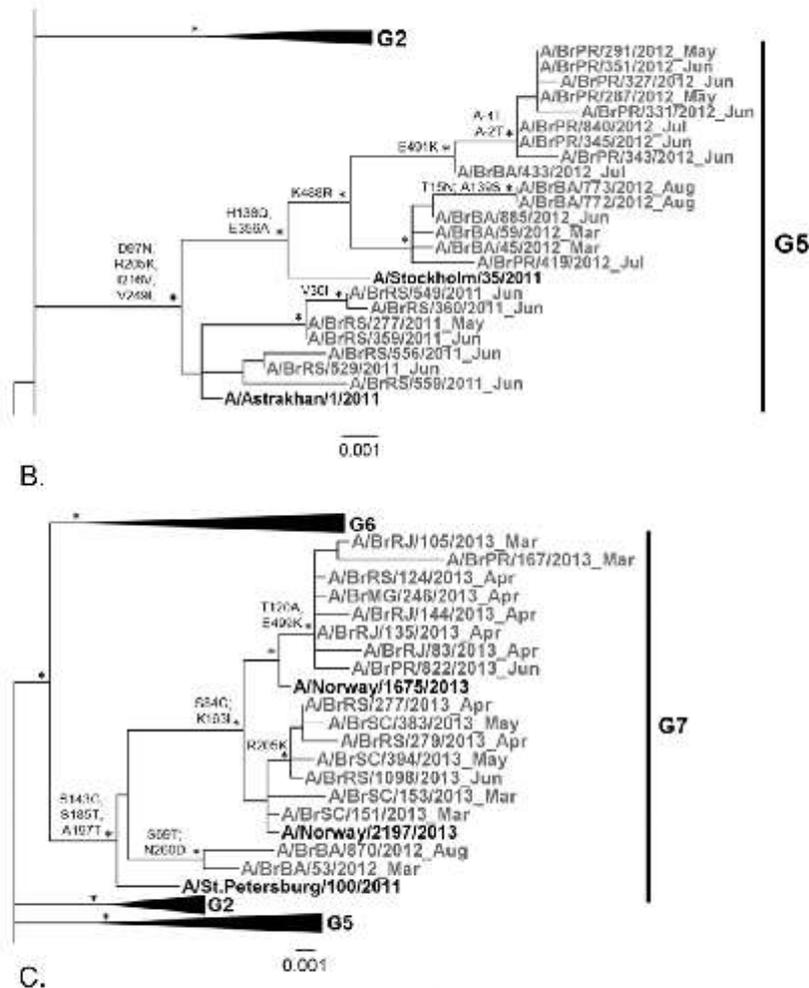


Fig. 1. (continued).

pandemic periods (G2 to G7, including 6A to 6C subgroups) (European Centre for Disease Prevention and Control, 2011, 2013; Nelson et al., 2009; Potdar et al., 2010). Sequences from unclassified phylogenetic groups found in this study were submitted to Basic Local Alignment Search Tool (BLAST) available at the NCBI database to obtain similar sequences circulating worldwide. The data set alignment was generated with Clustal W algorithm (integrated tool within Geneious 7.0 software) and edited manually for optimization.

Maximum Likelihood (ML) trees were inferred with the on-line PhyML platform (<http://www.atcg-montpellier.fr/phyml>), using the best-fitting nucleotide substitution model, Hasegawa–Kishino–Yano (HKY) + gamma distribution among the sites, which was estimated by means of Modeltest, integrated tool within the MEGA 6.0 software (Tamura et al., 2013). ML reliability of branches was evaluated using approximate likelihood-ratio test (aLRT) with an interior

branch cut-off value  $\geq 0.9$  and the tree was rooted with the vaccine strain A/California/7/2009.

#### 2.4. Analyses of amino acid substitutions and prediction of vaccine efficacy

Amino acid substitutions from Brazilian HA sequences were compared with vaccine strain using Sequencher 5.1 software (Gene Codes, Ann Arbor, MI). Amino acid distances among phylogenetic groups to vaccine strain were calculated in Mega 6.0 software (Tamura et al., 2013) using the *p*-distance method. In order to estimate vaccine efficacy (E), we used the equation  $E = 0.47 - 2.47 \times P_{\text{epitope}}$  described by Deem and Pan (2009).  $P_{\text{epitope}}$  means the largest *p*-distance value of antigenic sites, the proportion of different amino acids for each epitope between two strains. We considered the five epitopes Sa, Sb, Ca1, Ca2, and Cb

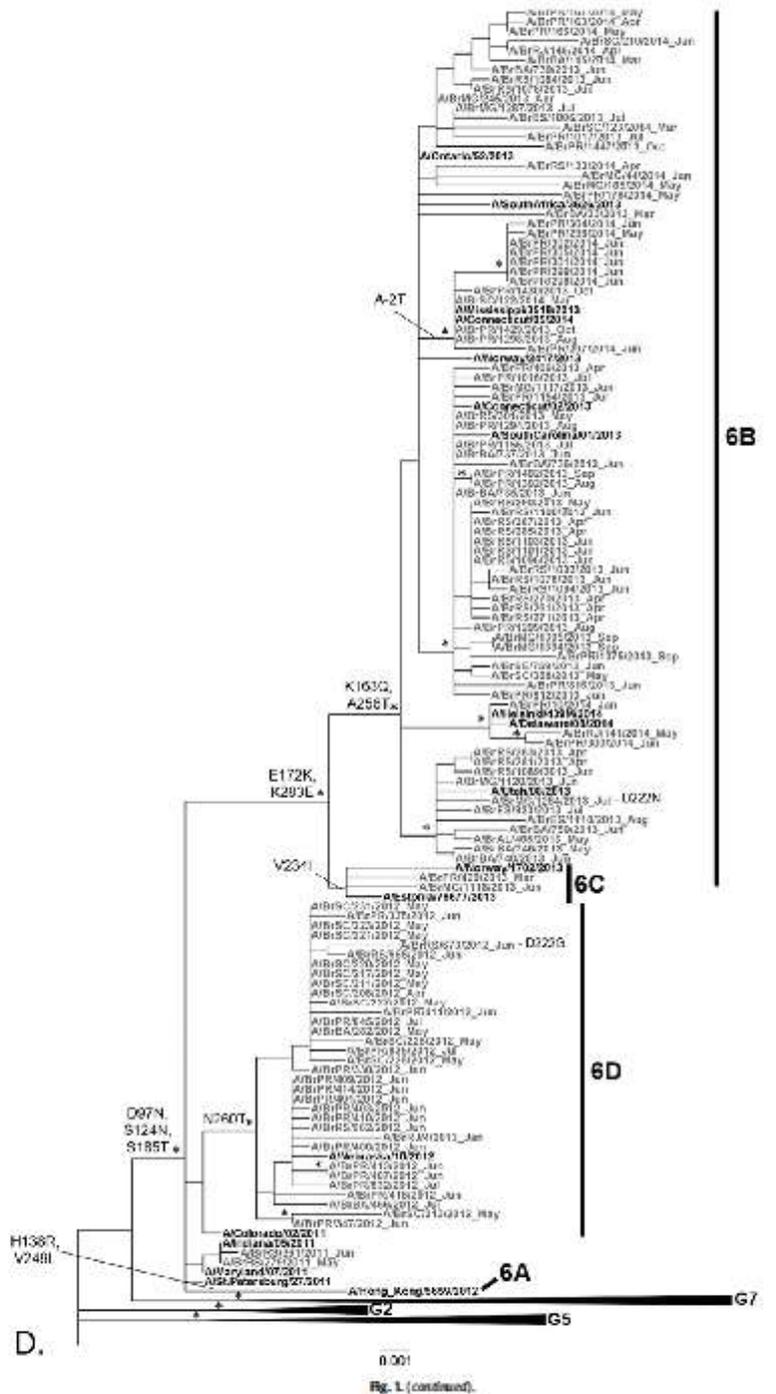


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of the HA1 globular domain, against which the majority of neutralizing antibodies are directed (Igarashi et al., 2010).

### 2.5. Statistical analysis

The software BaTS (Bayesian Tip-Significance testing) was used to test for significant phylogeny-trait correlations between virus sequences from mild and severe/fatal cases. It takes into account the statistical tests of Association Index (AI), the Parsimony Score (PS) and the Maximum Clade (MC), they provided 95% confidence intervals (CI) and significance estimation for these while accounting for phylogenetic uncertainty by the use of the posterior sets of trees obtained through earlier Bayesian phylogenetic analysis obtained by MrBayes software (Parke et al., 2008). Other statistical analyses were calculated by Fisher's exact test available at Graph Pad software an on-line tool (<http://www.graphpad.com/quickcalcs/contingency1/>). Statistical significance was considered only for p-values < 0.05.

## 3. Results

### 3.1. Phylogenetic analyses of the hemagglutinin gene

Molecular analyses of HA gene sequences obtained from 220 Brazilian A(H1N1)pdm09 strains revealed that at least eight genetic groups circulated in Brazil from 2009 to 2014 (Fig. 1A to D). The ML tree topology showed a clear temporal structure with co-circulation of phylogenetic groups and subgroups with replacement of genetic groups over time. Based only on the HA gene, we identified two phylogenetic groups that circulated early in the pandemic: G6-pdm, whose sequences clustered closely with A/New York/4735/2009-like with K15E and Q293H substitutions; and G7-pdm, similar to A/Shanghai/1/2009-like with S203T substitution. Four Brazilian strains (A/BrSC/6235/2009, A/BrRS/9116/2009, A/BrPR/2529/2009, A/BrMG/490/2009) were not clustered into G6-pdm and G7-pdm. Since we need the whole genome to classify the other early phylogenetic groups from pandemic period, these four sequences were considered ungrouped sequences from pandemic period (ungrouped-pdm). These two early phylogenetic groups and ungrouped-pdm were detected during May 2009 to June 2010. Of 65 strains analyzed from this period, the large majority (67.7%) were G7-pdm, followed by the G6-pdm (26.2%). The G7-pdm, was the origin of later phylogenetic groups (G2 to G7) identified in the post-pandemic period, they are shown collapsed at the ML tree (Fig. 1A, indicated by arrows). Post-pandemic Brazilian HA sequences from 2011 to 2014 epidemic seasons (Fig. 1A to D) were clustered in group 5 (G5), group 6 (G6), and group 7 (G7). The ML tree showed co-circulation of G5 and G6 during epidemic season 2011, of G5, G6 and G7 in 2012 and of G6 and G7 in 2013 (Fig. 1B and C). Only the G6 subgroup 6B was detected in 2014 epidemic season (Fig. 1D). G5 and G7 variants were less common than G6. The G5 A/Astrakhan/1/2011-like sequences were characterized by D97N, R205K, I216V and V249L substitutions. Twenty-two Brazilian strains clustered in this group, were collected from May 2011 to August 2012 (Fig. 1B). Seventeen G7 Brazilian sequences detected in 2012 and 2013 seasons were A/St.Petersburg/100/2011-like, being characterized by S143G, S185T and A197T signature substitutions. In addition to these signatures, strains from 2013 also exhibited the L-9 M, S84G and K163I substitutions (Fig. 1C). Regarding G6, represented by strains A/St.Petersburg/27/2011-like with the D97N and S185T substitutions, it has circulated widely in Brazil since 2011, represented by the strains A/BrRS/279/2011\_May and A/BrRS/361/2011\_Jun (Fig. 1D), and more than half of Brazilian viruses (n = 116) analyzed in this study belonged to it. This group also shows the wider diversity, being divided in four subgroups, 6A to 6D. The subgroup 6B was the most populated enclosing 79 Brazilian sequences from 2013 to 2014 and the signature substitutions K163Q and A256T. The 6D phylogenetic subgroup (n = 33), was defined by the N260I mutation. Only two samples were classified as

subgroup 6C at this collection and not one sample was clustered as subgroup 6A.

### 3.2. Amino acid substitutions and its association with clinical outcomes

In this study, we observed whether identified genetic groups were associated with severe/fatal clinical outcomes. In Table 1 we compared the number of mild versus severe/fatal cases in each phylogenetic group and this distribution was similar for both clinical outcomes. To analyze the statistical significance of this distribution we classified HA sequences in two traits: sequences obtained from mild cases (n = 44) and sequences obtained from severe/fatal cases (n = 67), and we constructed Bayesian trees. The phylogeny-trait associations were tested using BaTS software and the results confirmed the lack of association of clinical outcomes within genetic groups (Table 2).

Reviewing the main amino acid substitutions associated with an increase of severity in the literature, we observed that only the polymorphisms at residue 222 were statistically associated with severe/fatal cases (p-value = 0.016). However, these polymorphisms were not associated to any specific phylogenetic cluster, being found in several branches and groups throughout the tree (Fig. 1 and Table 1). The substitutions of D to G and/or asparagine (N) at residue 222 of the HA gene were found in 12 cases. Among these, 10 were fatal cases, one was severe case and another patient had no clinical outcome reported. The polymorphism D222G was found in four strains and D222N in one strain. However, we observed in the Sanger electropherograms of seven strains a double peak at the first and/or second base of the 222 codon (RRT or GRT, where R indicates the presence of nucleotides A or G), suggesting a presence of mixed viral subpopulations in these samples. To check the presence of viral subpopulations suggested by the Sanger electropherograms, we performed a customized PSQ protocol on residue 222 of the HA gene. This protocol allowed us to detect in RRT codons, one strain with D222G/D mutations and five with D222N/G mutations, and in GRT codon, one strain with D222D/G. Among the deceased patients, four were pregnant women and all presented mixed viral population D/G222 (n = 1) and N/G222 (n = 3).

### 3.3. Amino acid substitutions in antigenic sites and vaccine efficacy prediction

We identified 22 amino acid changes compared to the vaccine strain located at five different antigenic sites (1 to 5 mutations within antigenic sites). They were distributed in all phylogenetic groups, except in ungrouped-pdm. Groups from the pandemic period had 1 to 3 changes and all strains in groups from post-pandemic period contained 2 to 5 changes (Table 3).

The large majority of these mutations were found at a low frequency (0.5% to 0.9%) scattered throughout the years covered by our sampling, namely: T72I, A73S and S75T in Cb; P137S, A139S and A141R in Ca2; N137D; S174T and S179N in Sa; I183V, G187R and E235D in Ca1; A203T, D204Y, Q189K and S190N in Sb. However, other mutations at the five antigenic sites appeared in high proportions in the viral population. The S203T and R205K substitutions located at antigenic site Ca1, were found in 90.5% and 12.3% of strains respectively. In Sb 60.5% of strains showed substitutions at residue 185, S185T (n = 126) or S185A (n = 7). In Sa we observed K163Q or K163I substitutions in 42.7% of strains, H183Q was found in 6.8% and D222G, D222N or mixed viral populations D222D/G or D222N/G had a frequency of 5.5%.

The mean of global amino acid p-distance (number of amino acid substitutions per site) of each phylogenetic group was calculated in comparison to the vaccine strain (Table 3). It revealed that phylogenetic groups from the pandemic period had a low number of amino acid substitutions in comparison to phylogenetic groups from the post-pandemic period. We also calculated the antigenic distances of five HA epitopes from all sequences in relation to those from the vaccine strain A/California/7/2009 in order to estimate the theoretical vaccine efficacy

**Table 1**  
Comparison of phylogenetic groups and amino acid changes at the hemagglutinin gene of influenza A(H1N1)pdm09 strains in 138 Brazilian in patients with mild or severe/fatal clinical outcomes.

| Phylogenetic group<br>(amino acid signature) | Clinical outcome <sup>(a)</sup> |                                      | Polymorphisms at 222 residue of HA detected by pyrosequencing <sup>(b)</sup><br>(number of samples with mutation) |
|--|---------------------------------|--------------------------------------|---|
|  | Number of mild cases (X)        | Number of severe and fatal cases (X) |   |
| Ungrouped-pdm <sup>(c)</sup>                 | 1 (2.3%)                        | 2 (2.3%)                             |   |
| G6-pdm (K15E; Q310H)                         | 3 (6.8%)                        | 8 (8.5%)                             | D222G (2); D222N/G (1); and D222D/G (1)   |
| G7-pdm (S203T)                               | 6 (13.6%)                       | 31 (33.0%)                           | D222G (1) and D222N/G (4)   |
| G5 (D97N; R205K; I216V; V246I)               | 10 (22.7%)                      | 7 (7.4%)                             |   |
| G6 (D97N)                                    | 0 (0.0%)                        | 1 (1.1%)                             |   |
| 6B (K163Q; A256T)                            | 5 (11.4%)                       | 23 (24.5%)                           | D222N (1)   |
| 6C (V234I)                                   | 0 (0.0%)                        | 4 (4.3%)                             |   |
| 6D (N260T)                                   | 15 (34.1%)                      | 11 (11.7%)                           | D222G (1); D222D/G (1)  |
| G7 (S148G; A197T)                            | 4 (9.1%)                        | 7 (7.4%)                             |   |

<sup>(a)</sup> Only 138 from 220 patients included in this study had information about clinical outcome.

<sup>(b)</sup> Polymorphisms at residue 222 of HA gene were detected by Levine et al. (2011) protocol. The presence of these polymorphisms was significantly associated with severe/fatal clinical outcomes ( $p$ -value = 0.016), the statistical significance was calculated by Fisher's exact test.

<sup>(c)</sup> Samples from pandemic period that were not grouped into G6-pdm and G7-pdm.

(E) against Brazilian viruses using the  $P_{\text{epitope}}$  method (Deem and Pan, 2009). Phylogenetic groups from pandemic period presented a perfect theoretical vaccine match ( $P_{\text{epitope}} = 0$ ) with an E value of 0.470, which means 100% of predict efficacy against the vaccine. However, the phylogenetic groups with mutations in antigenic sites presented a decrease at the predict vaccine match (Table 3). The G5 presented the worst match with the vaccine with 4.5% of agreement, followed by 6C subgroup with 38.5% and others with 51.2%. Interestingly, from 53 individuals, who reported receiving vaccination on the epidemiological form, only 13 received vaccine in a period of one year (ranging from 3 to 334 days) prior their illness, 11 in 2013 and 2 in 2014 and their virus strains had a predicted vaccine match of 52.1% ( $E = 0.245$ ).

#### 4. Discussion

This study describes amino acid changes in the HA of 220 A(H1N1)pdm09 viruses circulating in Brazil during the pandemic period (April, 2009 to August, 2010) and in the following epidemic seasons (2011 to 2014). Phylogenetic analyses indicate that at least eight distinct genetic groups defined by specific mutations circulated in Brazil during this period. Previous evolutionary studies, conducted with A(H1N1)pdm09 that circulated worldwide during the initial phase of pandemic period, showed an early diversification based on molecular signatures of the whole genome, which characterized the seven initial phylogenetic groups (G1, G2-pdm to G7-pdm) (Nelson et al., 2009; Potdar et al., 2010). We identified two of these early phylogenetic groups, namely G6-pdm and G7-pdm, as well as four ungrouped-pdm circulating in Brazil. As this previous classification was based on whole genome sequence, because we have only HA sequences we need more information about the other genes to know exactly where these four sequences are clustering. In turn G6-pdm and G7-pdm co-circulated among Brazilian population confirming previous data (Goni et al., 2011; Oliveira et al., 2013; Sant'Anna et al., 2014). As observed worldwide, G7-pdm was also the most common phylogenetic group detected

in Brazil during the pandemic period (Nelson et al., 2009; Potdar et al., 2010). The global dominance of G7-pdm variant may be attributed to its initial foothold in New York city, which could have facilitated its rapid global spread due to its high international interconnectivity (Nelson et al., 2011). The end of pandemic period was declared on August, 2010 (World Health Organization, 2010a) and from mid-2010 to early 2011, ILL cases reduced in Brazil (Secretary of Health Surveillance – Brazilian MoH, 2012). We speculate that this may have been associated with increase of herd immunity following the mass vaccination campaign against A(H1N1)pdm09, together with the inter seasonality period in the Southern Hemisphere. Since then, A(H1N1)pdm09 has circulated as a seasonal virus together with influenza A/H3N2 and the two lineages of influenza B viruses causing annual epidemic seasons during the winter in Brazil with a lesser impact on morbidity and mortality than during the pandemic period (Secretary of Health Surveillance – Brazilian MoH, 2012, 2013a, 2013b, 2014).

However, after the pandemic period (2011 to 2014 epidemic seasons) new genetic groups emerged from G7-pdm (European Centre for Disease Prevention and Control, 2011, 2013). This increase of diversity was also observed in countries such as Turkey, India, Japan and Tunisia (Dakhave et al., 2013; Daput et al., 2012; El Moussi et al., 2013; Güldemir et al., 2013). At least six new phylogenetic groups were detected circulating worldwide and they were numbered from group 2 to 7 (G2 to G7), since vaccine strain A/California/7/2009 reminded as G1 (European Centre for Disease Prevention and Control, 2011, 2013). Brazilian samples were observed in three of these groups G5, G6 and G7 (Fig. 1A). Additionally, strains from epidemic seasons 2012/13 in Europe were classified in one of the G6 subgroups, 6A to 6C (European Centre for Disease Prevention and Control, 2013). Using the same approach, we describe a subgroup, with phylogenetic signature N260T, called 6D. This mutation, detected among 2012 Brazilian samples seems to be observed mostly in HA sequences in North and South America. Group co-circulation during epidemics in 2011 (G5 and G6), 2012 (G5, G6 and G7) and 2013 (G6 and G7) were observed, however samples of 2014 epidemic season were detected only at the subgroup 6B. According to data from Brazilian MoH, circulation of A(H1N1)pdm09 as a seasonal virus were more extensive during 2012 and 2013 epidemic seasons compared with the 2011 and 2014 epidemics (Secretary of Health Surveillance – Brazilian MoH, 2012, 2013a, 2013b, 2014). The increase in viral circulation is a contributory factor to increased viral diversity reflected in genetic drift and the emergence of new genetic groups as observed after the pandemic period. Some of these genetic groups were observed previously in Northern hemisphere before their detection in Brazil. Viruses collected during epidemic season 2010–2011 in European countries fall into the post-pandemic phylogenetic groups, and in Brazil, it occurred after May 2011 (European Centre for Disease Prevention and Control, 2011;

**Table 2**  
Comparison about the clustering of mild cases versus severe plus fatal cases using Bayesian Tip-Significance testing (BaTS).

| Statistic test            | Observed mean (95% CI) | Null hypothesis mean (95% CI) | Significance (p-value) |
|---------------------------|------------------------|-------------------------------|------------------------|
| AI                        | 3.9 (3.0–4.9)          | 5.6 (4.6–6.6)                 | 0.00                   |
| PS                        | 30.6 (28.0–32.0)       | 37.4 (34.0–40.3)              | 0.00                   |
| MC (mild cases)           | 7.0 (7.0–7.0)          | 2.6 (2.1–3.4)                 | 0.00                   |
| MC (severe + fatal cases) | 8.3 (7.0–13.0)         | 6.4 (4.5–9.9)                 | 0.27                   |

PS (parsimony score) statistic quantifies phylogeny-trait correlation; AI (association index) statistic takes into account the shape of the phylogeny by measuring the imbalance of internal phylogeny nodes; and MC (monophyletic clade).

**Table 3**  
Amino acid distance of the nine A(H1N1)pdm09 phylogenetic groups to the vaccine strain A/California/7/2009 and the substitutions frequency in antigenic sites into each group.

| Phylogenetic Group (amino acid signatures) | Number of sequences | Period of circulation in Brazil | Mean of a amino acid p-distance to A/California/7/2009 | Mean of predicted vaccine efficacy (E) | Number of changes in antigenic sites |
|--|---------------------|---------------------------------|--|--|--------------------------------------|
| Un grouped-pdm                             | 4                   | Jun 2009 to Aug 2009            | 0.005  | 0.470                                  | 0                                    |
| G6-pdm (K-15E; Q293H)                      | 17                  | May 2009 to Mar 2010            | 0.008  | 0.470                                  | 0 to 1                               |
| G7-pdm (S203T)                             | 44                  | Jun 2009 to Jun 2010            | 0.008  | 0.470                                  | 1 to 3                               |
| G5 (D97N; R205K; I216V; V249L)             | 22                  | May 2011 to Aug 2012            | 0.021  | 0.021                                  | 2 to 4                               |
| G6 (D97N)                                  | 2                   | May 2011 to Jun 2011            | 0.017  | 0.245                                  | 2                                    |
| 6B (K163Q; A256T)                          | 79                  | Mar 2013 to Jun 2014            | 0.021  | 0.245                                  | 3 to 5                               |
| 6C (V234I)                                 | 2                   | Mar 2013 to Jun 2013            | 0.019  | 0.181                                  | 2 to 3                               |
| 6D (N280T)                                 | 33                  | Apr 2012 to Jan 2013            | 0.018  | 0.245                                  | 2 to 3                               |
| G7 (S143G; A197T)                          | 17                  | Mar 2012 to Jun 2013            | 0.025  | 0.245                                  | 2 to 5                               |

H1 numbering without signal peptide; p-distance: proportion of different amino acids for each HA1 epitope between the strain of interest and the vaccine strain A/California/7/2009; vaccine efficacy (E) per each phylogenetic group.

Ledesma et al., 2012). It may suggest that the earlier epidemic in the North Hemisphere may predict and potentially seed the next epidemic season in Brazil, however more data is necessary to confirm this hypothesis.

In our study, sequences from post-pandemic strains showed more amino acid differences than pandemic strains from the vaccine and some of the amino acid substitutions occurred in antigenic sites (Ca1, Ca2, Cb, Sa and Sb) of the HA molecule (Sriwilaijaroen and Suzuki, 2012). Although the genetic groups identified in this study showed specific changes in different epitopes of HA, which contributed to a theoretical decrease in vaccine efficacy, the results obtained by formal antigenic characterization performed with similar variant viruses did not show a consistent marked change in antigenicity compared to vaccine virus A/California/7/2009 (European Centre for Disease Prevention and Control, 2011, 2013). Around 99.1% of Brazilian samples contained up to four mutations in HA antigenic sites. However, previous report indicates that such mutations did not contribute to a significant change in antigenicity (Dapat et al., 2012), indicating that the algorithms to evaluate genetic changes alone, needs future refinement before it can be used to infer vaccine choices.

None of the phylogenetic groups found in Brazil had a strong relationship with severe or fatal influenza disease, and our findings corroborates the lack of association with severity of K-15E and Q293H substitutions (G6-pdm) found previously (Graham et al., 2011; Lee et al., 2010; Mesquita et al., 2014). Otherwise, we observed a point mutation at residue 222 of the HA gene associated with an increase of severity of illness in humans (World Health Organization, 2010b). This mutation was detected across variant viral groups and in different seasons, which suggests it as a potential virulence marker. Associations of amino acid changes in the HA with a possible increase of virulence has been reported (El Mousi et al., 2013; Glinsky, 2010; Klönder et al., 2010; Maurer-Stroh et al., 2010; Melidou et al., 2010; Resende et al., 2014). Polymorphisms at residue 222 in Brazilian strains did not form a phylogenetic cluster, sequences with this mutation were observed in strains from two phylogenetic groups from the pandemic period, G6-pdm and G7-pdm, and it was observed in recent cases due to 6B and 6D viruses. The association of polymorphisms and the presence of quasispecies at residue 222 of the HA was widely explored in samples from pandemic period (Chen et al., 2010; Kuroda et al., 2010; Resende et al., 2014). This amino acid residue is located in the antigenic site Ca2 near from of the receptor-binding domain and local changes may influence binding preferences of the virus (Liu et al., 2010). The substitutions of D to G and/or N at residue 222 of the HA gene were found in 12 severe/fatal cases, among these, four fatal cases were pregnant

women and presented mixed viral populations D/G222 and N/G222. Our group had previously reported the detection of mixed viral polymorphism at this residue in samples from pregnant women (Resende et al., 2014). We suggest that the selection of mutant viruses may be related with an increase of sialic acid levels and expression of  $\alpha 2,3$  linkage in respiratory tract of pregnant women during pregnancy (Orczyk-Pawilowicz et al., 2012; Resende et al., 2014).

## 5. Conclusions

Five years after the pandemic the A(H1N1)pdm09 continues to circulate in the Brazilian population, eight influenza A(H1N1)pdm09 genetic groups defined by specific amino acid changes have been circulated in Brazil from 2009 to 2014. We have seen a regular replacement of variants by new phylogenetic groups over time and the presence of mutations in antigenic sites have been increasing. In accordance with antigenic results worldwide, none of these genetic groups seems to show significant antigenic differences with the vaccine strain A/California/7/2009, but theoretically the vaccine efficacy have been decreased due to changes in antigenic sites of these strains. This study reinforce the importance of continued laboratory based-surveillance to detect the emergence of new variants, which essential for reformulation of influenza vaccine, since the closer the vaccine strain is to the dominant circulating viral variant, the more effective is the vaccine is likely to be.

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## References

- Carrat, F., Flahault, A., 2007. Influenza vaccine: the challenge of antigenic drift. *Vaccine* 25, 6852–6862.
- Chen, H., Wen, X., To, K.K., Wang, P., Tse, H., Chan, J.F., Tsai, H.W., Fung, K.S., Tse, C.W., Lee, R.A., Chan, K.H., Yuen, K.Y., 2010. Quasispecies of the D225G substitution in the hemagglutinin of pandemic influenza A(H1N1) 2009 virus from patients with severe disease in Hong Kong, China. *J. Infect. Dis.* 201, 1517–1521.
- Dalrymple, M., Khirwal, A., Patel, K., Kadam, A., Pordar, V., 2013. Whole-genome sequence analysis of postpandemic influenza A(H1N1)pdm09 virus isolates from India. *Genome Announc.* 1.

- Dapat, I.C., Daplat, C., Banzrovich, T., Suzuki, Y., Kondo, H., Shobugawa, Y., Saito, R., Suzuki, H., 2012. Genetic characterization of human influenza viruses in the pandemic (2009–2010) and post-pandemic (2010–2011) periods in Japan. *PLoS One* 7, e36435.
- Dawood, F.S., Jain, S., Pflugh, L., Shaw, M.W., Lindstrom, S., Gerns, R.J., Galvani, L.V., Xu, X., Bridges, C.B., Iyeki, T.M., 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N. Engl. J. Med.* 360, 2605–2615.
- Deem, M.W., Pan, K., 2009. The epizootic regions of H1-subtype influenza A, with application to vaccine efficacy. *Protein Eng. Des. Sel.* 22, 540–546.
- El Moussati, A., Ben Hadj Kacem, M.A., Pozo, F., Linderma, J., Cuevas, M.T., Casas, I., Slim, A., 2013. Genetic diversity of HA1 domain of haemagglutinin gene of influenza A(H1N1)pdm09 in Tunisia. *Virus J.* 10, 150.
- European Centre for Disease Prevention and Control, 2011. *Influenza Virus Characterisation – Summary Europe, August and September 2011 Technical Document*.
- European Centre for Disease Prevention and Control, 2013. *Influenza Virus Characterisation – Summary Europe, September 2013. Surveillance Report*.
- Gilroy, G.V., 2010. Genetic analysis of pandemic (H1N1) 2009 reveals a association of increasing disease severity with emergence of novel haemagglutinin mutations. *Cell Cycle* 9, 958–970.
- Goni, M., Mouton, C., Ramas, V., Coppola, L., Chiaperelli, H., Cifolina, J., 2011. Phylogenetic analysis of pandemic 2009 influenza A virus circulating in the South American region: genetic relationships and vaccine strain match. *Arch. Virol.* 156, 47–54.
- Graham, M., Liang, B., Van Donkelaar, G., Bastien, N., Brundin, C., Tyler, S., Kaplan, B., Landry, E., Li, Y., 2011. National molecular surveillance of pandemic H1N1 influenza A virus genomes: Canada, 2009. *PLoS One* 6, e19287.
- Gökdemir, D., Kalyoncu, A.T., Altın, A.B., Koruyucu, G., Durmaz, R., 2013. Monitoring genetic diversity of influenza A(H1N1)pdm09 virus circulating during the post-pandemic period in Turkey. *Jpn. J. Infect. Dis.* 66, 299–305.
- Gupta, V., Earl, D.J., Deem, M.W., 2006. Quantifying influenza vaccine efficacy at a zoonotic distance. *Vaccine* 24, 3881–3884.
- Houser, K., Subhata, K., 2015. Influenza vaccines: challenges and inhibitors. *Clin. Hosp. Infect.* 17, 295–300.
- Ispahani, M., Ito, K., Yoshida, R., Tomihachi, D., Kida, H., Takada, A., 2010. Predicting the antigenic structure of the pandemic (H1N1) 2009 influenza virus haemagglutinin. *PLoS One* 5, e12553.
- Kilander, A., Ryhlmén, R., Dodman, S.G., Huang, O., 2010. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome. *Norway 2009–2010. Euro Surveill.* 15.
- Kuroda, M., Katano, H., Nakajima, N., Tobikane, M., Aizawa, T., Hasegawa, H., Tashiro, M., Sakaki, Y., Anzawa, Y., Hata, S., Watanabe, M., Sata, T., 2010. Characterization of pandemic 2009 influenza A virus (A/H1N1/2009) by de novo sequencing using a next-generation DNA sequencer. *PLoS One* 5, e12056.
- Lederman, J., Pozo, F., Reina, G., Bacon, M., Rodríguez, G., Monteiro, M., Lopez-Miragaya, I., Sánchez, C., Reina, J., Ortiz de Lejarza, R., Egido, P., Lopez Barba, J., Delgado, C., Cuevas, M.T., Casas, I., 2012. Genetic diversity of influenza A(H1N1) 2009 virus circulating during the season 2010–2011 in Spain. *J. Clin. Virol.* 53, 16–21.
- Lee, R.T., Santos, C.L., de Paiva, T.M., Chi, L., Simola, F.L., Eisenhaber, F., Maurer-Stroh, S., 2010. All that glitters is not gold—founder effects complicate associations of flu mutations to disease severity. *Virus J.* 7, 297.
- Levine, M., Shen, T.G., Gubareva, L.V., Mishin, V.P., 2011. Detection of haemagglutinin variants of the pandemic influenza A (H1N1) 2009 virus by pyrosequencing. *J. Clin. Microbiol.* 49, 1307–1312.
- Liu, Y., Chikis, R.A., Marzouk, T., Wharton, S., Palma, A.S., Chai, W., Danis, R., Gregory, V., Uhlendorf, J., Kiso, M., Klein, H.D., Hay, A., Feldt, T., Marzouk, M., 2010. A trend receptor specificity and cell tropism of D222G haemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. *J. Virol.* 84, 12069–12074.
- Maurer-Stroh, S., Lee, R.T., Eisenhaber, F., Chi, L., Phuah, S.P., Lin, R.T., 2010. A New Ginnomon Mutation in the Haemagglutinin of the 2009 (H1N1) Influenza A Virus. *PLoS Curr.* 2, RNN1162.
- Medina, R.A., Garcia-Sastre, A., 2011. Influenza A viruses: new research developments. *Nat. Rev. Microbiol.* 9, 590–603.
- Melidou, A., Gouka, G., Brikari, M., Chazidimitriou, D., Dika, E., Makris, N., 2010. Molecular and phylogenetic analysis of the haemagglutinin gene of pandemic influenza H1N1 2009 viruses associated with severe and fatal infections. *Virus Res.* 151, 192–199.
- Mezquita, M., Resende, P., Martorelli, A., Machado, V., Sacramento, C.Q., Figueira-Rodrigues, N., Abranches, J.L., Tavares, R., Schirmer, M., Siqueira, M.M., Souza, T.M., 2014. Detection of the influenza A(H1N1)pdm09 virus carrying the K-15E, P63S and Q203H mutations in patients who have undergone bone marrow transplant. *PLoS One* 9, e94822.
- Ministry of Health, 2015. SI-PNI – Sistema de Informação do Programa Nacional de Imunizações.
- Nelson, M., Spira, D., Wentworth, D., Beck, E., Fan, J., Ghedin, E., Halpin, R., Bera, J., Hine, E., Fausch, K., Stockwell, T., Liu, X., Griesemer, S., Kumar, S., Bose, M., Viboud, C., Holmes, E., Henrickson, K., 2009. The early diversification of influenza A(H1N1)pdm. *PLoS Curr.* 1, RNN1125.
- Nelson, M.I., Tan, Y., Ghedin, E., Wentworth, D.E., St George, K., Edelman, L., Beck, E.T., Fan, J., Lam, T.T., Kumar, S., Spira, D.J., Simonsen, L., Viboud, C., Holmes, E.C., Henrickson, K.J., Mouser, J.M., 2011. Phylogeography of the spring and fall waves of the H1N1/09 pandemic influenza virus in the United States. *J. Virol.* 85, 828–834.
- Oliveira, M.J., Mota, F.D., Siqueira, M.M., Resende, P.C., Bora, P.D., Souza, T.M., Mezquita, M., Oliveira, M.D., Carney, S., Mello, W.A., Magalhães, V., 2013. Molecular findings from influenza A(H1N1)pdm09 detected in patients from a Brazilian equatorial region during the pandemic period. *Mem. Inst. Oswaldo Cruz* 108, 912–917.
- Orczyk-Pawlowicz, M., Augustyniak, D., Haimle, L., Kamik-Pozoswicka, I., 2012. In-citro-based Analysis of Fucose and Sialic Acid Expressions on Human Amniotic IgA During Normal Pregnancy. *Glycoconj. J.*
- Parker, J., Rambaut, A., Pybus, O.G., 2008. Correlating viral phenotypes with phylogeny: accounting for phylogenetic uncertainty. *Infect. Genet. Evol.* 8, 239–246.
- Potter, V.A., Chadha, M.S., Jadhav, S.M., Mullick, J., Chakrabarti, S.S., Mishra, A.C., 2010. Genetic characterization of the influenza A pandemic (H1N1) 2009 virus isolates from India. *PLoS One* 5, e12623.
- Resende, P.C., Mota, F.C., Oliveira Mde, I., Gregianini, T.S., Fernandes, S.B., Cary, A.L., do Carmo, D.R.M., Souza, T.M., Siqueira, M.M., 2014. Polymorphism at residue 222 of the haemagglutinin of pandemic influenza A(H1N1)pdm09: association of quasi-species to morbidity and mortality in different risk categories. *PLoS One* 9, e102789.
- Sant'Anna, F.H., Borges, L.G., Fallavone, P.R., Gregianini, T.S., Moraes, F., Halpin, R.A., Wentworth, D., d'Azevedo, P.A., Veiga, A.B., 2014. Genetic analysis of pandemic and post-pandemic influenza A (H1N1) viruses isolated in Rio Grande do Sul, Brazil. *Arch. Virol.* 159, 621–630.
- Secretary of Health Surveillance – Brazilian MoH, 2010. *Influenza Pandêmica (H1N1) 2009 – Análise da situação epidemiológica e da resposta no ano de 2009*. Ministério da Saúde, Brasília, p. 21.
- Secretary of Health Surveillance – Brazilian MoH, 2012. *Influenza Recém de Influenza – Vigilância de Síndrome Respiratória Aguda Grave (SRAG) de Síndrome Gripal (SG) e de Interações por CID J09 e J18*. Ministério da Saúde, Brasília, p. 15.
- Secretary of Health Surveillance – Brazilian MoH, 2013a. *Boletim epidemiológico – Influenza (gripe) – Semana Epidemiológica (SE) 52 (atualizado em 08/01/2013)*. Ministério da Saúde, Brasília, p. 11.
- Secretary of Health Surveillance – Brazilian MoH, 2013b. *Boletim epidemiológico – Influenza: Monitoramento até a Semana Epidemiológica 52 de 2013*. Ministério da Saúde, Brasília, p. 10.
- Secretary of Health Surveillance – Brazilian MoH, 2014. *Boletim epidemiológico – Influenza: Monitoramento até a Semana Epidemiológica 46 de 2014*. Ministério da Saúde, Brasília, p. 11.
- Smith, D.J., Lapedes, A.S., de Jong, J.C., Beersma, T.M., Rimmelzwaan, G.F., Osterhaus, A.D., Raaijmakers, R.A., 2004. Mapping the antigenic and genetic evolution of influenza virus. *Science* 305, 371–376.
- Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghuwani, J., Bhatt, S., Peiris, J.S., Guan, Y., Rambaut, A., 2009. Origin and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* 459, 1122–1125.
- Souza, T.M., Resende, P.C., Figueira-Rodrigues, N., Gregianini, T.S., Bora, N., Fernandes, S.B., Cary, A.L., Rosa Mde, C., Siqueira, M.M., 2013. Detection of oseltamivir-resistant pandemic influenza A(H1N1)pdm09 in Brazil: can community transmission be ruled out? *PLoS One* 8, e60981.
- Sriwattanasakul, N., Suzuki, Y., 2012. Molecular basis of the structure and function of H1 haemagglutinin of influenza virus. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 88, 236–249.
- Tamura, K., Stecher, G., Peterson, D., Filipecki, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.
- Van Loveren, H., Van Amsterdam, J.G., Vandendriessche, R.J., Kimman, T.G., Runkle, H.C., Steenberg, P.S., Vos, J.G., 2001. Vaccine-induced antibody responses as parameters of the influence of endogenous and environmental factors. *Environ. Health Perspect.* 109, 757–764.
- World Health Organization, 2009. *Sequencing Primer and Protocol*. WHO Collaborating Centre for Influenza at CDC, Atlanta, GA.
- World Health Organization, 2010a. *Preliminary review of D222G amino acid substitution in the haemagglutinin of pandemic influenza A (H1N1) 2009 viruses*. *Wkly Epidemiol. Rec.* 85, 21–28.
- World Health Organization, 2010b. *Pandemic (H1N1) 2009 – update 112*. [http://www.who.int/csr/don/2010\\_08\\_05/en/index.html](http://www.who.int/csr/don/2010_08_05/en/index.html) (Geneva).
- World Health Organization, 2014a. *WHO Recommendations on the Composition of Influenza Virus Vaccines*.
- World Health Organization, 2014b. *Influenza (Seasonal)*. Fact sheet N°211. March 2014 ed. (<http://www.who.int/mediacentre/factsheets/fs211/en/index.html>).

## *REFERENCES*

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1. Man, W.H., W.A. de Steenhuijsen Piters, and D. Bogaert, *The microbiota of the respiratory tract: gatekeeper to respiratory health*. Nat Rev Microbiol, 2017. **15**(5): p. 259-270.
2. van den Bergh, M.R., et al., *Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria*. PLoS One, 2012. **7**(10): p. e47711.
3. WHO, W.H.O. *The top 10 causes of death*. 2017 [cited 2017 20/08/2017].
4. WHO, W.H.O. *Children: reducing mortality*. 2016; Available from: <http://www.who.int/mediacentre/factsheets/fs178/en/>.
5. Shaughnessy, E.E., E.L. Stalets, and S.S. Shah, *Community-acquired pneumonia in the post 13-valent pneumococcal conjugate vaccine era*. Curr Opin Pediatr, 2016. **28**(6): p. 786-793.
6. Tramper-Stranders, G.A., *Childhood community-acquired pneumonia: A review of etiology- and antimicrobial treatment studies*. Paediatr Respir Rev, 2017.
7. Lee, K.H., A. Gordon, and B. Foxman, *The role of respiratory viruses in the etiology of bacterial pneumonia: An ecological perspective*. Evol Med Public Health, 2016. **2016**(1): p. 95-109.
8. Figueiredo-Mello, C., et al., *Prospective etiological investigation of community-acquired pulmonary infections in hospitalized people living with HIV*. Medicine (Baltimore), 2017. **96**(4): p. e5778.
9. Nair, H., et al., *Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis*. Lancet, 2010. **375**(9725): p. 1545-55.
10. Benito, N., et al., *Pulmonary infections in HIV-infected patients: an update in the 21st century*. Eur Respir J, 2012. **39**(3): p. 730-45.
11. Stupka, J.E., et al., *Community-acquired pneumonia in elderly patients*. Aging Health, 2009. **5**(6): p. 763-774.
12. Feldman, C. and R. Anderson, *Recent advances in our understanding of Streptococcus pneumoniae infection*. F1000Prime Rep, 2014. **6**: p. 82.
13. Fukutani, K.F., et al., *Pathogen transcriptional profile in nasopharyngeal aspirates of children with acute respiratory tract infection*. J Clin Virol, 2015. **69**: p. 190-6.
14. Groothuis, J.R., J.M. Hoopes, and V.G. Jessie, *Prevention of serious respiratory syncytial virus-related illness. I: Disease pathogenesis and early attempts at prevention*. Adv Ther, 2011. **28**(2): p. 91-109.
15. Hancock, D.G., et al., *The heterogeneity of viral bronchiolitis: A lack of universal consensus definitions*. Pediatric Pulmonology, 2017.
16. Ralston, S.L., et al., *Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis*. Pediatrics, 2014. **134**(5): p. e1474-502.
17. *Bronchiolitis: diagnosis and management of bronchiolitis in children*. 2015.
18. Eugenia, M., et al., *Pathogenesis of Viral Respiratory Infection*. 2013.
19. Holt, P.G., J.W. Upham, and P.D. Sly, *Contemporaneous maturation of immunologic and respiratory functions during early childhood: implications for development of asthma prevention strategies*. J Allergy Clin Immunol, 2005. **116**(1): p. 16-24; quiz 25.
20. Nair, H., et al., *Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis*. The Lancet, 2013. **381**(9875): p. 1380-1390.
21. Pilie, P., et al., *Adult patients with respiratory syncytial virus infection: impact of solid organ and hematopoietic stem cell transplantation on outcomes*. Transpl Infect Dis, 2015. **17**(4): p. 551-7.
22. Falsey, A.R., et al., *Respiratory syncytial virus infection in elderly and high-risk adults*. N Engl J Med, 2005. **352**(17): p. 1749-59.
23. Musher, D.M. and A.R. Thorner, *Community-acquired pneumonia*. N Engl J Med, 2014. **371**(17): p. 1619-28.

24. Kim, G.L., S.H. Seon, and D.K. Rhee, *Pneumonia and Streptococcus pneumoniae vaccine*. Arch Pharm Res, 2017.
25. Troy, N.M. and A. Bosco, *Respiratory viral infections and host responses; insights from genomics*. Respir Res, 2016. **17**(1): p. 156.
26. Bogaert, D., R. De Groot, and P.W. Hermans, *Streptococcus pneumoniae colonisation: the key to pneumococcal disease*. Lancet Infect Dis, 2004. **4**(3): p. 144-54.
27. Jain, S., et al., *Community-acquired pneumonia requiring hospitalization among U.S. children*. N Engl J Med, 2015. **372**(9): p. 835-45.
28. Self, W.H., et al., *Respiratory Viral Detection in Children and Adults: Comparing Asymptomatic Controls and Patients With Community-Acquired Pneumonia*. J Infect Dis, 2016. **213**(4): p. 584-91.
29. WHO, W.H.O. *Influenza (Seasonal)*. 2016; Available from: <http://www.who.int/mediacentre/factsheets/fs211/en/>.
30. ICTV. *Orthomyxoviridae family*. 2010 2010 20/08/2017]; Available from: [https://talk.ictvonline.org/ictv-reports/ictv\\_9th\\_report/negative-sense-rna-viruses-2011/w/negrna\\_viruses/209/orthomyxoviridae](https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/209/orthomyxoviridae).
31. Wright, P.F., G. Neumann, and Y. Kawaoka, *Orthomyxoviruses*. 2013.
32. Hardelid, P., et al., *Effectiveness of pandemic and seasonal influenza vaccine in preventing pandemic influenza A(H1N1)2009 infection in England and Scotland 2009-2010*. Euro Surveill, 2011. **16**(2).
33. Salez, N., et al., *Influenza C virus high seroprevalence rates observed in 3 different population groups*. Journal of Infection, 2014. **69**(2): p. 182-189.
34. WHO, W.H.O. *WHO recommendations on the composition of influenza virus vaccines*. 2017; Available from: <http://www.who.int/influenza/vaccines/virus/recommendations/en/>.
35. Schrauwen, E.J., et al., *Reassortment between Avian H5N1 and human influenza viruses is mainly restricted to the matrix and neuraminidase gene segments*. PLoS One, 2013. **8**(3): p. e59889.
36. Wang, X., et al., *Epidemiology of avian influenza A H7N9 virus in human beings across five epidemics in mainland China, 2013–17: an epidemiological study of laboratory-confirmed case series*. The Lancet Infectious Diseases, 2017. **17**(8): p. 822-832.
37. Reperant, L.A., et al., *The immune response and within-host emergence of pandemic influenza virus*. Lancet, 2014.
38. Cardona, C.J., et al., *Avian influenza in birds and mammals*. Comp Immunol Microbiol Infect Dis, 2009. **32**(4): p. 255-73.
39. Moules, V., et al., *Importance of viral genomic composition in modulating glycoprotein content on the surface of influenza virus particles*. Virology, 2011. **414**(1): p. 51-62.
40. Einfeld, A.J., G. Neumann, and Y. Kawaoka, *At the centre: influenza A virus ribonucleoproteins*. Nat Rev Microbiol, 2015. **13**(1): p. 28-41.
41. Medina, R.A. and A. Garcia-Sastre, *Influenza A viruses: new research developments*. Nat Rev Microbiol, 2011. **9**(8): p. 590-603.
42. Wise, H.M., et al., *A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA*. J Virol, 2009. **83**(16): p. 8021-31.
43. Dubois, J., O. Terrier, and M. Rosa-Calatrava, *Influenza viruses and mRNA splicing: doing more with less*. MBio, 2014. **5**(3): p. e00070-14.
44. Gamblin, S.J. and J.J. Skehel, *Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins*. Journal of Biological Chemistry, 2010. **285**(37): p. 28403-28409.
45. Nayak, D.P., et al., *Influenza virus morphogenesis and budding*. Virus Res, 2009. **143**(2): p. 147-61.

46. Smith, G.J., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic*. Nature, 2009. **459**(7250): p. 1122-5.
47. Lofgren, E., et al., *Influenza seasonality: underlying causes and modeling theories*. J Virol, 2007. **81**(11): p. 5429-36.
48. Tamerius, J., et al., *Global influenza seasonality: reconciling patterns across temperate and tropical regions*. Environ Health Perspect, 2011. **119**(4): p. 439-45.
49. Karron, R.A. and R.E. Black, *Determining the burden of respiratory syncytial virus disease: the known and the unknown*. The Lancet, 2017.
50. Heikkinen, T., E. Ojala, and M. Waris, *Clinical and Socioeconomic Burden of Respiratory Syncytial Virus Infection in Children*. J Infect Dis, 2017. **215**(1): p. 17-23.
51. Scheltema, N.M., et al., *Global respiratory syncytial virus-associated mortality in young children (RSV GOLD): a retrospective case series*. Lancet Glob Health, 2017. **5**(10): p. e984-e991.
52. Perez-Yarza, E.G., et al., *The association between respiratory syncytial virus infection and the development of childhood asthma: a systematic review of the literature*. Pediatr Infect Dis J, 2007. **26**(8): p. 733-9.
53. Holtzman, M.J., *Asthma as a chronic disease of the innate and adaptive immune systems responding to viruses and allergens*. J Clin Invest, 2012. **122**(8): p. 2741-8.
54. Collins, P.L.a.K., R. A., *Respiratory Syncytial Virus and Metapneumovirus*, in *Fields Virology, 6th edition*. 2013, Wolters Kluwer Health Adis (ESP).
55. Falsey, A.R. and E.E. Walsh, *Respiratory syncytial virus infection in adults*. Clin Microbiol Rev, 2000. **13**(3): p. 371-84.
56. Hall, C.B., et al., *Respiratory syncytial viral infection in children with compromised immune function*. N Engl J Med, 1986. **315**(2): p. 77-81.
57. Afonso, C.L., et al., *Taxonomy of the order Mononegavirales: update 2016*. Arch Virol, 2016. **161**(8): p. 2351-60.
58. Huang, Y.T. and G.W. Wertz, *The genome of respiratory syncytial virus is a negative-stranded RNA that codes for at least seven mRNA species*. J Virol, 1982. **43**(1): p. 150-7.
59. Levine, S., *Polypeptides of respiratory syncytial virus*. J Virol, 1977. **21**(1): p. 427-31.
60. Huang, Y.T. and G.W. Wertz, *Respiratory syncytial virus mRNA coding assignments*. J Virol, 1983. **46**(2): p. 667-72.
61. Collins, P.L., Y.T. Huang, and G.W. Wertz, *Identification of a tenth mRNA of respiratory syncytial virus and assignment of polypeptides to the 10 viral genes*. J Virol, 1984. **49**(2): p. 572-8.
62. Bernstein, J.M. and J.F. Hruska, *Respiratory syncytial virus proteins: identification by immunoprecipitation*. J Virol, 1981. **38**(1): p. 278-85.
63. Dubovi, E.J., *Analysis of proteins synthesized in respiratory syncytial virus-infected cells*. J Virol, 1982. **42**(2): p. 372-8.
64. Fernie, B.F. and J.L. Gerin, *Immunochemical identification of viral and nonviral proteins of the respiratory syncytial virus virion*. Infect Immun, 1982. **37**(1): p. 243-9.
65. Collins, P.L., Crowe, J. E., *Respiratory Syncytial Virus and Metapneumovirus*, in *Fields Virology, 5th edition*, L.W. Wilkins, Editor. 2007.
66. Norrby, E., H. Marusyk, and C. Orvell, *Morphogenesis of respiratory syncytial virus in a green monkey kidney cell line (Vero)*. J Virol, 1970. **6**(2): p. 237-42.
67. Sullender, W.M., *Respiratory syncytial virus genetic and antigenic diversity*. Clin Microbiol Rev, 2000. **13**(1): p. 1-15, table of contents.
68. Crim, R.L., et al., *Identification of linear heparin-binding peptides derived from human respiratory syncytial virus fusion glycoprotein that inhibit infectivity*. J Virol, 2007. **81**(1): p. 261-71.
69. Tayyari, F., et al., *Identification of nucleolin as a cellular receptor for human respiratory syncytial virus*. Nature Medicine, 2011. **17**: p. 1132-1135.

70. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature, 1970. **227**(5259): p. 680-5.
71. Langedijk, J.P., et al., *Proposed three-dimensional model for the attachment protein G of respiratory syncytial virus*. J Gen Virol, 1996. **77 ( Pt 6)**: p. 1249-57.
72. Lentz, T.L., *The recognition event between virus and host cell receptor: a target for antiviral agents*. J Gen Virol, 1990. **71 ( Pt 4)**: p. 751-66.
73. Mettenleiter, T.C., et al., *Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus*. J Virol, 1990. **64**(1): p. 278-86.
74. Hallak, L.K., S.A. Kwilas, and M.E. Peebles, *Interaction between respiratory syncytial virus and glycosaminoglycans, including heparan sulfate*. Methods Mol Biol, 2007. **379**: p. 15-34.
75. Krusat, T. and H.J. Streckert, *Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells*. Arch Virol, 1997. **142**(6): p. 1247-54.
76. Teng, M.N., S.S. Whitehead, and P.L. Collins, *Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication in vitro and in vivo*. Virology, 2001. **289**(2): p. 283-96.
77. Fearn, R., M.E. Peebles, and P.L. Collins, *Mapping the transcription and replication promoters of respiratory syncytial virus*. J Virol, 2002. **76**(4): p. 1663-72.
78. Salter, A., B.N. Laoi, and B. Crowley, *Molecular epidemiology of human respiratory syncytial virus subgroups A and B identified in adults with hematological malignancy attending an Irish hospital between 2004 and 2009*. J Med Virol, 2011. **83**(2): p. 337-47.
79. Zhang, Z.Y., et al., *Genetic variability of respiratory syncytial viruses (RSV) prevalent in Southwestern China from 2006 to 2009: emergence of subgroup B and A RSV as dominant strains*. J Clin Microbiol, 2010. **48**(4): p. 1201-7.
80. Fujitsuka, A., et al., *A molecular epidemiological study of respiratory viruses detected in Japanese children with acute wheezing illness*. BMC Infect Dis, 2011. **11**: p. 168.
81. Law, B.J., X. Carbonell-Estrany, and E.A. Simoes, *An update on respiratory syncytial virus epidemiology: a developed country perspective*. Respir Med, 2002. **96 Suppl B**: p. S1-7.
82. Hillis, W.D., et al., *Respiratory syncytial virus infection in children in West Bengal*. Indian J Med Res, 1971. **59**(9): p. 1354-64.
83. Weber, M.W., E.K. Mulholland, and B.M. Greenwood, *Respiratory syncytial virus infection in tropical and developing countries*. Trop Med Int Health, 1998. **3**(4): p. 268-80.
84. Waris, M. and L.J. White, *Seasonality of respiratory syncytial virus infection*. Clin Infect Dis, 2006. **43**(4): p. 541.
85. Ramaekers, K., et al., *Prevalence and seasonality of six respiratory viruses during five consecutive epidemic seasons in Belgium*. J Clin Virol, 2017. **94**: p. 72-78.
86. Shorr, A.F., et al., *Viruses are prevalent in non-ventilated hospital-acquired pneumonia*. Respir Med, 2017. **122**: p. 76-80.
87. Abedi, G.R., et al., *Estimates of Parainfluenza Virus-Associated Hospitalizations and Cost Among Children Aged Less Than 5 Years in the United States, 1998-2010*. J Pediatric Infect Dis Soc, 2016. **5**(1): p. 7-13.
88. Pawelczyk, M. and M.L. Kowalski, *The Role of Human Parainfluenza Virus Infections in the Immunopathology of the Respiratory Tract*. Curr Allergy Asthma Rep, 2017. **17**(3): p. 16.
89. Vogel, M., et al., *What We Have Learned from the Influenza A pH1N1 2009/10 Pandemic: High Clinical Impact of Human Metapneumovirus and Respiratory Syncytial Virus in Hospitalized Pediatric Patients*. Jpn J Infect Dis, 2016. **69**(1): p. 6-11.
90. Principi, N. and S. Esposito, *Paediatric human metapneumovirus infection: epidemiology, prevention and therapy*. J Clin Virol, 2014. **59**(3): p. 141-7.

91. Kahn, J.S., *Epidemiology of human metapneumovirus*. Clin Microbiol Rev, 2006. **19**(3): p. 546-57.
92. Williams, J.V., et al., *Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children*. N Engl J Med, 2004. **350**(5): p. 443-50.
93. Guerrero-Plata, A., A. Casola, and R.P. Garofalo, *Human metapneumovirus induces a profile of lung cytokines distinct from that of respiratory syncytial virus*. J Virol, 2005. **79**(23): p. 14992-7.
94. Bao, X., et al., *Airway epithelial cell response to human metapneumovirus infection*. Virology, 2007. **368**(1): p. 91-101.
95. Edwards, K.M., et al., *Burden of human metapneumovirus infection in young children*. N Engl J Med, 2013. **368**(7): p. 633-43.
96. Kuypers, J., et al., *Clinical disease in children associated with newly described coronavirus subtypes*. Pediatrics, 2007. **119**(1): p. e70-6.
97. Talbot, H.K., et al., *The pediatric burden of human coronaviruses evaluated for twenty years*. Pediatr Infect Dis J, 2009. **28**(8): p. 682-7.
98. Gaunt, E.R., et al., *Epidemiology and clinical presentations of the four human coronaviruses 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method*. J Clin Microbiol, 2010. **48**(8): p. 2940-7.
99. van der Hoek, L., et al., *Burden of disease due to human coronavirus NL63 infections and periodicity of infection*. J Clin Virol, 2010. **48**(2): p. 104-8.
100. Prill, M.M., et al., *Human coronavirus in young children hospitalized for acute respiratory illness and asymptomatic controls*. Pediatr Infect Dis J, 2012. **31**(3): p. 235-40.
101. Pene, F., et al., *Coronavirus 229E-related pneumonia in immunocompromised patients*. Clin Infect Dis, 2003. **37**(7): p. 929-32.
102. Pavia, A.T., *What is the role of respiratory viruses in community-acquired pneumonia?: What is the best therapy for influenza and other viral causes of community-acquired pneumonia?* Infect Dis Clin North Am, 2013. **27**(1): p. 157-75.
103. Siegel, S.J. and J.N. Weiser, *Mechanisms of Bacterial Colonization of the Respiratory Tract*. Annu Rev Microbiol, 2015. **69**: p. 425-44.
104. Esposito, S., et al., *Characteristics of Streptococcus pneumoniae and atypical bacterial infections in children 2-5 years of age with community-acquired pneumonia*. Clin Infect Dis, 2002. **35**(11): p. 1345-52.
105. Heiskanen-Kosma, T., et al., *Etiology of childhood pneumonia: serologic results of a prospective, population-based study*. Pediatr Infect Dis J, 1998. **17**(11): p. 986-91.
106. Juven, T., et al., *Etiology of community-acquired pneumonia in 254 hospitalized children*. Pediatr Infect Dis J, 2000. **19**(4): p. 293-8.
107. Welte, T., A. Torres, and D. Nathwani, *Clinical and economic burden of community-acquired pneumonia among adults in Europe*. Thorax, 2012. **67**(1): p. 71-79.
108. <drijkoningen2014.pdf>.
109. Howard, L.S.G.E., et al., *Microbiological profile of community-acquired pneumonia in adults over the last 20 years*. Journal of Infection, 2005. **50**(2): p. 107-113.
110. Arnold, F.W., et al., *A Worldwide Perspective of Atypical Pathogens in Community-acquired Pneumonia*. American Journal of Respiratory and Critical Care Medicine, 2007. **175**(10): p. 1086-1093.
111. Black, S., *The volatile nature of pneumococcal serotype epidemiology: potential for misinterpretation*. Pediatr Infect Dis J, 2010. **29**(4): p. 301-3.
112. Wunderink, R.G. and G.W. Waterer, *Clinical practice. Community-acquired pneumonia*. N Engl J Med, 2014. **370**(6): p. 543-51.
113. Steel, H.C., et al., *Overview of community-acquired pneumonia and the role of inflammatory mechanisms in the immunopathogenesis of severe pneumococcal disease*. Mediators Inflamm, 2013. **2013**: p. 490346.

114. Bewick, T., et al., *Serotype prevalence in adults hospitalised with pneumococcal non-invasive community-acquired pneumonia*. Thorax, 2012. **67**(6): p. 540-5.
115. Restrepo, M.I., et al., *A comparative study of community-acquired pneumonia patients admitted to the ward and the ICU*. Chest, 2008. **133**(3): p. 610-7.
116. Gadsby, N.J., et al., *Comprehensive Molecular Testing for Respiratory Pathogens in Community-Acquired Pneumonia*. Clin Infect Dis, 2016. **62**(7): p. 817-823.
117. Kapatai, G., et al., *Whole genome sequencing of Streptococcus pneumoniae: development, evaluation and verification of targets for serogroup and serotype prediction using an automated pipeline*. PeerJ, 2016. **4**: p. e2477.
118. Calix, J.J. and M.H. Nahm, *A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene*. J Infect Dis, 2010. **202**(1): p. 29-38.
119. Johnson, H.L., et al., *Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project*. PLoS Med, 2010. **7**(10).
120. WHO, W.H.O. *Pneumonia*. 2016; Available from: <http://www.who.int/mediacentre/factsheets/fs331/en/>.
121. Simell, B., et al., *The fundamental link between pneumococcal carriage and disease*. Expert Review of Vaccines, 2012. **11**: p. 841-855.
122. Adegbola, R.A., et al., *Carriage of Streptococcus pneumoniae and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis*. PLoS One, 2014. **9**(8): p. e103293.
123. Kadioglu, A., et al., *The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease*. Nat Rev Microbiol, 2008. **6**(4): p. 288-301.
124. Abeyta, M., G.G. Hardy, and J. Yother, *Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of Streptococcus pneumoniae*. Infect Immun, 2003. **71**(1): p. 218-25.
125. Norskov-Lauritsen, N., *Classification, identification, and clinical significance of Haemophilus and Aggregatibacter species with host specificity for humans*. Clin Microbiol Rev, 2014. **27**(2): p. 214-40.
126. Zarei, A.E., H.A. Almehdar, and E.M. Redwan, *Hib Vaccines: Past, Present, and Future Perspectives*. J Immunol Res, 2016. **2016**: p. 7203587.
127. Hishiki, H., et al., *Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients*. J Infect Chemother, 2011. **17**(1): p. 87-90.
128. Avadhanula, V., et al., *Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner*. J Virol, 2006. **80**(4): p. 1629-36.
129. Avadhanula, V., et al., *Nontypeable Haemophilus influenzae and Streptococcus pneumoniae bind respiratory syncytial virus glycoprotein*. Journal of Medical Microbiology, 2007. **56**(9): p. 1133-1137.
130. Head, B.M., et al., *Atypical bacterial pneumonia in the HIV-infected population*. Pneumonia (Nathan), 2017. **9**: p. 12.
131. Mulcahy, M.E. and R.M. McLoughlin, *Staphylococcus aureus and Influenza A Virus: Partners in Coinfection*. MBio, 2016. **7**(6).
132. Li, Z., et al., *Immunomodulation and Disease Tolerance to Staphylococcus aureus*. Pathogens, 2015. **4**(4): p. 793-815.
133. Raygada, J.L. and D.P. Levine, *Methicillin-Resistant Staphylococcus aureus: A Growing Risk in the Hospital and in the Community*. Am Health Drug Benefits, 2009. **2**(2): p. 86-95.
134. Atkinson, T.P. and K.B. Waites, *Mycoplasma pneumoniae Infections in Childhood*. Pediatr Infect Dis J, 2014. **33**(1): p. 92-4.
135. Shankar, E.M., et al., *Epidemiological studies on pulmonary pathogens in HIV-positive and -negative subjects with or without community-acquired pneumonia with*

- special emphasis on Mycoplasma pneumoniae*. Jpn J Infect Dis, 2007. **60**(6): p. 337-41.
136. Burillo, A. and E. Bouza, *Chlamydomphila pneumoniae*. Infect Dis Clin North Am, 2010. **24**(1): p. 61-71.
  137. Cilloniz, C., et al., *Community-acquired pneumonia related to intracellular pathogens*. Intensive Care Med, 2016. **42**(9): p. 1374-86.
  138. Brogden, K.A., J.M. Guthmiller, and C.E. Taylor, *Human polymicrobial infections*. Lancet, 2005. **365**(9455): p. 253-5.
  139. Duttweiler, L., *Pulmonary and systemic bacterial co-infections in severe RSV bronchiolitis*. Archives of Disease in Childhood, 2004. **89**(12): p. 1155-1157.
  140. Thorburn, K., et al., *High incidence of pulmonary bacterial co-infection in children with severe respiratory syncytial virus (RSV) bronchiolitis*. Thorax, 2006. **61**(7): p. 611-5.
  141. Kneyber, M.C., et al., *Concurrent bacterial infection and prolonged mechanical ventilation in infants with respiratory syncytial virus lower respiratory tract disease*. Intensive Care Med, 2005. **31**(5): p. 680-5.
  142. Honkinen, M., et al., *Viruses and bacteria in sputum samples of children with community-acquired pneumonia*. Clin Microbiol Infect, 2012. **18**(3): p. 300-7.
  143. Bogaert, D., et al., *Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis*. PLoS One, 2011. **6**(2): p. e17035.
  144. Bellinghausen, C., et al., *Exposure to common respiratory bacteria alters the airway epithelial response to subsequent viral infection*. Respir Res, 2016. **17**(1): p. 68.
  145. Bosch, A.A.T.M., et al., *Viral and Bacterial Interactions in the Upper Respiratory Tract*. PLoS Pathogens, 2013. **9**: p. e1003057.
  146. Jansen, A.G., et al., *Invasive pneumococcal and meningococcal disease: association with influenza virus and respiratory syncytial virus activity?* Epidemiol Infect, 2008. **136**(11): p. 1448-54.
  147. Morens, David M., Jeffery K. Taubenberger, and Anthony S. Fauci, *Predominant Role of Bacterial Pneumonia as a Cause of Death in Pandemic Influenza: Implications for Pandemic Influenza Preparedness*. The Journal of Infectious Diseases, 2008. **198**(7): p. 962-970.
  148. Reid, A.H. and J.K. Taubenberger, *The 1918 flu and other influenza pandemics: "over there" and back again*. Lab Invest, 1999. **79**(2): p. 95-101.
  149. Dhanoa, A., et al., *Epidemiology and clinical characteristics of hospitalized patients with pandemic influenza A (H1N1) 2009 infections: the effects of bacterial coinfection*. Virol J, 2011. **8**: p. 501.
  150. Garcia-Rodriguez, J.A. and M.J. Fresnadillo Martinez, *Dynamics of nasopharyngeal colonization by potential respiratory pathogens*. J Antimicrob Chemother, 2002. **50 Suppl S2**: p. 59-73.
  151. Ruckinger, S., et al., *Reduction in the incidence of invasive pneumococcal disease after general vaccination with 7-valent pneumococcal conjugate vaccine in Germany*. Vaccine, 2009. **27**(31): p. 4136-41.
  152. Watson, M., et al., *The association of respiratory viruses, temperature, and other climatic parameters with the incidence of invasive pneumococcal disease in Sydney, Australia*. Clin Infect Dis, 2006. **42**(2): p. 211-5.
  153. Klugman, K.P. and S.A. Madhi, *Pneumococcal vaccines and flu preparedness*. Science, 2007. **316**(5821): p. 49-50.
  154. Koon, K., et al., *Co-detection of pandemic (H1N1) 2009 virus and other respiratory pathogens*. Emerg Infect Dis, 2010. **16**(12): p. 1976-8.
  155. O'Brien, K.L., et al., *Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection*. Clin Infect Dis, 2000. **30**(5): p. 784-9.
  156. Bakaletz, L.O., *Viral-bacterial co-infections in the respiratory tract*. Curr Opin Microbiol, 2016. **35**: p. 30-35.

157. Raza, M.W., et al., *Infection with respiratory syncytial virus enhances expression of native receptors for non-pilate Neisseria meningitidis on HEp-2 cells*. FEMS Immunol Med Microbiol, 1999. **23**(2): p. 115-24.
158. Hament, J.M., et al., *Enhanced adherence of Streptococcus pneumoniae to human epithelial cells infected with respiratory syncytial virus*. Pediatr Res, 2004. **55**(6): p. 972-8.
159. Smith, C.M., et al., *Respiratory syncytial virus increases the virulence of Streptococcus pneumoniae by binding to penicillin binding protein 1a. A new paradigm in respiratory infection*. Am J Respir Crit Care Med, 2014. **190**(2): p. 196-207.
160. McCullers, Jonathan A., et al., *Influenza Enhances Susceptibility to Natural Acquisition of and Disease due to Streptococcus pneumoniae in Ferrets*. The Journal of Infectious Diseases, 2010. **202**(8): p. 1287-1295.
161. Huber, V.C., et al., *Contribution of Vaccine-Induced Immunity toward either the HA or the NA Component of Influenza Viruses Limits Secondary Bacterial Complications*. Journal of Virology, 2010. **84**(8): p. 4105-4108.
162. Hament, J.M., et al., *Direct binding of respiratory syncytial virus to pneumococci: a phenomenon that enhances both pneumococcal adherence to human epithelial cells and pneumococcal invasiveness in a murine model*. Pediatr Res, 2005. **58**(6): p. 1198-203.
163. Lynch, S.V., *Viruses and microbiome alterations*. Ann Am Thorac Soc, 2014. **11 Suppl 1**: p. S57-60.
164. McNamee, L.A. and A.G. Harmsen, *Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection*. Infect Immun, 2006. **74**(12): p. 6707-21.
165. Colamussi, M.L., et al., *Influenza A virus accelerates neutrophil apoptosis and markedly potentiates apoptotic effects of bacteria*. Blood, 1999. **93**(7): p. 2395-403.
166. Engelich, G., M. White, and K.L. Hartshorn, *Neutrophil survival is markedly reduced by incubation with influenza virus and Streptococcus pneumoniae: role of respiratory burst*. J Leukoc Biol, 2001. **69**(1): p. 50-6.
167. Hendricks, M.R., et al., *Respiratory syncytial virus infection enhances Pseudomonas aeruginosa biofilm growth through dysregulation of nutritional immunity*. Proc Natl Acad Sci U S A, 2016. **113**(6): p. 1642-7.
168. Madhi, S.A., K.P. Klugman, and G. The Vaccine Trialist, *A role for Streptococcus pneumoniae in virus-associated pneumonia*. Nature Medicine, 2004. **10**(8): p. 811-813.
169. Nguyen, D.T., et al., *Streptococcus pneumoniae Enhances Human Respiratory Syncytial Virus Infection In Vitro and In Vivo*. PLoS One, 2015. **10**(5): p. e0127098.
170. Brealey, J.C., et al., *Viral bacterial co-infection of the respiratory tract during early childhood*. FEMS Microbiol Lett, 2015. **362**(10).
171. Nguyen, D.T., et al., *The synthetic bacterial lipopeptide Pam3CSK4 modulates respiratory syncytial virus infection independent of TLR activation*. PLoS Pathog, 2010. **6**(8): p. e1001049.
172. Basnayake, T.L. and G.W. Waterer, *Rapid diagnostic tests for defining the cause of community-acquired pneumonia*. Curr Opin Infect Dis, 2015. **28**(2): p. 185-92.
173. WHO, W.H.O., *Manual for the laboratory diagnosis and virological surveillance of influenza*. 2011.
174. Musher, D.M., R. Montoya, and A. Wanahita, *Diagnostic value of microscopic examination of Gram-stained sputum and sputum cultures in patients with bacteremic pneumococcal pneumonia*. Clin Infect Dis, 2004. **39**(2): p. 165-9.

175. Hasegawa, K., et al., *Respiratory syncytial virus genomic load and disease severity among children hospitalized with bronchiolitis: multicenter cohort studies in the United States and Finland*. J Infect Dis, 2015. **211**(10): p. 1550-9.
176. Gerna, G., et al., *Correlation of viral load as determined by real-time RT-PCR and clinical characteristics of respiratory syncytial virus lower respiratory tract infections in early infancy*. J Clin Virol, 2008. **41**(1): p. 45-8.
177. Herrera, M., et al., *Comparison of serological methods with PCR-based methods for the diagnosis of community-acquired pneumonia caused by atypical bacteria*. J Negat Results Biomed, 2016. **15**: p. 3.
178. Mahony, J.B., *Detection of respiratory viruses by molecular methods*. Clin Microbiol Rev, 2008. **21**(4): p. 716-47.
179. Biomarkers Definitions Working, G., *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework*. Clin Pharmacol Ther, 2001. **69**(3): p. 89-95.
180. Turner, R.B., et al., *Pneumonia in pediatric outpatients: cause and clinical manifestations*. J Pediatr, 1987. **111**(2): p. 194-200.
181. Wubbel, L., et al., *Etiology and treatment of community-acquired pneumonia in ambulatory children*. Pediatr Infect Dis J, 1999. **18**(2): p. 98-104.
182. Virkki, R., et al., *Differentiation of bacterial and viral pneumonia in children*. Thorax, 2002. **57**(5): p. 438-41.
183. Korppi, M., *Non-specific host response markers in the differentiation between pneumococcal and viral pneumonia: what is the most accurate combination?* Pediatr Int, 2004. **46**(5): p. 545-50.
184. Flood, R.G., J. Badik, and S.C. Aronoff, *The utility of serum C-reactive protein in differentiating bacterial from nonbacterial pneumonia in children: a meta-analysis of 1230 children*. Pediatr Infect Dis J, 2008. **27**(2): p. 95-9.
185. Korppi, M. and S. Remes, *Serum procalcitonin in pneumococcal pneumonia in children*. Eur Respir J, 2001. **17**(4): p. 623-7.
186. Korppi, M., et al., *The value of clinical features in differentiating between viral, pneumococcal and atypical bacterial pneumonia in children*. Acta Paediatr, 2008. **97**(7): p. 943-7.
187. Page, A.L., et al., *Diagnostic and prognostic value of procalcitonin and C-reactive protein in malnourished children*. Pediatrics, 2014. **133**(2): p. e363-70.
188. Van den Bruel, A., et al., *Diagnostic value of laboratory tests in identifying serious infections in febrile children: systematic review*. BMJ, 2011. **342**: p. d3082.
189. Esposito, S., et al., *Procalcitonin measurements for guiding antibiotic treatment in pediatric pneumonia*. Respir Med, 2011. **105**(12): p. 1939-45.
190. Baer, G., et al., *Procalcitonin guidance to reduce antibiotic treatment of lower respiratory tract infection in children and adolescents (ProPAED): a randomized controlled trial*. PLoS One, 2013. **8**(8): p. e68419.
191. Deliberato, R.O., et al., *Clinical and economic impact of procalcitonin to shorten antimicrobial therapy in septic patients with proven bacterial infection in an intensive care setting*. Diagn Microbiol Infect Dis, 2013. **76**(3): p. 266-71.
192. Hoshina, T., et al., *The utility of biomarkers in differentiating bacterial from non-bacterial lower respiratory tract infection in hospitalized children: difference of the diagnostic performance between acute pneumonia and bronchitis*. J Infect Chemother, 2014. **20**(10): p. 616-20.
193. Toikka, P., et al., *Serum procalcitonin, C-reactive protein and interleukin-6 for distinguishing bacterial and viral pneumonia in children*. Pediatr Infect Dis J, 2000. **19**(7): p. 598-602.
194. Schuetz, P., et al., *Procalcitonin to initiate or discontinue antibiotics in acute respiratory tract infections*. Evid Based Child Health, 2013. **8**(4): p. 1297-371.

195. Torres, A., et al., *Biomarkers and community-acquired pneumonia: tailoring management with biological data*. *Semin Respir Crit Care Med*, 2012. **33**(3): p. 266-71.
196. Don, M., et al., *Efficacy of serum procalcitonin in evaluating severity of community-acquired pneumonia in childhood*. *Scand J Infect Dis*, 2007. **39**(2): p. 129-37.
197. Zhu, F., et al., *Clinical significance of serum procalcitonin level monitoring on early diagnosis of severe pneumonia on children*. *Eur Rev Med Pharmacol Sci*, 2015. **19**(22): p. 4300-3.
198. Lee, J.Y., et al., *Clinical significance of serum procalcitonin in patients with community-acquired lobar pneumonia*. *Korean J Lab Med*, 2010. **30**(4): p. 406-13.
199. Zar, H.J., S. Andronikou, and M.P. Nicol, *Advances in the diagnosis of pneumonia in children*. *BMJ*, 2017. **358**: p. j2739.
200. Giulia, B., et al., *Procalcitonin and community-acquired pneumonia (CAP) in children*. *Clin Chim Acta*, 2015. **451**(Pt B): p. 215-8.
201. Elemraid, M.A., et al., *Utility of inflammatory markers in predicting the aetiology of pneumonia in children*. *Diagn Microbiol Infect Dis*, 2014. **79**(4): p. 458-62.
202. Cheng, C.W., et al., *New markers in pneumonia*. *Clin Chim Acta*, 2013. **419**: p. 19-25.
203. Lippi, G., T. Meschi, and G. Cervellin, *Inflammatory biomarkers for the diagnosis, monitoring and follow-up of community-acquired pneumonia: clinical evidence and perspectives*. *Eur J Intern Med*, 2011. **22**(5): p. 460-5.
204. Muller, B., et al., *Diagnostic and prognostic accuracy of clinical and laboratory parameters in community-acquired pneumonia*. *BMC Infect Dis*, 2007. **7**: p. 10.
205. Christ-Crain, M. and S.M. Opal, *Clinical review: the role of biomarkers in the diagnosis and management of community-acquired pneumonia*. *Crit Care*, 2010. **14**(1): p. 203.
206. Principi, N. and S. Esposito, *Biomarkers in Pediatric Community-Acquired Pneumonia*. *Int J Mol Sci*, 2017. **18**(2).
207. Martinez, I., et al., *Apoptosis, Toll-like, RIG-I-like and NOD-like Receptors Are Pathways Jointly Induced by Diverse Respiratory Bacterial and Viral Pathogens*. *Front Microbiol*, 2017. **8**: p. 276.
208. Hedlund, M., J.L. Larson, and F. Fang, *Antiviral strategies for pandemic and seasonal influenza*. *Viruses*, 2010. **2**(8): p. 1766-81.
209. Loregian, A., et al., *Antiviral strategies against influenza virus: towards new therapeutic approaches*. *Cell Mol Life Sci*, 2014. **71**(19): p. 3659-83.
210. Lackenby, A., C.I. Thompson, and J. Democratis, *The potential impact of neuraminidase inhibitor resistant influenza*. *Curr Opin Infect Dis*, 2008. **21**(6): p. 626-38.
211. Thorlund, K., et al., *Systematic review of influenza resistance to the neuraminidase inhibitors*. *BMC Infect Dis*, 2011. **11**: p. 134.
212. Yen, H.L., *Current and novel antiviral strategies for influenza infection*. *Curr Opin Virol*, 2016. **18**: p. 126-34.
213. Moss, R.B., et al., *A phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection*. *J Infect Dis*, 2012. **206**(12): p. 1844-51.
214. Hedlund, M., et al., *Sialidase-based anti-influenza virus therapy protects against secondary pneumococcal infection*. *J Infect Dis*, 2010. **201**(7): p. 1007-15.
215. Glatthaar-Saalmuller, B., K.H. Mair, and A. Saalmuller, *Antiviral activity of aspirin against RNA viruses of the respiratory tract-an in vitro study*. *Influenza Other Respir Viruses*, 2017. **11**(1): p. 85-92.
216. Koszalka, P., D. Tilmanis, and A.C. Hurt, *Influenza antivirals currently in late-phase clinical trial*. *Influenza Other Respir Viruses*, 2017. **11**(3): p. 240-246.
217. Ludwig, S., *Disruption of virus-host cell interactions and cell signaling pathways as an anti-viral approach against influenza virus infections*. *Biol Chem*, 2011. **392**(10): p. 837-47.

218. Brass, A.L., et al., *The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus*. *Cell*, 2009. **139**(7): p. 1243-54.
219. Sidwell, R.W. and D.L. Barnard, *Respiratory syncytial virus infections: recent prospects for control*. *Antiviral Res*, 2006. **71**(2-3): p. 379-90.
220. Chidgey, S.M. and K.J. Broadley, *Respiratory syncytial virus infections: characteristics and treatment*. *J Pharm Pharmacol*, 2005. **57**(11): p. 1371-81.
221. Gonzalez, P.A., et al., *Respiratory syncytial virus infection and immunity*. *Rev Med Virol*, 2012. **22**(4): p. 230-44.
222. Ventre, K. and A.G. Randolph, *Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children*. *Cochrane Database Syst Rev*, 2007(1): p. CD000181.
223. DeVincenzo, J.P., et al., *Oral GS-5806 activity in a respiratory syncytial virus challenge study*. *N Engl J Med*, 2014. **371**(8): p. 711-22.
224. Mackman, R.L., et al., *Discovery of an oral respiratory syncytial virus (RSV) fusion inhibitor (GS-5806) and clinical proof of concept in a human RSV challenge study*. *J Med Chem*, 2015. **58**(4): p. 1630-43.
225. DeVincenzo, J.P., M.W. McClure, and J. Fry, *ALS-008176 for Respiratory Syncytial Virus Infection*. *N Engl J Med*, 2016. **374**(14): p. 1391-2.
226. Korell, J., et al., *A human challenge model for respiratory syncytial virus kinetics, the pharmacological effect of a novel fusion inhibitor, and the modelling of symptoms scores*. *Eur J Pharm Sci*, 2017.
227. Coates, M., et al., *Preclinical Characterization of PC786, an Inhaled Small-Molecule Respiratory Syncytial Virus L Protein Polymerase Inhibitor*. *Antimicrob Agents Chemother*, 2017. **61**(9).
228. Challa, S., et al., *Mechanism of action for respiratory syncytial virus inhibitor RSV604*. *Antimicrob Agents Chemother*, 2015. **59**(2): p. 1080-7.
229. Williams, D.J., et al., *Antibiotic Choice for Children Hospitalized With Pneumonia and Adherence to National Guidelines*. *Pediatrics*, 2015. **136**(1): p. 44-52.
230. Zaman, S.B., et al., *A Review on Antibiotic Resistance: Alarm Bells are Ringing*. *Cureus*, 2017. **9**(6): p. e1403.
231. Bradley, J.S., et al., *The management of community-acquired pneumonia in infants and children older than 3 months of age: clinical practice guidelines by the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America*. *Clin Infect Dis*, 2011. **53**(7): p. e25-76.
232. Ambroggio, L., et al., *Comparative Effectiveness of Beta-lactam Versus Macrolide Monotherapy in Children with Pneumonia Diagnosed in the Outpatient Setting*. *Pediatr Infect Dis J*, 2015. **34**(8): p. 839-42.
233. Houser, K. and K. Subbarao, *Influenza vaccines: challenges and solutions*. *Cell Host Microbe*, 2015. **17**(3): p. 295-300.
234. WHO, W.H.O. *Influenza vaccine viruses and reagents*. 2017; Available from: <http://www.who.int/influenza/vaccines/virus/en/>.
235. Collins, P.L. and B.R. Murphy, *New generation live vaccines against human respiratory syncytial virus designed by reverse genetics*. *Proc Am Thorac Soc*, 2005. **2**(2): p. 166-73.
236. Elliott, M.B., et al., *Recombinant respiratory syncytial viruses lacking the C-terminal third of the attachment (G) protein are immunogenic and attenuated in vivo and in vitro*. *J Virol*, 2004. **78**(11): p. 5773-83.
237. Wright, P.F., et al., *Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy*. *J Infect Dis*, 2000. **182**(5): p. 1331-42.
238. Collins, P.L. and J.A. Melero, *Progress in understanding and controlling respiratory syncytial virus: still crazy after all these years*. *Virus Res*, 2011. **162**(1-2): p. 80-99.

239. Pliaka, V., Z. Kyriakopoulou, and P. Markoulatos, *Risks associated with the use of live-attenuated vaccine poliovirus strains and the strategies for control and eradication of paralytic poliomyelitis*. *Expert Rev Vaccines*, 2012. **11**(5): p. 609-28.
240. Etchart, N., et al., *Intranasal immunisation with inactivated RSV and bacterial adjuvants induces mucosal protection and abrogates eosinophilia upon challenge*. *Eur J Immunol*, 2006. **36**(5): p. 1136-44.
241. Voges, B., et al., *Recombinant Sendai virus induces T cell immunity against respiratory syncytial virus that is protective in the absence of antibodies*. *Cell Immunol*, 2007. **247**(2): p. 85-94.
242. Hancock, G.E., et al., *Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus*. *J Virol*, 1996. **70**(11): p. 7783-91.
243. Huang, Y., et al., *Murine host responses to respiratory syncytial virus (RSV) following intranasal administration of a Protollin-adjuvanted, epitope-enhanced recombinant G protein vaccine*. *J Clin Virol*, 2009. **44**(4): p. 287-91.
244. Bukreyev, A., et al., *Effect of coexpression of interleukin-2 by recombinant respiratory syncytial virus on virus replication, immunogenicity, and production of other cytokines*. *J Virol*, 2000. **74**(15): p. 7151-7.
245. Crowe, J.E., Jr., et al., *A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV*. *Vaccine*, 1993. **11**(14): p. 1395-404.
246. Bueno, S.M., et al., *Protective T cell immunity against respiratory syncytial virus is efficiently induced by recombinant BCG*. *Proc Natl Acad Sci U S A*, 2008. **105**(52): p. 20822-7.
247. Xie, C., et al., *Oral respiratory syncytial virus (RSV) DNA vaccine expressing RSV F protein delivered by attenuated Salmonella typhimurium*. *Hum Gene Ther*, 2007. **18**(8): p. 746-52.
248. Bueno, S.M., P.A. Gonzalez, and A.M. Kalergis, *Use of genetically modified bacteria to modulate adaptive immunity*. *Curr Gene Ther*, 2009. **9**(3): p. 171-84.
249. Cautivo, K.M., et al., *Efficient lung recruitment of respiratory syncytial virus-specific Th1 cells induced by recombinant bacillus Calmette-Guerin promotes virus clearance and protects from infection*. *J Immunol*, 2010. **185**(12): p. 7633-45.
250. Carrat, F. and A. Flahault, *Influenza vaccine: the challenge of antigenic drift*. *Vaccine*, 2007. **25**(39-40): p. 6852-62.
251. Hampson, A.W. and J. Wood, *Recent developments in seasonal and pandemic influenza vaccines*. *Influenza Other Respir Viruses*, 2008. **2**(6): p. 191-2.
252. Kapikian, A.Z., et al., *An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine*. *Am J Epidemiol*, 1969. **89**(4): p. 405-21.
253. Graham, B.S., *Vaccine development for respiratory syncytial virus*. *Curr Opin Virol*, 2017. **23**: p. 107-112.
254. Ausar, S.F., et al., *Analysis of the thermal and pH stability of human respiratory syncytial virus*. *Mol Pharm*, 2005. **2**(6): p. 491-9.
255. Caballero, M.T., F.P. Polack, and R.T. Stein, *Viral bronchiolitis in young infants: new perspectives for management and treatment*. *J Pediatr (Rio J)*, 2017.
256. Goldblatt, D., *Conjugate vaccines*. *Clin Exp Immunol*, 2000. **119**(1): p. 1-3.
257. Song, J.Y., et al., *Pneumococcal vaccine and opsonic pneumococcal antibody*. *J Infect Chemother*, 2013. **19**(3): p. 412-25.
258. Snapper, C.M., et al., *Bacterial lipoproteins may substitute for cytokines in the humoral immune response to T cell-independent type II antigens*. *J Immunol*, 1995. **155**(12): p. 5582-9.

259. WHO, W.H.O. *Pneumococcal conjugate vaccines*. 2012; Available from: <http://www.who.int/wer/2012/wer8714.pdf?ua=1>.
260. Simonsen, L., et al., *Impact of pneumococcal conjugate vaccination of infants on pneumonia and influenza hospitalization and mortality in all age groups in the United States*. *MBio*, 2011. **2**(1): p. e00309-10.
261. Vestheim, D.F., et al., *Impact of a pneumococcal conjugate vaccination program on carriage among children in Norway*. *Clin Vaccine Immunol*, 2010. **17**(3): p. 325-34.
262. Clarke, S.C., et al., *Pneumococci causing invasive disease in children prior to the introduction of pneumococcal conjugate vaccine in Scotland*. *J Med Microbiol*, 2006. **55**(Pt 8): p. 1079-84.
263. Rodenburg, G.D., et al., *Effects of pneumococcal conjugate vaccine 2 years after its introduction, the Netherlands*. *Emerg Infect Dis*, 2010. **16**(5): p. 816-23.
264. Guevara, M., et al., *Changing epidemiology of invasive pneumococcal disease following increased coverage with the heptavalent conjugate vaccine in Navarre, Spain*. *Clin Microbiol Infect*, 2009. **15**(11): p. 1013-9.
265. Brook, I., P.A. Foote, and J.N. Hausfeld, *Frequency of recovery of pathogens causing acute maxillary sinusitis in adults before and after introduction of vaccination of children with the 7-valent pneumococcal vaccine*. *J Med Microbiol*, 2006. **55**(Pt 7): p. 943-6.
266. Horacio, A.N., et al., *The majority of adult pneumococcal invasive infections in Portugal are still potentially vaccine preventable in spite of significant declines of serotypes 1 and 5*. *PLoS One*, 2013. **8**(9): p. e73704.
267. Spijkerman, J., et al., *Carriage of Streptococcus pneumoniae 3 years after start of vaccination program, the Netherlands*. *Emerg Infect Dis*, 2011. **17**(4): p. 584-91.
268. Ben-Shimol, S., et al., *Early impact of sequential introduction of 7-valent and 13-valent pneumococcal conjugate vaccine on IPD in Israeli children <5 years: an active prospective nationwide surveillance*. *Vaccine*, 2014. **32**(27): p. 3452-9.
269. Prymula, R., et al., *Impact of the 10-valent pneumococcal non-typeable Haemophilus influenzae Protein D conjugate vaccine (PHiD-CV) on bacterial nasopharyngeal carriage*. *Vaccine*, 2011. **29**(10): p. 1959-67.
270. Kim, G.L., et al., *Pneumococcal pep27 mutant immunization stimulates cytokine secretion and confers long-term immunity with a wide range of protection, including against non-typeable strains*. *Vaccine*, 2016. **34**(51): p. 6481-6492.
271. Pittet, L.F. and K.M. Posfay-Barbe, *Pneumococcal vaccines for children: a global public health priority*. *Clin Microbiol Infect*, 2012. **18 Suppl 5**: p. 25-36.
272. Minor, P.D., *Live attenuated vaccines: Historical successes and current challenges*. *Virology*, 2015. **479-480**: p. 379-92.
273. Wu, Q., et al., *Betulin protects mice from bacterial pneumonia and acute lung injury*. *Microb Pathog*, 2014. **75**: p. 21-8.
274. Choi, S.Y., et al., *Inactivated pep27 mutant as an effective mucosal vaccine against a secondary lethal pneumococcal challenge in mice*. *Clin Exp Vaccine Res*, 2013. **2**(1): p. 58-65.
275. Sun, Y. and C.B. Lopez, *The innate immune response to RSV: Advances in our understanding of critical viral and host factors*. *Vaccine*, 2017. **35**(3): p. 481-488.
276. Albiger, B., et al., *Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors*. *Journal of Internal Medicine*, 2007. **261**: p. 511-528.
277. Kumar, H., T. Kawai, and S. Akira, *Toll-like receptors and innate immunity*. *Biochem Biophys Res Commun*, 2009. **388**(4): p. 621-5.
278. Leulier, F. and B. Lemaitre, *Toll-like receptors--taking an evolutionary approach*. *Nat Rev Genet*, 2008. **9**(3): p. 165-78.
279. Kumar, N., et al., *Receptor tyrosine kinase inhibitors block multiple steps of influenza a virus replication*. *J Virol*, 2011. **85**(6): p. 2818-27.

280. van de Sandt, C.E., J.H. Kreijtz, and G.F. Rimmelzwaan, *Evasion of influenza A viruses from innate and adaptive immune responses*. *Viruses*, 2012. **4**(9): p. 1438-76.
281. Kreijtz, J.H., R.A. Fouchier, and G.F. Rimmelzwaan, *Immune responses to influenza virus infection*. *Virus Res*, 2011. **162**(1-2): p. 19-30.
282. Kim, T.H. and H.K. Lee, *Innate immune recognition of respiratory syncytial virus infection*. *BMB Rep*, 2014. **47**(4): p. 184-91.
283. Iwasaki, A. and P.S. Pillai, *Innate immunity to influenza virus infection*. *Nature Reviews Immunology*, 2014. **14**: p. 315-328.
284. Okabayashi, T., et al., *Type-III interferon, not type-I, is the predominant interferon induced by respiratory viruses in nasal epithelial cells*. *Virus Res*, 2011. **160**(1-2): p. 360-6.
285. Schwarze, J. and K.J. Mackenzie, *Novel insights into immune and inflammatory responses to respiratory viruses*. *Thorax*, 2013. **68**(1): p. 108-10.
286. Le Goffic, R., et al., *Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells*. *J Immunol*, 2007. **178**(6): p. 3368-72.
287. Guillot, L., et al., *Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus*. *J Biol Chem*, 2005. **280**(7): p. 5571-80.
288. Le Goffic, R., et al., *Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia*. *PLoS Pathog*, 2006. **2**(6): p. e53.
289. Branger, J., et al., *Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice*. *Infect Immun*, 2004. **72**(2): p. 788-94.
290. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5*. *Nature*, 2001. **410**(6832): p. 1099-103.
291. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7*. *Proc Natl Acad Sci U S A*, 2004. **101**(15): p. 5598-603.
292. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA*. *Science*, 2004. **303**(5663): p. 1529-31.
293. Pang, I.K. and A. Iwasaki, *Control of antiviral immunity by pattern recognition and the microbiome*. *Immunol Rev*, 2012. **245**(1): p. 209-26.
294. Albiger, B., et al., *Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice*. *Cell Microbiol*, 2005. **7**(11): p. 1603-15.
295. Takeuchi, O., et al., *Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins*. *J Immunol*, 2002. **169**(1): p. 10-4.
296. Mayer, A.K., et al., *Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells*. *J Immunol*, 2007. **178**(5): p. 3134-42.
297. Lester, S.N. and K. Li, *Toll-like receptors in antiviral innate immunity*. *J Mol Biol*, 2014. **426**(6): p. 1246-64.
298. Ioannidis, I., et al., *Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells*. *J Virol*, 2013. **87**(6): p. 3261-70.
299. Medzhitov, R., *Toll-like receptors and innate immunity*. *Nat Rev Immunol*, 2001. **1**(2): p. 135-45.
300. Man, S.M. and T.D. Kanneganti, *Regulation of inflammasome activation*. *Immunol Rev*, 2015. **265**(1): p. 6-21.
301. Sabbah, A., et al., *Activation of innate immune antiviral responses by Nod2*. *Nat Immunol*, 2009. **10**(10): p. 1073-80.
302. Broz, P., *Inflammasomes in Host Defense and Autoimmunity*. *Chimia (Aarau)*, 2016. **70**(12): p. 853-855.
303. Guarda, G., et al., *Differential expression of NLRP3 among hematopoietic cells*. *J Immunol*, 2011. **186**(4): p. 2529-34.

304. Segovia, J., et al., *TLR2/MyD88/NF-kappaB pathway, reactive oxygen species, potassium efflux activates NLRP3/ASC inflammasome during respiratory syncytial virus infection*. PLoS One, 2012. **7**(1): p. e29695.
305. Triantafyllou, K., et al., *Human respiratory syncytial virus viroporin SH: a viral recognition pathway used by the host to signal inflammasome activation*. Thorax, 2013. **68**(1): p. 66-75.
306. Ichinohe, T., I.K. Pang, and A. Iwasaki, *Influenza virus activates inflammasomes via its intracellular M2 ion channel*. Nat Immunol, 2010. **11**(5): p. 404-10.
307. Thomas, P.G., et al., *The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1*. Immunity, 2009. **30**(4): p. 566-75.
308. Mizgerd, J.P., *Acute lower respiratory tract infection*. N Engl J Med, 2008. **358**(7): p. 716-27.
309. Braciale, T.J., J. Sun, and T.S. Kim, *Regulating the adaptive immune response to respiratory virus infection*. Nat Rev Immunol, 2012. **12**(4): p. 295-305.
310. McNamara, P.S., et al., *Respiratory syncytial virus infection of airway epithelial cells, in vivo and in vitro, supports pulmonary antibody responses by inducing expression of the B cell differentiation factor BAFF*. Thorax, 2013. **68**(1): p. 76-81.
311. Yamaguchi, M., et al., *High frequency of repeated infections due to emerging genotypes of human respiratory syncytial viruses among children during eight successive epidemic seasons in Japan*. J Clin Microbiol, 2011. **49**(3): p. 1034-40.
312. Chang, J. and T.J. Braciale, *Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract*. Nat Med, 2002. **8**(1): p. 54-60.
313. DiNapoli, J.M., et al., *Impairment of the CD8+ T cell response in lungs following infection with human respiratory syncytial virus is specific to the anatomical site rather than the virus, antigen, or route of infection*. Virol J, 2008. **5**: p. 105.
314. Vallbracht, S., H. Unsold, and S. Ehl, *Functional impairment of cytotoxic T cells in the lung airways following respiratory virus infections*. Eur J Immunol, 2006. **36**(6): p. 1434-42.
315. Tavares, L.P., M.M. Teixeira, and C.C. Garcia, *The inflammatory response triggered by Influenza virus: a two edged sword*. Inflamm Res, 2017. **66**(4): p. 283-302.
316. Lowe, J.M., et al., *p53 and NF-kappaB coregulate proinflammatory gene responses in human macrophages*. Cancer Res, 2014. **74**(8): p. 2182-92.
317. Yoneyama, M., et al., *Viral RNA detection by RIG-I-like receptors*. Curr Opin Immunol, 2015. **32**: p. 48-53.
318. Chan, Y.K. and M.U. Gack, *Viral evasion of intracellular DNA and RNA sensing*. Nat Rev Microbiol, 2016. **14**(6): p. 360-73.
319. Durand, J.K. and A.S. Baldwin, *Targeting IKK and NF-kappaB for Therapy*. Adv Protein Chem Struct Biol, 2017. **107**: p. 77-115.
320. Kawai, T. and S. Akira, *Signaling to NF-kappaB by Toll-like receptors*. Trends in Molecular Medicine, 2007. **13**(11): p. 460-469.
321. Panday, A., et al., *Transcription Factor NF-kappaB: An Update on Intervention Strategies*. Arch Immunol Ther Exp (Warsz), 2016. **64**(6): p. 463-483.
322. Nakamura, S., K.M. Davis, and J.N. Weiser, *Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice*. Journal of Clinical Investigation, 2011. **121**(9): p. 3657-3665.
323. Short, K.R., et al., *Interactions between Streptococcus pneumoniae and influenza virus: a mutually beneficial relationship?* Future Microbiology, 2012. **7**: p. 609-624.
324. Parker, D., et al., *Streptococcus pneumoniae DNA initiates type I interferon signaling in the respiratory tract*. MBio, 2011. **2**(3): p. e00016-11.

325. Joyce, E.A., S.J. Popper, and S. Falkow, *Streptococcus pneumoniae nasopharyngeal colonization induces type I interferons and interferon-induced gene expression*. BMC Genomics, 2009. **10**: p. 404.
326. Barik, S., *Respiratory syncytial virus mechanisms to interfere with type 1 interferons*. Curr Top Microbiol Immunol, 2013. **372**: p. 173-91.
327. Teng, M.N. and P.L. Collins, *Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein*. J Virol, 1999. **73**(1): p. 466-73.
328. Jin, H., et al., *Recombinant respiratory syncytial viruses with deletions in the NS1, NS2, SH, and M2-2 genes are attenuated in vitro and in vivo*. Virology, 2000. **273**(1): p. 210-8.
329. Spann, K.M., et al., *Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]*. J Virol, 2004. **78**(8): p. 4363-9.
330. Swedan, S., A. Musiyenko, and S. Barik, *Respiratory syncytial virus nonstructural proteins decrease levels of multiple members of the cellular interferon pathways*. J Virol, 2009. **83**(19): p. 9682-93.
331. Goswami, R., et al., *Viral degradasome hijacks mitochondria to suppress innate immunity*. Cell Res, 2013. **23**(8): p. 1025-42.
332. Elliott, J., et al., *Respiratory syncytial virus NS1 protein degrades STAT2 by using the Elongin-Cullin E3 ligase*. J Virol, 2007. **81**(7): p. 3428-36.
333. Boyapalle, S., et al., *Respiratory syncytial virus NS1 protein colocalizes with mitochondrial antiviral signaling protein MAVS following infection*. PLoS One, 2012. **7**(2): p. e29386.
334. Honda, K., A. Takaoka, and T. Taniguchi, *Type I Inteferon Gene Induction by the Interferon Regulatory Factor Family of Transcription Factors*. Immunity, 2006. **25**(3): p. 349-360.
335. Osterlund, P.I., et al., *IFN Regulatory Factor Family Members Differentially Regulate the Expression of Type III IFN (IFN- $\gamma$ ) Genes*. The Journal of Immunology, 2007. **179**(6): p. 3434-3442.
336. Hillyer, P., et al., *Respiratory syncytial virus infection induces a subset of types I and III interferons in human dendritic cells*. Virology, 2017. **504**: p. 63-72.
337. Schoggins, J.W., *Interferon-stimulated genes: roles in viral pathogenesis*. Curr Opin Virol, 2014. **6**: p. 40-6.
338. Schoggins, J.W., et al., *A diverse range of gene products are effectors of the type I interferon antiviral response*. Nature, 2011. **472**(7344): p. 481-5.
339. Pavlovic, J., et al., *Enhanced virus resistance of transgenic mice expressing the human MxA protein*. J Virol, 1995. **69**(7): p. 4506-10.
340. Turan, K., et al., *Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome*. Nucleic Acids Res, 2004. **32**(2): p. 643-52.
341. Aylon, Y. and M. Oren, *The Paradox of p53: What, How, and Why?* Cold Spring Harb Perspect Med, 2016. **6**(10).
342. Lane, D.P. and L.V. Crawford, *T antigen is bound to a host protein in SV40-transformed cells*. Nature, 1979. **278**(5701): p. 261-3.
343. Linzer, D.I. and A.J. Levine, *Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells*. Cell, 1979. **17**(1): p. 43-52.
344. Lane, D.P., *Cancer. p53, guardian of the genome*. Nature, 1992. **358**(6381): p. 15-6.
345. Munoz-Fontela, C., et al., *Emerging roles of p53 and other tumour-suppressor genes in immune regulation*. Nat Rev Immunol, 2016. **16**(12): p. 741-750.
346. Maiuri, M.C., et al., *Autophagy regulation by p53*. Curr Opin Cell Biol, 2010. **22**(2): p. 181-5.

347. Qian, Y. and X. Chen, *Senescence regulation by the p53 protein family*. Methods Mol Biol, 2013. **965**: p. 37-61.
348. Kasthuber, E.R. and S.W. Lowe, *Putting p53 in Context*. Cell, 2017. **170**(6): p. 1062-1078.
349. Crawford, L.V., et al., *Detection of a common feature in several human tumor cell lines--a 53,000-dalton protein*. Proc Natl Acad Sci U S A, 1981. **78**(1): p. 41-5.
350. Hainaut, P., et al., *Modulation by copper of p53 conformation and sequence-specific DNA binding: role for Cu(II)/Cu(I) redox mechanism*. Oncogene, 1995. **10**(1): p. 27-32.
351. Mihara, M., et al., *p53 has a direct apoptogenic role at the mitochondria*. Mol Cell, 2003. **11**(3): p. 577-90.
352. Arrowsmith, C.H., *Structure and function in the p53 family*. Cell Death Differ, 1999. **6**(12): p. 1169-73.
353. Yamanishi, Y., et al., *Regulation of joint destruction and inflammation by p53 in collagen-induced arthritis*. Am J Pathol, 2002. **160**(1): p. 123-30.
354. Green, D.R. and G. Kroemer, *Cytoplasmic functions of the tumour suppressor p53*. Nature, 2009. **458**(7242): p. 1127-30.
355. Levine, A.J., *p53, the cellular gatekeeper for growth and division*. Cell, 1997. **88**(3): p. 323-31.
356. Meek, D.W., *Tumour suppression by p53: a role for the DNA damage response?* Nat Rev Cancer, 2009. **9**(10): p. 714-23.
357. Muller, P.A. and K.H. Vousden, *p53 mutations in cancer*. Nat Cell Biol, 2013. **15**(1): p. 2-8.
358. Juntila, M.R. and G.I. Evan, *p53--a Jack of all trades but master of none*. Nat Rev Cancer, 2009. **9**(11): p. 821-9.
359. Berkers, C.R., et al., *Metabolic regulation by p53 family members*. Cell Metab, 2013. **18**(5): p. 617-33.
360. Levine, A.J., et al., *The p53 family: guardians of maternal reproduction*. Nat Rev Mol Cell Biol, 2011. **12**(4): p. 259-65.
361. Vousden, K.H. and D.P. Lane, *p53 in health and disease*. Nat Rev Mol Cell Biol, 2007. **8**(4): p. 275-83.
362. Williams, A.B. and B. Schumacher, *p53 in the DNA-Damage-Repair Process*. Cold Spring Harb Perspect Med, 2016. **6**(5).
363. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network*. Nature, 2000. **408**(6810): p. 307-10.
364. Levine, A.J., W. Hu, and Z. Feng, *The P53 pathway: what questions remain to be explored?* Cell Death Differ, 2006. **13**(6): p. 1027-36.
365. Luo, Q., et al., *Dynamics of p53: A Master Decider of Cell Fate*. Genes (Basel), 2017. **8**(2).
366. Hock, A.K. and K.H. Vousden, *The role of ubiquitin modification in the regulation of p53*. Biochim Biophys Acta, 2014. **1843**(1): p. 137-49.
367. Momand, J., et al., *The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation*. Cell, 1992. **69**(7): p. 1237-45.
368. Haupt, Y., et al., *Mdm2 promotes the rapid degradation of p53*. Nature, 1997. **387**(6630): p. 296-9.
369. Honda, R., H. Tanaka, and H. Yasuda, *Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53*. FEBS Lett, 1997. **420**(1): p. 25-7.
370. Kubbutat, M.H., S.N. Jones, and K.H. Vousden, *Regulation of p53 stability by Mdm2*. Nature, 1997. **387**(6630): p. 299-303.
371. Barak, Y., et al., *mdm2 expression is induced by wild type p53 activity*. EMBO J, 1993. **12**(2): p. 461-8.
372. Meng, X., et al., *MDM2-p53 pathway in hepatocellular carcinoma*. Cancer Res, 2014. **74**(24): p. 7161-7.

373. Pant, V. and G. Lozano, *Limiting the power of p53 through the ubiquitin proteasome pathway*. *Genes Dev*, 2014. **28**(16): p. 1739-51.
374. Yamada, T., et al., *p28, a first in class peptide inhibitor of cop1 binding to p53*. *Br J Cancer*, 2013. **108**(12): p. 2495-504.
375. Harris, S.L. and A.J. Levine, *The p53 pathway: positive and negative feedback loops*. *Oncogene*, 2005. **24**(17): p. 2899-908.
376. Martins, C.P., L. Brown-Swigart, and G.I. Evan, *Modeling the therapeutic efficacy of p53 restoration in tumors*. *Cell*, 2006. **127**(7): p. 1323-34.
377. Vassilev, L.T., et al., *In vivo activation of the p53 pathway by small-molecule antagonists of MDM2*. *Science*, 2004. **303**(5659): p. 844-8.
378. Hasegawa, H., et al., *Activation of p53 by Nutlin-3a, an antagonist of MDM2, induces apoptosis and cellular senescence in adult T-cell leukemia cells*. *Leukemia*, 2009. **23**(11): p. 2090-101.
379. Issaeva, N., et al., *Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors*. *Nat Med*, 2004. **10**(12): p. 1321-8.
380. Vassilev, L.T., *MDM2 inhibitors for cancer therapy*. *Trends Mol Med*, 2007. **13**(1): p. 23-31.
381. Sakaguchi, K., et al., *DNA damage activates p53 through a phosphorylation-acetylation cascade*. *Genes Dev*, 1998. **12**(18): p. 2831-41.
382. Meek, D.W. and T.R. Hupp, *The regulation of MDM2 by multisite phosphorylation--opportunities for molecular-based intervention to target tumours?* *Semin Cancer Biol*, 2010. **20**(1): p. 19-28.
383. Waning, D.L., et al., *Controlling the Mdm2-Mdmx-p53 Circuit*. *Pharmaceuticals (Basel)*, 2010. **3**(5): p. 1576-1593.
384. Wade, M., Y.V. Wang, and G.M. Wahl, *The p53 orchestra: Mdm2 and Mdmx set the tone*. *Trends Cell Biol*, 2010. **20**(5): p. 299-309.
385. Chen, J., *The Roles of MDM2 and MDMX Phosphorylation in Stress Signaling to p53*. *Genes Cancer*, 2012. **3**(3-4): p. 274-82.
386. Ivanov, G.S., et al., *Methylation-acetylation interplay activates p53 in response to DNA damage*. *Mol Cell Biol*, 2007. **27**(19): p. 6756-69.
387. Huang, J., et al., *Repression of p53 activity by Smyd2-mediated methylation*. *Nature*, 2006. **444**(7119): p. 629-32.
388. Shi, X., et al., *Modulation of p53 function by SET8-mediated methylation at lysine 382*. *Mol Cell*, 2007. **27**(4): p. 636-46.
389. Cui, G., et al., *PHF20 is an effector protein of p53 double lysine methylation that stabilizes and activates p53*. *Nat Struct Mol Biol*, 2012. **19**(9): p. 916-24.
390. Li, M., et al., *Acetylation of p53 inhibits its ubiquitination by Mdm2*. *J Biol Chem*, 2002. **277**(52): p. 50607-11.
391. Yang, W.H., et al., *Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability*. *Nat Cell Biol*, 2006. **8**(10): p. 1074-83.
392. Bourdon, J.C., et al., *p53 isoforms can regulate p53 transcriptional activity*. *Genes Dev*, 2005. **19**(18): p. 2122-37.
393. Marcel, V., et al., *Biological functions of p53 isoforms through evolution: lessons from animal and cellular models*. *Cell Death Differ*, 2011. **18**(12): p. 1815-24.
394. Bourdon, J.C., *p53 Family isoforms*. *Curr Pharm Biotechnol*, 2007. **8**(6): p. 332-6.
395. Jorruiz, S.M. and J.C. Bourdon, *p53 Isoforms: Key Regulators of the Cell Fate Decision*. *Cold Spring Harb Perspect Med*, 2016. **6**(8).
396. Terrier, O., et al., *Influenza A viruses control expression of proviral human p53 isoforms p53beta and Delta133p53alpha*. *J Virol*, 2012. **86**(16): p. 8452-60.
397. Bourdon, J.C., et al., *p53 mutant breast cancer patients expressing p53gamma have as good a prognosis as wild-type p53 breast cancer patients*. *Breast Cancer Res*, 2011. **13**(1): p. R7.

398. Hafsi, H., et al., *Effects of Delta40p53, an isoform of p53 lacking the N-terminus, on transactivation capacity of the tumor suppressor protein p53*. BMC Cancer, 2013. **13**: p. 134.
399. Surget, S., M.P. Khoury, and J.C. Bourdon, *Uncovering the role of p53 splice variants in human malignancy: a clinical perspective*. Onco Targets Ther, 2013. **7**: p. 57-68.
400. Senturk, S., et al., *p53Psi is a transcriptionally inactive p53 isoform able to reprogram cells toward a metastatic-like state*. Proc Natl Acad Sci U S A, 2014. **111**(32): p. E3287-96.
401. Fujita, K., et al., *p53 isoforms Delta133p53 and p53beta are endogenous regulators of replicative cellular senescence*. Nat Cell Biol, 2009. **11**(9): p. 1135-42.
402. Marcel, V., et al., *Modulation of p53beta and p53gamma expression by regulating the alternative splicing of TP53 gene modifies cellular response*. Cell Death Differ, 2014. **21**(9): p. 1377-87.
403. Polyak, K., et al., *Genetic determinants of p53-induced apoptosis and growth arrest*. Genes Dev, 1996. **10**(15): p. 1945-52.
404. Janicke, R.U., D. Sohn, and K. Schulze-Osthoff, *The dark side of a tumor suppressor: anti-apoptotic p53*. Cell Death Differ, 2008. **15**(6): p. 959-76.
405. Clarke, A.R., et al., *Thymocyte apoptosis induced by p53-dependent and independent pathways*. Nature, 1993. **362**(6423): p. 849-52.
406. Lowe, S.W. and H.E. Ruley, *Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis*. Genes Dev, 1993. **7**(4): p. 535-45.
407. Rouault, J.P., et al., *Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway*. Nat Genet, 1996. **14**(4): p. 482-6.
408. Serrano, M., et al., *Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a*. Cell, 1997. **88**(5): p. 593-602.
409. Hay, S. and G. Kannourakis, *A time to kill: viral manipulation of the cell death program*. J Gen Virol, 2002. **83**(Pt 7): p. 1547-64.
410. Turpin, E., et al., *Influenza virus infection increases p53 activity: role of p53 in cell death and viral replication*. J Virol, 2005. **79**(14): p. 8802-11.
411. Kruiswijk, F., C.F. Labuschagne, and K.H. Vousden, *p53 in survival, death and metabolic health: a lifeguard with a licence to kill*. Nat Rev Mol Cell Biol, 2015. **16**(7): p. 393-405.
412. Floter, J., I. Kaymak, and A. Schulze, *Regulation of Metabolic Activity by p53*. Metabolites, 2017. **7**(2).
413. Takatori, H., et al., *Role of p53 in systemic autoimmune diseases*. Crit Rev Immunol, 2014. **34**(6): p. 509-16.
414. Kawashima, H., et al., *Tumor suppressor p53 inhibits systemic autoimmune diseases by inducing regulatory T cells*. J Immunol, 2013. **191**(7): p. 3614-23.
415. Thomasova, D., et al., *p53-independent roles of MDM2 in NF-kappaB signaling: implications for cancer therapy, wound healing, and autoimmune diseases*. Neoplasia, 2012. **14**(12): p. 1097-101.
416. Munoz-Fontela, C., et al., *Transcriptional role of p53 in interferon-mediated antiviral immunity*. J Exp Med, 2008. **205**(8): p. 1929-38.
417. Taura, M., et al., *p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines*. Mol Cell Biol, 2008. **28**(21): p. 6557-67.
418. Ak, P. and A.J. Levine, *p53 and NF- B: different strategies for responding to stress lead to a functional antagonism*. The FASEB Journal, 2010. **24**(10): p. 3643-3652.
419. Siegl, C. and T. Rudel, *Modulation of p53 during bacterial infections*. Nat Rev Microbiol, 2015. **13**(12): p. 741-8.
420. Shatz, M., D. Menendez, and M.A. Resnick, *The human TLR innate immune gene family is differentially influenced by DNA stress and p53 status in cancer cells*. Cancer Res, 2012. **72**(16): p. 3948-57.

421. Siegl, C., et al., *Tumor suppressor p53 alters host cell metabolism to limit Chlamydia trachomatis infection*. Cell Rep, 2014. **9**(3): p. 918-29.
422. Bhardwaj, V., et al., *Helicobacter pylori bacteria alter the p53 stress response via ERK-HDM2 pathway*. Oncotarget, 2015. **6**(3): p. 1531-43.
423. Gonzalez, E., et al., *Chlamydia infection depends on a functional MDM2-p53 axis*. Nat Commun, 2014. **5**: p. 5201.
424. Sato, Y., et al., *Transient increases in p53-responsible gene expression at early stages of Epstein-Barr virus productive replication*. Cell Cycle, 2010. **9**(4): p. 807-14.
425. Kraljevic Pavelic, S., et al., *Adenovirally mediated p53 overexpression diversely influence the cell cycle of HEp-2 and CAL 27 cell lines upon cisplatin and methotrexate treatment*. J Cancer Res Clin Oncol, 2009. **135**(12): p. 1747-61.
426. Havre, P.A., et al., *p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells*. Cancer Res, 1995. **55**(19): p. 4420-4.
427. Jiang, D., et al., *SV40 T antigen abrogates p53-mediated transcriptional activity*. Oncogene, 1993. **8**(10): p. 2805-12.
428. Yamada, T., et al., *The bacterial redox protein azurin induces apoptosis in J774 macrophages through complex formation and stabilization of the tumor suppressor protein p53*. Infect Immun, 2002. **70**(12): p. 7054-62.
429. Zhang, G., et al., *Borna Disease Virus Phosphoprotein Represses p53-Mediated Transcriptional Activity by Interference with HMGB1*. Journal of Virology, 2003. **77**(22): p. 12243-12251.
430. Park, S.W., et al., *Hantaan virus nucleocapsid protein stimulates MDM2-dependent p53 degradation*. J Gen Virol, 2013. **94**(Pt 11): p. 2424-8.
431. Ma-Lauer, Y., et al., *p53 down-regulates SARS coronavirus replication and is targeted by the SARS-unique domain and PLpro via E3 ubiquitin ligase RCHY1*. Proc Natl Acad Sci U S A, 2016. **113**(35): p. E5192-201.
432. Ghouzzi, V.E., et al., *ZIKA virus elicits P53 activation and genotoxic stress in human neural progenitors similar to mutations involved in severe forms of genetic microcephaly and p53*. Cell Death Dis, 2016. **7**(10): p. e2440.
433. Marques, J.T., et al., *Down-regulation of p53 by double-stranded RNA modulates the antiviral response*. J Virol, 2005. **79**(17): p. 11105-14.
434. Pampin, M., et al., *Cross talk between PML and p53 during poliovirus infection: implications for antiviral defense*. J Virol, 2006. **80**(17): p. 8582-92.
435. Yoo, N.K., et al., *Vaccinia virus-mediated cell cycle alteration involves inactivation of tumour suppressors associated with Brf1 and TBP*. Cell Microbiol, 2008. **10**(3): p. 583-92.
436. Huang, W.R., et al., *Avian Reovirus Protein p17 Functions as a Nucleoporin Tpr Suppressor Leading to Activation of p53, p21 and PTEN and Inactivation of PI3K/AKT/mTOR and ERK Signaling Pathways*. PLoS One, 2015. **10**(8): p. e0133699.
437. Izumi, T., et al., *HIV-1 viral infectivity factor interacts with TP53 to induce G2 cell cycle arrest and positively regulate viral replication*. Proc Natl Acad Sci U S A, 2010. **107**(48): p. 20798-803.
438. Munoz-Fontela, C., et al., *p53 serves as a host antiviral factor that enhances innate and adaptive immune responses to influenza A virus*. J Immunol, 2011. **187**(12): p. 6428-36.
439. Megyeri, K., L. Orosz, and L. Kemeny, *Vesicular stomatitis virus infection triggers apoptosis associated with decreased DeltaNp63alpha and increased Bax levels in the immortalized HaCaT keratinocyte cell line*. Biomed Pharmacother, 2007. **61**(5): p. 254-60.
440. Nailwal, H., et al., *The nucleoprotein of influenza A virus induces p53 signaling and apoptosis via attenuation of host ubiquitin ligase RNF43*. Cell Death Dis, 2015. **6**: p. e1768.

441. Groskreutz, D.J., et al., *Respiratory syncytial virus decreases p53 protein to prolong survival of airway epithelial cells*. J Immunol, 2007. **179**(5): p. 2741-7.
442. Eckardt-Michel, J., et al., *The fusion protein of respiratory syncytial virus triggers p53-dependent apoptosis*. J Virol, 2008. **82**(7): p. 3236-49.
443. Bian, T., et al., *Respiratory syncytial virus matrix protein induces lung epithelial cell cycle arrest through a p53 dependent pathway*. PLoS One, 2012. **7**(5): p. e38052.
444. Zhirnov, O.P. and H.D. Klenk, *Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling*. Apoptosis, 2007. **12**(8): p. 1419-32.
445. Technau-Ihling, K., et al., *Influenza A virus infection of mice induces nuclear accumulation of the tumorsuppressor protein p53 in the lung*. Arch Virol, 2001. **146**(9): p. 1655-66.
446. Julkunen, I., et al., *Inflammatory responses in influenza A virus infection*. Vaccine, 2000. **19 Suppl 1**: p. S32-7.
447. Lowy, R.J., *Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms*. Int Rev Immunol, 2003. **22**(5-6): p. 425-49.
448. He, Y., et al., *Influenza A virus replication induces cell cycle arrest in G0/G1 phase*. J Virol, 2010. **84**(24): p. 12832-40.
449. Martinez, I., et al., *Induction of DNA double-strand breaks and cellular senescence by human respiratory syncytial virus*. Virulence, 2016. **7**(4): p. 427-42.
450. Tian, B., et al., *BRD4 Couples NF-kappaB/RelA with Airway Inflammation and the IRF-RIG-I Amplification Loop in Respiratory Syncytial Virus Infection*. J Virol, 2017. **91**(6).
451. Gibbs, J.D., et al., *Cell cycle arrest by transforming growth factor beta1 enhances replication of respiratory syncytial virus in lung epithelial cells*. J Virol, 2009. **83**(23): p. 12424-31.
452. Terrier, O., et al., *Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway*. Virol J, 2011. **8**: p. 285.
453. Shen, Y., et al., *Influenza A virus induces p53 accumulation in a biphasic pattern*. Biochem Biophys Res Commun, 2009. **382**(2): p. 331-5.
454. Wang, X., et al., *The non-structural (NS1) protein of influenza A virus associates with p53 and inhibits p53-mediated transcriptional activity and apoptosis*. Biochem Biophys Res Commun, 2010. **395**(1): p. 141-5.
455. Terrier, O., et al., *Influenza NS1 interacts with p53 and alters its binding to p53-responsive genes, in a promoter-dependent manner*. FEBS Lett, 2013. **587**(18): p. 2965-71.
456. Bitko, V., et al., *Nonstructural proteins of respiratory syncytial virus suppress premature apoptosis by an NF-kappaB-dependent, interferon-independent mechanism and facilitate virus growth*. J Virol, 2007. **81**(4): p. 1786-95.
457. Wu, W., et al., *The interactome of the human respiratory syncytial virus NS1 protein highlights multiple effects on host cell biology*. J Virol, 2012. **86**(15): p. 7777-89.
458. Nakamura-Lopez, Y., N. Villegas-Sepulveda, and B. Gomez, *RSV P-protein impairs extrinsic apoptosis pathway in a macrophage-like cell line persistently infected with respiratory syncytial virus*. Virus Res, 2015. **204**: p. 82-7.
459. Schwarze, J. and K.J. Mackenzie, *Novel insights into immune and inflammatory responses to respiratory viruses*. Thorax, 2013. **68**: p. 108-110.
460. <Diamond\_et\_al-2000-Immunological\_Reviews.pdf>. 2000.
461. Quinton, L.J., et al., *Functions and regulation of NF-kappaB RelA during pneumococcal pneumonia*. J Immunol, 2007. **178**(3): p. 1896-903.
462. Kadioglu, A., et al., *The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease*. Nature Reviews Microbiology, 2008. **6**: p. 288-301.

463. Lijek, R.S. and J.N. Weiser, *Co-infection subverts mucosal immunity in the upper respiratory tract*. *Curr Opin Immunol*, 2012. **24**(4): p. 417-23.
464. Ballinger, M.N. and T.J. Standiford, *Postinfluenza bacterial pneumonia: host defenses gone awry*. *J Interferon Cytokine Res*, 2010. **30**(9): p. 643-52.
465. Rynda-Apple, A., K.M. Robinson, and J.F. Alcorn, *Influenza and Bacterial Superinfection: Illuminating the Immunologic Mechanisms of Disease*. *Infect Immun*, 2015. **83**(10): p. 3764-70.
466. McCullers, J.A., *The co-pathogenesis of influenza viruses with bacteria in the lung*. *Nat Rev Microbiol*, 2014. **12**(4): p. 252-62.
467. Belser, J.A., et al., *Complexities in Ferret Influenza Virus Pathogenesis and Transmission Models*. *Microbiol Mol Biol Rev*, 2016. **80**(3): p. 733-44.
468. Kash, J.C. and J.K. Taubenberger, *The role of viral, host, and secondary bacterial factors in influenza pathogenesis*. *Am J Pathol*, 2015. **185**(6): p. 1528-36.
469. O'Brien, K.L., et al., *Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates*. *Lancet*, 2009. **374**(9693): p. 893-902.
470. Simonsen, L., *The global impact of influenza on morbidity and mortality*. *Vaccine*, 1999. **17 Suppl 1**: p. S3-10.
471. Brundage, J.F. and G.D. Shanks, *Deaths from bacterial pneumonia during 1918-19 influenza pandemic*. *Emerg Infect Dis*, 2008. **14**(8): p. 1193-9.
472. Chien, Y.W., K.P. Klugman, and D.M. Morens, *Bacterial pathogens and death during the 1918 influenza pandemic*. *N Engl J Med*, 2009. **361**(26): p. 2582-3.
473. Hishiki, H., et al., *Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients*. *Journal of Infection and Chemotherapy: Official Journal of the Japan Society of Chemotherapy*, 2011. **17**: p. 87-90.
474. Randolph, A.G., L. Reder, and J.A. Englund, *Risk of bacterial infection in previously healthy respiratory syncytial virus-infected young children admitted to the intensive care unit*. *Pediatr Infect Dis J*, 2004. **23**(11): p. 990-4.
475. Esposito, S., et al., *Pneumococcal Bacterial Load Colonization as a Marker of Mixed Infection in Children With Alveolar Community-acquired Pneumonia and Respiratory Syncytial Virus or Rhinovirus Infection*. *The Pediatric Infectious Disease Journal*, 2013. **32**: p. 1199-1204.
476. Resch, B., W. Gusenleitner, and W.D. Mueller, *Risk of concurrent bacterial infection in preterm infants hospitalized due to respiratory syncytial virus infection*. *Acta Paediatr*, 2007. **96**(4): p. 495-8.
477. Kim, P.E., et al., *Association of invasive pneumococcal disease with season, atmospheric conditions, air pollution, and the isolation of respiratory viruses*. *Clin Infect Dis*, 1996. **22**(1): p. 100-6.
478. Hament, J.M., et al., *Respiratory viral infection predisposing for bacterial disease: a concise review*. *FEMS Immunol Med Microbiol*, 1999. **26**(3-4): p. 189-95.
479. Yokota, S.-i., et al., *Clarithromycin Suppresses Human Respiratory Syncytial Virus Infection-Induced Streptococcus pneumoniae Adhesion and Cytokine Production in a Pulmonary Epithelial Cell Line*. *Mediators of Inflammation*, 2012. **2012**: p. 1-7.
480. Pribul, P.K., et al., *Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development*. *J Virol*, 2008. **82**(9): p. 4441-8.
481. Kolli, D., et al., *Alveolar macrophages contribute to the pathogenesis of human metapneumovirus infection while protecting against respiratory syncytial virus infection*. *Am J Respir Cell Mol Biol*, 2014. **51**(4): p. 502-15.
482. Ali, F., et al., *Streptococcus pneumoniae-associated human macrophage apoptosis after bacterial internalization via complement and Fc gamma receptors correlates with intracellular bacterial load*. *J Infect Dis*, 2003. **188**(8): p. 1119-31.

483. Ghoneim, H.E., P.G. Thomas, and J.A. McCullers, *Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections*. J Immunol, 2013. **191**(3): p. 1250-9.
484. Sun, K. and D.W. Metzger, *Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection*. Nat Med, 2008. **14**(5): p. 558-64.
485. Ludewick, H.P., et al., *Long-term impairment of Streptococcus pneumoniae lung clearance is observed after initial infection with influenza A virus but not human metapneumovirus in mice*. J Gen Virol, 2011. **92**(Pt 7): p. 1662-5.
486. Sun, K. and D.W. Metzger, *Influenza infection suppresses NADPH oxidase-dependent phagocytic bacterial clearance and enhances susceptibility to secondary methicillin-resistant Staphylococcus aureus infection*. J Immunol, 2014. **192**(7): p. 3301-7.
487. Arruvito, L., S. Raiden, and J. Geffner, *Host response to respiratory syncytial virus infection*. Curr Opin Infect Dis, 2015. **28**(3): p. 259-66.
488. Marchant, D., et al., *Toll-like receptor 4-mediated activation of p38 mitogen-activated protein kinase is a determinant of respiratory virus entry and tropism*. J Virol, 2010. **84**(21): p. 11359-73.
489. Dey, N., et al., *TAK1 regulates NF-KappaB and AP-1 activation in airway epithelial cells following RSV infection*. Virology, 2011. **418**(2): p. 93-101.
490. Grandin, C., et al., *Evidence for an intranasal immune response to human respiratory syncytial virus infection in cynomolgus macaques*. J Gen Virol, 2015. **96**(Pt 4): p. 782-92.
491. Patton, K., et al., *Enhanced immunogenicity of a respiratory syncytial virus (RSV) F subunit vaccine formulated with the adjuvant GLA-SE in cynomolgus macaques*. Vaccine, 2015.
492. Klugman, K.P., Y.-W. Chien, and S.A. Madhi, *Pneumococcal pneumonia and influenza: a deadly combination*. Vaccine, 2009. **27 Suppl 3**: p. C9-C14.
493. Vu, H.T.T., et al., *Association Between Nasopharyngeal Load of Streptococcus pneumoniae, Viral Coinfection, and Radiologically Confirmed Pneumonia in Vietnamese Children*. The Pediatric Infectious Disease Journal, 2011. **30**(1): p. 11-18.
494. Techasaensiri, B., et al., *Viral coinfections in children with invasive pneumococcal disease*. The Pediatric Infectious Disease Journal, 2010. **29**: p. 519-523.
495. Franz, A., et al., *Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection*. J Clin Virol, 2010. **48**(4): p. 239-45.
496. Stockman, L.J., et al., *Respiratory syncytial virus and Staphylococcus aureus coinfection in children hospitalized with pneumonia*. Pediatr Infect Dis J, 2010. **29**(11): p. 1048-50.
497. Ghani, A.S., et al., *An investigation into the prevalence and outcome of patients admitted to a pediatric intensive care unit with viral respiratory tract infections in Cape Town, South Africa*. Pediatr Crit Care Med, 2012. **13**(5): p. e275-81.
498. Kwofie, T.B., et al., *Respiratory viruses in children hospitalized for acute lower respiratory tract infection in Ghana*. Virol J, 2012. **9**: p. 78.
499. Okada, T., et al., *A practical approach estimating etiologic agents using real-time PCR in pediatric inpatients with community-acquired pneumonia*. J Infect Chemother, 2012. **18**(6): p. 832-40.
500. Klein, E.Y., et al., *The frequency of influenza and bacterial coinfection: a systematic review and meta-analysis*. Influenza Other Respir Viruses, 2016. **10**(5): p. 394-403.
501. Sharma-Chawla, N., et al., *Influenza A Virus Infection Predisposes Hosts to Secondary Infection with Different Streptococcus pneumoniae Serotypes with Similar Outcome but Serotype-Specific Manifestation*. Infect Immun, 2016. **84**(12): p. 3445-3457.

502. Wolter, N., et al., *High Nasopharyngeal Pneumococcal Density, Increased by Viral Coinfection, Is Associated With Invasive Pneumococcal Pneumonia*. Journal of Infectious Diseases, 2014: p. jiu326.
503. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation*. Nat Rev Immunol, 2008. **8**(12): p. 958-69.
504. Murray, P.J., et al., *Macrophage activation and polarization: nomenclature and experimental guidelines*. Immunity, 2014. **41**(1): p. 14-20.
505. Labadie, K., et al., *Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages*. J Clin Invest, 2010. **120**(3): p. 894-906.
506. Le Douce, V., et al., *Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage*. Retrovirology, 2010. **7**: p. 32.
507. Subrata, L.S., et al., *Interactions between innate antiviral and atopic immunoinflammatory pathways precipitate and sustain asthma exacerbations in children*. J Immunol, 2009. **183**(4): p. 2793-800.
508. Melgert, B.N., et al., *More alternative activation of macrophages in lungs of asthmatic patients*. J Allergy Clin Immunol, 2011. **127**(3): p. 831-3.
509. Nagarkar, D.R., et al., *Rhinovirus infection of allergen-sensitized and -challenged mice induces eotaxin release from functionally polarized macrophages*. J Immunol, 2010. **185**(4): p. 2525-35.
510. Keegan, A.D., et al., *Enhanced allergic responsiveness after early childhood infection with respiratory viruses: Are long-lived alternatively activated macrophages the missing link?* Pathog Dis, 2016. **74**(5).
511. Haeberle, H.A., et al., *Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways*. J Infect Dis, 2002. **186**(9): p. 1199-206.
512. Reed, J.L., et al., *Macrophage impairment underlies airway occlusion in primary respiratory syncytial virus bronchiolitis*. J Infect Dis, 2008. **198**(12): p. 1783-93.
513. Makris, S., et al., *Alveolar Macrophages Can Control Respiratory Syncytial Virus Infection in the Absence of Type I Interferons*. J Innate Immun, 2016. **8**(5): p. 452-63.
514. Tripathi, S., M.R. White, and K.L. Hartshorn, *The amazing innate immune response to influenza A virus infection*. Innate Immun, 2015. **21**(1): p. 73-98.
515. Morales-Nebreda, L., et al., *The heterogeneity of lung macrophages in the susceptibility to disease*. European Respiratory Review, 2015. **24**(137): p. 505-509.
516. Gordon, S.B. and R.C. Read, *Macrophage defences against respiratory tract infections*. Br Med Bull, 2002. **61**: p. 45-61.
517. Byrne, A.J., et al., *Pulmonary macrophages: key players in the innate defence of the airways*. Thorax, 2015. **70**(12): p. 1189-1196.
518. Rivera-Toledo, E., et al., *Conditioned medium from persistently RSV-infected macrophages alters transcriptional profile and inflammatory response of non-infected macrophages*. Virus Res, 2017. **230**: p. 29-37.
519. Cooper, G.E., et al., *Viral Inhibition of Bacterial Phagocytosis by Human Macrophages: Redundant Role of CD36*. PLoS One, 2016. **11**(10): p. e0163889.
520. Qi, F., et al., *Respiratory macrophages and dendritic cells mediate respiratory syncytial virus-induced IL-33 production in TLR3- or TLR7-dependent manner*. Int Immunopharmacol, 2015. **29**(2): p. 408-15.
521. Gaona, J., et al., *Respiratory syncytial virus persistence in macrophages upregulates Fcgamma receptors expression*. Viruses, 2014. **6**(2): p. 624-39.
522. Guerrero-Plata, A., E. Ortega, and B. Gomez, *Persistence of respiratory syncytial virus in macrophages alters phagocytosis and pro-inflammatory cytokine production*. Viral Immunol, 2001. **14**(1): p. 19-30.
523. Camberlein, E., et al., *Importance of Bacterial Replication and Alveolar Macrophage-Independent Clearance Mechanisms during Early Lung Infection with Streptococcus pneumoniae*. Infection and Immunity, 2015. **83**(3): p. 1181-1189.

524. Kopf, M., C. Schneider, and S.P. Nobs, *The development and function of lung-resident macrophages and dendritic cells*. *Nature Immunology*, 2014. **16**(1): p. 36-44.
525. Schleimer, R.P., et al., *Epithelium: at the interface of innate and adaptive immune responses*. *J Allergy Clin Immunol*, 2007. **120**(6): p. 1279-84.
526. Lambrecht, B.N. and H. Hammad, *Biology of lung dendritic cells at the origin of asthma*. *Immunity*, 2009. **31**(3): p. 412-24.
527. Paats, M.S., et al., *Local and systemic cytokine profiles in nonsevere and severe community-acquired pneumonia*. *Eur Respir J*, 2013. **41**(6): p. 1378-85.
528. Menendez, R., et al., *Cytokine activation patterns and biomarkers are influenced by microorganisms in community-acquired pneumonia*. *Chest*, 2012. **141**(6): p. 1537-1545.
529. Rendon, A., E.J. Rendon-Ramirez, and A.G. Rosas-Taraco, *Relevant Cytokines in the Management of Community-Acquired Pneumonia*. *Curr Infect Dis Rep*, 2016. **18**(3): p. 10.
530. Turner, T., et al., *Respiratory syncytial virus: current and emerging treatment options*. *ClinicoEconomics and Outcomes Research*, 2014: p. 217.
531. Dufour, J.H., et al., *IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking*. *J Immunol*, 2002. **168**(7): p. 3195-204.
532. Hayney, M.S., et al., *Serum IFN-gamma-induced protein 10 (IP-10) as a biomarker for severity of acute respiratory infection in healthy adults*. *J Clin Virol*, 2017. **90**: p. 32-37.
533. Karron, R.A. and P.L. Collins, *Parainfluenza viruses*, in *Fields Virology, 6th edition*. 2013, Wolters Kluwer Health Adis (ESP).
534. Ellis, G.T., et al., *TRAIL+ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-Streptococcus pneumoniae coinfection*. *EMBO Rep*, 2015.
535. Itoh, Y., et al., *In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses*. *Nature*, 2009. **460**(7258): p. 1021-5.
536. Rivera-Toledo, E., L. Torres-Gonzalez, and B. Gomez, *Respiratory Syncytial Virus Persistence in Murine Macrophages Impairs IFN-beta Response but Not Synthesis*. *Viruses*, 2015. **7**(10): p. 5361-74.
537. Ravi, L.I., et al., *A systems-based approach to analyse the host response in murine lung macrophages challenged with respiratory syncytial virus*. *BMC Genomics*, 2013. **14**: p. 190.
538. Bates, J.T., et al., *Immunogenicity and efficacy of alphavirus-derived replicon vaccines for respiratory syncytial virus and human metapneumovirus in nonhuman primates*. *Vaccine*, 2016. **34**(7): p. 950-6.
539. Graham, B.S., *Biological challenges and technological opportunities for respiratory syncytial virus vaccine development*. *Immunol Rev*, 2011. **239**(1): p. 149-66.
540. Stittelaar, K.J., et al., *Ferrets as a Novel Animal Model for Studying Human Respiratory Syncytial Virus Infections in Immunocompetent and Immunocompromised Hosts*. *Viruses*, 2016. **8**(6).
541. Taylor, G., *Animal models of respiratory syncytial virus infection*. *Vaccine*, 2017. **35**(3): p. 469-480.
542. Domachowske, J.B., C.A. Bonville, and H.F. Rosenberg, *Animal models for studying respiratory syncytial virus infection and its long term effects on lung function*. *Pediatr Infect Dis J*, 2004. **23**(11 Suppl): p. S228-34.